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Composición bioquímica y crecimiento de paralarvas de pulpo (*Octopus vulgaris* Cuvier, 1797), alimentadas con juveniles de *Artemia* enriquecidos con microalgas y otros suplementos nutricionales.

Pedro Fernandes Seixas

Tesis Doctoral / PhD Thesis

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Composición bioquímica y crecimiento de paralarvas de pulpo (*Octopus vulgaris* Cuvier, 1797), alimentadas con juveniles de *Artemia* enriquecidos con microalgas y otros suplementos nutricionales.

Memoria que para optar
al Grado de Doctor en Biología presenta
Pedro Fernandes Seixas

Santiago de Compostela, a 8 de Abril de 2009

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Fdo: Pedro Fernandes Seixas

Manuel Rey Méndez, Profesor Titular del Departamento de Bioquímica y Biología Molecular de la Facultad de Biología, Ana María Otero Casal, Profesora Titular del Departamento de Microbiología y Parasitología de la Facultad de Biología, ambos de la Universidad de Santiago de Compostela, y Luísa Maria Pinheiro Valente, Profesora Asociada del Departamento de Producción Acuícola de la Universidad de Porto (Portugal),

HACEN CONSTAR:

Que la memoria titulada "**Composición bioquímica y crecimiento de paralarvas de pulpo (*Octopus vulgaris* Cuvier, 1797), alimentadas con juveniles de *Artemia* enriquecidos con microalgas y otros suplementos nutricionales**", que presenta D. Pedro Fernandes Seixas para optar al Grado de Doctor en Biología, fue realizada bajo nuestra dirección en ambos departamentos de la Universidad de Santiago de Compostela y en el CIIMAR (Portugal), y autorizamos su presentación.

Y para que así conste, firmamos la presente en Santiago de Compostela, a 8 de Abril de 2009.

Fdo: Dr. Manuel Rey Méndez	Fdo: Dra. Ana Maria Otero Casal	Fdo: Dra. Luísa Maria Pinheiro Valente

À memória dos meus avós
Constância, Joaquim, Francisca e Sebastião

Mar Português

"…

Valeu a pena? Tudo vale a pena

Se a alma não é pequena.

Quem quer passar além do Bojador

Tem que passar além da dor.

Deus ao mar o perigo e o abismo deu,

Mas nele é que espelhou o céu."

Fernando Pessoa (*Mensagem*)

El trabajo contenido en la presente memoria ha sido cofinanciado por JACUMAR (Junta Nacional Asesora de Cultivos Marinos), Secretaria General de Pesca Marítima, España, a través del proyecto titulado “Optimización del engorde del pulpo (*Octopus vulgaris*)”, 2007-2009.

Pedro Fernandes Seixas ha sido becario predoctoral de la “Fundação para a Ciência e a Tecnologia”, en el período 2004-2008 (beca con referencia: SFRH/BD/16419/2004).

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Introducción / Introduction

1. Potencial del pulpo común (*Octopus vulgaris* Cuvier, 1797) para la acuicultura

1.1 Biología y ciclo de vida del pulpo común (*Octopus vulgaris*)

El pulpo común (*Octopus vulgaris* Cuvier, 1797), también denominado pulpo de roca (Fig. 1), es uno de los moluscos cefalópodos mejor estudiados en la actualidad, siendo el más destacado de entre las más de 100 especies del género *Octopus* descritas. Según una revisión reciente (Current Classification of Recent Cephalopoda, 2001), esta especie se clasifica de la siguiente forma:

Reino.....ANIMALIA
Filo.....MOLLUSCA
Clase.....CEPHALOPODA Cuvier, 1797
Subclase.....Coleoidea
Superorden.....Octopodiformes
Orden.....Octopoda
Suborden.....Incirrata
Familia.....**Octopodidae**
Subfamilia.....**Octopodinae**
Género.....*Octopus* Cuvier, 1797
Especie..... *Octopus vulgaris* Cuvier, 1797

Figura 1 – Ejemplar adulto del pulpo común (*Octopus vulgaris*). Foto: Pedro Seixas

El pulpo *O. vulgaris* ha sido modelo de investigación en estudios de neurobiología, fisiología animal y bioquímica, y también de comportamiento animal (Young, 1971; Wells, 1978; Boyle, 1983). Además, en los últimos 15 años se ha enfatizado el estudio de su biología y ciclo de vida en cautividad, con el objetivo de aplicar estos conocimientos a su cultivo integral (Iglesias *et al.*, 2007a). El pulpo es un animal solitario y territorial, que presenta migraciones estacionales, principalmente cuando se aproxima la época reproductiva. Esta especie costera vive en rocas, fondos arenosos o entre algas, desde la superficie hasta los 200 metros de profundidad. Son animales que pasan gran parte del día escondidos entre grietas o en agujeros de rocas, o en materiales de origen humano

desechados al mar, siendo igualmente verdaderos maestros del camuflaje, ya que tienen la capacidad de cambiar el color y la textura de la piel en perfecta sintonía con el entorno. Su ciclo de vida es relativamente corto, estimándose entre uno y dos años en el medio natural, aunque según diferentes autores este período puede variar en función de la zona geográfica: entre 12 y 20 meses en el Mediterráneo (Mangold y Boletzky, 1973); de 18 a 24 meses en la costa NW de África (Hatanaka, 1979); de 9 a 15 meses en la costa NE de África (Smale y Buchan, 1981); o de 14 a 20 meses en la costa NW de África (Domain *et al*., 2000). Los machos suelen presentar mayor longevidad que las hembras, ya que éstas al final del periodo de incubación de los huevos acaban por perecer.

El interés comercial del desarrollo del cultivo del pulpo se debe a que esta especie es muy demandada en varios países de Europa y en Asia y posee un elevado valor comercial. Presenta, además, algunas características biológicas muy interesantes para ser considerado un serio candidato para la acuicultura: ciclo de vida corto (1-2 años), elevadas tasas de crecimiento (entre el 1,0 y el 11,5% peso corporal dia^{-1} a lo largo de toda su vida), alta tasa de conversión alimentaria (30-60%), elevada fertilidad y muy alta viabilidad de los huevos, fácil adaptación a la cautividad, aceptación de alimentos de bajo valor comercial, elevado contenido proteico (70-85% del peso seco) y, por último, prácticamente ausencia de patologías (Mangold y Boletzky, 1973; Mangold, 1983; Boucher-Rodoni *et al*., 1987; Lee, 1994; Villanueva, 1995; Villanueva *et al*., 1995; Iglesias *et al.*, 2000, 2007a; Vaz-Pires *et al.*, 2004). El ciclo de vida del pulpo común se representa en la figura 2.

El pulpo es una especie gonocórica, es decir, tiene los sexos separados, y aunque no se aprecian diferencias considerables de tamaño y peso corporal entre machos y hembras, presentan un claro dimorfismo sexual a partir del comienzo de la maduración sexual. El aparato reproductor de los machos posee un único testículo, localizado en la parte anterior del cuerpo, donde se producen los espermatozoides, que salen por el conducto seminal deferente que a su vez se conecta con una serie de glándulas. En éstas, los espermatozoides producidos son empaquetados y rodeados por membranas, dando lugar a los espermatóforos, que una vez completamente formados se almacenan en el saco espermatofórico o bolsa de Needhan, de la cual salen a través del conducto seminal aferente y del órgano terminal (o pene). La estructura más característica del macho es el tercer brazo derecho, que presenta ciertas modificaciones morfológicas, y que por eso recibe el nombre de hectocótilo (Fig. 3). Este brazo, que tiene la función de transferir/depositar los espermatóforos en los conductos oviductales de la hembra, posee un

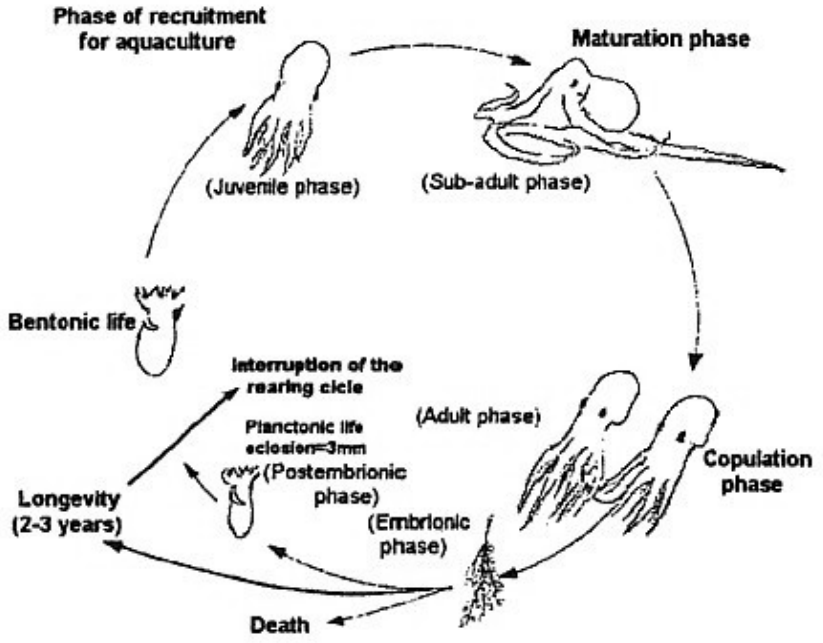

Figura 2 - Esquema del ciclo de vida del pulpo. Tomado de Boucaud-Camou (1989).

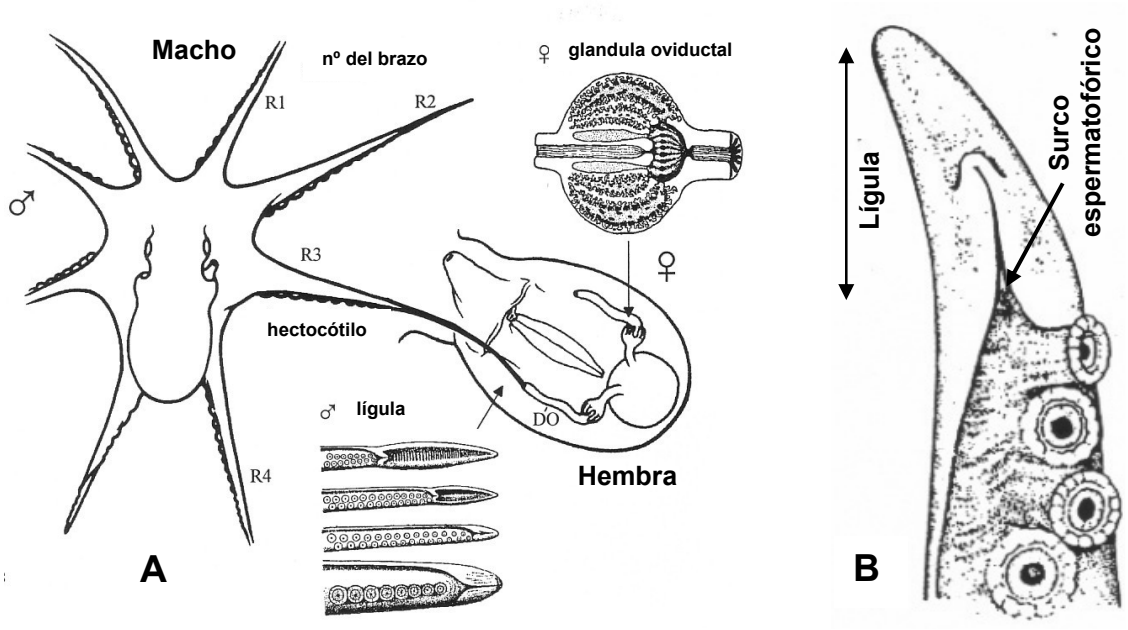

Figura 3 – (A) Esquema de la transferencia de los espermatóforos a través del hectocotilo hasta los conductos oviductales (DO) de la hembra. (Tomado de Hanlon y Messenger, 1996). (B) Aspecto detallado de la región distal del brazo hectocotilizado de *Octopus vulgaris*, mostrando la lígula.

surco a lo largo de toda su cara ventral, por donde van a deslizar los espermatóforos en el momento de la cópula, acabando en una zona lisa y aplanada (lígula), a diferencia de los otros brazos que acaban en punta y con ventosas. Otra de las diferencias morfológicas entre machos y hembras en la edad adulta, y quizás la más fácil de identificar, es que los machos poseen unas pocas ventosas de tamaño destacado en los segundos y terceros pares de brazos, mientras que en las hembras las ventosas son de tamaño más uniforme a lo largo de todo el brazo. Algunos autores apuntan a que estas grandes ventosas sirven para reconocimiento sexual en el medio natural, antes de que se inicie cualquier tipo de actividad copulatoria (Hanlon y Messenger, 1996). El sistema reproductor de las hembras consta de un ovario que desemboca en dos oviductos y de las glandulas oviductales (Fig. 3). El ovario se sitúa en la parte posterior de la cavidad del manto y en él se forman los ovocitos. Cuando la hembra es fecundada, los espermatóforos quedan almacenados en los receptáculos seminales de las glándulas oviductales. Se desconoce, o al menos no hay evidencias claras, de que los pulpos tengan un comportamiento de cortejo, habiendo sido descritas situaciones de múltiple transferencia de espermatóforos por varios machos a una sóla hembra (Hanlon y Messenger, 1996). En un estudio reciente realizado en nuestro laboratorio (Departamento de Bioquímica y Biología Molecular), se confirmó que existe efectivamente paternidad múltiple en puestas de huevos de varias hembras, ya que por identificación genética de los huevos se encontraron genes de distintos progenitores machos, mientras que el gen materno era el mismo en todos los huevos de cada puesta (datos no publicados). Estos descubrimientos tienen su importancia a la hora de aclarar el tema de la competencia entre machos para dejar sus espermatóforos *versus* existencia de paternidad multiple, ya que prevalecía la idea de que los machos antes de transferir sus espermatóforos, se aseguraban de la presencia de espermatóforos dejados por otros machos, y en caso de que así fuera, los removían antes de dejar los suyos.

La época reproductiva de *O. vulgaris* es bastante amplia y varía con la zona geográfica. En el Mediterraneo, Mangold y Boletzky (1973) observaron hembras con puestas de marzo a octubre, mientras que machos maduros se encontraban a lo largo de todo el año. Tanaka (1958) registró resultados similares para *O. vulgaris* en la península de Boso en Japón. Sin embargo, dependiendo de la zona geográfica de donde provienen, se han identificado dos picos de puestas al año: el primero corresponde a la época marzo-mayo, más importante en el Atlántico/Mediterráneo, y la segunda al comienzo del otoño (septiembre-octubre), más importante en Japón (FAO, 2003). El pulpo común realiza una puesta única – semelparía – en la cual el ovario se desarrolla de forma sincrónica y la ovulación se da de una sóla vez,

no existiendo desarrollo y maduración de nuevos ovocitos después de la puesta. El número de huevos que las hembras depositan está estimado entre 100.000 y 500.000 (Mangold, 1987), aunque otros autores han descrito puestas de mayor tamaño (605.000 huevos) de hembras grandes mantenidas en laboratorio (Iglesias *et al.*, 1997). La fecundación en los pulpos es interna y se da a medida que los ovocitos van pasando por el oviducto proximal hasta la zona distal, fertilizándose a su paso por la glándula oviductal, donde están alojados los espermatóforos. La puesta de los huevos puede tardar entre 15 y 30 días. Posteriormente, la hembra pasa a ejercer cuidados maternales como protección contra depredadores, y limpieza y oxigenación de los huevos, pudiendo este periodo prolongarse de 25 a 125 días, dependiendo de la temperatura del agua (Mangold y Boletzky, 1973). Durante este tiempo los huevos de pulpo han de pasar por varias fases de desarrollo (Fig. 4), las cuales han sido clasificadas en XX estadios por Naef (1928).

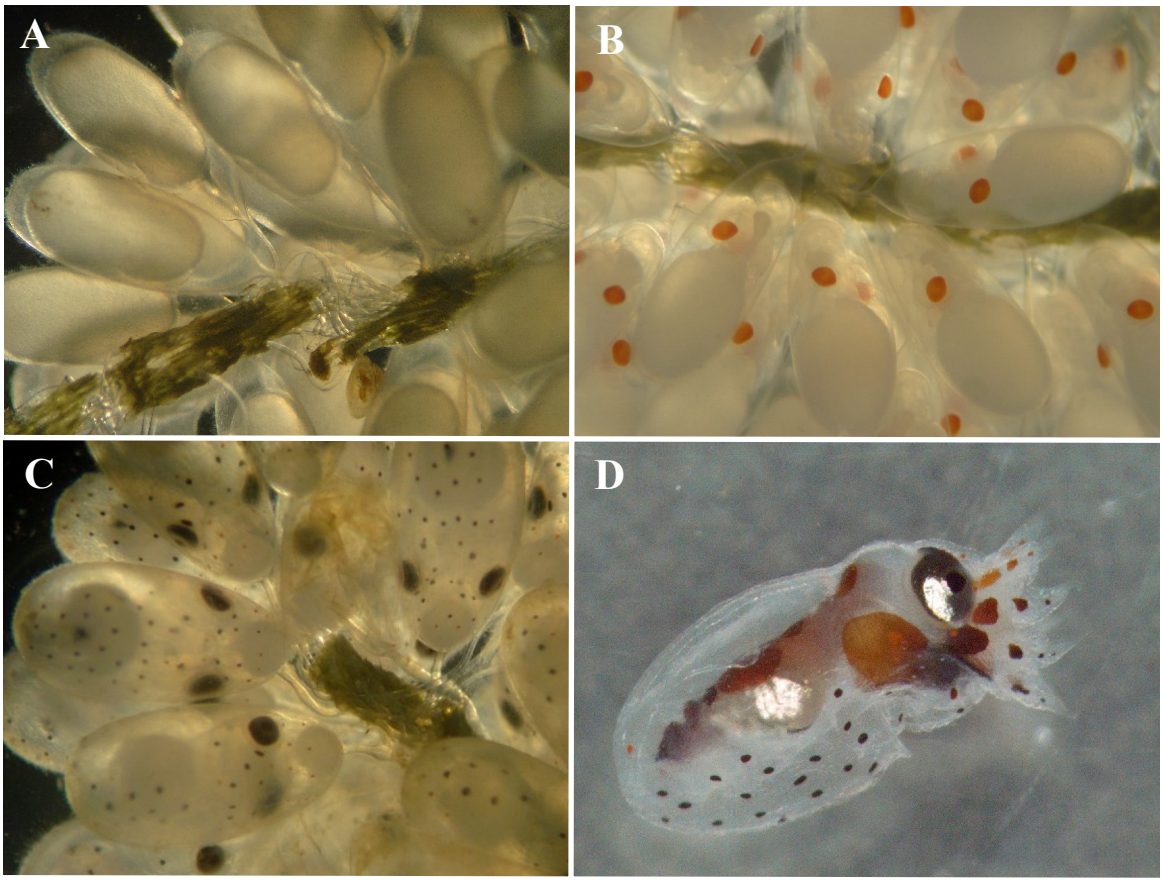

Figura 4 - Fotografías a la lupa de huevos de pulpo en diferentes estadios de desarrollo y de una paralarva de pulpo. (A) huevos de pulpo (≈2 x 1 mm) recién depositados; (B) huevos en una etapa de desarrollo intermedia (estadio X-XII); (C) huevos en un estado de desarrollo avanzado (estadio XVIII); (D) paralarva de pulpo recién eclosionada (tamaño total ≈ 3 mm). Fotos: Pedro Seixas.

A lo largo de este periodo las hembras dejan de alimentarse, pudiendo perder hasta el 60% de su peso corporal inicial antes de haber iniciado la puesta (Wodinsky, 1978). Mangold (1987) observó que los ejemplares capturados en fase de post-desove tenían el hepatopáncreas reducido, y el aspecto del animal mostraba signos de agotamiento, lo que le produciría la muerte. La eclosión de los huevos libera las paralarvas (Fig. 4), termino propuesto por Young y Harman (1988), que así se denominan porque en realidad no van a sufrir una verdadera metamorfosis antes de convertirse en juveniles, aunque al contrario que en la vida adulta, son planctónicas en sus primeros días de vida. Una vez finalizado el periodo de incubación y liberación de las paralarvas, las hembras mueren. El tamaño del manto de las paralarvas recién eclosionadas es de alrededor de 2 mm y en cada brazo poseen 3 ventosas (Boletzky, 1987; Villanueva, 1995). Se estima que el periodo de vida planctónico de las paralarvas es de 30 a 60 días de vida, dependiendo de la temperatura del agua y de la zona geográfica (Itami *et al.*, 1963; Mangold y Boletzky, 1973; Villanueva *et al*., 1995). Se cree que las paralarvas de pulpo, al igual que otras larvas de cefalópodos estudiadas en su medio natural (Vecchione, 1987; Passarella y Hopkins, 1991), se alimentan de pequeños crustáceos que forman parte del zooplancton. Estas criaturas son depredadoras activas desde el primer día de vida (Iglesias *et al*., 2006), a pesar de que poseen reservas vitelinas internas que les permiten sobrevivir en ausencia de alimento durante algunos días (Boletzky, 1975). La absorción de determinados nutrientes por la piel también ha sido señalada como un factor importante en los primeros estadios de vida de las larvas de cefalópodos (Lee, 1994), mientras que en otras especies de pulpos como el *Octopus dofleini,* se ha descrito un comportamiento de alimentación neustónico, es decir, las paralarvas se colocan con los brazos hacía la superficie aprovechando la tensión superficial del agua, alimentándose de los detritos organicos o presas de esta interfase (Marliave, 1981). De los trabajos realizados en laboratorio de cultivo de paralarvas de *O. vulgaris* (Villanueva, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006) se sabe que a medida que éstas van creciendo, la proporción de los brazos frente al manto va aumentando, y una vez alcanzado un determinado tamaño, realizan el asentamiento en el fondo, convirtiéndose así en juveniles bentónicos de morfología igual que los adultos. El crecimiento de los juveniles hasta la fase adulta puede tardar de 8 a 15 meses, dependiendo mayormente de la temperatura y de la disponibilidad de alimento (Mangold y Boletzky, 1973; Forsythe y Van Heukelem, 1987; Semmens *et al.*, 2004; Leporati *et al*., 2007). Al igual que muchos otros cefalópodos, *O. vulgaris* es una especie carnívora durante todo su ciclo de vida. En las fases de juvenil hasta la edad adulta, los pulpos se alimentan de una

gran variedad de presas que están representadas por la mayor parte de los filums marinos (Mangold, 1983), de los cuales varias especies de crustáceos, peces, y otros moluscos constituyen las presas más comunes, siendo los cangrejos una de sus favoritas (Nixon, 1987). Esta especie presenta, además, un comportamiento de canibalismo frecuente en el medio natural.

A pesar del gran interés en torno a esta especie para su cultivo industrial, persisten dos grandes problemas por resolver: por un lado la obtención de juveniles bentónicos resultantes del cultivo de las paralarvas, y por otro la optimización de los procesos de engorde de sub-adultos capturados en la naturaleza. En el primer caso, en general, se observa una mortalidad casi siempre masiva de las paralarvas, antes de que éstas alcancen la fase de transición hacía la vida bentónica, o sea, cuando se convierten en juveniles. Este tema será abordado en detalle a continuación (apartado 1.2). En cuanto al engorde de ejemplares sub-adultos, todavía hace falta optimizar y buscar alternativas a muchos aspectos de los métodos de engorde que se emplean. Algunos de los problemas a resolver son la búsqueda de alimentos alternativos a la dieta a base de pescado y/o crustáceos, la búsqueda de nuevos sistemas de engorde, la disminución de la mortalidad observada durante el proceso de engorde, la optimización de la rentabilidad económica del proceso, etc. En el apartado 1.3 (Engorde de pulpos procedentes del medio natural) se presentará un resumen general del proceso de engorde del pulpo y del potencial de crecimiento de esta especie, y en el capitulo 6 se describirán con más detalle algunos de los resultados obtenidos en experimentos anteriores, además de los trabajos de engorde realizados por nuestro grupo (Departamento de Bioquímica y Biología Molecular) en los últimos dos años en la ría de Vigo, en el marco del proyecto nacional JACUMAR (Optimización del engorde del pulpo *Octopus vulgaris*).

1.2 Obtención de juveniles bentónicos: el cuello de botella del cultivo integral de *Octopus vulgaris*

Actualmente el principal problema para el desarrollo integral del cultivo del pulpo es la elevada mortalidad observada durante el cultivo de las paralarvas planctónicas. A pesar de los intentos llevados a cabo por diferentes grupos de trabajo, en los que se han utilizado distintas metodologías de alimentación, pocos fueron los experimentos en los que se han obtenido ejemplares de juveniles bentónicos (revisado por Iglesias *et al*., 2007a). Uno de

los primeros trabajos, o quizás el primero, que describió la obtención de juveniles bentónicos de *O. vulgaris*, fue publicado en 1963 por Itami *et al*., que utilizando zoeas de camarón (*Palaemon serrifer*), y a una temperatura media de cultivo de 24,7 ºC, lograron conseguir juveniles bentónicos al cabo de 45 días, con una supervivencia del 8%. Las zoeas de camarón resultaban ser presas adecuadas, aunque su tamaño representase del 60% al 100% del tamaño de las paralarvas. Años más tarde, Imamura (1990) describía un conjunto de experimentos llevados a cabo a lo largo de los años 80 en tres estaciones experimentales de Japón, haciendo una revisión de los avances y logros obtenidos en el cultivo larvario del pulpo. El autor refirió que por primera vez se había demostrado la posibilidad de cultivar paralarvas sólo con *Artemia*, resultado de gran importancia por ser ésta una presa fácil de producir, y cuyo tamaño se puede controlar para su suministro a las paralarvas de pulpo, a lo largo del periodo de cultivo. Hamazaki *et al*. (1991) publicaron por aquellas fechas la obtención de juveniles de pulpo bentónicos en condiciones controladas, empleando como alimento vivo juveniles de *Artemia* (1,5-2 mm) enriquecidos con *Nannochloropsis* sp. y añadiendo esta misma microalga al tanque de cultivo, alcanzando en uno de sus experimentos en un tanque de 20 m^3 un número de 23.700 juveniles bentónicos (supervivencia del 29%) al cabo de 25 días de cultivo, a una temperatura media del agua de 26,9 ºC. Estos autores, mediante un conjunto de experimentos con aguas verdes o claras, observaron efectos positivos sobre el crecimiento y la supervivencia de las paralarvas, al añadir *Nannochloropsis* sp. a los tanques de cultivo, y enriqueciendo *Artemia* (1,5-2 mm) con esta misma microalga. Imamura (1990) hacía referencia a las supuestas causas que podrían justificar los buenos resultados encontrados por aquellos autores, al utilizar *Nannochloropsis* sp. en el proceso de cultivo de paralarvas, como el continuo enriquecimiento de las presas vivas, y la suavización de las condiciones de luminosidad en los tanques, que podrían reducir el estrés de las paralarvas. Sin embargo, y a pesar de los resultados prometedores publicados por estos autores, el cultivo integral de *O. vulgaris* en la costa noroeste del Pacífico no sufrió avances relevantes hasta hoy en día.

Al comienzo de los años 90, se iniciaron también en España un conjunto de importantes trabajos relacionados con el cultivo del pulpo *O. vulgaris.* Los primeros trabajos publicados sobre el cultivo larvario de cefalópodos en laboratorio fueron llevados a cabo por Villanueva (1994, 1995), que probando zoeas de distintos crustáceos decápodos (*Pagurus prideaux*, *Liocarcinus depurator* y *Dardanus arrosor*), logró obtener juveniles de pulpo bentónicos, confirmando a las zoeas como un alimento apropiado para suministrar a

larvas de cefalópodos. Sin embargo, el autor ya ponía de manifiesto la dificultad en obtener zoeas en momentos precisos y los altos costes de mantenimiento y mano de obra que implicaba esta práctica. Por otro lado, en el IEO de Vigo, el grupo liderado por José Iglesias iniciaba un conjunto de experimentos de engorde de pulpo en laboratorio, afrontando entre los años 1997 y 2000, el problema del cultivo de la fase planctónica del pulpo, ya que éste era el punto crítico del cultivo integral de la especie en laboratorio. En los primeros experimentos realizados (Iglesias *et al*., 1999; 2000), estos autores probaron distintos tipos de alimento tales como: zooplancton del medio natural (copépodos, zoeas de crustáceos y misidáceos), ictioplancton, huevos de peces, micropellets, *Artemia* y rotíferos, y más tarde zoeas de crustáceos obtenidas en laboratorio a partir de stocks de reproductores de cangrejo (*Carcinus maenas*), nécora (*Necora puber*) y camarón (*Palaemon serratus*). Sin embargo, la mortalidad de las paralarvas fue casi total en los primeros días de vida, alcanzándose supervivencias máximas del 10% a los 32 días al emplear como alimento metanauplios y a continuación *Artemia* adulta enriquecida con microalgas, pero sin que se lograse alcanzar la fase de pre-asentamiento de las paralarvas.

La puesta en marcha de un conjunto de proyectos cuyo objetivo era solucionar el cultivo integral del pulpo, generó una buena cantidad de información sobre el cultivo larvario. Básicamente, los cultivos experimentales de paralarvas de pulpo incidieron sobre la utilización de monodietas, o de dietas mixtas a base de *Artemia*, zoeas o microdietas artificiales como alimento para suministrar a las paralarvas, con el fin de cubrir sus requerimientos nutricionales. En los intentos llevados a cabo en el IEO de Vigo, y más tarde en el Centro de Experimentación Pesquera de Asturias, se obtuvieron algunos ejemplares de juveniles bentónicos que han llegado a alcanzar la edad adulta, utilizando como dieta para suministrar a las paralarvas *Artemia* enriquecida con microalgas, complementada con zoeas de centolla *Maja brachydactyla* (Fig. 5) en momentos de disponibilidad de zoeas (Iglesias *et al*., 2002, 2004; Carrasco *et al.*, 2003, 2006), a temperaturas medias del agua de 21-22ºC. Las primeras pruebas en las que se ha empleado esta mezcla de presas vivas (Moxica *et al*., 2002), fueron llevadas a cabo en el IEO de Vigo en un tanque de 9 m^3, al que se aportó inicialmente nauplios de *Artemia* y más tarde juveniles de *Artemia* (1-4 mm) a una densidad de presas de 0,1 *Artemia* ml^{-1}, complementada con zoeas a partir de la tercera semana de cultivo (3000 a 5000 zoeas al día). Al tanque se le aportaba a diario una mezcla de microalgas (40% de *Isochrysis galbana* Parke, 40% de *Tetraselmis suecica* Kylin y 20% de *Chaetoceros* sp.) con el fin de mantener las presas vivas constantemente alimentadas. Aunque no se han obtenido pulpos

bentónicos en este experimento, los autores refirieron la obtención de paralarvas en fase de asentamiento con elevado número de ventosas en los brazos (17-18) y un peso seco de 9,2 ± 0,9 mg a los 52 días de vida. La supervivencia en este experimento fue de 8,3% al mes de vida y de 0,2% al cabo de 52 días. En un experimento posterior, en el que se redujo la escala y se empleó un tanque de 1 m^3, las paralarvas se alimentaron con *Artemia* (1-4 mm) enriquecida en las últimas 24 h con *Chlorella* sp., añadiendo diariamente al tanque de cultivo una mezcla de microalgas (*Chlorella* sp., *Isochrysis galbana* y *Chaetoceros* sp.). Como complemento de *Artemia* se suministraron zoeas de centollo (*Maja brachydactyla*) cuatro veces a la semana, a una concentración de 0,01-0,1 zoeas ml^{-1}, alcanzándose así una supervivencia del 31,5% al día 40, y un reducido número de juveniles bentónicos dos semanas más tarde (Iglesias *et al.*, 2002, 2004). El seguimiento de los únicos pulpos supervivientes de este experimento hasta la fase adulta, y la obtención de una puesta de huevos por parte de una hembra, originó el cierre del ciclo de vida del pulpo en laboratorio por primer vez (Iglesias *et al*., 2004).

De forma similar, Carrasco *et al*. (2003, 2006), utilizando un protocolo de alimentación semejante al del grupo del IEO de Vigo, pero usando tanques convexos de 30 l de volumen, lograron obtener en uno de sus experimentos pulpos bentónicos a los 60 días, con una supervivencia del 3,4%. En Canarias, el grupo de investigación de Juan Roo ha utilizado zoeas de *Grapsus grapsus* o de *Plagusia depressa,* complementadas con metanauplios de *Artemia* enriquecidos con A1 Selco (INVE), para cultivar paralarvas de pulpo. Sin embargo, a pesar de la mejor supervivencia y peso seco de las paralarvas a los 28 días de vida, en comparación con el grupo alimentado únicamente con *Artemia*, los autores no han descrito la obtención de juveniles bentónicos (revisado por Iglesias *et al*., 2007a). Sin embargo, la utilización de zoeas de crustáceos supone un elevado riesgo, ya que no se controla la obtención de las zoeas en momentos precisos ni la cantidad a producir, además de los elevados costes que conlleva esta práctica por la necesidad de disponer de más recursos materiales, de espacio y de personal (Navarro y Villanueva, 2000). *Artemia*, en cambio, es un crustáceo fácil de producir, los nauplios o metanauplios se obtienen en 24-72 h, y su composición bioquímica se puede modular mediante técnicas de enriquecimiento, al ser un filtrador continuo obligado y no selectivo. La utilización de *Artemia* como presa viva se consolida, por tanto, como una de las mejores alternativas para el cultivo de paralarvas, aunque sea necesario seguir trabajando en la modulación de su valor nutritivo, de acuerdo con los requerimientos nutricionales de las paralarvas.

Varios autores han utilizado nauplios de *Artemia* enriquecidos con distintos productos comerciales (Navarro y Villanueva, 2000, 2003; Villanueva *et al*., 2004; Okumura *et al*., 2005) en sus experimentos de cultivo de paralarvas de pulpo, siendo el tamaño de estas presas (0,5-1,2 mm) adecuado hasta determinado momento del cultivo. Sin embargo, se ha demostrado que las paralarvas tienen una preferencia clara por *Artemia* de mayor tamaño (1,4 mm) sobre la más pequeña (0,8 mm), desde los primeros días de vida (Iglesias *et al*., 2006). De entre los productos o suplementos nutricionales empleados por diversos autores para enriquecer *Artemia* se encuentran emulsiones lipídicas de la gama Selco (INVE) u otros aceites de pescado, vitaminas, harina de huevos de pescado (BASF), harinas de cereales, o productos de la gama Ori (Skretting) (Navarro y Villanueva, 2000, 2003; Villanueva *et al*., 2002; Iglesias *et al*., 2004; Okumura *et al*., 2005; Izquierdo *et al*., 2008), casi todos diseñados para enriquecer nauplios de *Artemia*. No obstante, de los varios intentos de cultivo de paralarvas realizados por los autores antes mencionados a base de *Artemia* enriquecida con productos comerciales, ninguno ha originado tasas de supervivencia o pesos secos de las paralarvas semejantes a los obtenidos con la utilización de zoeas de crustáceos.

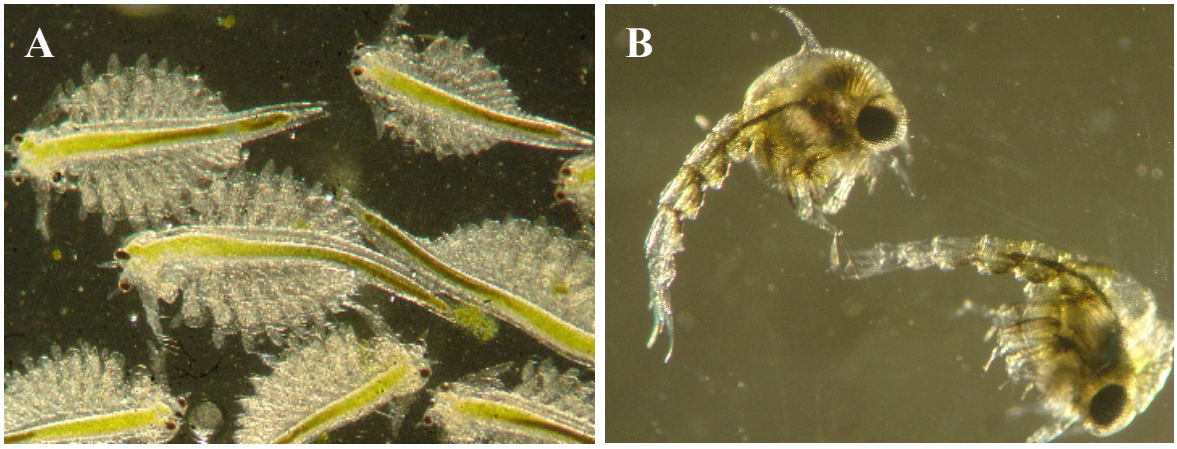

Figura 5 - (A) Juveniles de *Artemia* (≈ 1,7-2,8 mm) enriquecidos con *Tetraselmis suecica*, y (B) zoeas de centollo (*Maja brachydactyla*) recién eclosionadas (≈ 2,5 mm). Fotos: Pedro Seixas.

Como alternativa a las zoeas o a la *Artemia*, también se han probado otros tipos de presas vivas o dietas artificiales para suministrar a las paralarvas, aunque en todo caso este tipo de pruebas se hicieron complementadas con *Artemia* enriquecida. Dentro de las presas vivas alternativas, se han realizado algunos ensayos de cultivo de paralarvas en la Universidad Federal do Rio Grande (FURG, Brasil) con copépodos adultos (*Acartia tonsa*) cultivados

en laboratorio (revisado por Iglesias *et al*., 2007a). Estas presas, con un tamaño aproximado de 1 mm, han sido suministradas a una densidad de 80 copepodos l^{-1} como complemento de nauplios de *Artemia* enriquecidos en los primeros 15 días de cultivo. La supervivencia de las paralarvas a los 40 días de vida fue del 20 al 38%, con una tasa de crecimiento especifico del 4,6% peso seco dia^{-1}, lo que demostraron el potencial de estas presas para el cultivo larvario del pulpo. Dentro de las dietas artificiales, Navarro y Villanueva (2000) formularon micropellets de 250-500 μm (con un 6% de humedad) a base de eufasiaceos congelados, harina de pota, hidrolizado de pescado, DC Super-Selco (INVE) y otros suplementos nutricionales, para suministrar como complemento de nauplios enriquecidos. El porcentaje de paralarvas capturando y/o manejando los pellets fue del 49%, aunque las tasas de supervivencia y crecimiento de las paralarvas al día 30 (6,7% y 0,7 mg, respectivamente) no fueron muy buenas. Sin embargo, el porcentaje de paralarvas que habían ingerido efectivamente los pellets fue del 18%. En otro intento de utilización de microdietas artificiales, Villanueva *et al*. (2002) probaron distintas milicápsulas producidas a través de procesos de gelificación-coacervación, haciendo variar su composición nutricional, el color y la humedad, usando ingredientes a base de harinas de pota y crustáceos y otros suplementos nutricionales. Las milicápsulas tenían forma oval y un tamaño aproximado de 1,3-2,0 mm. De forma similar a los resultados obtenidos con micropellets secos, estos autores observaron paralarvas capturando e ingiriendo las milicápsulas durante el cultivo, pero el crecimiento de las paralarvas no fué significativamente más elevado que con la utilización de *Artemia* enriquecida como alimento único. Los autores referían razones de orden fisiológico, relacionadas con la digestión/absorción de nutrientes, o carencias nutricionales en las microdietas, que podrían estar detrás de los pobres resultados obtenidos. En Japón, Okumura *et al*. (2005), utilizando nauplios de *Artemia* enriquecidos de diferentes tamaños, complementados con copos de pescado (*Ammodytes personatus*) raspados sobre el agua de los tanques, lograron mejorar el perfil de ácidos grasos de las paralarvas de pulpo, pero no referieron la obtención de juveniles bentónicos.

Otro de los "suplementos" empleados muy a menudo por diversos autores para enriquecer/cultivar *Artemia* son las microalgas, de las que las más comúnmente utilizadas fueron las siguientes: *Nannochloropsis* sp., *Dunaliella viridis, Tetraselmis suecica* Kylin, *Isochrysis galbana* Parke, *Chaetoceros* sp., *Chlorella* sp. (Hamazaki *et al*., 1991; Navarro y Villanueva, 2000; Moxica *et al*., 2002; Iglesias *et al*., 2002, 2004; Carrasco *et al*., 2003, 2006; Moxica *et al*., 2006). Sin embargo, los cultivos de microalgas utilizados por estos

investigadores siempre provenían de cultivos de microalgas en discontinuo (o tipo "batch") de donde se suelen cosechar microalgas cuando éstas están en la fase final del crecimiento logarítmico o ya en fase estacionaria. Es durante estas fases de los cultivos microalgales que la composición nutricional de la biomasa es peor, ya que debido al agotamiento de los nutrientes del medio, las microalgas acumulan mayor cantidad de carbohidratos y lípidos de reserva (en general triglicéridos saturados), bajando a su vez la fracción proteica y la de lípidos estructurales (Otero *et al*., 2002). Además, en esta clase de cultivos no se ejerce ningún control sobre la composición bioquímica de las microalgas, las productividades alcanzadas son muy bajas en comparación con cultivos continuos, y la cantidad de bacterias contaminantes es más alta (Otero *et al*., 2002). En el apartado 2 (Alimento vivo) se detallarán más las diferencias entre los cultivos de microalgas en "batch" o en continuo, enumerándose todas las ventajas que tiene la utilización de éstos últimos para la acuicultura. La variabilidad generada en la composición nutricional de una misma especie de microalga, cultivada en semicontinuo con diferentes tasas de renovación y a diferentes concentraciones de nutrientes, se refleja a su vez de forma notable en el crecimiento y en la composición bioquímica de *Artemia* adulta (Fábregas *et al.*, 1996b, 2001).

Hay un cierto consenso entre los grupos de investigadores que se dedican al cultivo larvario del pulpo, sobre que los aspectos nutricionales están en la base de la elevada mortalidad observada durante su cultivo (revisado por Iglesias *et al*., 2007a). En la tabla I se presenta un resumen de los diferentes trabajos llevados a cabo por varios grupos de investigadores que se dedican al cultivo larvario de pulpo. Uno de los problemas que dificulta la interpretación de los resultados descritos y el avance en la nutrición del cultivo larvario del pulpo, es que muy pocos autores presentan datos de la composición bioquímica de las dietas utilizadas y de sus efectos sobre la composición de las paralarvas (Moxica *et al*., 2002; Navarro *et al*., 2000, 2003; Villanueva *et al*., 2004; Okumura *et al.*, 2005), cuando esta información es crucial para interpretar los resultados obtenidos, y así poder relacionarlos con la supervivencia y el crecimiento de las paralarvas. No obstante, se ha generado una importante cantidad de información sobre la composición bioquímica de paralarvas de pulpo recién eclosionadas, y, en algunos casos, también de juveniles salvajes, que permite disponer de un punto de partida para la formulación de microdietas, o para el enriquecimiento selectivo de *Artemia*. En concreto, la publicación de los perfiles de ácidos grasos y de las clases de lípidos (Navarro *et al.*, 2000, 2003; Okumura *et al*., 2005), del perfil de aminoácidos totales y de aminoácidos libres (Villanueva *et al.*, 2004), de macro- y de oligo-elementos (Villanueva y Bustamante, 2006), y de las vitaminas A y E (Villanueva

et al., 2009) de paralarvas de pulpo, han sido fundamentales para la modelización de los posibles requerimientos nutricionales de esta especie en los estadios iníciales de su ciclo de vida.

Tabla I. Resumen de las condiciones de cultivo de paralarvas de pulpo (*Octopus vulgaris*) realizados por varios grupos de investigación (tomada de Iglesias *et al*., 2007a).

Parameters	Barcelona ICM-CSIC	Vigo IEO	Canary Islands ICCM
Tank volume (L)	25–50	1000	100
Tank colour	Black	Black	Grey
Tank shape	Cylindrical and parabolic	Cylindrical	Cylindrical
Water system	Open	1st week stagnant, 2nd semi-open (3–4 h = 100%)	Open 25% day^{-1}
Aeration	No	Yes, intermediate	Yes, gentle
Light	24 h bulb 60 w 900 lx at 1 cm under the water surface	24 h fluorescent (2) 36 W 2000 lx	Natural photoperiod
Temperature	19–23	20–22	21.5–22.5
Clear/green water	Clear	Green *Isochrysis*+ *Nannochloropsis*	Clear
Paralarvae density (ind. L^{-1})	13–48	5	25
Type and prey density (ind. mL^{-1})	Zoeae *Liocarcinus* and *Pagurus*) nauplii *Artemia* (2–6) and *Artemia* biomass	Zoeae *Maja* (0.01–0.1) (when available)+ *Artemia* (0.05–0.1)	Zoeae *Grapsus* (15 ind. L^{-1})+ *Artemia* (72 h) (2)
Size of prey	Zoeae (1.3–3.1 mm TL) *Artemia* nauplii to 1–3 mm *Artemia* biomass	Zoeae: 1 mm TL *Artemia*: 2–3 mm TL	Zoeae: 1.5 mm TL *Artemia*: 0.85 mm TL
Artemia enrichment	DCSuperSelco, Metionine	Reared in commercial cereal flour, enriched with *Nannochloropsis* (5.10^6 cell mL^{-1})	*Artemia* enrichment (A_1 Selco Inve)
Sampling	Every 7–10 days	Every 7 days	Every 7 days
Survival	0.8% at day 60 with zoeae, and 54% at day 20 with *Artemia* nauplii (with poor growth)	31.5% at day 40	11–27% at day 30
Cleaning	Daily tank bottom siphoning	No bottom cleaning until day 30	No bottom cleaning

Tabla I. Continuación (tabla tomada de Iglesias *et al*., 2007).

Asturias CEP	Andalusia IFAPA	Brazil FURG	Japan YS
30	400	100	500
White	Black, grey	Black	Orange
Parabolic	Cylindrical and rectangular	Cylindrical	Cylindrical
Open (recirculation)	Open	Closed (recirculation)	5 first days stagnant then open system
Yes, gentle (cleaning)	Yes, gentle	No	Yes, gentle
12 h light–12 h darkness; fluorescent (1) 40 W	Natural photoperiod	10 h light–14 h darkness; natural + cold light	Fluorescent (1) 36 W
20–22	19–22	19–24	25
Clear	Green *Tetraselmis* + *Isochrysis*	Clear	Freshwater *Chlorella*
25	20	5–30	3
Zoeae *Maja* (0.7–1) + *Artemia* (3 times/week) (0.5–0.7)	Zoeae (*Carcinus*, *Palaemon* and *Maja*) (<0.1) + *Artemia* + *Moina* (4–5 day-old) (1.0)	Crustacean zoeae, copepods, mysids, nauplii and adult *Artemia* 0.15–0.3 (4–5 takes)	*Artemia* nauplii + fish flakes from 5th day
Zoeae: 1 mm TL *Artemia* retained in 300 μm sieve	Zoeae: 0.8–1.0 mm Moina: 1.0–1.2 mm *Artemia*: 1–3 mm	0.4–8 mm	650 μm (*Artemia*) 1–2 cm diameter, 0.5–1 mm thickness (FF)
Reared and enriched with *Tetraselmis*	Reared and enriched with *Tetraselmis* + *Isochrysis* SuperSelco, Prolon	Super SELCO and DHA SELCO Inve	Fish egg powder (Plus Aquaran, BASF Japan)
Every 10 days	Every 7–10 days	Daily up to day 7 and every 5 days thereafter	Every 5 days
89.6–93.5% at day 20 and 3.4% at day 60	5–15% at day 35	1–20% at day 40 with *Artemia* and from 20–39% at day 40 with *Artemia* and copepods	10–30% at day 30
Every 20 days changing tank by pipetting and checking the survival	Daily tank bottom siphoning	Bottom siphoning daily or every other day	Daily tank bottom siphoning after 5th day

Por ejemplo, se ha demostrado que las paralarvas de pulpo poseen un elevado contenido de ácidos grasos altamente insaturados (HUFA) como el 22:6n-3 (DHA, ≈ 20% del total de ácidos grasos), el 20:5n-3 (EPA, ≈ 13%), o el 20:4n-6 (ARA, ≈ 7%), y que los lípidos polares representan alrededor del 60% del total de lípidos, mientras que el colesterol puede suponer más de un 20% del total de lípidos (Navarro y Villanueva, 2000). En cuanto al contenido en proteínas y aminoácidos totales, se sabe que la fracción proteica en las paralarvas es de alrededor de un 70%, y que los aminoácidos lisina, arginina y leucina representan cerca de la mitad del total de los aminoácidos esenciales (Villanueva *et al*., 2004).

A pesar de los continuos intentos en cerrar el ciclo de vida del pulpo (*Octopus vulgaris)* en cautividad, la mortalidad de las paralarvas durante la fase planctónica sigue siendo casi total. La formulación de microdietas inertes, la búsqueda de nuevas presas vivas y la mejora de la composición de *Artemia* sp. mediante las técnicas de enriquecimiento apropiadas, han sido señaladas como áreas prioritarias para solucionar los problemas del cultivo de paralarvas (revisado por Iglesias *et al*., 2007a). Por ello, este trabajo se ha enfocado principalmente en la mejora de la composición bioquímica de juveniles de *Artemia*, mediante la utilización de microalgas de composición controlada y optimizada, como alimento vivo para suministrar a paralarvas de pulpo. Por otro lado, se ha intentando formular una microdieta en forma de pellets con una composición nutricional basada en la composición corporal de las paralarvas, para suministrar como complemento del alimento vivo.

1.3 Engorde de pulpos procedentes del medio natural

El pulpo es una especie de gran valor comercial en los mercados de Ásia y del sur de Europa. Los principales países del mundo con extracción pesquera son China, Japón, Marruecos, Tailandia, Corea, España, México, Senegal y Mauritania. Entre los países consumidores, Japón se sitúa a la cabeza de la lista, seguido por España e Italia (Globefish, 2005). El precio de venta del pulpo varían en función de su tamaño y del país donde se comercializa, pero suele estar comprendido entre los 2 y los 8 euros kg^{-1} (Globefish, 2005). El gran interés por esta especie en los países del sur de Europa con fuerte tradición en su consumo, ha generado una importante cantidad de trabajos relacionados con el tema del engorde del pulpo, con el fin de averiguar su potencial para la diversificación del sector de

la acuicultura. Aunque la mayor parte de los trabajos tengan su origen en España (Iglesias *et al*., 1997, 1999, 2000, 2003, 2007b; Rama-Villar *et al.*, 1997; Luaces-Canosa y Rey-Méndez, 1999; Tuñón *et al.*, 1999, 2000, 2001, 2003; Aguado y García-García, 2002; García-García y Aguado, 2002; Oltra *et al.*, 2005; Socorro *et al*., 2005; Rodríguez *et al*., 2006), también en Portugal (Gonçalves, 1993; Sendão *et al*., 1998; Vaz-Pires *et al*., 2004), en Italia (Cagnetta, 1999; Cagnetta y Sublimi, 2000) y en Grecia (Miliou *et al*., 2005, 2006) se han realizado experimentos de engorde. Sin embargo, el engorde de pulpo había sido ya puesto en práctica en Japón casi tres décadas antes, donde la producción en los años 1967-1971 era de alrededor de 100 t año^{-1}, bajando hasta 50 t a mediados de los años 70, utilizandose como alimento caballa u otros pescados de descarte, con tasas de conversión alimentaria de entre 2,5-5,0 (Boletzky y Hanlon, 1983).

De los trabajos de engorde de pulpo realizados por los diferentes grupos de investigación en Europa, se destacan las siguientes conclusiones generales: 1) las altas tasas de crecimiento de la especie; 2) la aceptación de alimentos de bajo valor comercial; 3) la elevada tasa de ingestión; 4) la fácil adaptación del pulpo a la cautividad; y 5) las tasas de crecimiento más elevadas a temperaturas comprendidas entre los 18 y los 22 ºC. Entre los problemas identificados por varios autores se citan: 1) tasas de mortalidad muy variables, que en ciclos cortos de 3 meses difícilmente bajan del 15%, pudiendo alcanzar hasta un 50% a medida que se aumenta el tiempo de cultivo; 2) la dificultad de consecución de pulpos pequeños, tanto por la reticencia de los pescadores a proporcionar ejemplares vivos por miedo a que el aumento de producción pueda hacer descender los precios, como por la falta de ejemplares en el medio natural en algunas épocas; 3) la poca resistencia del pulpo a variaciones de salinidad, lo que limita el proceso de engorde de pulpo a ciertas zonas de la costa; y 4) la elevada cantidad de materia orgánica que genera la utilización de alimentos naturales, que conllevaría en el futuro problemas de impacto ambiental que tendrían que ser solucionados y/o legislados.

En estudios sobre el contenido estomacal del pulpo, Guerra (1978) constató que en individuos del Mar de Cataluña, el 80% del alimento era a base de crustáceos, el 12% de pescado y el 8% de otras especies de cefalópodos. Este autor también verificó que la variabilidad de crustáceos ingeridos variaba según la profundidad y el sustrato. En la costa NW de África, Nigmatullin y Ostapenko (1976) analizaron la dieta de 2025 pulpos *O. vulgaris* y encontraron predominantemente crustáceos (54%), peces (25%), moluscos (9,5%) e individuos de la misma especie (canibalismo, 7,5%). Sin embargo, mientras en algunas áreas los crustáceos son la presa más importante, en otras, los moluscos parecen

ser el recurso alimenticio más dominante. Hatanaka (1979), también en la costa NW de África, analizó el contenido estomacal del pulpo y verificó que los gasterópodos y bivalvos eran las presas más importantes (45-60%), mientras peces (19-34%), crustáceos (7-16%) y otros cefalópodos (4-13%) completaban el resto.

Galicia es la comunidad autonóma de España con mayor tradición de consumo de pulpo, ya sea por la abundancia de este recurso en la costa, como por la exquisitez de las muchas recetas y formas de preparar este cefalópodo. La creciente demanda de esta especie en países asiáticos y mediterráneos desde los años 90, con el consiguiente aumento del precio, impulsaron al Instituto Español de Oceanografía (IEO) de Vigo y a la Universidad de Santiago de Compostela a realizar, durante el período 1995-1999, una serie de experimentos de engorde de pulpo para evaluar la posibilidad de cultivo de esta especie a escala comercial. Desde el año 1995 se realizaron numerosas experiencias de engorde, con el fin de aplicar a la explotación industrial los conocimientos y avances logrados. Los trabajos experimentales desarrollados, por un lado, por el grupo liderado por José Iglesias, en el IEO de Vigo, y por otro, por Manuel Rey Méndez, en la Universidad de Santiago de Compostela, han dado lugar a resultados que permiten tener una visión optimista en lo referente a su aplicación industrial. En la introducción del capítulo 6 se describirán algunos de los trabajos más importantes llevados a cabo por estos dos grupos, y se presentaran resultados recientes sobre el engorde de pulpo en jaulas suspendidas de batea, realizados en el marco de proyecto JACUMAR (Optimización del engorde del pulpo *Octopus vulgaris*).

Actualmente, el engorde de pulpo se basa en la captura de individuos adultos del medio natural, con el peso mínimo permitido por ley (entre 750 y 1000 g, dependiendo de épocas o zonas de extracción), seguida de su distribución en jaulas flotantes o suspendidas en bateas (Fig. 6). Un ciclo de engorde típico comprende un periodo de 3 a 4 meses, a lo largo del cual los pulpos son alimentados a diario (excepto los domingos) con combinaciones variables de pescado, crustáceos y mejillón, según la empresa que lo desarrolla, alcanzándose al final del proceso pesos medios de alrededor de 3 kg, altamente cotizados en el mercado. Los alimentos más comúnmente utilizados por varios autores en trabajos experimentales, o por las empresas dedicadas al proceso de engorde del pulpo, fueron distintos tipos de pescado, como el lirio (*Micromesistius poutassou*), la caballa (*Scomber scombrus*), la boga (*Boops boops*), la sardina (*Sardina pilchardus*), el jurel (*Trachurus trachurus*), el cangrejo (*Carcinus maenas*), el patexo (*Polybius henslowii*) y el mejillón (*Mytilus* sp). De todos ellos, los crustáceos suelen dar mejores resultados de crecimiento (Sendão *et al*., 1998; Cagnetta y Sublimi, 2000; García-García y Cerezo, 2006).

En un estudio comparativo de las tasas de crecimiento de pulpos alimentados con monodietas de cangrejo o de pescado, o con mezclas variables de los dos alimentos, García-García y Cerezo (2006) concluyeron que la mejor relación "coste del alimento/biomasa obtenida" se conseguía con la mezcla de un 25% cangrejo y 75% de pescado. La formulación de dietas artificiales para pulpos que puedan sustituir el alimento fresco es objeto de estudios intensos hoy día, en un intento de abaratar los costes de engorde y facilitar la tarea de alimentación de los pulpos. Sin embargo, los resultados alcanzados hasta la fecha no son muy alentadores, debido al escaso crecimiento de los pulpos con dietas húmedas observado por varios autores (Cerezo *et al*., 2008; Quintana *et al*., 2008).

El esquema general de explotación de una empresa en funcionamiento consiste en jaulas de sección cuadrada o rectangular con capacidad para albergar de 100 a 200 pulpos (Fig.6).

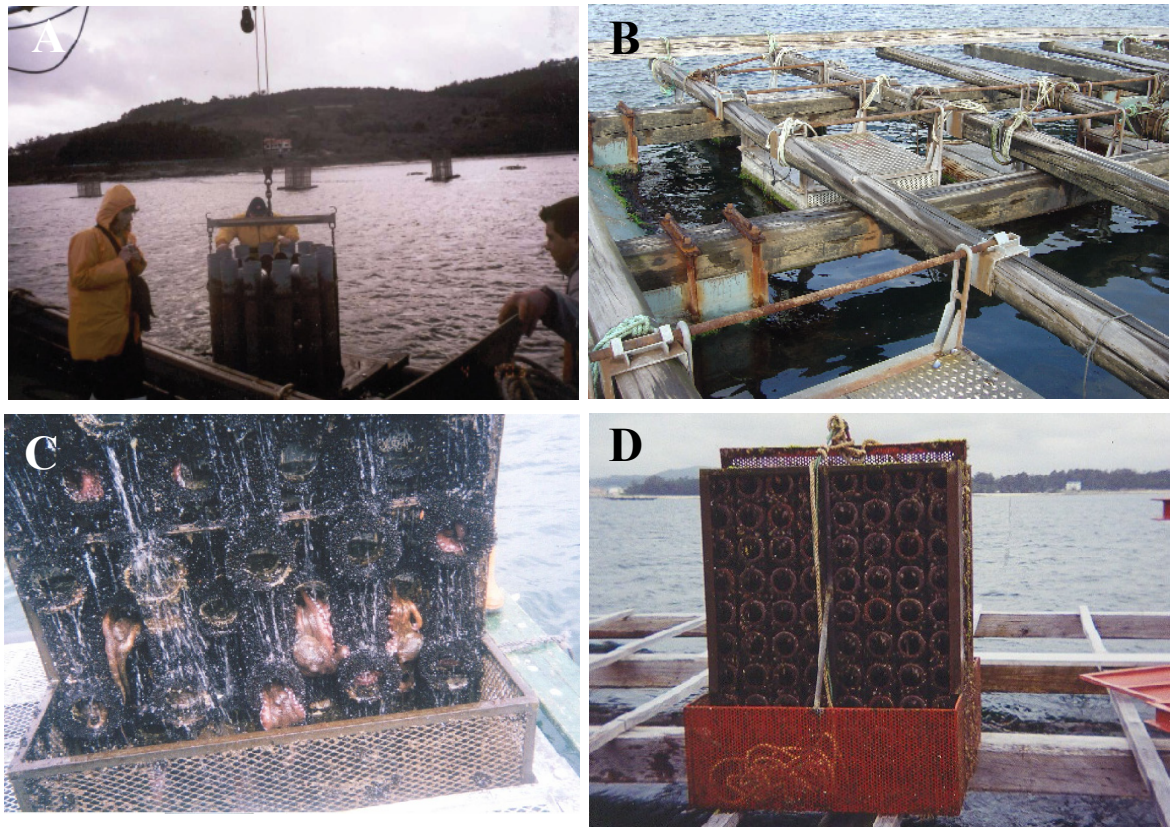

Figura 6 – Sistemas de engorde de pulpo. (A) Jaulas flotantes de sección circular. (B) Jaulas suspendidas de batea de sección rectangular o cuadrada. (C) Levantamiento de una jaula de engorde con refugios para pulpos. (D) Sistema de jaula con malla de hierro perforada y nasas de plástico negro con lastre. Fotos: Manuel Rey Méndez.

Estas jaulas pueden ser unidades individuales con sistema de flotación propio o acoplarse en una plataforma flotante común. El proceso de engorde, al tener una duración de 3 a 4 meses, con el objectivo de alcanzar el tamaño comercial óptimo de alrededor de 3,0 kg, permitiría que se realizasen tres ciclos de engorde al año, por lo que una empresa con 25 jaulas podría engordar unos 9.000-12.000 pulpos al año. De los estudios económicos sobre la explotación del pulpo, García-García *et al*. (2004) refirieron que una explotación empresarial de pulpo tendría que tener un mínimo de 43 jaulas y una producción anual estimada en 38 t para ser rentable, aunque estos valores son discutibles teniendo en cuenta los precios de adquisición de los ejemplares, del alimento y de venta del pulpo en lonja.
En el año 1999 existían en Galicia un total de cinco concesiones experimentales para engorde de pulpo con fines comerciales y otra destinada a trabajos de investigación (Tabla II), de las que hoy día sólo dos siguen realizando el engorde, existiendo una nueva explotación ubicada en Cangas.

Tabla II. Concesiones para engorde de pulpo en Galicia (1999). Datos suministrados por la Xunta de Galicia. (Tomada de: Iglesias *et al*., 2003)

Empresa	Polígono de ubicación	Modalidad de cultivo autorizada	Producción máxima autor. (Tm/año)
Ameixa de Carril, S.A.	Cambados-A	2 bateas con jaulas	90
Arrecifes del Atlántico, S.L.	Camariñas-A	50 jaulas independientes	45
Asociación profesional de naseiros "Samertolomeu"	Cangas-C	1 vivero con 10 jaulas	17
Blanco Míguez, Mª Begoña	Grove-C	1 batea con 63 jaulas	55
Marfrío Marín, S.A.	Cangas-B	3 plataformas con jaulas	68
Universidade de Santiago de Compostela	Noia-A	1 vivero para investigación	No destinado a producción

El pulpo está considerado como una especie de interés prioritario de cara a su potencial en acuicultura, estando previstos proyectos de engorde empresariales en otras CC.AA. de España, como Canarias, Valencia o Andalucía, en estructuras flotantes o en tanques. En Galicia, la producción total en 1998 fue de 72 t, frente a las 500 t anuales de capacidad con la que contaban las instalaciones con permiso de explotación. En los años siguientes la

producción bajó considerablemente con ≈30 t en 2000 y ≈15 t en 2001, mientras que en el 2002 y 2003 hubo ausencia de producción a causa del accidente del Prestige. Actualmente la producción media anual ronda las 10-20 t. Sin embargo, esta actividad no puede consolidarse en el mercado debido a la dificultad en la obtención de juveniles a partir de las paralarvas. Aunque se haya demostrado la alta rentabilidad de esta especie para acuicultura, y los resultados de engorde sean muy interesantes, el cuello de botella en el cultivo integral de esta especie en cautiverio es el ya citado de la supervivencia de las paralarvas.

2. Alimento vivo

2.1 Utilización de *Artemia* sp. en acuicultura

2.1.1 Generalidades

El crustáceo branquiópodo *Artemia* sp. es probablemente la presa viva que más se utiliza en acuicultura a nivel mundial. Su utilización con fines de sustitución de presas naturales de larvas de peces se remonta a principios de 1930 (Dhont y Van Stappen, 2003). A mediados del siglo XIX había únicamente dos fuentes comerciales de quistes de este crustáceo: el Gran Lago Salado de Utah y la Bahía de San Francisco, ambos en EE.UU. Debido al creciente desarrollo de la acuicultura en los años 60 y 70, y a la fuerte escalada de los precios de los quistes, la explotación de nuevos bancos naturales surgió en otros puntos del globo, como por ejemplo en China, Argentina, Canadá, Colombia, Australia y Francia, o en países con gestión controlada de la producción de *Artemia*, como Brasil y China (Dhont y Van Stappen, 2003). En 1997 el consumo de quistes a nivel mundial ascendía a unas 1500 t y los precios variaban entre los USD 20 kg^{-1} y los 200 kg^{-1}, en función de la calidad y de la abundancia en cada año. Aunque el consumo de quistes sigue subiendo a causa del continuado desarrollo de la acuicultura, desde los años 2000-2001 su gasto por unidad de larva producida disminuyó considerablemente debido a la mejor eficacia en la utilización de este producto y a la sustitución parcial de *Artemia* por microdietas formuladas (Sorgeloos *et al*., 2001). Aunque no haya dudas de que *Artemia* será gradualmente sustituida por piensos artificiales formulados, su utilización aún seguirá siendo imprescindible en los próximos años.

Los nauplios y metanauplios de *Artemia*, en general de tamaño inferior a 1,5 mm, son la forma comúnmente utilizada en acuicultura (Fig. 7). Pueden ser suministrados directamente a las especies diana, o pueden ser enriquecidos durante un determinado periodo de tiempo, en general desde algunas horas hasta 48 h (Sorgeloos *et al*., 2001). En estos estadios de desarrollo, *Artemia* posee movimientos relativamente lentos y constituye una presa adecuada para ser fácilmente capturada e ingerida. Entre las especies diana para las que se utilizan nauplios de *Artemia* se encuentran varios esparídos y morónidos, el bacalao, la palometa, el rodaballo y otros pleuronéctidos, esturiones, carpas, pez-gato, además de varias especies de crustáceos.

La utilización de juveniles o adultos de *Artemia* como presas vivas, se reserva a determinadas especies de crustáceos decápodos (Dhert *et al*., 1993; Conklin, 1995; Ritar *et al*., 2003; Tlusty *et al*., 2005), peces en su mayoría ornamentales (Lim *et al*., 2001; Woods, 2003) y estadios iníciales de varias especies de cefalópodos (Domingues *et al*., 2001; Iglesias *et al*., 2007a).

Artemia se presenta como una opción muy viable para la alimentación de especies acuícolas desde sus primeras fases larvarias, debido a determinadas características que hacen su utilización sencilla y adecuada:

a) la forma de resistencia de los embriones de *Artemia*, conocida como quiste, puede ser tratada como un material inerte y en condiciones de almacenamiento adecuado se pueden mantener en buen estado durante años, sin que se vea afectado su valor nutricional o se deteriore su viabilidad;

b) la manipulación necesaria para provocar la eclosión de los quistes es un proceso muy simple y económico, mediante el cual, en pocas horas, se obtiene gran número de presas vivas;

c) el cultivo de nauplios de *Artemia* hasta el estadio de juvenil o adulto es relativamente sencillo y permite obtener, con bajos costes, gran cantidad de biomasa con unos tamaños adecuados a la alimentación de los diferentes estados de desarrollo de la especie diana a cultivar;

d) existe gran cantidad de información disponible acerca de *Artemia*: biología, ecología, composición bioquímica, características del cultivo, etc.;

e) se ha demostrado que *Artemia* puede ser utilizada como vehículo para suministrar a las larvas de especies de interés, sustancias que son fundamentales para su desarrollo. Esto se consigue proporcionando a *Artemia* estas sustancias disueltas en su medio de cultivo o bien

microencapsuladas, ya que al poco tiempo las ingiere y puede ser suministrada como presa a esas especies.

Uno de los pocos factores en contra que tiene *Artemia* en la actualidad es el elevado precio que alcanzan los quistes, debido a su fuerte demanda por la industria de la acuicultura, que sigue creciendo en todo el mundo.

La incubación de quistes de *Artemia* es una tarea sencilla que se puede llevar a cabo sin grandes problemas. Sin embargo, para optimizar este proceso a gran escala, como es el caso de los criaderos, diversos factores han de ser cumplidos rigorosamente: la temperatura debe rondar los 25-28 ºC, la salinidad debe estar comprendida entre 15-35 ppt, el pH mínimo recomendado es de 8,0, el oxígeno en el agua debe estar cerca de la saturación, se recomienda una iluminación de por lo menos 2000 lux y la densidad máxima de quistes debe ser de 2 g l^{-1} (Van Stappen, 1996). La desinfección o descapsulación de los quistes de forma previa a su incubación es igualmente recomendada en caso de necesidad de grandes cantidades de nauplios.

Figura 7 - (A) Quistes de *Artemia* sp. hidratados vistos a la lupa (≈ 200 µm); (B) Nauplio de *Artemia* (≈ 500 µm); (C) Metanauplio enriquecido con *Nannochloropsis gaditana* (≈ 900 µm); (D) *Artemia* en estadio de pre-adulto enriquecida con *Rhodomonas lens* (≈ 4,5 mm). Fotos: Pedro Seixas.

El valor nutritivo de *Artemia* varía en función del estadio de desarrollo del individuo, de la cepa y origen geográfico de los quistes, y del alimento que se le suministra cuando empiezan a filtrar. Los nauplios recién eclosionados, al no tener el aparato digestivo abierto, poseen una composición bioquímica que varía únicamente con la cepa u origen geográfico, y con el gasto energético realizado a lo largo del proceso de eclosión. Sin embargo, es frecuente encontrar diferencias considerables en la composición bioquímica de quistes con la misma procedencia, pero en años distintos, a raíz de la alimentación de la población adulta.

2.1.2 Producción y enriquecimiento de Artemia *sp.*

En comparación con otros crustáceos, *Artemia* tiene un mecanismo de alimentación muy primitivo ya que es un filtrador fagotrófico obligado, no selectivo y continuo (Provasoli *et al*., 1959). Las partículas en suspensión, de un tamaño adecuado, son retiradas continuamente del medio de cultivo por el movimiento de los toracópodos, sin importar cual sea su naturaleza (Reeve, 1963). Debido a estas características, se consideran factores críticos en la selección de la dieta de *Artemia* los siguientes: tamaño de la partícula, que no ha de ser mayor de 50 μm, digestibilidad y valor nutritivo del alimento y solubilidad de las partículas. El tamaño de las partículas que ingiere *Artemia* puede variar de 1 μm hasta cerca de 25 μm para los metanauplios (Takano, 1967) y de 1 a 50 μm para los adultos (D'Agostino, 1980; Van Stappen, 1996). También Fernández (2001) refería tamaños de partículas entre 7 y 28 μm como preferentes, habiendo descrito incluso un tamaño óptimo de 16 μm. Esta característica de filtrador no selectivo permite que se enriquezca el tubo digestivo de *Artemia* con productos ricos en compuestos tan variados como nutrientes esenciales (ácidos grasos, fosfolípidos, vitaminas, proteínas, aminoácidos), profilácticos, antibióticos o pigmentos (Merchie, 1996; Tonheim *et al*., 2000; Sorgeloos *et al*., 2001; Monroig *et al*., 2007). En la figura 8 se presenta un esquema del concepto de la utilización de *Artemia* como vehículo de suministro de diferentes componentes en el cultivo larvario.

Para el crecimiento de *Artemia* hasta las fases de juvenil o adulto se han empleado dietas tan distintas como levaduras, microalgas, bacterias, protozoos, harina de algas, de arroz, o de distintos cereales, o detritos orgánicos procedentes de la industria (revisado por Dhont y Lavens, 1996), obteniéndose los mejores resultados de crecimiento con microalgas. Sin embargo, no todas las microalgas son adecuadas para su cultivo, ya que algunas especies originan resultados de muy bajo crecimiento o elevada mortalidad, como por ejemplo, microalgas pertenecientes a los géneros *Chlorella* y *Stichococcus*, que resultaron inadecuadas para la producción de biomasa de *Artemia* (Sick, 1976; Dhont y Lavens, 1996), ya sea debido a la producción de sustancias gelatinosas que interfieren con la filtración o por problemas de digestibilidad de las microalgas.

La tasa de crecimiento de *Artemia* depende de varios factores bióticos y abióticos, siendo la cantidad y calidad del alimento disponible y la temperatura de cultivo considerados como los más importantes (Dhont y Lavens, 1996). Aunque en la naturaleza difícilmente se produzcan situaciones de alimentación basadas en monodietas, este tipo de cultivos

suele realizarse en laboratorio para estudios fisiológicos, o cuando la dieta es lo suficientemente rica y adecuada para un determinado organismo. La obtención de biomasa de *Artemia*, ya sea en la forma de juveniles o de adultos, tiene importancia fundamental en acuicultura a la hora de optimizar los procesos de alimentación de las especies diana. Por otro lado, la composición bioquímica de la biomasa de *Artemia* juega igualmente un papel fundamental en el normal desarrollo y supervivencia de las larvas marinas. El crecimiento y la composición bioquímica de *Artemia* sp. pueden ser modificados mediante la utilización de microalgas de composición bioquímica mejorada producidas en cultivos semicontinuos (Fábregas *et al.*, 1996b, Fábregas *et al*., 2001). Estos autores han demostrado que al cultivarse *Artemia* con *Tetraselmis suecica*, la tasa de crecimiento, la mortalidad y la composición bioquímica de los individuos adultos estaban influenciadas por la composición nutricional de las microalgas, que a su vez variaba con la tasa de renovación y la concentración de nutrientes utilizados para cultivar las microalgas.

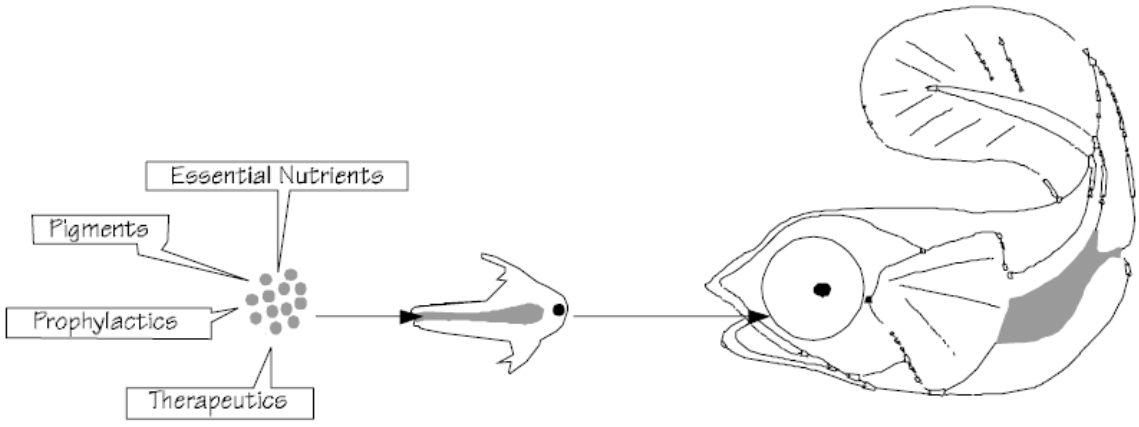

Figura 8 - Esquema de la utilización de *Artemia* como vector de transferencia de nutrientes específicos para las larvas marinas. Tomado de Merchie (1996).

En el caso de las paralarvas de pulpo, se ha verificado que un tamaño de los juveniles de *Artemia* de 1,5 a 4,0 mm, es el más adecuado para suministrar a lo largo de su cultivo (Iglesias *et al*., 2004, 2006, 2007a; Carrasco *et al*., 2006). Los tamaños más pequeños se utilizarían en los primeros días de vida, incrementando el tamaño de las presas a medida que las paralarvas se hacen más grandes. Aunque otros autores hayan observado que tamaños inferiores a 1,5 mm son aptos para suministrar a las paralarvas en sus primeros días de vida (Navarro y Villanueva, 2000, 2003; Villanueva *et al*., 2002, 2004; Okumura

et al., 2005), ese tamaño se vuelve poco atractivo a partir de los 20 días de cultivo. Por ello, la optimización del crecimiento de nauplios de *Artemia* hasta los tamaños más adecuados y la mejora del perfil nutricional de los juveniles de *Artemia* son importantes metas a alcanzar en el cultivo larvario del pulpo, con el fin de facilitar la gestión y disponibilidad del alimento vivo durante los experimentos y mejorar las tasas de crecimiento y supervivencia de las paralarvas.

2.2 Microalgas

2.2.1 Generalidades

Las microalgas son microorganismos eucariotas que realizan fotosíntesis oxigénica, y que aprovechando la energía solar son capaces de sintetizar nueva materia orgánica a partir de sustratos inorgánicos, tales como sales solubles, dióxido de carbono y agua. Poseen una maquinaria fotosintética cuya capacidad para convertir la energía solar en biomasa, presenta una eficiencia de 2 a 5 veces mayor que la de las plantas superiores (Thomas *et al.*, 1984). Presentan altas tasas de producción, se adaptan a distintas condiciones ambientales, y se encuentran en cualquier medio acuático donde exista una fuente de carbono, nutrientes y luz suficiente, junto con el rango apropiado de temperaturas (Shelef y Soeder, 1980). En el medio acuático, sea marino o limnológico, existe una gran diversidad de microalgas representada por miles de especies, que suponen un importante potencial como fuente de proteínas, lípidos, carbohidratos simples o complejos, vitaminas y minerales. Asimismo, se confirman como una fuente de sustancias de uso industrial o farmacológico de gran valor económico, pudiéndose extraer compuestos tales como: β-caroteno, astaxantina, ácidos grasos (DHA, EPA, ARA), pigmentos, flavonoides, ficobilinas, polisacáridos, enzimas, tocoferol, etc. Existen hoy día numerosas aplicaciones comerciales para el empleo de microalgas, como por ejemplo: pueden ser utilizadas para el enriquecimiento nutritivo de alimentos para consumo humano o en piensos para animales (debido a su elevado valor nutricional), desempeñan un papel fundamental en acuicultura y son incorporadas directamente o se utilizan extractos de microalgas en productos cosméticos o farmacéuticos (Spolaore *et al*., 2006). A pesar de la existencia de miles de especies de microalgas distribuidas por todo el mundo, pocas son en realidad las utilizadas en acuicultura. En la tabla III se hace referencia a los principales géneros de microalgas y cianobacterias producidos en acuicultura a nivel mundial.

Las microalgas son la base de la cadena trófica en muchos procesos de la acuicultura. Son imprescindibles para alimentar directamente todas las fases de desarrollo de moluscos bivalvos y gasterópodos (Brown *et al*., 1997; Otero *et al*., 2002) y etapas larvarias de determinadas especies de crustáceos y peces (Reitan *et al*., 1997; Piña *et al*., 2006). Son igualmente importantes para el cultivo y enriquecimiento de las presas vivas más comúnmente utilizadas en acuicultura, como los rotíferos y *Artemia* (Dhert *et al*., 2001; Aragão *et al*., 2004a), e imprescindibles para el cultivo de copépodos (Støttrup y Jensen, 1990; Støttrup, 2003).

La composición bioquímica de las microalgas varía mucho en función de la especie, pero en condiciones normales de producción se admite que los valores de proteína puedan alcanzar hasta un 60% de su peso seco, los lípidos entre un 7 y un 23% y los carbohidratos entre un 5 y un 23% (Becker, 2004). Sin embargo, haciendo variar determinados factores como la luz, la concentración de nutrientes, la temperatura y la tasa de crecimiento de las microalgas se puede manipular de forma considerable la proporción de proteínas, lípidos y carbohidratos en éstas (Otero y Fábregas, 1997; Otero *et al*., 2002; Renaud *et al*., 2002; Fábregas *et al*., 2004).

Tabla III. Principales géneros de microalgas y cianobacterias utilizados en acuicultura a nivel mundial (Becker, 2004).

Bacillariophyceae	**Haptophyceae**	**Chryptophyceae**	**Cyanophyceae**	**Chlorophyceae**	**Eustigmatophy**
Skeletonema	*Isochrysis*	*Cryptomonas*	*Spirulina*	*Tetraselmis*	*Nannochloropsis*
Phaeodactylum	*Pavlova*	*Rhodomonas*		*Chlorella*	*Nannochloris*
Thalassiospira		*Chroomonas*		*Scenedesmus*	
Chaetoceros				*Dunaliella*	
Nitzschia				*Chlamydomonas*	

2.2.2 Importancia de las microalgas en larvicultura marina

Las microalgas constituyen el alimento directo en cultivos de moluscos bivalvos, u otros moluscos filtradores, y en cultivos larvarios de determinados crustáceos. En el caso de la mayor parte de los cultivos de larvas de peces marinos, el suministro de microalgas a los tanques de cultivos no tiene por objetivo alimentarlas directamente, ya que éstas no son filtradoras de microalgas y no pueden vivir exclusivamente de esta dieta. Sin embargo, se ha demostrado que la introducción de microalgas en los tanques de cultivo, una práctica

conocida como cultivo en "aguas verdes", mejora la supervivencia, el crecimiento y el factor de conversión alimentario de más de 40 especies, en comparación con condiciones de "aguas claras" (revisado por Muller-Feuga *et al*., 2003). Las razones por las que se observan estos efectos positivos sobre los cultivos larvarios no son todavía muy claras, debido al gran numero de factores en los que pueden influir las microalgas. Las siguientes hipótesis han sido sugeridas por varios autores para explicar los fenómenos de mejora de los cultivos larvarios con microalgas: los parámetros del agua se estabilizan mejor o incluso se mejoran, la luz incidente provoca un mayor contraste de las presas, las microalgas pueden servir directamente de alimento (a través del agua ingerida o por retención en las branquias) o indirectamente (vía las presas enriquecidas), provocan el estimulo de procesos fisiológicos o de ingestión de presas, regulan el crecimiento bacteriano oportunista por acción antibacteriana o probiótica, incrementan la cantidad y la calidad de las presas vivas (Muller-Feuga *et al*., 2003).

La designación de técnica de "aguas verdes" se utiliza ampliamente en acuicultura, aunque existen definiciones más precisas sobre los métodos que originan la presencia de microalgas en los tanques de cultivo. Así, la técnica de "aguas verdes" consiste en provocar un bloom de microalgas y de rotíferos en los tanques de cultivo; la técnica de "pseudo aguas verdes" consiste en el aporte diario de microalgas y rotíferos a los tanques de cultivo; mientras que el mesocosmos se basa en provocar un "bloom" de una cadena trófica pelágica de origen natural (Divanach y Kentouri, 2000). En cualquiera de los casos, la presencia de microalgas en los sistemas de cultivo larvario de peces mejora considerablemente la supervivencia de varias especies. Por ejemplo, en especies consideradas difíciles de cultivar en aguas claras, como el rodaballo, la dorada o la palometa, el incremento en la supervivencia puede ser del 100-500% (Naas *et al*., 1992; Reitan *et al*., 1993; Papandroulakis *et al*., 2002). Otro de los efectos positivos observados en larvas de peces, en presencia de microalgas, es el incremento de la producción de enzimas digestivas, mejorando además la flora intestinal de las larvas. En larvas de lubina (*Dicentrarchus labrax*), Cahu *et al*. (1998) encontraron que la presencia de *Isochrysis galbana* T-ISO estimulaba la producción de enzimas digestivas, tanto pancreáticas como intestinales, facilitando así el desarrollo de las funciones hidrolíticas de las membranas celulares en las microvellosidades intestinales.

En la figura 9 se presentan fotografías de cuatro especies de microalgas utilizadas en el presente trabajo pertenecientes a géneros distintos.

2.2.3 Condiciones de cultivo de microalgas

La actividad fotosintética de un cultivo microalgal depende de distintos factores, de entre los cuales los más importantes son la luz, la temperatura, la concentración y formulación de nutrientes, el pH, condiciones fisiológicas de las microalgas y concentraciones de CO_2 y O_2. Es conveniente una suficiente agitación del cultivo microalgal, que en general es producida por la aireación introducida en él, y que proporciona un movimiento turbulento del agua. Esta agitación permite que la exposición de las células a la luz sea más unifome y evita que éstas se depositen en el fondo del recipiente de cultivo, desplazando igualmente el O_2 producido y evitando así procesos de foto-oxidación.

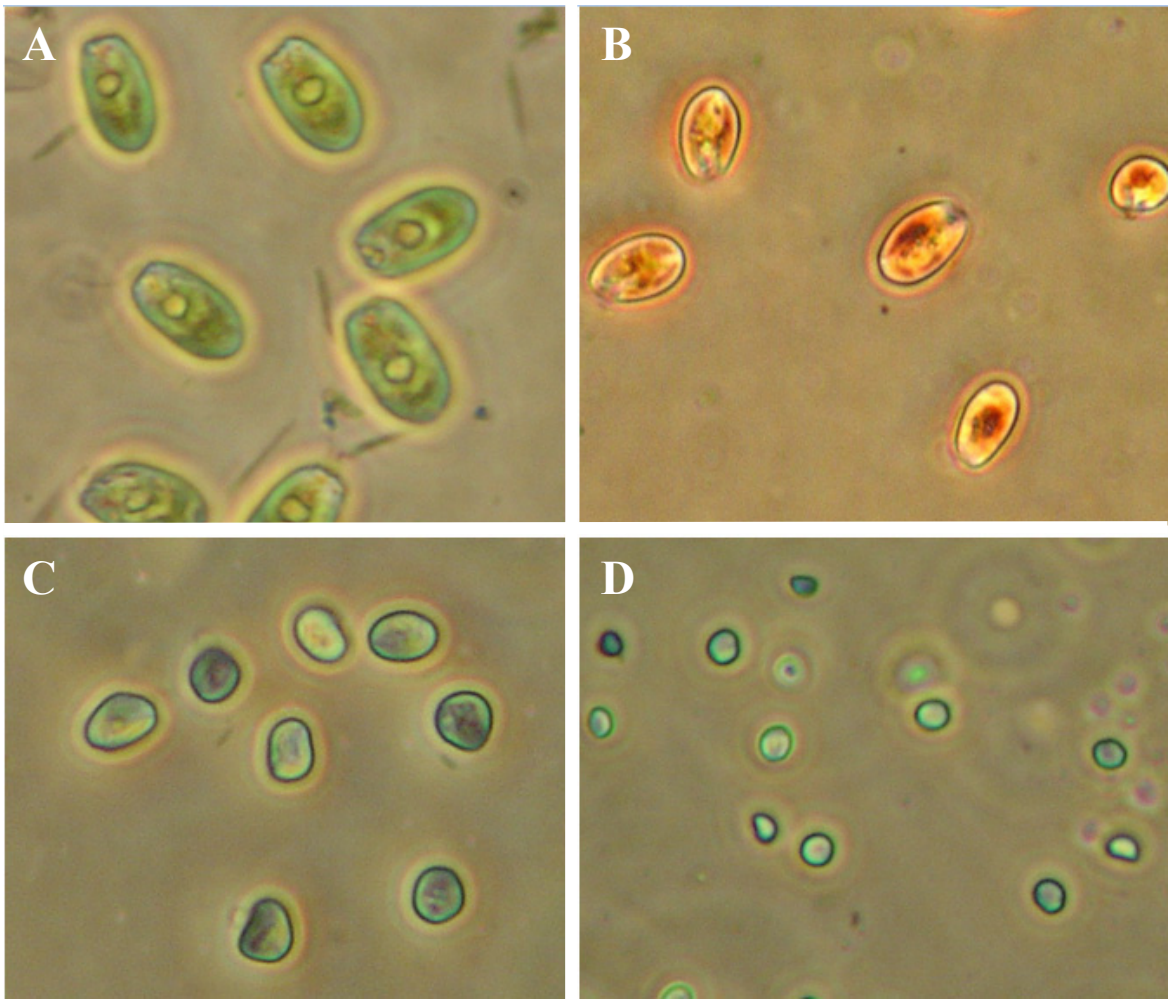

Figura 9 - Fotografías al microscopio óptico de cuatro especies de microalgas marinas utilizadas a menudo en acuicultura, y en el presente trabajo. A - *Tetraselmis suecica* (tamaño ≈ 15 x 9 µm); B - *Rhodomonas lens* (≈ 8 x 12 µm); C - *Isochrysis galbana* Parke (≈ 5 x 3 µm); D - *Nannochloropsis gaditana* (≈ 3 µm). Tamaños de las microalgas según Brown *et al*. (1997). Fotos: Pedro Seixas.

En el momento de plantear un cultivo masivo de microalgas, hay que considerar la concentración inicial óptima (o tamaño del inóculo), que juega un importante papel en el desarrollo posterior del cultivo. Concentraciones demasiado bajas pueden perderse por foto-inibición u otras causas, debiendo el cultivo iniciarse con una densidad celular mínima que permita el comienzo rápido de su crecimiento exponencial. Luz, temperatura, pH y concentración de nutrientes son los factores más importantes que se consideran como limitantes del crecimiento de las microalgas, de forma que la mayoría de los estudios se centran sobre el modo en que cada uno de estos factores afecta la composición y fisiología de las células.

La cantidad de luz que debe llegar a las microalgas es un factor fundamental a la hora de establecer un cultivo de microalgas. Demasiada luz al comienzo puede provocar foto-inhibición de las células, mientras que poca luz tendrá un efecto de retraso sobre su crecimiento. La utilización de ciclos de luz/oscuridad en el cultivo es también un factor muy importante en los sistemas de producción masiva. Se han descrito rendimientos máximos por hora de luz y tasas de crecimiento máximas con fotoperiodos de longitud intermedia, reduciéndose en luz continua, así como tasas de crecimiento similares en cultivos estáticos bajo condiciones de iluminación continua e iluminación circadiana (Maseda, 2002; Fábregas *et al.*, 2004).

Las temperaturas óptimas de crecimiento de las microalgas varían mucho según la especie y la zona geográfica de donde proceden. Para especies provenientes de zonas templadas las temperaturas óptimas suelen estar comprendidas entre los 18 y 21 ºC, mientras que para especies tropicales el rango puede estar entre los 25 y los 35 ºC. Se sabe que la temperatura puede influir considerablemente sobre el contenido proteico, de carbohidratos y de lípidos en las microalgas. Algunos autores han descrito un incremento del contenido proteico y una disminución de los lípidos y carbohidratos en microalgas cultivadas a temperaturas más elevadas dentro de su rango de cultivo, mientras que otros han observado lo contrario, lo que hace suponer que la respuesta de la composición bioquímica de las células a altas o bajas temperaturas varía de especie a especie (Renaud *et al*., 2002).

En cuanto a los nutrientes, se sabe que los cultivos de microalgas requieren macronutrientes, como nitrógeno y fósforo, además de varios micronutrientes y vitaminas. Cada microalga tiene sus necesidades específicas que han de ser calculadas, siendo la composición bioquímica de las microalgas altamente dependiente de los nutrientes que tenga a su disposición (Otero, 1994).

El pH es otro de los factores más importantes en el cultivo de microalgas. Determina la disponibilidad del CO_2 y los minerales en el medio de cultivo e influye directa o

indirectamente en el metabolismo de las microalgas. Cada microalga tiene un óptimo de pH para su cultivo, que en la mayor parte de las especies utilizadas en acuicultura es inferior a 8. A su vez, el pH de los cultivos se ve afectado o puede estar influido por varios factores, como la composición y la capacidad tampón del medio de cultivo, la cantidad de CO_2 disuelto, la temperatura (que a su vez influye en la solubilidad del CO_2) y la actividad metabólica de las microalgas.

En cuanto a la salinidad, las microalgas marinas se consideran generalmente tolerantes y adaptables a un amplio rango de salinidades, soportando posibles cambios a través de la regulación de su presión osmótica interna, mediante la acumulación de distintos metabolitos (Ben-Amotz y Avron, 1983).

En el presente trabajo los cultivos de microalgas se han realizado en régimen semicontinuo, utilizando tasas de renovación intermedias y en saturación de nutrientes, con el fin de obtener biomasa de composición estable y mejorada (Otero, 1994; Otero *et al.*, 2002). A continuación se presentan las definiciones y características de los cultivos de microorganismos en discontinuo o "batch", en semicontinuo y en continuo, que se aplican igualmente a los cultivos de microalgas, y que serán tratados en este trabajo:

a) Cultivos en "batch": también denominados cultivos discontinuos, cerrados o estáticos. Durante el proceso de operación en "batch" no se añade ningún sustrato a la carga inicial ni se retira ningún producto hasta el final del proceso. Los cultivos discontinuos (Fig. 10) son sistemas cerrados (a excepción de entrada de gases en aquellos sometidos a aireación) donde las células se multiplican hasta que alguno de los nutrientes se agota o algún metabolito se acumula hasta alcanzar un nivel tóxico. Como resultado, en los cultivos discontinuos, la concentración de nutrientes en el medio cambia a lo largo del tiempo, como consecuencia del crecimiento del microorganismo.

b) Cultivo continuo (quimiostatos o turbidostatos): en este tipo de cultivos el sustrato es añadido de forma continuada y el producto se retira también de forma continuada, permaneciendo constante el volumen del cultivo, al igual que la concentración de sustrato y microorganismos una vez alcanzado el estado de equilibrio. En la figura 11 se ilustran diferentes tipos de fotobioreactores que se utilizan para la producción de microalgas el cultivo continuo.

c) Cultivo semi-continuo: este tipo de cultivo es una aproximación a la producción de flujo continuo, la diferencia es que una porción del cultivo es retirada y reemplazada por medio fresco a intervalos de tiempo circadianos (Fig. 11). Por lo tanto, a pesar de que la producción es continua, la concentración de sustrato y microorganismos no es constante a lo largo del tiempo, presentando ciclos de 24h. En el caso de los cultivos de microalgas sometidos a ciclos de luz/oscuridad, y debido a que en estas condiciones la división celular se produce de forma sincronizada cada 24 h, los cultivos semi-continuos en las que la renovación se realiza a intervalos de 24 h son conceptualmente similares a los cultivos continuos clásicos, habiendo sido denominados "ciclostatos".

2.2.4 Composición bioquímica de las microalgas y productividad

El papel crucial que las microalgas juegan en la cadena trófica de la producción en acuicultura, se basa en que éstas, al servir de alimento directa, o indirectamente, a las especies diana a producir, van a influir en su supervivencia y crecimiento, ya que son la fuente primaria de nutrientes esenciales. Por otro lado, la composición bioquímica de las microalgas pude variar enormemente en función de las condiciones de cultivo establecidas. Se han realizado numerosos trabajos sobre el efecto que distintos nutrientes y factores ambientales tienen sobre la composición bioquímica de las microalgas (revisado por Richmond, 2004). Factores como la intensidad de luz, la concentración y composición de nutrientes, la temperatura, el aporte de CO_2, la salinidad, etc., influyen enormemente en el crecimiento y composición de las microalgas. Sin embargo, el método de cultivo puede ser tan o más importante que la especie seleccionada para la obtención del valor nutritivo de la biomasa (Wikfors *et al*., 1984; Wikfors, 1986; Fábregas *et al*., 1984, 1986). La importancia de los sistemas de cultivo continuo como herramienta manipuladora de la composición bioquímica de las microalgas fue puesta de manifiesto por primera vez por Scott (1980) y Taub (1980), que encontraron que se puede producir un rango tan importante de variabilidad bioquímica con una sola especie microalgal, mediante la alteración de los parámetros del cultivo continuo, como el rango de variabilidad encontrado para un amplio espectro de especies microalgales. La productividad de los cultivos continuos de microalgas es también considerablemente más alta que la obtenida con los métodos convencionales, pudiendo alcanzar rendimientos hasta 10 veces superiores.

Figura 10 - Ejemplos de cultivos de microalgas en discontinuo o en "batch". Cultivo en tanques de sección circular (izquierda), o en bolsas de plástico de volumen variable. Fotos tomadas de Coutteau (1996).

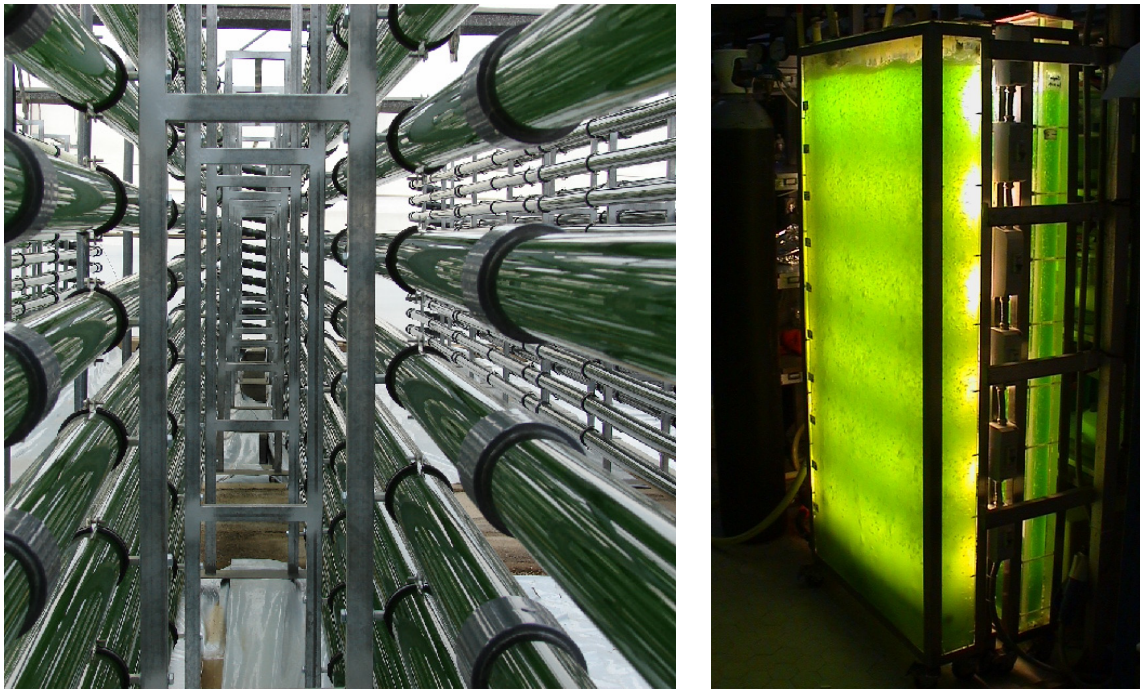

Figura 11 - Cultivo de microalgas en contínuo en fotobiorreactores tubulares (izquierda) y en paneles verticales de sección rectangular (derecha). Fotos: Gabriel Acién y Ana Otero.

Existen paralelamente otras características que hacen los cultivos continuos más adecuados para la producción de microalgas:

a) la tasa de crecimiento y la producción pueden mantenerse cerca del máximo (sobretodo en volúmenes pequeños) cuando las condiciones de cultivo son adecuadas, estando la población microalgal en fase de crecimiento exponencial permanente, lo que proporciona una biomasa de mayor calidad nutritiva;

b) se produce biomasa más controlada y de calidad uniforme, siendo instrumentos óptimos para la manipulación de la composición bioquímica;

c) el sistema de cultivo facilita la automatización (diseño de fotobiorreactores de alto rendimiento) reduciéndose los costes de mano de obra.

Objetivos / Objectives

Los principales objetivos del presente trabajo han sido:

1) La mejora del crecimiento y la supervivencia de paralarvas de pulpo (*Octopus vulgaris*), utilizando distintas dietas (alimento vivo sólo o complementado con microdietas) que han sido formuladas o moduladas teniendo en cuenta la composición bioquímica de estadios iniciales de *O. vulgaris*, y que consistieron en:

a) juveniles de *Artemia* enriquecidos con microalgas de composición optimizada y controlada, o con otros suplementos nutricionales tales como emulsiones lipídicas comerciales, compuestos purificados, etc.;

b) microdietas artificiales formuladas específicamente para las paralarvas de pulpo.

2) La evaluación de nuevas estrategias para mejorar el cultivo de pulpo en jaulas flotantes a escala industrial.

The main objectives of the present work were:

1) The improvement of *Octopus vulgaris* paralarvae growth and survival rates through the use of different dietary regimes (live prey alone or complemented with microdiets), which were modulated or formulated taking into consideration the biochemical composition of *O. vulgaris* early life stages, consisting of:

a) *Artemia* juveniles enriched with either microalgae of optimal and controlled composition, or other nutrient supplements such as commercial lipid emulsions, purified compounds, etc.;

b) artificial pellets formulated specifically for paralarvae.

2) The evaluation of new strategies to improve the rearing conditions of adult octopuses at an industrial scale in floating cages.

Producing juvenile *Artemia* as prey for *Octopus vulgaris* paralarvae with different microalgal species of controlled biochemical composition

Capítulo 1 / Chapter 1

Abstract

The major bottleneck of *Octopus vulgaris* culture is the rearing of its paralarval life stage, being the obtainment of adequate live prey to feed paralarvae one of the key issues for the success of the culture of this valuable species. *Artemia* has been widely used as a single prey or in combination with crustacean zoeae as food items for paralarvae, but few works have reported the biochemical composition of this prey. *Artemia* juveniles of two different sizes (1.5–2.0 mm and 3.0–3.5 mm), appropriate to feed *O. vulgaris* paralarvae, were obtained by growing *Artemia* nauplii with *Tetraselmis suecica* for 2 and 4 days, being then further enriched for 26 h with four different microalgal species: *T. suecica*, *Isochrysis galbana*, *Isochrysis* aff. *galbana* (T-ISO) and *Rhodomonas lens*. Microalgae were cultured semi-continuously in nutrient saturated conditions and with a daily renewal rate of 30% of the volume of cultures, in order to achieve biomass of constant and optimal biochemical composition. The gross composition and the fatty acid (FA) profiles of the enriched *Artemia* juveniles were assessed in order to evaluate their nutritional value for octopus paralarvae. The FA composition of newly hatched *O. vulgaris* paralarvae and of wild *Maja brachydactyla* zoeae, a prey that has been described as suitable to rear paralarvae, were also analysed with the aim of establishing comparisons of FA profiles. The total amino acid (AA) composition of big *Artemia* juveniles (3.0-3.5 mm) was also analyzed and compared with data previously published by other authors concerning the total AA composition of octopus hatchlings. The protein content of *R. lens* (62% of dry weight) was considerably higher than that of the remaining microalgae (42-44%, $P<0.001$), whereas lipid and carbohydrate were significantly higher in both T-ISO and *I. galbana* (20-21% and 17-19%, respectively) ($P<0.05$). Small juvenile *Artemia* (1.5–2.0 mm) contained nearly 51% protein regardless the enrichment diet used, with the exception of individuals enriched with *I. galbana* (group AISO) which contained a lower protein content (41%, $P<0.01$). In these juveniles, lipid percentages were higher when enriched with T-ISO (AT-ISO) or with *R. lens* (ARHO), both with circa 16% ($P<0.05$); whereas carbohydrate was higher in juveniles from groups AISO or AT-ISO (11%, $P<0.05$). Large juvenile *Artemia* (3.0–3.5 mm) contained higher protein levels than small juveniles with values ranging between 64 and 68% for all treatments, whereas the lipid fraction among groups increased

in the order: ARHO (10%) < ATET = AT-ISO (16%) < AISO (18%) ($P<0.05$). The lowest percentage of carbohydrate was found in group ARHO (6%, $P<0.01$). The maximum protein/energy ratio was observed in 5-day old juveniles from group ARHO (P/E ratio=31.7). The highest percentage (% total FA) of eicosapentaenoic acid (EPA, 20:5n-3) in small juvenile *Artemia* was found in individuals from groups AISO or ARHO (circa 9%), whereas in 5-day old juveniles the highest value was found in group AISO (14.6%, $P<0.05$). Regarding docosahexaenoic acid (DHA, 22:6n-3), small juveniles from groups AT-ISO or AISO had higher values (1.9 and 1.5%, respectively) than juveniles from group ARHO (1.0%, $P<0.05$), whereas in 5-day old *Artemia* maximum percentage of DHA was found in group AT-ISO (3.9%, $P<0.05$). DHA was absent in *Artemia* juveniles enriched with *T. suecica*. The FA composition of *O. vulgaris* paralarvae revealed much higher percentages of DHA (19.7%) and arachidonic acid (ARA, 20:4n-6) (3.4%), and in general also of EPA (14.7%) than values found in *Artemia* juveniles. In *M. brachydactyla* zoeae, the percentages of those FA were, in the same order: 8.7%, 7.8% and 24.3%. These results suggest that *Artemia* may have a deficit of highly unsaturated fatty acids (HUFA) to cover paralarvae needs. In this study we found that lysine could be a limiting AA in *Artemia* juveniles, but this observation needs further studies based in more replicate analysis and appropriate experimental trials of octopus paralarvae, to evaluate the possible effects of lysine supplementation. If the general composition of *Artemia* juveniles (gross composition and FA profiles of both *Artemia* sizes) is taken into consideration, the enrichment with *R. lens* provided the best results among groups, though the highest sum of EPA and DHA was found in *Artemia* juveniles enriched with *I. galbana*.

1. Introduction

The control of *Octopus vulgaris* life cycle in captivity for rearing purposes has long been a subject of research, with first experiments being carried out in the 60's in Japan (Itami *et al*., 1963). Yet, it was only in the middle of the 90's that intensive experiments to evaluate the potential of this species as a new candidate for aquaculture started to be carried out in countries worldwide. Due to some interesting biological characteristics and to high market demand and price, this species was latter considered as a strong candidate for aquaculture diversification (Iglesias *et al*., 2000; Navarro and Villanueva, 2000; Vaz-Pires *et al*., 2004). However, and despite the various attempts to rear the planktonic life stage of *O. vulgaris*, which is the bottleneck of this species for aquaculture development, researchers still did not succeeded in overcoming the high mortality encountered during paralarvae rearing (reviewed by Iglesias *et al*., 2007a).

Field studies on the feeding habits of early life stages of cephalopods have shown that small planktonic crustaceans constitute major diets for these organisms (Vecchione, 1987; Passarella and Hopkins, 1991) and indeed, most of the successful rearing experiments of squid and cuttlefish hatchlings, relied on the harvesting of natural zooplankton to provide as main prey (Yang *et al*., 1986; Forsythe *et al*., 1994; Domingues *et al*., 2004). Few researchers have succeeded in achieving settled *O. vulgaris* juveniles in captivity (reviewed by Iglesias *et al*., 2007a), and in all cases zoeae of different crustacean alone or combined with enriched *Artemia* of different sizes, according to authors, were used as food items. As far as we know, only one research group has reported the success of rearing octopus paralarvae on *Artemia* as single prey (Hamazaki *et al*., 1991). In general, it is recognized that *O. vulgaris* paralarvae may require diets rich in phospholipids, cholesterol, and in the polyunsaturated fatty acids (PUFAs) EPA and DHA; but also very rich in protein and in essential amino acids (Navarro and Villanueva, 2000, 2003; Villanueva *et al*., 2004). Analysis of the biochemical composition of decapod zoeae have shown that their lipid classes and FA composition are undoubtedly more in accordance to *O. vulgaris* hatchlings and wild juveniles than *Artemia per se*, due to higher levels of phospholipids and DHA and EPA levels as well (Navarro and Villanueva, 2000, 2003). However, the use of parallel cultures of crustaceans to obtain newly hatched zoeae as live prey would be impractical beyond the experimental scale as cost effectiveness and risks implied would be limiting issues (Navarro and Villanueva, 2000).

The enrichment of *Artemia* (1-4 mm) with microalgae for first feeding of *O. vulgaris* or the addition of microalgae to tanks to keep prey fully enriched is a common practice so far, and microalgae species like *Nannochloropsis* sp., *Isochrysis galbana*, *Chaetoceros* sp., *Chlorella* sp., and *Tetraselmis suecica* have previously been used (Hamazaki *et al*., 1991; Moxica *et al*., 2002, Iglesias *et al*., 2002, 2004; Okumura *et al*., 2005; Carrasco *et al*., 2006). Commercial lipid emulsions, cereal mixes, and fish egg powder were also tried to enrich *Artemia* nauplii or juveniles to feed paralarvae (Navarro and Villanueva, 2000, 2003; Villanueva *et al*., 2002; Iglesias *et al*., 2004; Okumura *et al*., 2005). In spite of the common utilization of enriched *Artemia* (nauplii or juveniles) for the rearing of *O. vulgaris* paralarvae, few authors (Navarro and Villanueva, 2000; Villanueva *et al*., 2004; Okumura *et al*., 2005) have reported biochemical composition data of these prey, even if this information is crucial to understand possible nutritional deficiencies in paralarvae rearing.

Dramatic changes in the biochemical composition of microalgae can be induced through the use of continuous culture techniques, which have been used to produce microalgal biomass of constant and controlled composition, which in turn lead to improvement in the growth and survival of filter-feeder species used in aquaculture (Scott, 1980; Taub, 1980; Fábregas *et al*., 1996a, 2001). Semi-continuous cultures are a variant of continuous cultures in which cultures are maintained under light:dark cycles and culture medium is renewed at a fixed rate every 24h, and have been demonstrated to be as efficient as standard continuous cultures in controlling the nutritional value of microalgae (Fábregas *et al*., 1996a; Otero and Fábregas, 1997; Otero *et al*., 1997; Otero *et al*., 2002). Remarkable improvements in *Artemia* length, dry weight and survival were observed when *T. suecica* supplied to *Artemia* was cultured under nutrient saturated conditions (Fábregas *et al*., 1996b) or high renewal rates (Fábregas *et al*., 2001). Besides improvement of growth and survival, important changes in the gross biochemical composition of *Artemia* fed *T. suecica* cultured through semi-continuous culture at different renewal rates have been demonstrated (Fábregas *et al*., 2001). The standardization of culture conditions to control the biochemical composition of the microalgae used in feeding and/or enrichment experiments is therefore crucial in order to make results obtained with different species comparable and repeatable.

With the aim of evaluating which microalgal species would be more suitable to improve the biochemical composition of juvenile *Artemia* as prey for *O. vulgaris* paralarvae, we analysed the biochemical composition of juvenile *Artemia* of two different sizes, commonly used in paralarvae rearing (1.5–2.0 and 3.0–3.5 mm), enriched for 26 h with

four different microalgae cultured semi-continuously in nutrient saturated conditions. Two microalgal species were selected due to either high protein content (*Rhodomonas lens*) or high DHA content (*Isochrysis* aff. *galbana* T-ISO) and the others (*Isochrysis galbana* Parke and *Tetraselmis suecica*) because they have been previously used by different authors and are hereby compared. Analysis of the FA composition of *O. vulgaris* hatchlings and of *Maja brachydactyla* zoeae were also carried out to establish nutritional comparisons with the results found for *Artemia* juveniles. *M. brachydactyla* zoeae were chosen as this prey was shown to constitute a suitable prey to rear paralarvae until the settlement stage by two research groups (Iglesias *et al*., 2004; Carrasco *et al*., 2006), but no information about its FA composition was reported.

2. Materials and methods

2.1 Microalgae cultures

Non-axenic monoalgal cultures of *Tetraselmis suecica* Kylin, *Isochrysis galbana* Parke (both strains isolated from Ría de Arousa, Spain), *Isochrysis* aff. *galbana* (T−ISO) CCMP 1324, and *Rhodomonas lens* Pascher et Ruttner CCMP 739, were carried out in 1-l glass bottles (Fig. 1).

Figure 1 - Production of the different microalgal species in 1-l glass bottles. Cultures were daily renewed at 30% of the total volume with a nutrient concentration of 4 mM $NaNO_3$ in order to ensure nutrient saturated conditions.

Sea water was adjusted to a salinity of 35 ppt and autoclaved at 121 ºC for 15 min, before nutrients were added to attain a final concentration of 4 mM $NaNO_3$ (Fábregas *et al*., 1986), which ensured saturation of nutrients. Cultures were provided with constant aeration through capillary tubes and submitted to 12h:12h light/dark photoperiod with an irradiance of 148 µmol photon m^{-2} s^{-1} under and 141 µmol photon m^{-2} s^{-1} beneath cultures, provided by day light fluorescent lamps (OSRAM L36W). Irradiance was measured with a luxmeter Neurtex HD8366 followed by conversion according to the formula proposed by Ginzburg (1987). The pH of all cultures was kept between 7.5 and 8.3 through pulses of CO_2 during the light period. The temperature of the culture chamber was kept at 21.0±1.5 ºC. Once cultures reached early stationary phase, daily renewal rates of 30% of the volume of cultures were carried out during the first hour of the light period with sterilized seawater and the same nutrient concentration. When cellular density attained the steady state, the harvested cultures were used to enrich different *Artemia* groups.

Cell density was calculated in the harvested culture by microscope counting using an improved Neubauer haemacytometer. Microalgae dry weight was determined by filtering 2 ml of cultures through carbonised Whatman GF/C glass fibre filters (Whatman, Brentford, UK). Filters were washed twice with ammonium formiate (0.5 M) and dried at 80 ºC until constant weight (Utting, 1985). In order to assess the stability of the biochemical composition of microalgae along the steady state, several samples (8 ml each) of the different microalgal cultures were obtained on three different days, centrifuged and immediately frozen at -18 ºC for later biochemical analysis.

2.2 *Artemia* sp. growth and enrichment

Artemia sp. cysts (AF, INVE, Dendermonde, Belgium) were incubated in seawater adjusted to a salinity of 30 ppt, with constant aeration, temperature of 28±1 ºC, and exposed to an irradiance of 39 µmol photon m^{-2} s^{-1}. Newly hatched nauplii were transferred into glass-flasks containing 700 ml of filtered and autoclaved seawater, also adjusted to 30 ptt salinity, and placed in the following conditions: initial density of 2.0 nauplii ml^{-1}, water temperature of 26.5±0.5 ºC, constant aeration provided by capillary tubes, and dim light for 24 h. Nauplii were initially fed *T. suecica* with a food ration established at 25 µg dry weight of microalgae per nauplii (equivalent to $125x10^3$ cells $nauplii^{-1}$), increasing the amount of supplied food along the experiment, depending on the transparency of the

culture media, so that almost all food supplied was ingested (Sorgeloos *et al*., 1986). At days 2 and 4 of *Artemia* growth (at day 2 individuals had a total length of 1.2±0.1 mm and a survival of 76±2%, and at day 4 2.7±0.4 mm and a survival of 74±4%), different groups of *Artemia* in triplicate were enriched with different microalgal species for 26 h. Four hours before the end of the enrichment process, a second dose of microalgae was supplied in order to ensure a complete enrichment. Group AISO was enriched with *Isochrysis galbana* Parke, group AT-ISO with *Isochrysis* aff. *galbana* T–ISO, group ARHO with *Rhodomonas lens*, and group ATET continued to receive *T. suecica*. Before carrying out the enrichment process, water was completely renewed in order to remove any remaining food and *Artemia* faeces. The same amount of food was provided to all groups and was calculated according to the dry weight of each microalgal species (obtained from previous experiments in semi-continuous culture regime). The length of *Artemia* juveniles was measured under a stereoscope using a calibrated ocular micrometer (25 individuals per replicate). Dry weight was determined by washing *Artemia* juveniles with distilled water (samples of 10 individuals placed in fibre-glass filters, n=5), followed by 24 h drying at 100 ºC. Survival was calculated to verify possible effects of each enrichment treatment. Samples of *Artemia* juveniles were collected (60 juveniles per sample were individually counted on day 3, and 50 juveniles per sample on day 5) for gross composition analysis and also for total FA analysis, being briefly washed with distilled water and immediately frozen at -18 ºC. Samples of 30-40 mg DW of 5-day old juveniles were also collected for total amino acid (AA) composition.

2.3 Hatchlings of *Octopus vulgaris* for fatty acid analysis

A female *O. vulgaris* with eggs was transported to the facilities of the University of Santiago de Compostela (Spain) and placed in a tank of a recirculation water circuit with mean water temperature of 17.5±1.0 ºC. On the fifth day after the first paralarvae started to hatch, newly octopus hatchlings where collected, briefly washed with distilled water and immediately frozen at -18 ºC for fatty acid analysis. This day was chosen in order to collect hatchlings in a moment of massive paralarvae hatching.

2.4 Collection of *Maja brachydactyla* zoeae for fatty acid analysis

Several ovigerous females of spider-crab (*Maja brachydactyla*) were caught between 10 and 20 m depth in the north coast of Portugal, between Viana do Castelo (41º 41'N; 8º 52'O) and Caminha (41º49'N; 8º53'W). Females were transported to an enclosed culture system in the shore of Viana, and when zoeae started to hatch they were collected with a hand net, followed by a brief wash with distilled water and preserved at -18 ºC for fatty acid analyses. Spider-crab zoeae (n=20) were measured as described above for *Artemia*.

2.5 Biochemical composition analysis

Protein content was determined by the method of Lowry (Lowry *et al*., 1951), after hydrolysis with NaOH 1.0 M at 95 ºC. Carbohydrate was determined by the phenol/sulphuric acid method (Kochert, 1978), and lipid was quantified by carbonization at 200 ºC (Marsh and Weinstein, 1966) after extraction of total lipid (Bligh and Dyer, 1959). Determination of C-H-N in the different microalgal species was carried out on freeze-dried samples (n=3 from three different days) using an autoanalyzer (Carlo Erba EA 1108, Rodano, Italy). This method is based in the instantaneous combustion of all organic and inorganic compounds in its combustion end products, which is quantified by a thermic conductivity detector. The equipment was calibrated with the standard compound acetanilide, normally recommended when samples with high organic content are to be measured. Caloric values were calculated using the conversion factors proposed by the National Research Council (1993) for protein (5.64), lipid (9.44) and carbohydrate (4.11). Fatty acids were identified and quantified using a gas chromatograph-mass spectrograph (GC-MS Fisons Instruments, MD-800, Beverly, MA.) equipped with a Omegawax^{TM} 250 column 30 m x 0.25 mm (Supelco, Inc.), using helium as gas carrier, after methanolysis of the lipid extracts with 5% HCl in methanol at 85 ºC during 2.5 h and recovery of the methyl esters with hexane (Sato and Murata, 1988). Triheptadecanoin (Sigma, St. Louis, MO.) was used as internal standard. To determine the total AA composition of 5-day old *Artemia* juveniles (3.0-3.5 mm), samples of 30-40 mg of freeze-dried biomass were hydrolysed in 25 ml of 6 M HCl for 24 h at 105 ºC. The obtained hydrolyzed solutions and amino acids standards (Waters, standards WAT 088122) were derivatized using AccQ-Tag® System for amino acid analysis (Water, Milford, MA) and run on a modification of

the reversed-phase HPLC system (Waters Associates). To separate the different amino acids, a reverse-phase column (AccQ Tag, 150 mm long, 3.9 mm internal diameter) was used. Samples of 10 µl were injected by autosampler, and the eluting products were measured with a fluorescent detector at excitation wavelengths of 250 and 395 nm. Chromatograms were recorded using the software program Breeze (Waters, USA). The following essential amino acids (EAA) were determined: leucine, lysine, arginine, threonine, valine, isoleucine, phenylalanine, histidine and methionine were analysed; as well as the non essential amino acids (NEAA): alanine, cysteine, glycine, proline, serine, tyrosine, glutamine and glutamic acid, and asparagine plus aspartic acid. Results for tryptophan are not reported since this AA is destroyed by acid hydrolysis. All biochemical analyses were carried out in triplicate except for total amino acids for which a single analysis was done.

2.6 Statistical analysis

Statistical analyses were performed using the software SPSS V 14.0.1 statistical package (SPSS, Inc.). After verifying that data of *Artemia* length met the requirements of normality (Kolmogorov-Smirnov test), the comparisons among groups were performed by an analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05. The same statistical tests were carried out to compare the productivities of the different microalgal species. After log-transformation of dry weight data, and arcsine-√ transformation of survival and biochemical composition percentages, ANOVA and Tukey-Kramer HSD tests for post-hoc multiple comparisons (α=0.05) were also performed (Zar, 1999). The percentages of EPA and DHA found in the enrichment diets and in *Artemia* juveniles were correlated using a linear regression model (Zar, 1999).

3. Results

3.1 Biochemical composition of microalgae

Steady state cell densities and daily productivities (mg dry weight l^{-1} of culture day^{-1}) obtained in the different microalgal semi-continuous cultures are shown in table I. *I.*

galbana and T-ISO cultures attained overall 30% higher productivities than *R. lens* and *T. suecica* ($P<0.01$). Since microalgae used to enrich juvenile *Artemia* were considerably different in size, no direct comparisons of the content in protein, lipid and carbohydrate per single cell could be established, and thus comparisons were done as percentages of dry weight (Fig. 2). The protein content in *R. lens* (62%) was considerable higher than in the remaining microalgal species (42-44%, $P<0.001$). Lipid levels were nearly the same in *R. lens* and in *T. suecica* (12 and 13%, respectively), being significantly higher in both T-ISO and *I. galbana* (20-21%, $P<0.001$); whereas the content of carbohydrates varied significantly in all specis and increased in the order: *R. lens* (11%) < *T. suecica* (16%) < T-ISO (17%) < *I. galbana* (19%) ($P<0.05$). The biochemical composition of microalgae did not change along the course of the experiment, supporting the statement that the harvested biomass was stable in composition and was cultured in controlled conditions.

Table I. Steady state cell density ($x10^6$ ml^{-1}), dry weight (pg $cell^{-1}$), organic weight (OW as sum of protein, lipid and carbohydrate, pg $cell^{-1}$) and productivity (mg dry weight l^{-1} of culture per day) of the different microalgal species used to enrich juvenile *Artemia*, produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures.

	TET	T-ISO	ISO	RHO
Steady state cell density	2.1 ± 0.4^a	20.8 ± 2.3^b	22.8 ± 1.8^b	3.5 ± 0.4^a
Cell dry weight	220.7 ± 11.5^a	30.0 ± 2.8^b	26.8 ± 1.1^b	133.5 ± 5.4^c
OW	157.7 ± 5.1^a	24.3 ± 0.8^b	22.1 ± 0.5^b	112.5 ± 2.0^c
Daily productivity	139 ± 23^a	187 ± 21^b	183 ± 14^b	140 ± 14^a

TET (*Tetraselmis suecica*), T-ISO (*Isochrysis* aff. *galbana* T–ISO), ISO (*Isochrysis galbana*), RHO (*Rhodomonas lens*). Data are means ± S.D. (n=3 for dry weigth and OW, harvested in different days; n=6 for steady state density and daily productivity, harvested in different days). Different superscript letters within the same line indicate significant differences among microalgal species (α=0.05).

The C:N ratios found in microalgal species confirmed the gross biochemical composition data, as this ratio reflects the proportion of protein to lipid plus carbohydrate (Fig. 2). Significant differences were observed among all species, with the C:N ratio increasing in the order: *R. lens* (4.4) < *T. suecica* (5.2) < T-ISO (7.0) < *I. galbana* (8.0) ($P<0.05$). The high protein and low lipid and carbohydrate percentages found in *R. lens* reflected the lowest C:N ratio found in this species. The higher C:N ratios in both T-ISO and *I. galbana* are also in agreement with the higher percentages of carbohydrate and lipid found in these two species.

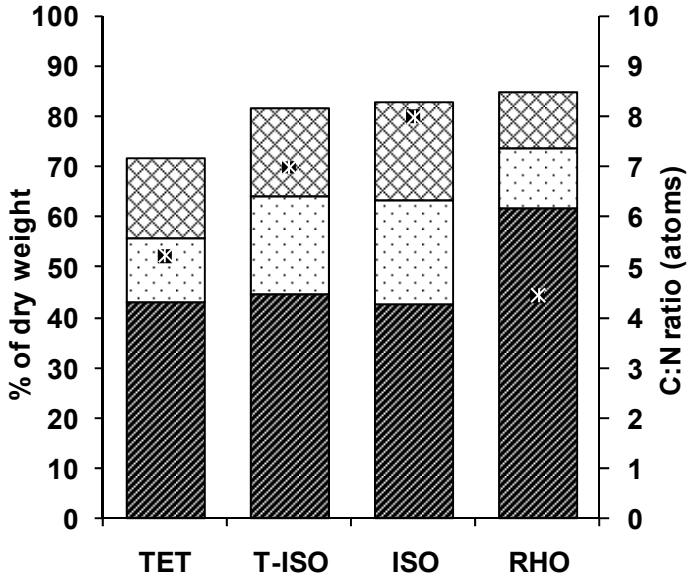

Figure 2 – Gross biochemical composition (as % of dry weight) and C:N ratio of the different microalgal species used to enrich juvenile *Artemia*. ▨ Protein, ⬚ Lipid, ⊠ Carbohydrate, ▩ C:N ratio. Abbreviations of TET, T-ISO, ISO and RHO are like in Table I. Data are mean values (n=3, harvested in three different days). Standard deviations were all below 10% and are not shown.

The FA composition (% of total FA) of the different microalgae used to enrich juvenile *Artemia* revealed several differences at both quantitative and qualitative levels (Table II). All microalgae had a predominant saturated FA in its composition, except *R. lens* which had the PUFA 18:3n-3 (32%) as the major FA. Both *T. suecica* and *I. galbana* contained 16:0 as the main FA (35 and 28%, respectively), whereas 14:0 was the major FA found in T-ISO (25%). The sum of saturated FA was clearly lower in *R. lens* (21%) than in the remaining species (42-47%). Due to the high percentages of the PUFAs 18:3n-3 and 18:4n-3 in *R. lens* (32% and 20%, respectively) this microalgal doubled the proportion of PUFAs when compared to values found in *I. galbana* and T-ISO (34-36%). *T. suecica* contained 42% of PUFA, but the FA 16:4n-3 accounted for 15% of the total FA. The n-6 class of FA represented less than 2% in all microalgae, except in T-ISO which contained a considerable higher proportion (7%). The highest percentage of DHA was found in T-ISO (10.7%, $P<0.001$), followed by *R. lens* (7.4%) and *I. galbana* (6.4%), whereas no DHA was found in *T. suecica* (Table II). The percentage of EPA was significantly different among all species ($P<0.001$), with the maximum value being found in *I. galbana* (19%), followed by *R. lens* (9.7%), *T. suecica* (5.5%) and T-ISO (0.7%).

Table II. Fatty acid (FA) composition (% of total FA) and FA content (% of the dry weight) of the different microalgal species used to enrich *Artemia* juveniles, produced in semi-continuous regimen in nutrient saturated conditions and with a daily renewal of 30% of the volume of cultures.

Fatty acid	TET	T-ISO	ISO	RHO
14:0	6.7 ± 0.2^{a}	24.8 ± 1.3^{b}	19.2 ± 1.2^{b}	7.4 ± 0.4^{a}
16:0	35.2 ± 3.3^{a}	16.3 ± 0.2^{b}	27.6 ± 1.2^{c}	12.8 ± 0.2^{d}
16:1n-9	1.6 ± 0.1^{a}	6.8 ± 0.3^{b}	17.7 ± 0.7^{c}	1.0 ± 0.1^{d}
16:1n-7	2.1 ± 0.1^{a}	0.0	n.f.	2.2 ± 0.1^{a}
16:4n-3	15.2 ± 1.8	n.f.	n.f.	n.f.
18:0	0.0	2.6 ± 0.1^{a}	0.1 ± 0.1^{b}	0.4 ± 0.0^{c}
18:1n-9	9.8 ± 0.6^{a}	12.0 ± 1.2^{b}	0.2 ± 0.0^{c}	0.3 ± 0.0^{c}
18:1n-7	0.9 ± 0.3^{a}	0.7 ± 0.2^{a}	0.4 ± 0.0^{a}	3.3 ± 0.1^{b}
18:2n-6	1.3 ± 0.5^{a}	6.3 ± 0.2^{b}	0.6 ± 0.0^{c}	1.7 ± 0.1^{a}
18:3n-3	11.7 ± 1.1^{a}	10.1 ± 0.9^{a}	1.1 ± 0.0^{b}	31.7 ± 0.5^{c}
18:4n-3	7.9 ± 0.7^{a}	7.1 ± 0.4^{a}	5.9 ± 0.1^{a}	20.3 ± 0.6^{b}
20:1n-9	1.2 ± 0.1^{a}	0.5 ± 0.1^{b}	n.f.	n.f.
20:4n-6	0.3 ± 0.0^{a}	0.2 ± 0.1^{a}	0.2 ± 0.0^{a}	0.0
20:4n-3	0.5 ± 0.1^{a}	n.f.	n.f.	1.0 ± 0.1^{b}
20:5n-3	5.5 ± 0.1^{a}	0.7 ± 0.2^{b}	19.0 ± 0.3^{c}	9.7 ± 0.2^{d}
22:5n-6	n.f.	0.9 ± 0.4^{a}	1.2 ± 0.0^{a}	0.0
22:6n-3	n.f.	10.7 ± 0.3^{a}	6.4 ± 0.4^{b}	7.4 ± 0.3^{c}
Others	0.0	0.0	0.0	0.1
Saturated	41.9 ± 5.8^{a}	43.7 ± 1.5^{a}	46.9 ± 0.2^{a}	20.6 ± 0.6^{b}
Monoenes	15.7 ± 1.1^{b}	20.0 ± 1.5^{a}	18.3 ± 0.7^{a}	6.8 ± 0.1^{c}
PUFA	42.4 ± 4.9^{b}	$36.0 \pm 0.6^{b,c}$	34.4 ± 0.6^{c}	71.8 ± 0.5^{a}
n-3	40.8 ± 4.4^{b}	28.6 ± 0.8^{d}	32.4 ± 0.6^{c}	70.1 ± 0.4^{a}
n-6	1.6 ± 0.5^{b}	7.4 ± 0.3^{a}	2.0 ± 0.1^{b}	1.7 ± 0.1^{b}
DHA/EPA	-	15.3	0.3	0.8
FA content (% of DW)	4.6 ± 0.4^{d}	11.1 ± 0.7^{b}	13.8 ± 0.3^{a}	7.3 ± 0.1^{c}

Abbreviations of TET, T-ISO, ISO and RHO are the same as in Table I. Data are means ± S.D. (n=3). n.f.: not found. Values referred as 0.0 are below 0.05. Different superscript letters within the same line indicate significant differences among microalgal species (α=0.05).

The maximum DHA/EPA ratio was found in T-ISO (15.3). The content of FA (% dry weight) found in microalgae followed the same tendency as lipid levels, being higher in both *I. galbana* and T-ISO than in *R. lens* and *T. suecica* (Table II).

The total amount and composition of the food (expressed as dry weight) supplied to 2-day old *Artemia* for 26 h is shown in figure 3.

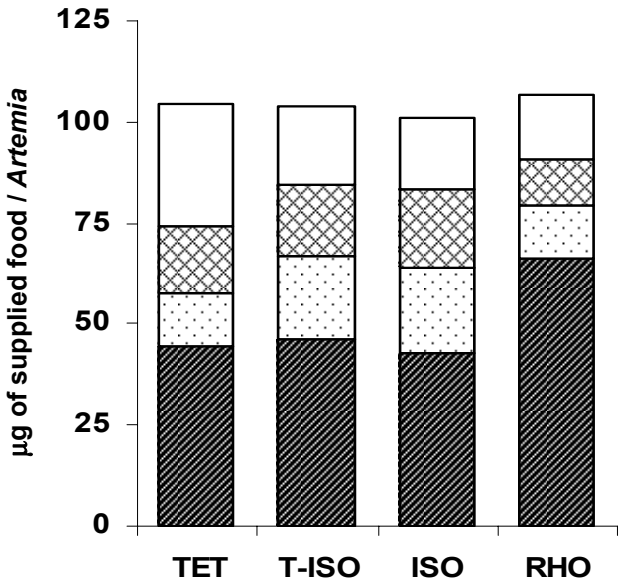

Figure 3 - Composition and total amount of food (µg dry weight microalgae *Artemia*$^{-1}$) supplied to 2-day old *Artemia* for 26 h during the enrichment process. ▨ Protein, ⁘ Lipid, ▩ Carbohydrate, ☐ Total amount of food supplied. Abbreviations are the same as in figure 1.

The slight differences observed in the total food supplied (µg of food per *Artemia*) to each group were due to differences in the dry weight of the microalgae obtained in the present work and the theoretical dry weights of each microalgal species that were considered when carrying out the calculations for food distribution. Even so, it should be kept in mind that the amount of food supplied for enrichment was in excess in all cases, and enough to guarantee the correct enrichment of *Artemia* juveniles.

The total amount of EPA and DHA in the diets (corresponding to the different microalgal species) supplied to 2-day old juvenile *Artemia* for 26 h is shown in figure 4. Group enriched with *I. galbana* received considerable higher amounts of EPA in the diet than the remaining groups, whereas group enriched with T-ISO received much more DHA than the other groups. Four-day old juvenile *Artemia* received 15% more food than 2-day old juveniles when enrichment was carried out, since individuals were bigger.

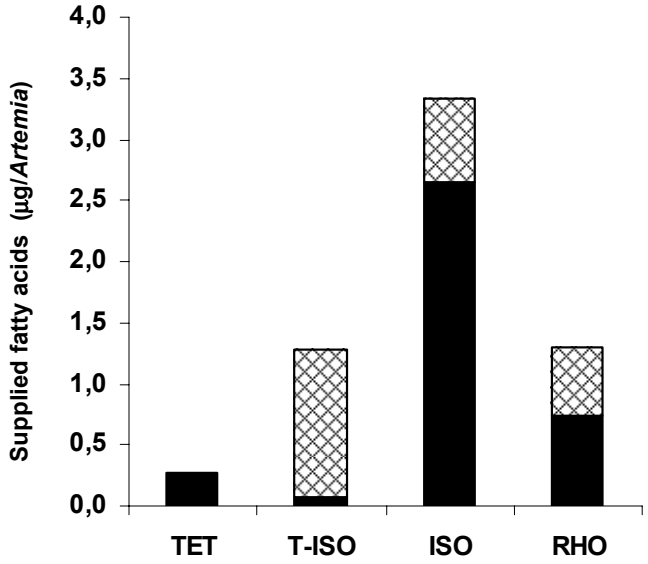

Figure 4 - Total amount of the highly unsaturated fatty acids 20:5n-3 (EPA, ■) and 22:6n-3 (DHA, ⊠) supplied to 2-day old *Artemia* juveniles for 26 h for the enrichment with the different microalgal species.

3.2 Growth and survival of enriched *Artemia* juveniles

The enrichment of *Artemia* with the different microalgal species for 26 h, at days 2 and 4, did not cause any significant difference in the survival of individuals and was higher than 95% in all treatments. On the other hand, significant differences in the dry weight of *Artemia* juveniles could be observed (Fig. 5) with only 26 h of enrichment period, with differences being more evident among 5-day old juveniles.

Regarding the total length of *Artemia* juveniles, significant differences among groups were observed only for 3-day old juveniles (Table III). Individuals enriched with *R. lens* (group ARHO) or with *T. suecica* (ATET) were larger than those enriched with T-ISO (AT-ISO) or with *I. galbana* (AISO) ($P<0.001$ for comparisons with group ARHO; $P<0.01$ for group ATET).

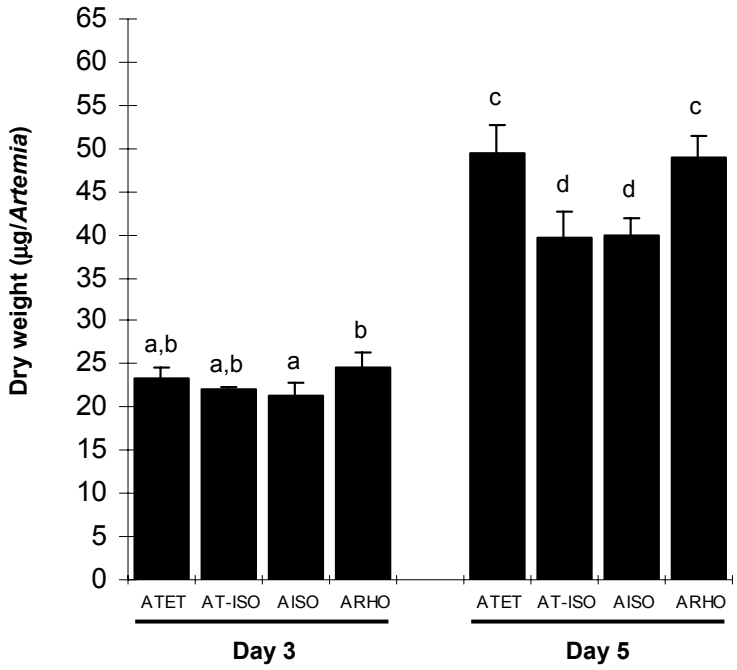

Figure 5 - Dry weight (µg *Artemia*$^{-1}$) of 3 and 5-day old juvenile *Artemia* enriched with different microalgal species for 26 h. ATET: *Artemia* enriched with *T. suecica*; AT-ISO: enriched with T-ISO; AISO: enriched with *I. galbana*; ARHO: enriched with *R. lens*. Data are means ± S.D. (n=3, 25 individuls per replicate). Different letters within the same day indicate significant differences among groups (P<0.05).

3.3 Gross composition of *Artemia* juveniles

The enrichment process modified the biochemical composition of *Artemia* juveniles and important differences were observed among groups (Table III). In 3-day old juvenile *Artemia*, protein content was roughly the same in all groups (51% of dry weight), with the exception of individuals from group AISO which contained a lower protein content (41%, P<0.01). The highest lipid percentages were found in juveniles from groups AT-ISO and ARHO (both with circa 16%), whereas groups AISO and ATET had 10 and 13%, respectively (Table III). Juveniles from groups AISO and AT-ISO had almost the same percentage of carbohydrate (11%), which was higher than values found in juveniles from groups ARHO or ATET (P<0.05). The protein percentage in 5-day old juvenile *Artemia* ranged between 64 and 68%, and in contrast to 3-day old juvenile, no significant

differences were observed among treatments. As for lipid, 5-day old juveniles from group AT-ISO contained 17.5%, followed by groups ATET and AISO (both with circa 15.5%, $P<0.05$), whereas juveniles from group ARHO had the lowest lipid content (10.1%, $P<0.001$). The lowest carbohydrate percentage was found in individuals from group ARHO (6.1%, $P<0.01$), whereas juveniles from group AISO, AT-ISO and ATET had considerable more carbohydrate (Table III). The protein/energy ratio (P/E, expressed in mg protein KJ^{-1}) in 3-day old juveniles ranged between 25 and 27, whereas in 5-day old juveniles ranged between 27-32 (Table III). The maximum P/E ratio was observed in juveniles enriched with *R. lens* (31.7).

Table III. Gross biochemical composition (% of dry weight), energy (J *Artemia*$^{-1}$), protein/energy ratio (P/E, mg protein KJ^{-1}) and length (mm) of juvenile *Artemia* enriched with different microalgae produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures.

Group	ATET	AT-ISO	AISO	ARHO
3-day old *Artemia*				
Total length (mm)	1.7 ± 0.2^{a}	1.6 ± 0.2^{b}	1.5 ± 0.2^{b}	1.8 ± 0.2^{a}
Protein (%)	50.4 ± 1.2^{a}	50.7 ± 0.4^{a}	41.3 ± 1.4^{b}	50.7 ± 1.7^{a}
Lipid (%)	12.8 ± 1.2^{a}	16.1 ± 1.0^{b}	10.1 ± 0.7^{c}	15.9 ± 0.4^{b}
Carbohydrate (%)	9.3 ± 0.2^{a}	10.7 ± 0.3^{b}	11.0 ± 0.9^{b}	8.4 ± 0.4^{a}
Energy	0.43	0.44	0.33	0.49
P/E ratio	27.2	25.2	26.4	25.8
5-day old *Artemia*				
Total length (mm)	3.2 ± 0.3	3.1 ± 0.4	3.2 ± 0.4	3.1 ± 0.3
Protein (%)	63.6 ± 2.6	64.7 ± 2.7	67.7 ± 0.5	63.7 ± 1.9
Lipid (%)	15.6 ± 0.7^{a}	17.5 ± 1.4^{b}	15.5 ± 0.7^{a}	10.1 ± 0.5^{c}
Carbohydrate (%)	$10.3 \pm 1.2^{a,b}$	10.9 ± 0.3^{b}	9.0 ± 0.6^{a}	6.1 ± 0.4^{c}
Energy	1.16	0.95	0.94	0.98
P/E ratio	28.0	26.9	28.8	31.7

ATET: *Artemia* enriched with *T. suecica*; AT-ISO: enriched with T-ISO; AISO: enriched with *I. galbana*; ARHO: enriched with *R. lens*. Data are means ± S.D. (n=3). Different superscript letters within the same line and for the same day indicate statistical differences among treatments (α=0.05).

The enrichment of the digestive tract of *Artemia* juveniles can be clearly seen in figure 6, where the different colours exhibited are related with the corresponding filtered microalgal species.

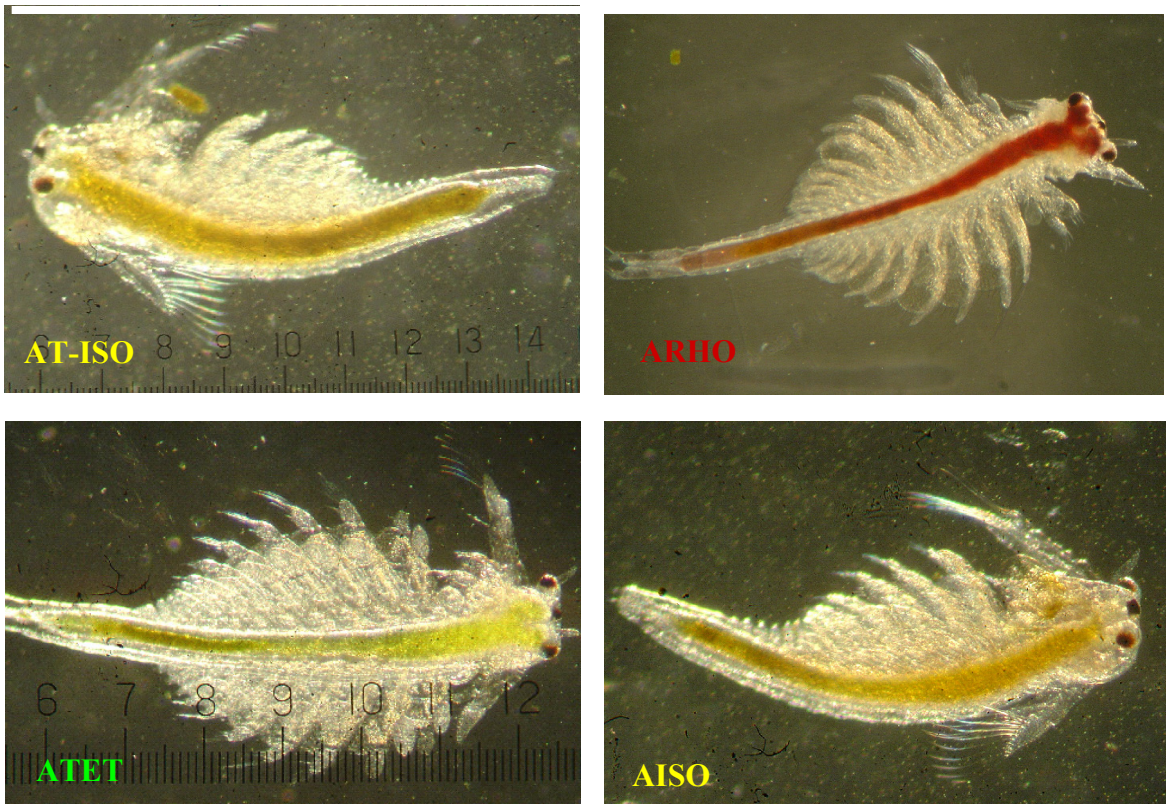

Figure 6 – Photographs of 5-day old juvenile *Artemia* (3.0-3.5 mm) enriched with different microalgal species under a stereoscope. Abbreviations of ATET, AT-ISO, AISO and ARHO are the same as in figure 5.

3.4 Fatty acid composition of *Artemia* juveniles, octopus hatchlings and spider-crab zoeae

The total FA composition of 3 and 5-day old juvenile *Artemia* reflected to a certain extent the FA composition of the ingested microalgae (see tables II and IV). In small juveniles (3-day old), palmitic acid (16:0) was the major FA found in groups ATET, AT-ISO and AISO (23-33%), whereas group ARHO had 18:3n-3 as main FA (27%). The sum of saturated FA found in small juveniles (40-48%) was similar to values found in *O. vulgaris* hatchlings (42%), except in juveniles from group ARHO which had a lower value (33%, Table IV). In these juveniles only group ARHO had similar percentages of total monoenes as in hatchlings (14.3%), whereas the other groups roughly doubled this percentage. The higher percentage of PUFAs found in group ARHO (50%) compared to the other groups was mainly due to the presence of 18:3n-3 in its composition (as observed in *R. lens*).

Arachidonic acid (ARA, 20:4n-6) was present in very low percentages in small juveniles (≤0.4%) compared to octopus hatchlings (3.2%). Important differences in the percentages of EPA and DHA were found among small *Artemia* juveniles. Individuals from groups AISO and ARHO had significantly higher values of EPA (nearly 9%) than groups ATET (4.3%) and AT-ISO (2.6%, Table IV). The highest percentage of DHA was found in groups AT-ISO and AISO (1.9% and 1.5%, respectively), followed by group ARHO (1.0%, $P<0.05$). No DHA was found in juveniles from group ATET. When comparing these values with the percentages of EPA and DHA found in *O. vulgaris* hatchlings remarkable differences could be observed. Octopus hatchlings contained 14.7% of EPA, 19.7% of DHA and 3.4% of ARA, whereas *Artemia* juveniles contained much lower percentages of DHA and ARA. The ratio DHA/EPA in small *Artemia* juveniles also reflected the imbalance that exists compared to octopus hatchlings. The amount of FA (expressed as % of dry weight) in *Artemia* juveniles (Table IV) was quite similar to values found in hatchlings (4.0%), except for group AT-ISO which had a higher value (6.6%).

Five-day old *Artemia* (large juveniles) showed some differences in their FA composition when compared with 3-day old juveniles (Table IV). The saturated FA 16:0 was clearly the major FA found in all *Artemia* groups, with values ranging between 30-34%. The percentage of PUFA found in groups ARHO and AT-ISO was lower than the values observed in small juveniles, mainly due to a drop in the percentage of 18:3n-3 found in large juveniles. In groups ATET and AISO the sum of PUFA remained roughly the same. Like observed for small *Artemia*, large juveniles contained only minor levels of ARA (≤0.3%) when compared to octopus hatchlings. The percentage of EPA in juveniles from group AISO (14.6%) was similar to values observed in octopus hatchlings, and unlike observed for small juveniles, individuals from group ARHO had a lower percentage of EPA (7%) in comparison with AISO ($P<0.001$). As previously observed for small juveniles, no DHA was found in juveniles from group ATET, whereas group AT-ISO contained the highest amount of DHA (3.9%) among groups ($P<0.05$). The DHA/EPA ratio in juveniles from group AT-ISO was the same as in octopus hatchlings, but it should be kept in mind that the levels of HUFAs were very different. The amount of FA (% of DW) in *Artemia* juveniles was higher in group ATET (5.6%) than in the remaining groups (3.3 to 4.2%, $P<0.05$). In comparison with octopus hatchlings juveniles from group ATET showed a slightly higher content whereas juveniles from ARHO showed a slightly lower content (Table IV).

Table IV. Fatty acid (FA) composition (% of total FA) and total FA (% of dry weight) of 3 and 5-day old juvenile *Artemia* enriched with different microalgal species produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures, and also of *Octopus vulgaris* hatchlings and *Maja brachydactyla* zoeae to establish comparisons of FA profiles.

	3-day old *Artemia* (1.5-2.0 mm)				*O. vulgaris* hatchlings	*Maja brachydactyla* zoeae (2.5 mm)
Fatty acid	ATET	AT-ISO	AISO	ARHO		
14:0	0.8 ± 0.1^{a}	11.9 ± 0.4^{b}	2.9 ± 1.0^{c}	1.4 ± 0.6^{a,c}	3.1 ± 0.3	1.0 ± 0.1
15:0	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.3	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
16:0	33.1 ± 0.4^{a}	23.4 ± 0.7^{b}	31.3 ± 2.3^{a}	21.6 ± 1.6^{b}	27.1± 1.3	17.8 ± 1.0
16:1n-9	1.8 ± 0.1^{a}	9.2 ± 0.4^{b}	12.4 ± 1.4^{c}	1.9 ± 0.1^{a}	0.1 ± 0.0	n.f.
16:1n-7	0.9 ± 0.1^{a}	0.1 ± 0.0^{b}	0.3 ± 0.0^{c}	1.3 ± 0.2^{d}	1.2 ± 0.3	1.8 ± 0.3
16:4n-3	2.5 ± 0.4	n.f.	n.f.	n.f.	n.f.	n.f.
18:0	8.2 ± 0.4^{a}	3.9 ± 0.4^{b}	13.0 ± 2.6^{c}	10.2 ± 1.1^{a,c}	12.3 ± 0.3	16.0 ± 1.1
18:1n-11	n.f.	n.f.	n.f.	n.f.	1.0 ± 0.1	n.f.
18:1n-9	17.8 ± 0.5^{a}	16.1 ± 0.4^{a}	10.8 ± 1.3^{b}	6.7 ± 0.4^{c}	3.4 ± 0.1	9.7 ± 0.3
18:1n-7	3.0 ± 0.4^{a}	3.5 ± 0.1^{a}	7.0 ± 0.6^{b}	6.4 ± 0.2^{b}	1.8 ± 0.2	7.6 ± 0.3
18:2n-6	2.9 ± 0.2^{a}	7.6 ± 0.1^{b}	2.3 ± 0.9^{a}	2.2 ± 0.1^{a}	0.7 ± 0.0	0.6 ± 0.0
18:3n-3	14.8 ± 0.2^{a}	12.6 ± 0.3^{b}	4.3 ± 0.8^{c}	26.7 ± 0.4^{d}	n.f.	n.f.
18:4n-3	7.5 ± 0.5^{a}	5.3 ± 0.1^{b}	2.6 ± 0.3^{c}	8.9 ± 0.8^{a}	n.f.	n.f.
20:1n-9	0.4 ± 0.0^{a}	0.1 ± 0.0^{b}	0.3 ± 0.1^{c}	0.2 ± 0.0^{c}	5.5 ± 0.6	0.8 ± 0.2
20:2n-6	0.3 ± 0.1^{a}	0.5 ± 0.0^{b}	0.5 ± 0.1^{b}	0.4 ± 0.1^{a,b}	n.f.	1.1 ± 0.2
20:4n-6	0.1 ± 0.0^{a}	0.0	0.0	0.4 ± 0.0^{b}	3.4 ± 0.1	7.8 ± 0.4
20:3n-3	n.f.	n.f.	n.f.	n.f.	1.4 ± 0.0	0.1 ± 0.1
20:4n-3	0.4 ± 0.0^{a}	0.3 ± 0.0^{a}	0.4 ± 0.2^{a}	1.0 ± 0.1^{b}	n.f.	0.2 ± 0.0
20:5n-3	4.3 ± 0.3^{a}	2.6 ± 0.1^{b}	9.3 ± 0.2^{c}	8.7 ± 0.9^{c}	14.7 ± 0.5	24.3 ± 0.4
22:1	0.2 ± 0.0^{a,b}	0.1 ± 0.0^{a}	0.4 ± 0.2^{b}	0.3 ± 0.0^{a,b}	1.4 ± 0.2	0.5 ± 0.1
22:5n-6	n.f.	0.4 ± 0.0^{a}	0.1 ± 0.0^{b}	n.f.	0.0	n.f
22:5n-3	n.f.	n.f.	n.f.	n.f.	1.5 ± 0.2	0.3 ± 0.0
22:6n-3	n.f.	1.9 ± 0.1^{a}	1.5 ± 0.3^{a}	1.0 ± 0.1^{b}	19.7 ± 1.6	8.7 ± 0.9
Others	0.1	0.0	0.0	0.0	0.0	0.3
Saturated	42.5 ± 0.1^{a}	39.5 ± 0.7^{b}	47.7 ± 4.2^{a}	33.5 ± 1.8^{c}	43.1 ± 0.9	36.1 ± 1.8
Monoenes	24.1 ± 0.6^{b}	29.0 ± 0.3^{a}	31.2 ± 2.6^{a}	16.8 ± 0.2^{c}	13.4 ± 0.2	20.5 ± 0.5
PUFA	30.3 ± 0.7^{b}	31.2 ± 0.5^{b}	21.0 ± 1.7^{c}	49.7 ± 1.6^{a}	43.6 ± 1.1	43.4 ± 1.5
n-3	29.5 ± 0.6^{b}	22.7 ± 0.5^{c}	18.1 ± 0.7^{d}	46.7 ± 1.5^{a}	37.3 ± 1.1	33.6 ± 1.0
n-6	3.3 ± 0.2^{b}	8.5 ± 0.1^{a}	2.9 ± 0.9^{b}	3.0 ± 0.1^{b}	6.2 ± 0.2	9.7 ± 0.6
DHA/EPA	0.0	0.7	0.2	0.1	1.3	0.4
Total FA (% DW)	3.9 ± 0.4^{b}	6.6 ± 0.1^{a}	3.7 ± 0.2^{b}	4.1 ± 0.8^{b}	4.0 ± 0.2	4.3 ± 0.3

Abbreviations of ATET, AISO, AT-ISO and ARHO are the same as in Table III. Data are means ± S.D. (n=3). n.f.: not found. Values referred as 0.0 are below 0.05. Different superscript letters within the same line indicate significant differences among *Artemia* groups (α=0.05).

Table IV. (continued)

	5-day old *Artemia* (3.0-3.5 mm)				*O. vulgaris* hatchlings	*Maja brachydactyla* zoeae (2.5 mm)
Fatty acid	ATET	AT-ISO	AISO	ARHO		
14:0	0.6 ± 0.1^{a}	16.0 ± 1.3^{b}	6.2 ± 1.1^{c}	2.7 ± 0.8^{d}	3.1 ± 0.3	1.0 ± 0.1
15:0	0.3 ± 0.0^{a}	0.6 ± 0.1^{b}	0.3 ± 0.0^{a}	0.5 ± 0.2^{a,b}	0.6 ± 0.1	0.6 ± 0.1
16:0	30.4 ± 1.4	33.5 ± 3.0	33.9 ± 3.1	34.5 ± 1.9	27.1± 1.3	17.8 ± 1.0
16:1n-9	1.3 ± 0.3^{a}	5.2 ± 1.1^{b}	15.7 ± 1.6^{c}	1.6 ± 0.3^{a}	0.1 ± 0.0	n.f.
16:1n-7	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	1.2 ± 0.2^{b}	1.2 ± 0.3	1.8 ± 0.3
16:4n-3	3.7 ± 1.0	n.f.	n.f.	n.f.	n.f.	n.f.
18:0	4.7 ± 1.2^{a}	5.0 ± 0.6^{a}	6.3 ± 1.3^{a}	10.3 ± 1.2^{b}	12.3 ± 0.3	16.0 ± 1.1
18:1n-11	n.f.	n.f.	n.f.	n.f.	1.0 ± 0.1	n.f.
18:1n-9	20.1 ± 1.1^{a}	13.3 ± 1.2^{b}	7.3 ± 1.7^{c}	10.2 ± 1.3^{b,c}	3.4 ± 0.1	9.7 ± 0.3
18:1n-7	5.9 ± 0.6^{a,c}	3.8 ± 0.1^{b}	5.2 ± 1.0^{b,c}	7.4 ± 0.6^{a}	1.8 ± 0.2	7.6 ± 0.3
18:2n-6	1.9 ± 0.1^{a}	3.0 ± 0.2^{b}	0.9 ± 0.1^{c}	1.0 ± 0.2^{c}	0.7 ± 0.0	0.6 ± 0.0
18:3n-3	15.8 ± 1.1^{a}	5.9 ± 0.7^{b}	2.6 ± 0.4^{c}	14.7 ± 1.1^{a}	n.f.	n.f.
18:4n-3	9.8 ± 1.0^{a}	4.3 ± 0.4^{b}	2.5 ± 0.3^{c}	4.5 ± 0.5^{b}	n.f.	n.f.
20:1n-9	0.4 ± 0.1^{a}	0.1 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	5.5 ± 0.6	0.8 ± 0.2
20:2n-6	0.2 ± 0.1^{a}	0.5 ± 0.1^{b}	0.3 ± 0.1^{a,b}	0.3 ± 0.0^{a}	n.f.	1.1 ± 0.2
20:4n-6	0.1 ± 0.0^{x}	0.0	0.0	0.3 ± 0.0^{b}	3.4 ± 0.1	7.8 ± 0.4
20:3n-3	n.f.	n.f.	n.f.	n.f.	1.4 ± 0.0	0.1 ± 0.1
20:4n-3	0.4 ± 0.1^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.6 ± 0.0^{a}	n.f.	0.2 ± 0.0
20:5n-3	3.9 ± 0.7^{c}	2.9 ± 0.5^{c}	14.6 ± 0.7^{a}	7.0 ± 0.9^{b}	14.7 ± 0.5	24.3 ± 0.4
22:1	0.1 ± 0.0^{a}	0.3 ± 0.0^{a,b}	0.3 ± 0.1^{b}	0.4 ± 0.1^{b}	1.4 ± 0.2	0.5 ± 0.1
22:5n-6	n.f.	0.7 ± 0.3^{a}	0.3 ± 0.0^{b}	n.f.	0.0	n.f
22:5n-3	n.f.	n.f.	n.f.	n.f.	1.5 ± 0.2	0.3 ± 0.0
22:6n-3	n.f.	3.9 ± 0.5^{a}	2.9 ± 0.2^{b}	1.9 ± 0.2^{c}	19.7 ± 1.6	8.7 ± 0.9
Others	0.0	0.0	0.0	0.3	0.0	0.3
Saturated	36.0 ± 0.2^{c}	55.1 ± 2.2^{a}	46.7 ± 3.0^{b}	48.0 ± 2.5^{b}	43.1 ± 0.9	36.1 ± 1.8
Monoenes	28.1 ± 1.9^{a}	23.0 ± 2.2^{b}	28.7 ± 1.4^{a}	21.0 ± 1.4^{b}	13.4 ± 0.2	20.5 ± 0.5
PUFA	32.1 ± 1.9^{a}	21.5 ± 2.2^{b}	24.3 ± 1.7^{b}	30.3 ± 2.4^{a}	43.6 ± 1.1	43.4 ± 1.5
n-3	33.6 ± 1.8^{a}	17.3 ± 2.0^{d}	22.8 ± 1.5^{c}	28.7 ± 2.5^{b}	37.3 ± 1.1	33.6 ± 1.0
n-6	2.2 ± 0.1^{b}	4.2 ± 0.5^{a}	1.5 ± 0.2^{c}	1.6 ± 0.1^{c}	6.2 ± 0.2	9.7 ± 0.6
DHA/EPA	0.0	1.3	0.2	0.3	1.3	0.4
Total FA (% DW)	5.6 ± 0.2^{a}	4.2 ± 0.5^{b}	4.0 ± 0.5^{b}	3.3 ± 0.7^{b}	4.0 ± 0.2	4.3 ± 0.3

Abbreviations of ATET, AISO, AT-ISO and ARHO are the same as in Table III. Data are means ± S.D. (n=3). n.f.: not found. Values referred as 0.0 are below 0.05. Different superscript letters within the same line mean significant differences among *Artemia* groups (α=0.05).

Regarding the FA profile of *M. brachydactyla* zoeae (Table IV), higher levels of EPA, DHA and ARA were found in its composition in comparison with *Artemia* juveniles. The levels of DHA (8.7%) and EPA (24.3%) were in general twice to four-times higher than in *Artemia* juveniles, whereas ARA was found in much higher amounts (7.8%) than in *Artemia*. Despite considerable differences in the FA profile of zoeae and of octopus hatchlings were also observed, it was clear that the content of HUFAs in zoeae was much higher than in *Artemia* juveniles. In addition, the sum of PUFAs and the relative proportions of n-3 and n-6 FA in zoeae were similar to values found in hatchlings. Regarding the FA profile of the enriched *Artemia* juveniles, we observed that when plotting the levels of EPA and DHA found in the enrichment-diet (i.e., microalgal species supplied) and in *Artemia* juveniles, positive linear correlations could be found for both 3-day old and 5-day old juveniles (Fig. 7).

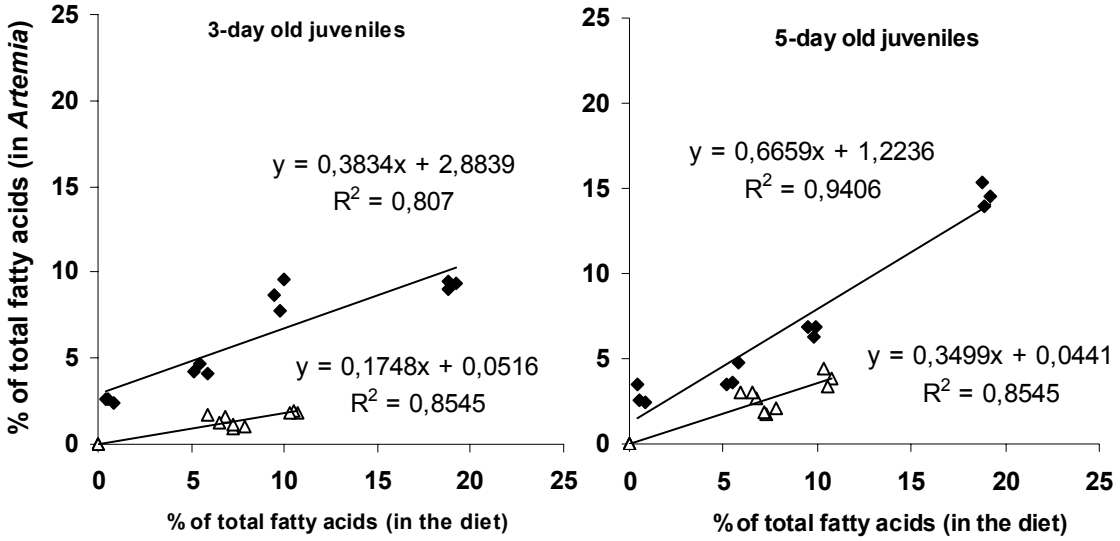

Figure 7 - Relationships between the percentages of the highly unsaturated fatty acids EPA (♦) and DHA (△) found in the enrichment diets and in 3-day old and 5-day old *Artemia* juveniles.

3.5 Total amino acid composition of *Artemia* juveniles

In this study we analysed the total amino acid composition of large *Artemia* juveniles (3.0-3.5 mm) to compare its composition with data from *O. vulgaris* hatchlings reported previously by Villanueva *et al*. (2004). Since a single analysis were carried out, it is risky to withdraw conclusions from these comparisons, but in a roughly way we observed that

Artemia juveniles from groups AISO and ARHO contained higher amounts of all essential amino acids (EAA) than paralarvae, except for lysine (Fig. 8). In contrast, *Artemia* juveniles from groups ATET or AT-ISO contained lower amounts of the EAA lysine, arginine, valine, leucine, isoleucine, threonine and histidine.

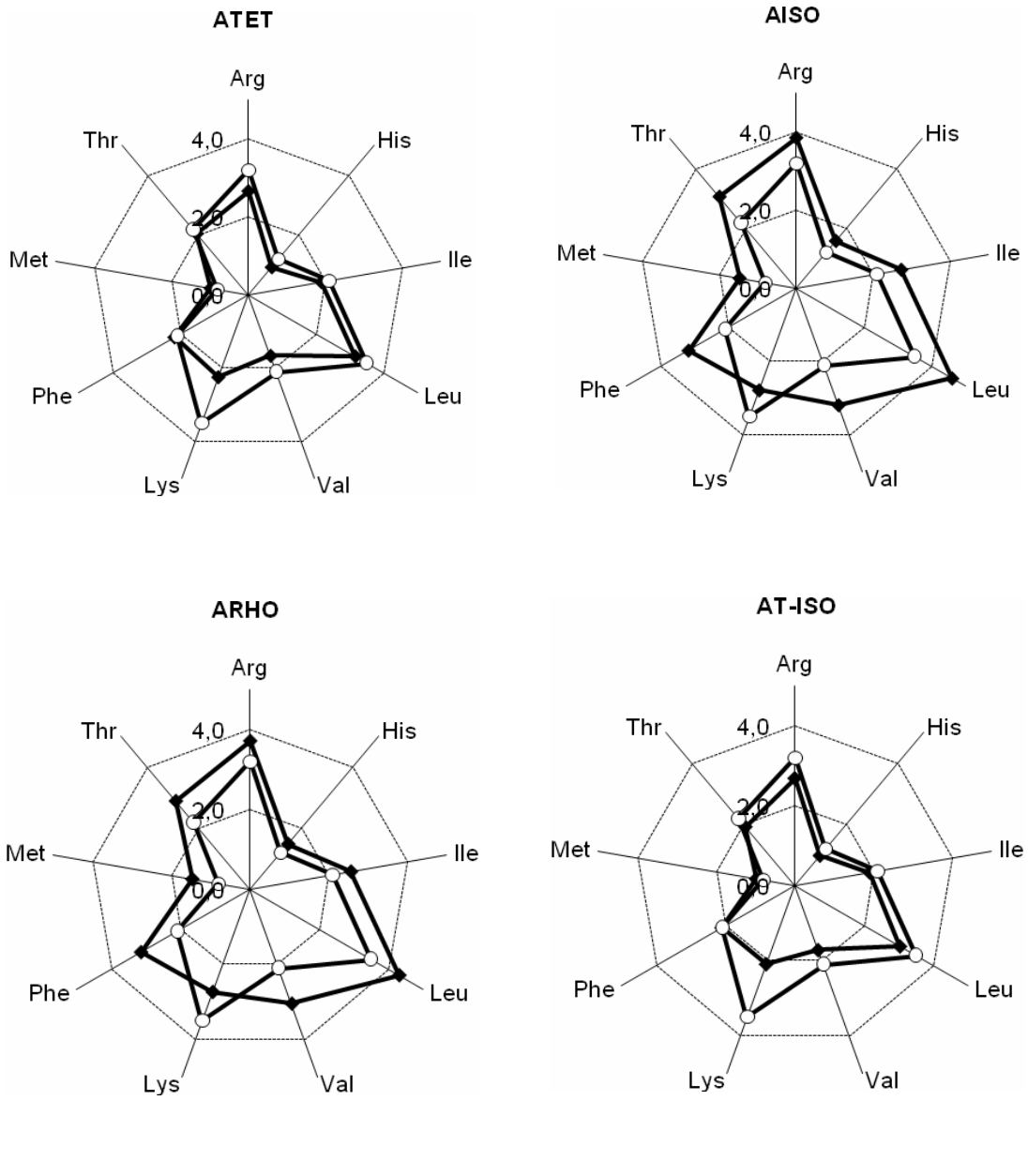

Figure 8 - Comparison of the total essential amino acid (EAA) composition (mg AA/100 mg dry weight) of juvenile *Artemia* (3.0-3.5 mm) enriched with different microalgal species and with the total EAA of *O. vulgaris* hatchlings. (*) EAA values of *O. vulgaris* hatchlings are from Villanueva *et al*. (2004).

Regarding the non esential amino acids (NEAA), only juveniles from ATET contained in broad terms less NEAA than octopus paralarvae, whereas the remaining juveniles showed similar results for almost all NEAA (Fig. 9).

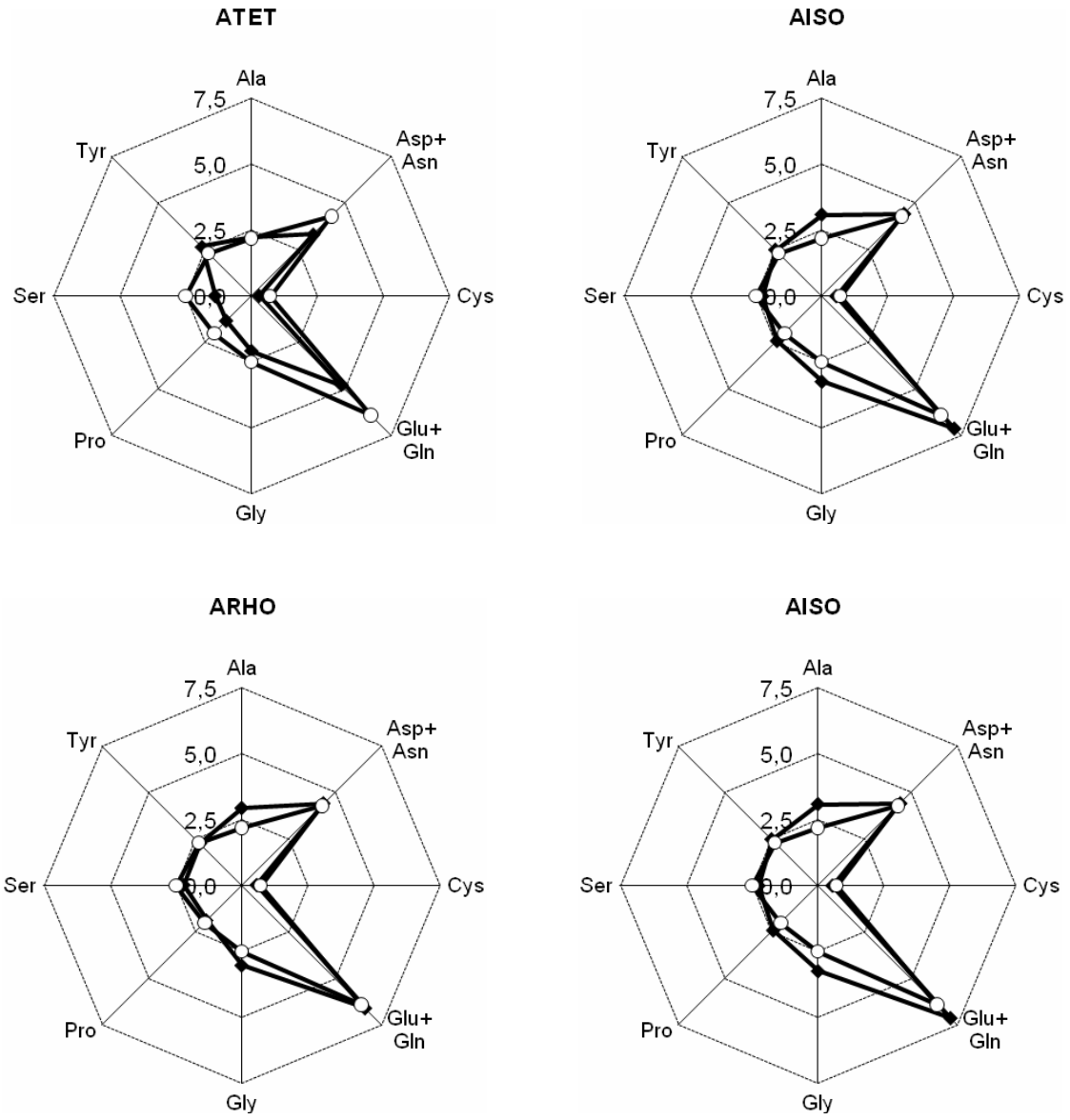

Figure 9 - Comparison of the total non essential amino acid (NEAA) composition (mg AA/100 mg dry weight) of *Artemia* juveniles (3.0-3.5 mm) enriched with different microalgal species and the total NEAA of *O. vulgaris* hatchlings. (*) NEAA values of *O. vulgaris* hatchlings are from Villanueva *et al*. (2004).

4. Discussion

The production of *Artemia* biomass depends to a great extent on the quantity and quality of the food supplied and on rearing conditions such as temperature among others factors (Sick, 1976; Dhont and Lavens, 1996). In the present work significant differences in the dry weight of 3 and 5-day old juveniles, and in the length of 3-day old juvenile *Artemia*, were observed with different microalgae with only 26 h of enrichment period. Lower growth of *Artemia* juveniles was observed in groups enriched with T-ISO or *I. galbana* when compared to those fed *T. suecica* or *R. lens*. T-ISO was already reported to produce lower growth when compared with *Chaetoceros* sp. for the feeding of *Artemia franciscana* (Lora-Vilchis *et al*., 2004). The total length (TL) and dry weight (DW) of 3 and 5-day old juveniles found in the present work were superior to values reported by Evjemo and Olsen (1999) for *A. franciscana* fed a monodiet of T-ISO: 1.3 mm TL and 3.5 µg individual^{-1} DW for 3-day old *Artemia*; and 2.4 mm TL and 15.9 µg individual^{-1} DW for 5-day old *Artemia*. Naegel (1999) obtained a TL of 1.97 mm for 6-day old *Artemia* fed *Chaetoceros* sp. at 25 ºC rearing temperature. Growth results in the same range than those reported in the present work were obtained by Lora-Vilchis *et al*. (2004) for *A. franciscana* fed *Chaetoceros* sp. or T-ISO, but cultures were maintained at a slightly higher temperature (27.5±0.5 ºC).

The concept of "enrichment" is generally associated with a period of exposure (e.g. 12-48 h) of *Artemia* nauplii to a certain diet that is supposed to modify principally its gut content. Due to the higher filtration capacity of *Artemia* juveniles, this type of enrichment can be achieved in much shorter periods (1-4 h) than for nauplii (Dhont and Lavens, 1996). Longer enrichment periods of juveniles will allow the incorporation of the diet compounds to the body tissue as well, producing a more stable enrichment, even though some degradation/conversion of essential nutrients can be produced (Navarro *et al*., 1999; Dhont and Lavens, 1996). In our case, as demonstrated by differences in the dry weight of the *Artemia* juveniles depending on the algal diet during the 26 h enrichment period, the "enrichment" process included both gut content and changes in the body tissues of the *Artemia*. A similar result was found for the enrichment of the rotifer *Brachionus plicatilis*, since changes in the body composition of rotifers enriched for 24 h were described, that could hardly be explained by the concept of rotifers acting as simple "carriers" of ingested microalgae (Ferreira *et al*., 2008).

Only limited information exists concerning the nutritional requirements of cephalopods early life stages, though it is generally recognized that diets provided to these fast-growing carnivoroes must be rich in protein and in essential amino acids (particularly lysine, leucine and arginine), and in phospholipids, cholesterol, and particularly in DHA and EPA, as well as in copper (Navarro and Villanueva, 2000, 2003; Villanueva *et al*., 2004; Villanueva and Bustamante, 2006). Protein percentage in *O. vulgaris* hatchlings and in small wild juveniles was found to be around 70% (Villanueva *et al*., 2004), which suggests the importance of high protein levels in the diets for these animals. In fact, the high requirement that cephalopods show for protein are due not only to their rapid growth along all life cycle (3-10% body weigh day^{-1}), but also because they mainly rely on amino acids as a source of energy, even for routine metabolism (Lee, 1994). This extraordinary capacity of growing is further explained by their high retention efficiency of protein (up to 90%) as shown for *O. vulgaris* (Houlihan *et al*., 1990).

In the present work the protein content observed in 3-day old *Artemia* juveniles was circa 51% (except AISO with 41%), which is less than the above mentioned 70% protein found in hatchlings and wild juvenile octopus. However, protein percentages found in 5-day old *Artemia* juveniles ranged between 64-68% and seem to be more in accordance with the needs of octopus hatchlings. Andrés *et al*. (2007) reported a protein content of 13-24% for *Maja brachydactyla* zoeae reared in laboratory, a prey that has given good results for the rearing of octopus paralarvae, but this low value derived from the use of a methodology specific for determination of soluble protein. When comparing the total AA composition of *O. vulgaris* hatchlings with that of *M. squinado* and *P. prideaux* zoeae and also with enriched *Artemia* nauplii, Villanueva *et al*. (2004) found few differences between *Artemia* nauplii and the zoeae of both decapod crabs. In addition, the EAA profiles of octopus hatchlings and of those preys revealed that enriched *Artemia* nauplii contained only lower levels of histidine in comparison with hatchlings, whereas *M. squinado* zoeae showed lower levels of more EAA. In this study, we found that lysine could be a limiting AA in *Artemia* juveniles, but this observation needs further studies based in more replicate analysis and an appropriate trial of paralarvae rearing, to evaluate the possible effects of lysine supplementation.

Three and 5-day old *Artemia* juveniles enriched with *R. lens* contained the lowest percentages of carbohydrate, reflecting the biochemical composition of the ingested microalga. Since carbohydrate is of minor importance for cephalopods and represents less than 1% of their composition (Lee, 1994), it may be important to minimize this source of

energy in diets for paralarvae to avoid halt food intake before the protein requirement is fulfilled. The content of lipid in 3-day old *Artemia* ranged between 10-16%, and similar values were found for 5-day old juveniles (10-17%). Lipid percentage in *O. vulgaris* hatchlings was found to represent nearly 13% of the dry weight (Navarro and Villanueva, 2000) and tended to decrease in small wild juveniles from roughly 12.5 to 6.6% as body weight increased (Navarro and Villanueva, 2003). Total lipid in *Artemia* juveniles was within the same range or in some cases superior to values described for octopus hatchlings. The enrichment of *Artemia* with T-ISO gave the highest percentage of lipid in both sizes of juveniles (16 to 17.5%), which may be in excess for paralarvae. In the remaining groups the percentage of lipid varied randomly between 10 and 16% in both sizes of *Artemia* juveniles. The optimum protein/energy ratio (P/E, mg protein KJ energy^{-1}) for fishes and aquatic crustaceans has been reported to be between 20 and 30 (reviewed by Lee, 1994), which is much higher than optimal values for homoeothermic vertebrates (10-15), but not as high as the best P/E ratio that promotes maximum growth of the cuttlefish *Sepia officinalis* (P/E=50), as stated by that author. The P/E ratios found in 3-day old juveniles ranged between 25 and 27, whereas in 5-day old juveniles it increased to 27-32 (being maximum in juveniles enriched with *R. lens*= 31.7), which are not as high as the best value reported for cuttlefish (50), but was shown to be influenced by the composition of the microalgal diet supplied to *Artemia*.

The FA composition of *O. vulgaris* hatchlings found in the present work was similar to data previously reported by other authors, even though some differences were found. The following percentages of the FA 16:0 (27%), EPA (14.7%) and DHA (19.7%) were observed in the present work, in comparison to the percentages described by Navarro and Villanueva (2000) (17.5, 12.6, and 21.2%, by same order of FA), or by Okumura *et al*. (2005) (18.6, 17.7, and 27.0%, respectively). As for *M. brachydactyla* zoeae, the percentages of EPA, DHA and ARA found in this study (24%, 9% and 8%, respectively), were in the same range, with the exception of DHA, than the values reported by Navarro and Villanueva (2000) for *Pagurus prideaux* zoeae and for the mysidacean shrimp *Acanthomysis longicornis* (15-22% of EPA, 18-24% of DHA and 1-5% of ARA), preys that were successfully used by Villanueva (1994) to feed octopus paralarvae until the benthic stage. Nevertheless, the sum of those HUFA was very similar among these preys.

The percentages of EPA and DHA found in 3 and in 5-day old juvenile *Artemia* enriched with *I. galbana* and *R. lens*, were similar or even higher than values described by Navarro and Villanueva (2000) for 1-3 mm *Artemia* enriched with commercial lipid emulsions

(EPA ≈ 7 to 8% and DHA ≈ 2%). Other authors found similar percentages of EPA (15%) and DHA (0.4%) in 1.6-1.8 mm *Artemia* juveniles enriched for 24 h with *Chaetoceros muelleri* (Ritar *et al*., 2004). Five-day old juveniles enriched with T-ISO contained the maximum percentage of DHA (3.9%) found in the present work, which is similar to values described by Ritar *et al*. (2003) in 1.5 mm and 2.5 mm *Artemia* enriched with the same microalgal species (1.2 to 3.4%). The evidence that both size of juvenile *Artemia* enriched with T-ISO contained such high percentage of EPA (circa 3%) compared to the percentage of EPA found in the correspondent microalgal ingested (0.7%) could be partially explained by the capacity of *Artemia* sp. to retroconvert DHA to EPA, as described by Navarro *et al*. (1999). Higher percentages of DHA and EPA have been obtained by enriching *Artemia* nauplii with commercial lipid emulsions or marine oils (Smith *et al*., 2002; Bell *et al*., 2003); however, these products are often quite low in protein and much more susceptible to peroxidation and rancidity during the enrichment period, in addition to generating high levels of contaminant bacteria in *Artemia* (McEvoy *et al*., 1995; Ritar el al., 2004). Moreover, the use of commercial lipid emulsions designed for nauplii for the enrichment of *Artemia* juveniles for 24 h caused high mortality (>90%, unpublished results), as also described by other authors (Ritar *et al*., 2004), indicating the need for the development of specific enrichment emulsions for this purpose. Enrichment of juvenile *Artemia* with those products should therefore be considered as useful to enrich only a proportion of the juvenile *Artemia* supplied to paralarvae, in order to provide the necessary amount of n-3 HUFA, but it is advisable to carry out short time enrichment periods (i.e.: 6-12 h) in order to avoid peroxidation processes (McEvoy *et al*., 1995) and also mortality of juvenile *Artemia*. Sargent *et al*. (1999) suggested the enrichment of nauplii for early feeding of marine fish larvae with highly purified preparations of DHA and EPA or the use of single cell triacylglycerol oils (e.g. from the heterotrophic dinoflagellate *Crypthecodinium cohnii*, or others) as sources of single HUFA, rather than using commercial fish oils which contain only moderate levels of n-3 HUFA.

In conclusion, the FA composition of juvenile *Artemia* enriched with microalgae was very different from the FA profile of *O. vulgaris* paralarvae, mainly due to the very low percentages of DHA and ARA, and to moderate levels of EPA, and might be unsufficient to cover paralarvae needs. However, since *Artemia* will continue to play an important role as prey in the rearing of octopus paralarvae, while efforts to develop inert micro-diets are done, the variability generated in the biochemical composition through the use of different microalgal species highlights the importance of optimising its biochemical composition, in

particularly the P/E ratio and its HUFA composition. Juveniles enriched with *R. lens* contained the best general composition as prey for paralarvae, due to high protein content, low carbohydrate and moderate lipid levels, as well as high levels of HUFA among the juveniles analysed. Yet, the highest sum of the HUFAs EPA and DHA was found in *Artemia* juveniles enriched with *I. galbana*. The identification of alternative live prey, the formulation of suitable and digestible micro-diets for octopus paralarvae and the improvement of the biochemical composition of *Artemia* have all been reported as key issues for the future development of *O. vulgaris* early life-stage rearing (Iglesias *et al*., 2007a), together with further studies on zootechnical conditions. The improvements obtained in the biochemical composition of *Artemia* through the use of selected microalgal species produced under controlled conditions will provide an understanding tool for the study of nutritional requirements of *O. vulgaris* paralarvae.

Growth and changes in the fatty acid composition of *Octopus vulgaris* paralarvae fed on different diets

Capítulo 2 / Chapter 2

Abstract

The rearing of *Octopus vulgaris* paralarvae during their planktonic stage is a major challenge in the culture of this species, as mortality is currently very high and unpredictable. The improvements of the biochemical composition of *Artemia* and the development of inert diets have been pointed out as key issues in paralarvae rearing. In this study we examined the survival and growth rates of *O. vulgaris* paralarvae, as well as its fatty acid composition, fed on three different dietary treatments: group ADHA was fed juvenile *Artemia* enriched with a commercial lipid emulsion (DHA-Selco®) rich in docosahexaenoic acid (DHA, 22:6n-3); group AR+I was fed juvenile *Artemia* enriched with a mixed diet of microalgae (70%:30% of *Rhodomonas lens* and *Isochrysis galbana*, respectively, in a dry weight basis) produced semi-continuously to achieve biomass of optimal and controlled composition; and group P+AR+I received the same *Artemia* as group AR+I complemented with artificial pellets. The survival rates of 15-days post hatch (-dph) paralarvae from groups AR+I (19±8%) and P+AR+I (17±4%) tended to be higher than in group ADHA (13±5%), though these differences were not statistically different. The increase in the dry weight (DW) from hatchlings to 15-dph paralarvae was almost 60% in groups AR+I and P+AR+I and nearly 40% in group ADHA. At day 10, significant differences in the DW of paralarvae could already be noticed, with individuals from groups AR+I and P+AR+I showing higher values than paralarvae from ADHA ($P<0.05$). However, significant differences in the DW of 15-dph paralarvae were only found between groups P+AR+I and ADHA, being higher in group P+AR+I ($P<0.05$), despite the trend of higher DW also observed in paralarvae from AR+I. Regarding the total length (TL) and mantle length (ML) of paralarvae, higher values of TL and ML were found in 10-dph and 15-dph paralarvae from groups AR+I and P+AR+I, in comparison with individuals from ADHA ($P<0.05$). Analysis of the fatty acid (FA) composition of paralarvae showed a remarkable drop of DHA from hatchlings (19.2% of total FA) to 15-dph paralarvae in all groups ($P<0.05$). However, paralarvae from group ADHA contained higher levels of DHA (12.5%) than paralarvae from groups AR+I and P+AR+I (both with circa 10%, $P<0.05$). As for eicosapentaenoic acid (EPA, 20:5n-3), the percentage found in hatchlings (14.3% of total FA) did not change with time in paralarvae from group ADHA (14.5% in 15-dph

paralarvae), but increased significantly in paralarvae from groups AR+I and P+AR+I (16.1% and 17.2%, respectively) ($P<0.05$). Even though paralarvae from group ADHA displayed a FA composition more closely related with the values found in hatchlings, the growth of these individuals was worst than paralarvae from groups AR+I and P+AR+I. Moreover, despite *Artemia* enriched with DHA-Selco® contained three-times more DHA than *Artemia* enriched with microalgae, we did not observe any clear positive effects over the growth and survival of paralarvae. Results also suggest that the protein:lipid ratio of the diet is an important factor to improve the growth of *O. vulgaris* paralarvae, but efforts to obtain an optimal balance of FA in *Artemia* should continue with the aim of avoiding FA deficiencies in octopus paralarvae.

1. Introduction

The rearing of *Octopus vulgaris* paralarvae during its planktonic stage is still a major bottleneck in the development of this species for aquaculture (reviewed by Iglesias *et al*., 2007a). As far as we know, Itami *et al*. (1963) were the first authors to report the success of *O. vulgaris* paralarvae rearing until the benthic stage, by using zoeae of the shrimp *Palaemon serrifer* as live prey. More recent works on which zoeae of decapod crustaceans alone, or in combination with enriched *Artemia* (1-4 mm) were used as food items, have also been successful, tough high mortality of paralarvae was still observed (Villanueva, 1994, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006). Yet, the constant supply of decapod zoeae to feed paralarvae is limited and uncertain (Navarro and Villanueva, 2000), as no control on the production of zoeae can be exerted and costs to obtain substantial quantities of those preys would hardly be realistic.

So far only one work has reported the rearing of *O. vulgaris* paralarvae until the benthic stage using *Artemia* as single live prey (Hamazaki *et al*., 1991). In contrast, previous works on which enriched *Artemia* alone (either nauplii or juveniles), or in co-feeding regimen with micropellets, has been used to rear *O. vulgaris* paralarvae, resulted in mass mortalities (Iglesias *et al*., 2000; Navarro and Villanueva, 2000; Villanueva *et al*., 2002). Other authors, combining enriched *Artemia* nauplii with frozen-flakes of pacific sandeel (*Ammodytes personatus*) sliced above the surface of tanks, improved the growth and development of paralarvae, but did not attained benthic juveniles (Okumura *et al*., 2005).

One of the main subjects of study in octopus paralarval rearing is related with the influence that the FA composition of the diets has on the FA composition of paralarvae (Navarro and Villanueva, 2000, 2003; Okumura *et al*., 2005). When taking a first insight of the lipid composition of *O. vulgaris* hatchlings (i.e. the main lipid classes and FA profiles), Navarro and Villanueva (2000) found that phospholipids and cholesterol accounted for, respectively, nearly 50% and 25% of the total lipids, whereas the main FA were the polyunsaturated fatty acids DHA and EPA (21% and 13% of the total FA, respectively) and the saturated palmitic acid (18%). In later studies of octopus paralarvae rearing, Navarro and Villanueva (2003) have shown clear changes in the lipid and FA composition of paralarvae fed enriched *Artemia* nauplii alone or complemented with micropellets, in comparison with the lipid composition of *O. vulgaris* early life stages, leading these

authors to suggest that the high mortalities of paralarvae could be related with imbalances in the lipid composition of the diets, especially in the FA profile. The essentiality of n-3 HUFA in diets for crustacean and marine fish larvae to promote a correct development is well known (Coutteau *et al*., 1997; Sargent *et al*., 1999; Tocher *et al*., 2008). Several works have reported poor growth or related deficiencies associated with qualitative lipid imbalances in diets for marine-fish larvae such as gilthead seabream (*Sparus aurata*), red seabream (*Pagrus major*), turbot (*Scophtalmus maximus*) and Senegalese sole (*Solea senegalensis*) (Mourente *et al*., 1993; Watanabe, 1993; Rodríguez *et al*., 1994; Estévez *et al*., 1999; Morais *et al*., 2005; Izquierdo, 2006).

Despite the lipid composition of live food and its effects in the reared paralarvae has been the main subject of research, another feature that recently received attention was the total amino acid composition and protein content of *O. vulgaris* early life stages (Villanueva *et al*., 2004).

The aim of this study was to test the effects of feeding *O. vulgaris* paralarvae with *Artemia* juveniles either enriched with a commercial lipid emulsion rich in DHA (DHA-Selco®) or with microalgae of optimal and controlled biochemical composition. From previous data related with the improvement of *Artemia* composition, using different microalgal species cultured semi-continuously, in order to ensure biomass of constant and controlled composition (chapter 1, Seixas *et al*., 2008), a mixed diet of two microalgae (70% *Rhodomonas lens* and 30% *Isochrysis galbana*) was chosen to enrich *Artemia* juveniles. *R. lens* was selected due to its very high protein content (62%) in comparison with the remaining species tested (42-44%), and moderate levels of EPA (10%) and DHA (7%), whereas *I. galbana* was chosen because of its very high levels of EPA (19%) and moderate of DHA (6%). In addition, it was observed that the *Artemia* juveniles enriched with *R. lens* contained the best general composition (highest protein/energy ratio and moderate levels of EPA and DHA) to meet the possible nutrient needs of octopus paralarvae, while *Artemia* juveniles enriched with *I. galbana* (in particular the large-size) had the highest sum of the fatty acids EPA and DHA (see table IV of chapter 1). The proportion of each microalga (70%:30% of *R. lens* and *I. galbana*, respectively) was decided taking into account that protein is the major constituent of octopus hatchlings (70% of its DW) and thus this fraction should be maximized.

In this work, artificial pellets specifically formulated for *O. vulgaris* paralarvae, based on the whole body composition of octopus hatchlings and wild juveniles (reported by Navarro and Villanueva, 2000; Villanueva *et al*., 2004), were also tested in co-feeding regime with

the *Artemia* juveniles. The effects of the different dietary treatments on the survival and growth rates of *O. vulgaris* paralarvae, as well as on its FA composition, were assessed.

2. Material and methods

2.1 Formulation of artificial pellets for paralarvae

Dry pellets were specifically formulated to feed one of the groups of paralarvae as a complement of juvenile *Artemia* enriched with microalgae (AR+I). The ingredients used for the preparation of the artificial pellets are shown in table I. Pellets had a size of approximately 1 mm and were produced by Aphytec (France). Biochemical composition analyses of the artificial pellets are shown in the section of results (Tables II and III).

Table I. Ingredients used in the formulation of the artificial pellets for paralarvae, taking into account the whole body composition of early life stages of *Octopus vulgaris*.

Ingredients	For 1 kg diet (in g)
Fish meal Norvik 70	130
CPSP G[a]	301.5
Squid meal	400
Yellow pea	100
Fish oil (36% n-3)	50
Choline chloride	1
Lutavit C35	0.5
Lutavit E50	1
Mineral and vitamin mix	5
EYPL[b] 85%	10
Bethaine	1
TOTAL	1000

[a] Fish soluble protein concentrate (Sopropèche)
[b] Egg Yolk Phospholipids

2.2 Growth and enrichment of *Artemia* juveniles

Non-axenic cultures of *Rhodomonas lens* and *Isochrysis galbana* were carried out in 6-l glass flasks of flat bottom (Fig. 1) in semi-continuous regimen. Nutrients were added at a final concentration of 2 mM in *I. galbana* culture and of 4mM $NaNO_3$ in *R. lens* culture, to ensure nutrient saturation conditions, which was confirmed by determination of the NO_3^{-2} concentration (Clesceri *et al*., 1989) present in the harvested cultures. The higher requirement for nutrients that *R. lens* shows to attain saturation conditions is probably related to the accumulation of the pigment phycoerythrin, responsible for the redish color of this alga (for further details see Seixas *et al*., submitted, Annex I). Cultures were daily renewed at 30% of the volume of the cultures, which ensured biomass of constant and controlled composition, and the daily harvested biomass was used for the on-growing and enrichment processes of *Artemia* juveniles.

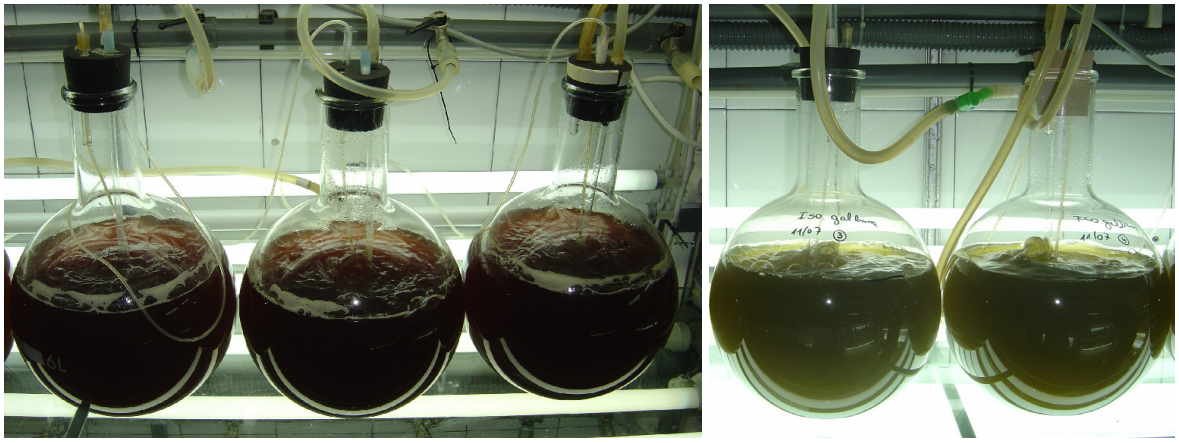

Figure 1 - Culture of the microalgal species *Rhodomonas lens* (on the left) and *Isochrysis galbana* (on the right) in semi-continuous regimen in 6-l glass flasks, with a daily renewal rate of 30% of the volume of cultures.

Newly hatched *Artemia* nauplii (AF, INVE, Belgium) were initially grown with *R. lens* in 12-L plastic tanks and water temperature of 26.5 ± 0.5 ºC (Fig. 2). This microalga was chosen for the on-growing of nauplii until the juvenile stage, since it clearly gave the best results of *Artemia* growth in comparison with other species such as *Tetraselmis suecica*, *I. galbana* and *Nannochloropsis gaditana* (Seixas *et al*., submitted, Annex I). In that study, we also observed that *N. gaditana* is ineffective for the on-growing of *Artemia* (individuals

reach a size of 1.5±0.2 mm after 8 days, in comparison with 4.9±0.6 mm in group fed *R. lens*), and the survival rate was also much lower (18±3% at day 8) in comparison with the remaining microalgal diets (69 to 88%). The main reason for these findings is probably associated with the difficult digestion of this microalga, which is known to contain a very thick cell-wall (for more details see Annex I, Seixas *et al*., submitted).

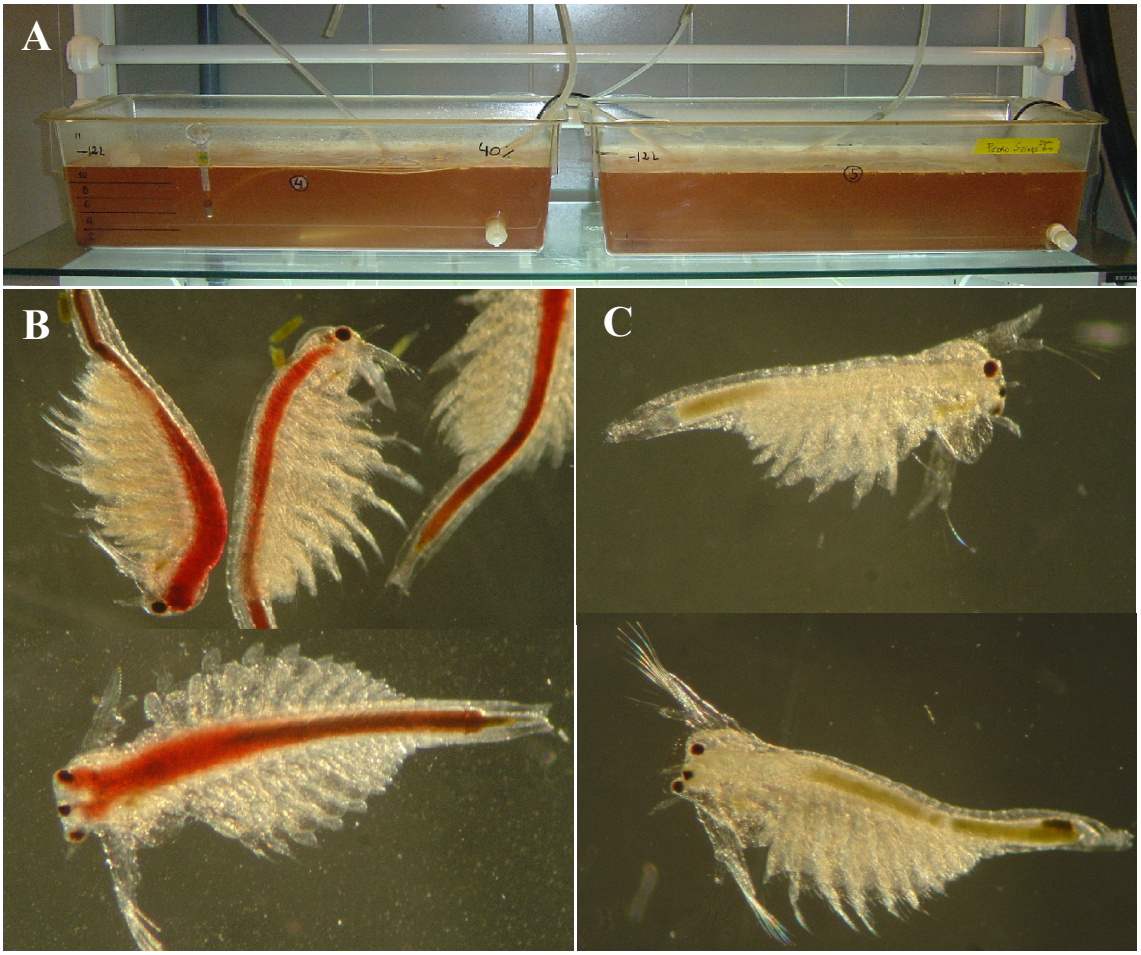

Figure 2 - (A) Growth of *Artemia* nauplii with *Rhodomonas lens* in 12-L plastic tanks, at a temperature of 26.5±0.5 ºC, to achieve juvenile stages. *Artemia* juveniles (1.5-2.3 mm) enriched with: (B) either a mixed diet of *Rhodomonas lens* and *Isochrysis galbana* - group AR+I; or (C) with DHA-Selco® (INVE) - group ADHA.

The two-day old *Artemia* grown with *R. lens* were then enriched for further 4 h or 24 h, to feed octopus paralarvae, with one of the following diets: either DHA-Selco® (INVE, Belgium) at half of the concentration recommended by the manufacturer (group ADHA), since the use of a higher concentration produced high mortality (nearly 90%) of *Artemia*

juveniles (as mentioned in chapter 1, data not shown); or with the mixed diet of *R. lens* and *I. galbana* (70%:30%) - group AR+I. Figure 2 shows the on-growing conditions of *Artemia* nauplii, and the appearance of the digestive tracts of juveniles enriched with either DHA-Selco® or microalgae. The length of *Artemia* juveniles was measured under a stereoscope using a calibrated ocular micrometer (n=40). Samples of juveniles (70 individuals per sample) were individually counted, washed with distilled water and immediately frozen at -18 ºC for later biochemical analysis.

2.3 Experiment of *Octopus vulgaris* paralarvae rearing

Newly hatched paralarvae from an egg mass of *O. vulgaris* kept in a closed water circuit in the facilities of the University of Santiago de Compostela (Spain), were individually counted and transferred to 50-l conical fibre glass tanks, with 50 cm diameter and white walls (Fig. 3). Illumination was provided by day-light lamps placed 40 cm above the water surface, establishing a photoperiod of 18 h light:6 h dark. Tanks were provided with gentle aeration, and 25% of the water was renewed every 3 days. Before entering the tanks, seawater (salinity of 34 ppt) was filtered through 50 μm cartridge filters and disinfected with UV lamps. Water temperature was kept constant between 17.0 and 18.0 ºC in a stable climatized room. Paralarvae density was established at 25 individuals l^{-1} and food was provided since the very first day. Three dietary treatments, each in triplicate, were tested: group ADHA was fed juvenile *Artemia* enriched with DHA-Selco®; group AR+I was fed juvenile *Artemia* enriched with the mixed diet of microalgae (as described above in section 2.2); and group P+AR+I was fed the same amount of *Artemia* as group AR+I plus artificial pellets of 1 mm, which were distributed automatically for 15 min, each 3 h, during the light period (3 g of pellets day^{-1}). *Artemia* juveniles were supplied in two meals, at 9:30 a.m. and 03:00 p.m., after being enriched for 24 h or for 4 h, respectively, and the total daily ration was established at 0.1 *Artemia* mL^{-1} day^{-1}.

Octopus paralarvae were measured under a stereoscope using a calibrated ocular micrometer (30 hatchlings and 20 paralarvae per replicate on days 10 and 15). Total length and mantle length were measured as described by Villanueva (1995). Dry weight (DW) of paralarvae was determined by weighing samples of 10 paralarvae (n=5 per replicate) after washing individuals with distilled water and drying them in a stove at 101±1 ºC for 24 h. Specific growth rate (SGR, % day^{-1}) was calculated as follows: $100 \times [(\ln DW_2 - \ln DW_1)/t_2$-

t_1], where DW_2 and DW_1 represent the DW of paralarvae at sampling days t_2 and t_1, and ln the natural logarithm. Samples of hatchlings and of 10-dph and 15-dph paralarvae (70 to 90 individuals) were collected and immediately frozen at -18 ºC for later biochemical composition analyses (total lipid and fatty acid profiles).

Figure 3 – Conical fiber-glass tanks with 50-l volume used for the rearing experiments of *Octopus vulgaris* paralarvae. Tanks were established in closed systems without recirculation, but water was renewed at 25% of the total volume every 3 days. Gentle aeration was provided through air-stones and light was placed 40 cm above the water surface.

2.4 Biochemical composition analysis

Protein content was determined by the Folin-phenol method (Lowry *et al*., 1951), after hydrolysis with 1.0 M Na OH at 95 ºC for 1 h, whereas carbohydrate was determined by the phenol/sulphuric acid method (Kochert, 1978). Total lipid was determined gravimetrically after extraction of lipids with chloroform/methanol (2:1 v/v) according to Bligh and Dyer (1959). The fatty acid composition of *Artemia* juveniles and paralarvae was determined by submitting lipid extracts to methanolysis (5% HCl in methanol) at 85 ºC during 2.5 h (Sato and Murata, 1988), followed by extraction of methyl esters with hexane, and analysed in a GC-MS (Fisons Instruments, MD-800) using a column Omegawax 250 (Supelco) 30 m x 0.25 mm using helium as gas carrier. Triheptadecanoin (Sigma, St. Louis, Mo.) was used as internal standard. All biochemical analyses were carried out in triplicate.

2.5 Statistical analysis

Statistical analyses were carried out with the software SPSS V 14.0.1 (SPSS, Inc.). Total length and mantle length of paralarvae were compared by analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05. After log-transformation of dry weight data and arcsine-√ transformation of survival and biochemical composition percentages, the same statistical tests were carried out (Zar, 1999). Statistical comparisons of dietary P:L ratios and of SGR among groups were carried out by the non-parametric test of Kruskal-Wallis (Zar, 1999).

3. Results

3.1 Biochemical composition of the diets

The gross biochemical composition (% of DW) of *Artemia* juveniles and artificial pellets supplied to paralarvae is shown in Table II. No statistically significant differences were found in the protein content of the enriched *Artemia* juveniles, though individuals from AR+I had a slightly higher value than juveniles from ADHA. Pellets contained the highest protein content (62%) of all diets ($P<0.001$).

Table II. Gross biochemical composition (% of dry weight) of *Artemia* juveniles (1.5-2.3 mm) and of the artificial pellets used to feed *Octopus vulgaris* paralarvae. Composition data are expressed as % of dry weight. Values of the protein:lipid ratio and protein:energy ratio (g protein MJ energy^{-1}) found in the diets are also shown.

	Juvenile *Artemia* (1.5-2.3 mm)		
	ADHA	AR+I	Artificial Pellets
	Mean ± S.D.	Mean ± S.D.	
Protein (%)*	46.2 ± 4.7^{b}	50.7 ± 1.7^{b}	62.5 ± 2.9^{a}
Lipid (%)*	22.4 ± 1.6^{a}	12.6 ± 0.9^{c}	16.4 ± 1.0^{b}
Carbohydrate (%)*	5.8 ± 1.1^{b}	8.9 ± 0.2^{a}	6.9 ± 0.0^{b}
P:L ratio**	2.1 ± 0.2^{a}	4.0 ± 0.4^{b}	3.8 ± 0.2^{b}
P:E ratio**	22.2 ± 0.8^{b}	27.4 ± 0.8^{a}	27.9 ± 0.4^{a}

ADHA: *Artemia* enriched with DHA-Selco®; AR+I: *Artemia* enriched *Rhodomonas lens* and *Isochrysis galbana*. Different superscript letters within the same line indicate significant differences. *ANOVA followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons (α=0.05); ** Non-parametric test of Kruskal-Wallis ($P<0.05$).

Lipid levels were considerably higher in *Artemia* juveniles from ADHA (22%) than in pellets (16%) or in juveniles from AR+I (13%, P<0.001). Carbohydrate content in the diets increased in the sense: *Artemia* from ADHA < artificial pellets < *Artemia* from AR+I (P<0.01) (Table II). The protein:energy (P:E) and protein:lipid (P:L) ratios were found to be higher in *Artemia* juveniles from group AR+I and in pellets than in ADHA (Table II). The fatty acid (FA) composition of *Artemia* juveniles revealed important differences between groups and also in comparison with the artificial pellets (Table III).

Table III. Fatty acid (FA) composition (% of total FA) and total FA (% of DW) of juvenile *Artemia* enriched with the different diets and of the artificial pellets used to feed *O. vulgaris* paralarvae.

	Juvenile *Artemia* (1.5-2.3 mm)		
Fatty acid	ADHA	AR+I	Artificial pellets
14:0	2.8 ± 0.3^{b}	2.1 ± 0.2^{c}	6.2 ± 0.4^{a}
15:0	0.7 ± 0.0^{a}	0.4 ± 0.0^{c}	0.6 ± 0.0^{b}
16:0	18.0 ± 1.0^{a}	15.2 ± 1.0^{b}	18.9 ± 0.9^{a}
16:1n-7	6.2 ± 0.8^{b}	2.8 ± 0.6^{c}	8.8 ± 0.1^{a}
16:4n-3	n.f.	n.f.	1.1 ± 0.1
18:0	8.8 ± 0.4^{b}	11.9 ± 1.0^{a}	5.1 ± 0.3^{c}
18:1n-9	16.7 ± 1.8^{a}	3.2 ± 0.1^{b}	16.2 ± 1.7^{a}
18:1n-7	6.3 ± 0.4^{b}	9.0 ± 1.1^{a}	3.6 ± 0.4^{c}
18:2n-6	6.3 ± 0.3^{a}	1.7 ± 0.1^{c}	2.8 ± 0.1^{b}
18:3n-6	n.f.	n.f.	0.9 ± 0.1
18:3n-3	7.3 ± 1.9^{b}	18.6 ± 1.9^{a}	1.7 ± 0.1^{c}
18:4n-3	4.4 ± 1.5^{c}	14.3 ± 1.8^{a}	7.8 ± 0.6^{b}
20:1n-9	1.6 ± 0.3^{a}	0.5 ± 0.1^{b}	0.3 ± 0.0^{c}
20:4n-6	1.3 ± 0.3^{a}	0.4 ± 0.1^{b}	0.9 ± 0.0^{a}
20:3n-3	0.4 ± 0.1^{b}	0.9 ± 0.1^{a}	n.f.
20:4n-3	1.2 ± 0.3^{b}	2.6 ± 0.2^{a}	0.6 ± 0.0^{c}
20:5n-3	9.8 ± 0.7^{b}	13.0 ± 0.8^{a}	9.6 ± 0.2^{b}
22:1	0.8 ± 0.4^{b}	0.7 ± 0.1^{b}	4.0 ± 0.3^{a}
22:5n-3	n.f.	n.f.	1.2 ± 0.0
22:6n-3	6.7 ± 0.6^{b}	2.0 ± 0.3^{c}	9.0 ± 0.1^{a}
∑ Saturated	33.1 ± 3.3	29.5 ± 2.2	30.8 ± 1.5
∑ Monoenes	30.9 ± 2.3^{a}	16.9 ± 2.2^{b}	32.9 ± 1.8^{a}
∑ PUFA	36.0 ± 5.6^{b}	53.5 ± 4.4^{a}	35.6 ± 0.6^{a}
∑ n-3	29.4 ± 4.8^{b}	51.5 ± 4.2^{a}	30.9 ± 0.6^{b}
∑ n-6	6.6 ± 0.8^{a}	2.1 ± 0.1^{c}	4.6 ± 0.2^{b}
DHA/EPA	0.7	0.2	0.9
Total FA	9.9 ± 1.4^{a}	6.9 ± 0.8^{b}	8.8 ± 0.4^{a}

Data are means ± S.D. of triplicate analyses. Different superscript letters within the same line indicate significant differences among the diets (P<0.05).

Similar levels of total saturated FA, monoenes and polyunsaturated fatty acids (PUFAs) were found in *Artemia* from ADHA and pellets, whereas juveniles from AR+I contained higher levels of total PUFA, at the expense of a decrease in monoenes. The higher content of PUFA in *Artemia* from AR+I was mainly due to the high percentages of 18:3n-3 and 18:4n-3 found in this group. Maximum DHA levels were found in pellets (9%), being lower in *Artemia* juveniles from ADHA (6.8%) and finally in juveniles from AR+I (2%, $P<0.001$). The highest value of EPA was found in juveniles from AR+I (13%, $P<0.001$), whereas maximum arachidonic acid (20:4n-6) value was found in juveniles from ADHA (1.3%, $P<0.05$). As found for the lipid levels, the highest amount of FA (% of DW) was found in juveniles from ADHA, followed by the artificial pellets and finally in juveniles from AR+I (Table III).

3.2 Survival, growth and fatty acid composition of *O. vulgaris* paralarvae

After 15 days of rearing, a tendency for higher survival rates of paralarvae from groups AR+I and P+AR+I (20% and 17%, respectively), was observed in comparison with group ADHA (13%), though these differences were not statistically significant. Paralarvae were found to grow regularly along the experiment, increasing in both DW and size (Table IV). Results showed that 15-dph paralarvae from groups AR+I and P+AR+I increased their DW by almost 60% with respect to the initial DW, whereas the increase in paralarvae from group ADHA was nearly 40%. The higher DW of paralarvae from groups AR+I and P+AR+I, in comparison with individuals from ADHA ($P<0.01$), could already be observed at day 10 (Table IV). The same trend for higher DW in paralarvae from groups AR+I and P+AR+I remained unchanged at day 15, though statistical analysis revealed no significant differences in the DW of paralarvae between groups AR+I and ADHA ($P=0.080$). Regarding the total length (TL) and mantle length (ML) of paralarvae, a general tendency for better TL and ML in paralarvae fed microalgae-enriched *Artemia* alone or complemented with pellets was observed, in comparison with individuals being fed juveniles enriched with DHA-Selco® (Table IV). The SGR of paralarvae from the different groups is shown in table IV. Despite the clear tendency for higher values in groups AR+I and P+AR+I, in comparison with ADHA, significant differences were only found at day 10 of rearing ($P\leq0.05$).

Paralarvae were seen to actively attack *Artemia* juveniles as soon as they were supplied to tanks, especially in the first ten minutes after being distributed. Competition for the same prey was sometimes observed between two paralarvae. In contrast, the ingestion of artificial pellets was not observed during the distribution period, mainly due to their fast sinking. Even if paralarvae were seen to display pursuing behaviour toward the pellets, they eventually abandoned swimming as the speed of pellets going down was faster. Once pellets touched the bottom of the tanks, paralarvae were no more interested in catching them. However, pellets were left in the bottom of tanks until the next day, when cleaning of the tanks by siphoning was carried out, and therefore some nutrient leaching into the water could possibly happen.

Regarding paralarvae composition, the initial lipid content in hatchlings was almost 12%, decreasing slightly with time in paralarvae from groups AR+I and P+AR+I (Table V). An inverse tendency was observed in paralarvae from group ADHA, with lipids increasing slightly in 10-dph and 15-dph paralarvae. In 15-dph paralarvae, the lipid content of individuals from ADHA was significantly higher than in groups AR+I and P+AR+I ($P<0.05$).

The saturated palmitic acid 16:0 was the major FA found in hatchlings (Table V), representing 28% of the total FA, followed by DHA (19.5%) and EPA (14.5%). The sum of saturated FA and of PUFA was very similar (nearly 43% each), whereas monoenes accounted for nearly 13% of the total FA. In general, the changes observed in the FA composition of the reared paralarvae, with respect to the FA composition of hatchlings, reflected the FA composition of the ingested diet (Table V). The percentage of DHA dropped in 10-dph paralarvae from all groups, whereas EPA remained stable in group ADHA and increased slightly in groups AR+I and P+AR+I. After 15 days of rearing, the levels of DHA decreased further in all groups, but paralarvae from group ADHA evidenced an higher percentage of DHA (12.6%) than paralarvae from groups AR+I and P+AR+I (9.9-10.6%, $P<0.05$). A significant drop in the ratio DHA/EPA was observed from hatchlings (1.3) to 10-dph paralarvae (0.9-1.0), decreasing further in 15-dph paralarvae. However, this decrease was less evident in paralarvae from ADHA than in paralarvae from the remaining groups (Table V). The levels of arachidonic acid (ARA, 20:4n-6) remained similar to initial values in group ADHA, decreasing in groups AR+I and P+AR+I. The sum of monounsaturated FA increased in paralarvae from all groups at the expense of slight decreases of both saturated FA and PUFA.

Table IV. Survival (%), total length (TL), dorsal mantle length (ML), dry weight (DW) and specific growth rate (SGR x 100, % day^{-1}) of *Octopus vulgaris* paralarvae fed on three different diets.

		10-dph paralarvae			15-dph paralarvae		
	Initial	ADHA	AR+I	P+AR+I	ADHA	AR+I	P+AR+I
Survival (%)*		49.3 ± 2.6	59.4 ± 9.7	51.5 ± 7.3	13.3 ± 4.7	19.7 ± 7.5	17.3 ± 4.0
TL (mm)*	3.00 ± 0.13	3.20 ± 0.13^{a}	3.30 ± 0.16^{b}	3.40 ± 0.16^{c}	3.40 ± 0.13^{x}	3.57 ± 0.13^{y}	3.61 ± 0.22^{y}
ML (mm)*	1.97 ± 0.10	2.13 ± 0.09^{a}	2.20 ± 0.12^{b}	2.23 ± 0.14^{b}	2.24 ± 0.13^{x}	2.28 ± 0.11^{x,y}	2.34 ± 0.13^{y}
DW (µg ind^{-1})*	317.5 ± 20.7	388.5 ± 28.8^{a}	423.7 ± 28.7^{b}	430.9 ± 35.2^{b}	441.7 ± 63.9^{x}	497.2 ± 64.5^{x,y}	504.5 ± 79.7^{y}
SGR**		2.2 ± 0.1^{a}	3.2 ± 0.2^{b}	3.4 ± 0.4^{b}	2.5 ± 1.6	3.2 ± 0.7	3.3 ± 0.2

ADHA: group fed juvenile *Artemia* enriched with DHA-Selco® (INVE); AR+I: group fed juvenile *Artemia* enriched with a mixed diet of *R. lens* and *I. galbana*; P+AR+I: group fed the same *Artemia* as group AR+I plus artificial pellets of 1 mm. –dph: days post hatch. Data are means ± S.D. Different superscript letters within the same day among groups indicate significant differences. *ANOVA followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons (α=0.05); **Non-parametric test of Kruskal-Wallis (P≤0.05).

Table V. Fatty acid composition (% of total FA), fatty acid content (% of the dry weigth) and total lipid (% of DW) of *Octopus vulgaris* hatchlings and of 10-dph and 15-dph paralarvae from the three different dietary treatments (ADHA, AR+I and P+AR+I).

		10-dph paralarvae			15-dph paralarvae		
Fatty acid	Hatchlings	ADHA	AR+I	P+AR+I	ADHA	AR+I	P+AR+I
14:0	3.1±0.3	1.5±0.2	1.7±0.1	1.9±0.2	1.5±0.1	1.4±0.0	1.5±0.3
15:0	0.6±0.0	0.5±0.0^{a}	0.4±0.0^{b}	0.5±0.0^{a,b}	0.3±0.1	0.3±0.0	0.4±0.0
16:0	28.0±1.0	25.2±0.3	25.7±0.8	26.5±0.5	23.1±1.8	23.0±0.7	22.8±1.1
16:1n-7	1.2±0.3	2.1±0.1^{a}	1.3±0.1^{b}	1.4±0.4^{b}	1.7±0.6^{x}	1.2±0.1^{y}	1.4±0.1^{y}
18:0	12.1±0.2	13.5±0.9	14.6±0.1	14.6±0.6	14.6±0.7^{y}	17.4±0.3^{x}	16.9±0.8^{x}
18:1n-11	n.f.	1.0±0.1^{a}	1.2±0.0^{b}	1.2±0.1^{a,b}	0.7±0.1^{y}	1.1±0.1^{x}	1.1±0.0^{x}
18:1n-9	3.4±0.2	6.2±0.4^{a}	3.1±0.1^{b}	3.3±0.2^{b}	8.1±1.0^{x}	3.6±0.1^{y}	3.6±0.3^{y}
18:1n-7	1.8±0.2	5.1±0.6	5.0±0.1	4.9±0.8	6.6±0.7^{y}	8.0±0.1^{x}	8.3±0.8^{x}
18:2n-6	0.7±0.1	1.6±0.2^{a}	0.8±0.0^{b}	0.8±0.1^{b}	2.1±0.4^{x}	0.8±0.0^{y}	0.9±0.1^{y}
18:3n-3	n.f.	1.1±0.2^{b}	2.5±0.2^{a}	2.1±0.5^{a}	2.1±0.5^{y}	3.6±0.3^{x}	3.9±0.8^{x}
18:4n-3	n.f.	0.3±0.1^{b}	0.8±0.0^{a}	0.9±0.1^{a}	0.5±0.1^{y}	0.9±0.1^{x}	1.2±0.2^{x}
20:1n-9	5.5±0.5	3.6±0.1	3.7±0.2	3.7±0.5	3.1±0.4	3.2±0.2	2.9±0.2
20:2n-6	0.7±0.0	0.6±0.0^{a}	0.4±0.1^{b}	0.5±0.0^{a}	0.6±0.0	0.5±0.0	0.5±0.0
20:4n-6	3.4±0.1	3.8±0.1^{a}	2.3±0.6^{b}	3.3±0.3^{a}	3.5±0.1^{x}	2.8±0.0^{y}	2.9±0.0^{z}
20:3n-3	1.4±0.0	1.3±0.1	1.2±0.4	1.4±0.2	1.0±0.1^{y}	1.4±0.1^{x}	1.3±0.1^{x}
20:5n-3	14.5±0.8	14.2±0.6^{b}	16.0±0.7^{a}	15.4±0.7^{a,b}	14.6±0.5^{y}	16.4±0.5^{x}	17.6±0.7^{x}
22:1	1.3±0.2	1.3±0.1	1.4±0.1	1.3±0.2	1.1±0.2	1.1±0.2	0.9±0.1
22:4n-6	1.0±0.1	1.1±0.1	1.3±0.1	1.1±0.2	0.8±0.1^{x,y}	0.9±0.1^{x}	0.6±0.1^{y}
22:5n-6	0.5±0.1	0.5±0.1^{b}	0.7±0.0^{a}	0.5±0.1^{a,b}	0.4±0.1	0.4±0.0	0.3±0.0
22:5n-3	1.5±0.2	1.1±0.1	1.4±0.0	1.2±0.3	0.9±0.2	1.1±0.2	0.8±0.1
22:6n-3	19.5±1.3	14.4±0.4	14.5±0.9	13.7±0.3	12.6±0.7^{x}	10.6±0.2^{y}	9.9±0.5^{y}
∑ Saturated	43.7±0.9	40.7±1.4	42.4±0.7	43.4±0.3	39.5±2.6	42.2±0.5	41.7±1.6
∑ Monoenes	13.2±0.2	19.3±0.6	15.7±0.2	15.8±0.6	21.3±1.1	18.3±0.4	18.3±0.8
∑ PUFA	43.0±1.1	40.0±0.9	41.8±0.7	40.8±0.3	39.2±1.5	39.5±0.2	40.1±1.1
∑ n-3	36.9±1.1	32.5±1.0	36.4±1.3	34.6±0.6	31.9±1.2	34.0±0.2	34.7±1.0
∑ n-6	6.2±0.2	7.5±0.1	5.4±0.7	6.2±0.6	7.3±0.3	5.5±0.1	5.3±0.2
DHA/EPA	1.3	1.0	0.9	0.9	0.9	0.6	0.6
FA content (% of DW)	3.9±0.2	3.6±0.3	3.4±0.1	3.1±0.5	4.2±0.4^{x}	3.1±0.1^{y}	3.2±0.2^{y}
Total lipid (% of DW)	11.9±1.2	12.6±0.5	11.6±1.2	10.3±1.2	12.4±0.7^{x}	10.3±0.4^{y}	10.6±0.6^{y}

Abbreviations of ADHA, AR+I and P+AR+I are like in Table IV. Data are means± S.D. (n=3). Values coted as 0.0 were below 0.05. Different superscript letters within the same day of paralarval rearing indicate significant differences among groups ($P<0.05$).

4. Discussion

The gross composition of the *Artemia* juveniles (1.5-2.3 mm) enriched with the mixed diet of 70% *R. lens* and 30% *I. galbana* (AR+I) was closely related with the composition of juveniles (1.5-2.0 mm) enriched with monodiets of *R. lens* (ARHO) or *I. galbana* (AISO), previsouly analyzed in chapter 1. The protein content found in juveniles from AR+I in the present work (51%) was equal to the levels previsouly found in ARHO (51%), whereas lipid and carbohydrate levels (13% and 9%, respectively) were within the ranges found in juveniles from ARHO and AISO (10-16% of lipid and 8-11% of carbohydrate). Moreover, the FA profile of the juveniles from AR+I, especially the PUFA, reflected the mixture of the two microalgal species, as can be confirmed by direct comparisons with the FA profiles of juveniles from ARHO and AISO in chapter 1. *Artemia* juveniles enriched with DHA-Selco® had an average lipid content of 22%, which is similar to values reported by Navarro and Villanueva (2000) for 1-3 mm *Artemia* enriched with Super Selco® (25% lipid), but *Artemia* enriched with DHA-Selco® displayed higher DHA and EPA levels (6.7% and 10%, respectively) than *Artemia* enriched the Selco-product used by those authors (2.3% of DHA and 8.2% of EPA), supporting the effectiveness of increasing the percentage of DHA in *Artemia* through the use of products richer in DHA. However, it should be kept in mind that it is necessary to adjust the concentration of these products for the enrichment of *Artemia* juveniles, as the concentrations recommended by the manufacturer for nauplii enrichment produce high mortality of juveniles.

The DW of paralarvae obtained in the present work was in general slightly inferior to values reported by other authors (Villanueva *et al*., 2002, 2004; Iglesias *et al*., 2000), which could be explained by the different rearing temperatures used in this work (mean of 17.5 ºC) and in those experiments (mean temperature of 20 ºC). Temperature is a major factor influencing cephalopod growth rates when food is not a limiting issue (Leporati *et al*., 2007). The best DW of 10-dph and 15-dph paralarvae obtained in this work (424-431 μg ind^{-1} and about 500 μg ind^{-1}, respectively) was thus inferior to values reported by Villanueva *et al*. (2002, 2004) for paralarvae fed enriched *Artemia* (nauplii or juvenile) or co-fed with microdiets (470 to 660 μg ind^{-1} in 10-dph paralarvae and from 540 to 880 μg ind^{-1} in 15-dph paralarvae), or than values found by Iglesias *et al*. (2000) for 12-dph paralarvae (750 μg ind^{-1}). Carrasco *et al*. (2006) also obtained higher paralarval DW (700

to 820 µg ind^{-}1) at day 10 when using spider crab (*Maja brachydactyla*) zoeae and juvenile *Artemia* enriched with *Tetraselmis suecica* at mean water temperature of 21 ºC.

Regarding the composition of paralarvae, total lipid found in *O. vulgaris* hatchlings (12% of DW) was similar to values previously reported by other authors: 13.4% and 11% of the DW (Navarro and Villanueva, 2000; Okumura *et al*., 2005, respectively). In this study the lipid content of the reared paralarvae reflected somehow the ingested diet, as a slight increase of lipid was observed in paralarvae from group ADHA, which were fed *Artemia* containing 22% lipid, whereas a slight decrease of lipid was observed in paralarvae from groups AR+I and P+AR+I. Navarro and Villanueva (2003) had also reported increased lipid levels in the body composition of paralarvae fed high-lipid *Artemia* in comparison with octopus hatchlings, diverging with the general tendency for a progressive reduction of the lipid content in wild octopus juveniles with increasing weight (from 12% to circa 7%), also described by those authors.

Certain PUFAs such as 18:3n-3 and 18:4n-3, initially not found in hatchlings, tended to increase in the reared paralarvae due to its presence in the supplied *Artemia* juveniles, indicating the incorporation of these PUFAs in paralarval tissues. Despite a remarkable drop of DHA was observed in paralarvae from all groups, the 15-dph individuals from group ADHA were shown to contain the highest levels of DHA and similar levels of EPA in comparison with hatchlings. However, no beneficial effects derived from this "better" FA profile in comparison with the remaining groups, as the growth rate of paralarvae from ADHA was the worst of all groups and the survival rate tended to be inferior, though no significant differences were detected. Navarro and Villanueva (2003) had previously described that the levels of DHA decreased significantly in paralarvae fed enriched *Artemia* nauplii alone or in combination with microdiets rich in DHA, suggesting that the poor growth and high mortality of paralarvae could be related with imbalances in the dietary lipid composition, especially in its FA profile. The decrease of DHA observed in paralarvae could be related with inadequate levels of this FA in the supplied prey, as DHA levels were still low in comparison with octopus hatchlings, but results drive us to speculate if in fact is the presentation mode of DHA a key factor for its correct absorption, instead of the total amount of DHA available. In contrast to crustacean zoeae and copepods, which contain HUFA mainly within phospholipids, in *Artemia* they are located predominantly in the triglyceride fraction (Navarro and Villanueva, 2000; Bell *et al*., 2003), which could interfere with its absorption or incorporation into body membranes. In fact, this could be one of the reasons why crustacean zoeae have been previously used with

success in the rearing of octopus paralarvae until settlement, whereas *Artemia* alone failed in most cases.

Previous works with marine-fish larvae (gilthead seabream, red seabream, turbot and Senegalese sole) have shown poor larval growth or deficiencies related with qualitative lipid imbalances in the diets (Mourente *et al*., 1993; Watanabe, 1993; Rodríguez *et al*., 1994; Estévez *et al*., 1999; Morais *et al*., 2005; Izquierdo, 2006). However, other works showed the ineffectiveness of increasing n-3 HUFA levels in enriched rotifers and *Artemia* to improve the survival and growth of turbot larvae (Rainuzzo *et al*., 1994; Reitan *et al*., 1994). Similarly, experiments carried out with Senegal sole (*Solea senegalensis*) larvae, on which *Artemia* was enriched with graded levels of DHA and EPA to feed larvae, failed to improve the survival and growth of larvae, in comparison with a group fed non enriched *Artemia* (Morais *et al*., 2004; Villalta *et al*., 2005). Moreover, Villalta *et al*. (2005) found that Senegal sole larvae fed *Artemia* without any DHA content, could grow until 36-dph at growth and survival rates as good as larvae fed on *Artemia* containing medium to high DHA levels. In the present work, despite *Artemia* enriched with DHA-Selco® contained three-times more DHA than *Artemia* enriched with microalgae, we did not observe any clear positive effects over the growth and survival of paralarvae. These observations need further studies, in order to understand the role of DHA in paralarval nutrition and the implications that diets rich, or poor in this FA, may have in the development of paralarvae.

The inert pellets formulated resulted ineffective for the proposed objective due to inadequate physical properties, as they were sinking too fast and paralarvae did not have time to catch them. Even if paralarvae were not seen to ingest the pellets, paralarvae from this group had a slight higher DW than group fed AR+I. This difference could be due to increased dissolved organics in the water leaching from pellets that could be directly absorbed by the skin of paralarvae, as this phenomenon has been described for cephalopod hatchlings (reviewed by Lee, 1994). Further works in the field of inert diets development for paralarvae should be undertaken, as previous works have demonstrated active capture of microdiets by *O. vulgaris* paralarvae (Villanueva *et al*., 2002) and reasonable growth rates were achieved when feeding *S. officinalis* with semi-purified diets (Castro *et al*., 1993, 1994).

Protein is the major component of cephalopods body composition (70 to 85% of DW), but in contrast to fishes they contain overall 20% more protein and 50-100% less lipid and carbohydrate (Lee, 1994). The high requirement that cephalopods have for protein is primarily fulfilled by high ingestion rates and high digestion efficiency (Forsythe and Van

Heukelem, 1987; Lee, 1994). The better growth rate found in paralarvae fed juvenile *Artemia* enriched with microalgae (AR+I), in comparison with paralarvae fed *Artemia* enriched with DHA-Selco®, could be related with the higher P:E ratio, or P:L ratio, found in the diet. It has been shown that diets containing maximum protein:energy ratios (P:E) promoted higher growth rates of *Sepia officinalis* juveniles (Lee, 1994), and in other molluscs such as the green abalone *Haliotis fulgens* the same remarks were described (Gómez-Montes *et al*., 2003). On the other hand, since the carbohydrate content in *Artemia* juveniles enriched with either microalgae or DHA-Selco® ranged between 6 and 9%, and this source of energy is of minor importance for cephalopods (Lee, 1994), the P:L ratio of the diet should be used instead of the P:E ratio, to carry out comparisons of the effects that different diets have on paralarval performance, with the additional advantage of being easily calculated. However, this simplified ratio could only be used whenever carbohydrate levels in prey are similar, and do not represent a major component of the diet. Further studies are needed to clarify the importance of the dietary P:L ratio for the improvement of *O. vulgaris* paralarvae growth. In addition, since the enrichment of juvenile *Artemia* with microalgae, cultured in controlled conditions, was shown to promote a better growth of paralarvae than juveniles enriched with commercial lipid emulsions, this methodology of prey enrichment can now be used in future works as control group. New enrichment diets for juveniles should be tested, together with combinations of *Artemia* juveniles enriched with either microalgae or other products/diets, with the aim of feeding paralarvae with live prey enriched with different essential nutrients.

Rearing of *Octopus vulgaris* paralarvae in clear or green water conditions

Capítulo 3 / Chapter 3

Abstract

The use of green water technique or pseudo-green water conditions to rear marine larval species is a well known practice in aquaculture, and it has been shown to improve the survival, growth and food conversion index of more than 40 species in comparison with clear-water conditions. In this study we analysed the effects of adding *Nannochloropsis gaditana* to the rearing tanks of octopus paralarvae, since a species of the same microalgal genera was previously used at large scale by other authors with good results. Two groups of paralarvae were set, each in triplicate: one group was maintained in clear water conditions, whereas the other was established in green waters conditions, adding *N. gaditana* to tanks at an initial concentration of $200x10^3$ cells ml^{-1}. The effects of green-waters were analyzed on the growth and survival rates of paralarvae. The diet consisted of a combination of *Artemia* juveniles enriched with either a mixed diet of microalgae (*Rhodomonas lens* and *Isochrysis galbana*, in a proportion of 70%:30% dry weight basis), or with DHA-Selco® (INVE). In this work the gross biochemical composition and the fatty acid profiles of *N. gaditana* and of two sizes of *Artemia* juveniles (1.6 mm and 2.3 mm) enriched with *N. gaditana* were also addressed, as few data about their composition was previously reported, to evaluate which properties may be behind the positive effects that this genera of microalga produces in paralarvae rearing. In clear water tanks survival rate at day 15 was 38%, decreasing to nearly 3% at day 25. Unexpectedly and due to unknown reasons, a sudden mass mortality of paralarvae was observed in the green water tanks between days 9 and 11, even if paralarvae displayed healthy behaviour, good survival and normal capture of prey in the previous days. The initial dry weight (DW) of hatchlings was 330±20 μg $paralarva^{-1}$, whereas 25-day post-hatch paralarvae maintained in clear waters attained a DW of 840±150 μg $paralarva^{-1}$. In this study the utilization of green water conditions in low-volume tanks (50-l) in closed systems without re-circulation was not successful, but further experiments using green water conditions with this microalga, or with other microalgal species, should be carried out in order to elucidate if environmental conditions (light diffusion in tanks, gut flora enhancement, anti-bacterial effects on water) and/or nutritional issues promote higher growth and survival of paralarvae. Regarding the biochemical composition of the small *Artemia* juveniles enriched with *N. gaditana* (≈ 1.6

mm), protein was found to represent 49% of its DW, whereas in big size juveniles (≈ 2.3 mm) protein accounted for 65% of DW. Lipid levels ranged between 10 and 12% in both *Artemia* juveniles, whereas carbohydrates ranged between 8 and 10%. The major fatty acids (FAs) found in *N. gaditana* were the saturated palmitic acid (16:0) and eicosapentaenoic acid (EPA, 20:5n-3), each accounting 25% of the total FA. Arachidonic acid (ARA, 20:4n-6) represented 4.4% of total FAs, whereas docosahexaenoic acid (DHA, 22:6n-3) was not found in this microalga. EPA and ARA levels found in both sizes of *Artemia* juveniles represented 14-15% and 2%, respectively, of the total FAs. As observed for *N. gaditana*, no DHA was found in *Artemia* juveniles. The major differences between *Artemia* juveniles enriched with *N. gaditana* or enriched with *R. lens* and *I. galbana* were related with the lower content of ARA in this last group (0.4% of total FAs).

1. Introduction

The use of green water techniques to rear marine larval species is a well known practice in aquaculture. The addition of microalgae to larval rearing tanks, defined as pseudo-green-water by Divanach and Kentouri (2000), has been shown to improve the survival, growth and feed conversion index of more than 40 species in comparison with clear-water conditions (reviewed by Muller-Feuga *et al*., 2003). Although the reasons for the apparently positive effects of microalgae on fish larvae are not fully understood, some hypotheses have been proposed to explain this phenomenon: the stabilisation or improvement of water quality, the light contrast, the role of direct (via drinking and gill retention) or indirect (via enriched live prey) nutrition, the micronutrient stimulus for feeding behaviour or physiological processes, the regulation of bacterial opportunistic populations and antibacterial or probiotic action, and the improvement of live prey nutrition and availability (Muller-Feuga *et al*., 2003). In order to simply the terminology used in this work, the pseudo-green water conditions will be just referred as green water.

The use of green water conditions to rear *Octopus vulgaris* paralarvae was first attempted by Hamazaki *et al*. (1991), who succeeded in attaining benthic octopus after 25 days post-hatching at a mean water temperature of 26.9 ºC. These authors used *Artemia* juveniles (1.5-2 mm) enriched with *Nannochloropsis* sp. as food item, adding this same microalga to the larval tanks at a concentration of $1x10^6$ cells ml^{-1}. Imamura (1990) pointed out that the reasons behind the positive effects of *Nannochloropsis* sp. in the rearing tanks could be related with the constant enrichment of live prey and to a reduction of light intensity that could diminish paralarvae stress. Recently, Moxica *et al*. (2006) also reported the positive effects of enriching *Artemia* with *Nannochloropsis* sp. and creating green waters with the same microalga, attaining paralarvae in the pre-settlement stage after 70 days of rearing at mean water temperature of 22.4±0.8 ºC. Even though the survival rate obtained was low (0.9%), paralarvae displayed 17 suckers per arm and attained a dry weigth of 7.00±0.95 mg. However, previous works on which octopus paralarvae were fed *Artemia* alone enriched with either commercial products or microalgae, or in co-feeding regime with artificial microdiets, resulted in mass mortalities (Iglesias *et al*., 2000; Navarro and Villanueva, 2000; Villanueva *et al*., 2002, 2004).

In a previous work on *O. vulgaris* paralarvae rearing, it was shown that *Artemia* enriched with a mixed diet of *Rhodomonas lens* and *Isochrysis galbana* could promote better

performance of paralarvae, than *Artemia* enriched with DHA-Selco. However, paralarvae fed Selco-enriched *Artemia* displayed a FA profile more closely related with the FA composition of hatchlings, as eicosapentaenoic acid (EPA, 20:5n-3) levels remained stable and the decrease of docosahexaenoic acid (DHA, 22:6n-3) was less intense than in paralarvae fed *Artemia* enriched with *R. lens* and *I. galbana*. In view of these findings, in this study octopus paralarvae were fed a combination of juvenile *Artemia* enriched with either microalgae or DHA-Selco®, in order to supply a more balanced diet, and two rearing conditions were tested: clear or green water conditions, in order to assess the effects that green waters may have on the growth and survival rates of paralarvae.

In addition, a parallel experiment was carried out to analyse the composition of *Artemia* juveniles of two different sizes enriched with *N. gaditana.* In previous works carried out by Seixas *et al*. (submitted, Annex I) in which *Artemia* nauplii were fed different microalgal species (*Tetraselmis suecica*, *R. lens*, *I. galbana* and *N. gaditana*), we observed that the group fed *N. gaditana* grew very poorly and mortality was very high after 8 days of rearing, in comparison with *Artemia* fed the other microalgal species, which could be related to the difficult digestibility of this species. Neverthless, the gross composition and the fatty acid profiles of both *N. gaditana* and enriched *Artemia* juveniles were assessed, with the aim of gathering more information about the nutritional composition of this prey, as Hamazaki *et al*. (1991) did not reported any information on this subject, and Moxica *et al*. (2006) only provided some data of the lipid composition of enriched *Artemia*.

2. Material and methods

2.1 Microalgae cultures

The marine microalgae *Rhodomonas lens* Pascher et Ruttner CCMP 739, *Isochrysis galbana* Parke (strain isolated from Ría de Arousa, Spain) and *Nannochloropsis gaditana* CCMP 527 were grown semi-continuously in glass flasks with flat bottom, containing 5-l of sterilized sea water (salinity of 35 ppt), and in nutrient saturated conditions. For *R. lens*, nutrients were added at a concentration of 4 mM N l^{-1}, whereas for *I. galbana* and *N. gaditana* a concentration of 2 mM N l^{-1} was used, as this was enough to ensure N saturation, as confirmed by determination of the NO_3^{-2} concentration (Clesceri *et al*., 1989) left in the harvested cultures of microalgae. Cultures were submitted to 12h:12h light/dark

cycle periods and an irradiance of 197 µmol photon m^{-2} s^{-1} in the rear and of 166 µmol photon m^{-2} s^{-1} under flasks. Daily renewal rates of 30% of the volume of cultures were carried out once cultures approached late-logarithmic growth, adding sterilized seawater enriched with the same corresponding nutrient concentration. Once the steady-state was achieved, as assessed by steady cell density, the daily harvested cultures were used to enrich *Artemia* juveniles or to add to the rearing tanks.

2.2 Production and enrichment of *Artemia* juveniles

Newly hatched *Artemia* nauplii (AF, INVE, Belgium) were initially grown for two days with *Rhodomonas lens* in 12-L plastic tanks and water temperature of 26.5±0.5 ºC, until attaining a size of nearly 1.4 mm, as this microalga was shown to provide the best results of *Artemia* growth (see chapter 2 or Annex I). The brine shrimp was finally enriched with one of the following diets: a mixed diet of *Rhodomonas lens* and *Isochrysis galbana* (70%:30% dry weight basis); or with DHA-Selco® (INVE, Dendermonde, Belgium), t half of the concentration recommended by the manufacturer (as described in chapter 2). Average *Artemia* length was determined by measuring 40 individuals for each group.
In a parallel experiment, *Artemia* juveniles of two different sizes (≈ 1.4 mm and ≈ 2.2 mm) previously grown with *R. lens*, were distributed in 1-l glass flasks containing 700 ml of seawater (30 ppt), initial density of 3.0 *Artemia* ml^{-1}, water temperature of 26.5±0.5 ºC, and enriched for 24 h with either *N. gaditana* (ANANO) or the mixed diet of *R. lens* and *I. galabana* (AR+I). Samples of 60 juveniles were individually counted, briefly washed with distilled water and immediately frozen at -18 ºC for later biochemical composition analysis.

2.3 Experiment of *Octopus vulgaris* paralarvae rearing

Newly hatched paralarvae from an egg mass of *O. vulgaris* kept in a 1500-l water circuit in Viana do Castelo (Portugal), were transported within 2 h to the facilities of the University of Santiago de Compostela (Spain). Upon arrival hatchlings were individually counted and transferred to conical fiber glass tanks with 50 cm diameter and white walls containing a total volume of 50 l. Light was provided by day-light lamps placed 40 cm above the water surface, establishing a photoperiod of 18 h light:6 h darkness. Water temperature was

raised and kept constant at 20.5±0.5 ºC through the use of thermo heaters RENA 100W. Tanks were provided with gentle aeration and seawater (34 ppt) was renewed at 30% of the volume of tanks every 3 days, being filtered through 50 μm filter-cartridges and disinfected with UV before entering the tanks. Paralarvae were established at 10 individuals l^{-1} and food was provided since the first day. Meals consisted of a combination of juvenile *Artemia* enriched as follows: 30% of the supplied *Artemia* was enriched with DHA-Selco®, whereas the remaining 70% of *Artemia* juveniles were enriched with the mixed diet of microalgae. *Artemia* juveniles were distributed twice a day in equal proportions (10:00 a.m. and 06:00 p.m.), in a total of 0.05 *Artemia* mL^{-1} day^{-1}. Two groups, each in triplicate, were set in clear or green water conditions, to test the effects of adding *N. gaditana* to the rearing tanks on the growth and survival of paralarvae (Fig. 1). *N. gaditana* was added to the tanks established in green waters at an initial concentration of $200x10^3$ cells of ml^{-1}.

Octopus paralarvae were measured under a stereoscope using a calibrated ocular micrometer (30 hatchlings and 20 individuals per replica on days 10 and 15). Total length and mantle length were measured as described by Villanueva (1995). Dry weight (DW) of paralarvae was determined by weighing samples of 10 paralarvae (n=5 per replicate) after washing individuals with distilled water and placed in a stove at 101±1 ºC for 24 h.

2.4 Biochemical composition analysis

Protein content was determined by the Folin-phenol method (Lowry *et al*., 1951), after hydrolysis with NaOH 1.0 M at 95 ºC; carbohydrates were analyzed by the phenol/sulphuric acid method (Kochert *et al*., 1978) and lipids were quantified by the charring method (Marsh and Weinstein, 1966) after extraction of total lipids (Bligh and Dyer, 1959). Fatty acid composition analyses were carried out using a gas chromatograph-mass spectrograph (GC-MS Fisons Instruments, MD-800, Beverly, Mass.), equipped with an Omegawax™ 250 column 30m x 0.25mm (Supelco, Inc.), after methanolysis of the lipid extracts with 5% HCl in methanol at 85 ºC, during 2.5 h, followed by extraction with hexane (Sato and Murata, 1988). Triheptadecanoin (Sigma®, St. Louis, Mo.) was used as internal standard. All biochemical composition analyses were carried out in triplicate.

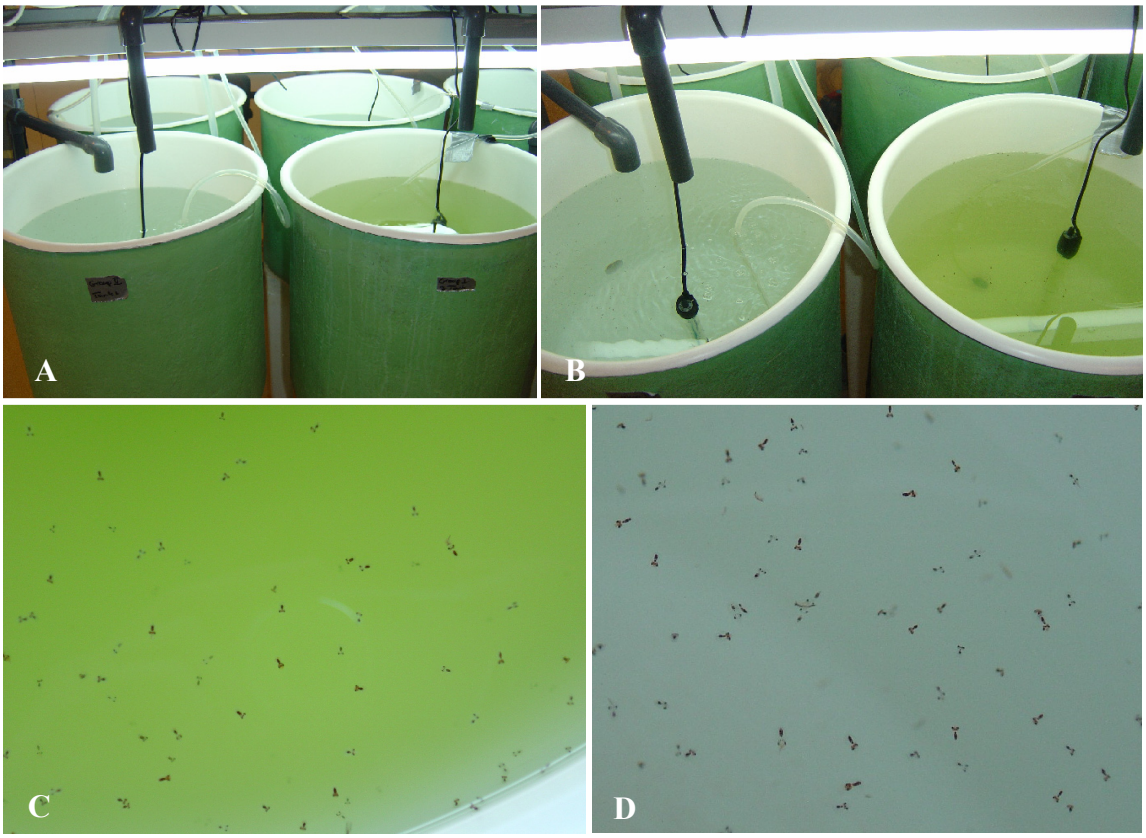

Figure 1 - *Octopus vulgaris* paralarvae rearing conditions in 50-l volume tanks without recirculation. (A) General view of the conical fiber-glass tanks with white walls and provided with gentle aeration. (B) Detail of thermo-heaters and of fluorescent lamps placed 40 cm above water surface. (C) - (D) Detail of paralarvae in tanks established in green or clear water conditions.

3. Results

3.1 Survival and growth of *Octopus vulgaris* paralarvae

In the first week of the experiment almost no mortality of paralarvae was observed in both treatments (Fig. 2). After 15 days of rearing the survival rate in the group maintained in clear water conditions was 38%, decreasing to nearly 3% at day 25. Unexpectedly and due to unknown reasons, in the green-waters group a sudden mortality of paralarvae was observed in two of the tanks between days 9 and 11, and in the third tank at day 12 (Fig. 2), even if paralarvae displayed healthy behaviour and normal capture of prey in the previous days. Unfortunately no comparisons of paralarval dry weigth (DW) could be done

between the two treatments, as the first sampling point was carried out after 15 days of rearing. However, it was decided to continue the experiment to analyze the growth and survival of paralarvae maintained in clear waters, as well as its fatty acid composition. As observed in figure 3, paralarvae increased in both size and DW along the experiment. From an initial DW of 330±20 µg ind.$^{-1}$ at day 0 (hatchlings), paralarvae attained 840±150 µg ind.$^{-1}$ after 25 of rearing.

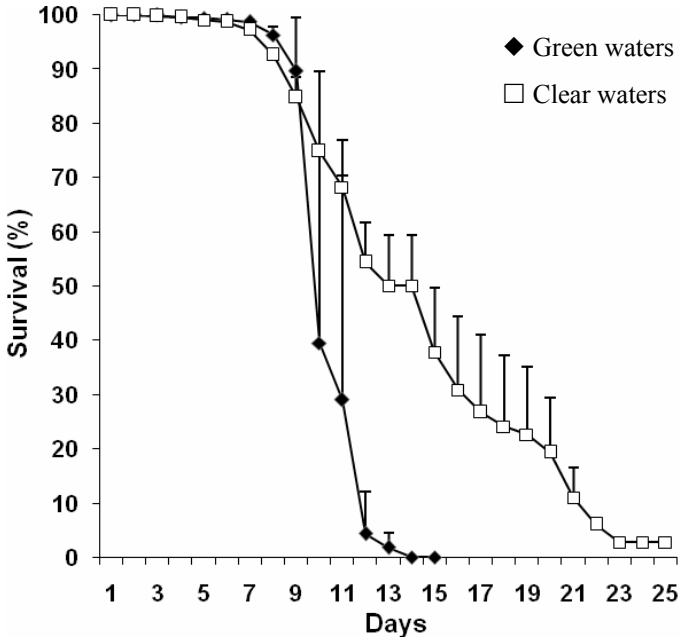

Figure 2 - Survival rate of *Octopus vulgaris* paralarvae reared in green- or in clear water conditions in the course of the experiment. Means ± S.D. (n=3).

3.2 Fatty acid composition of *O. vulgaris* paralarvae

The main FAs found in octopus hatchlings were the saturated palmitic acid (16:0, 28.0% of the total FA), DHA (19.4%) and EPA (16.8%). The FA composition of paralarvae maintained in clear water conditions is shown in table I. A remarkable drop of DHA was observed from hatchlings to 15-dph paralarvae, the same being observed for arachidonic acid (ARA, 20:4n-6). EPA levels increased slightly in 15-dhp paralarvae, but decreased to lower levels in 25-dph paralarvae (Table I). The PUFAs 18:3n-3 and 18:4n-3, initially not found in hatchlings, represented circa 5% and 1.2% of the total FA, respectively, reflecting the composition of the ingested prey (see the FA composition of *Artemia* juveniles enriched with *R. lens* and *I. galbana* in table II of this chapter or the profiles of *Artemia* in

chapter 2). The levels of saturated FA remained stable in 15-dph paralavae, in comparison with the initial levels found in hatchlings, but increased in 25-dph paralarvae, whereas the sum of monoenes increased slightly in 15-dph paralarvae, remaining stable thereafter. This increase of both saturated and monoenes was due at the expense of a decrease of PUFA levels in the reared paralarvae, affecting both n-3 and n-6 classes of FAs.

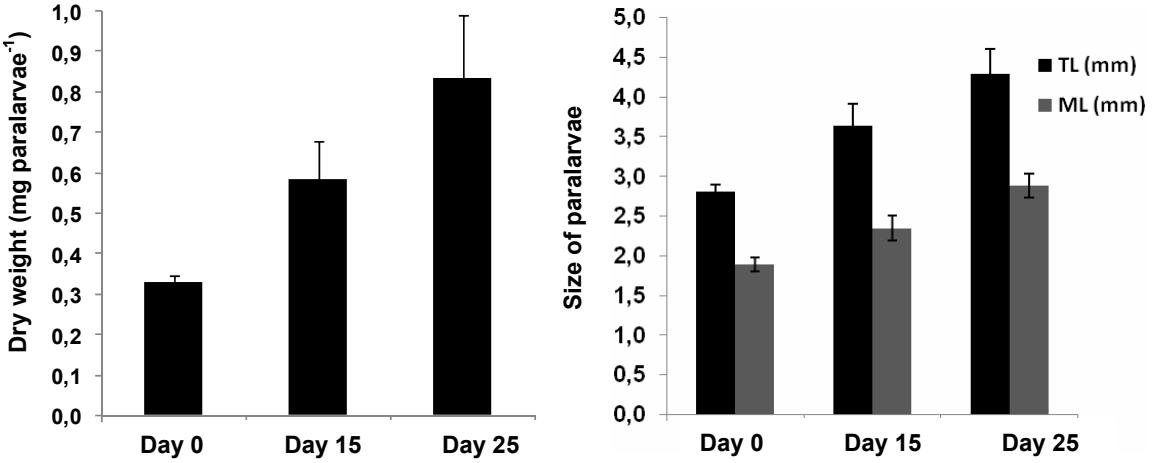

Figure 3 – Dry weight, total length (TL) and mantle length (ML) of *Octopus vulgaris* paralarvae maintained in clear water conditions, in the course of the experiment.

3.3 Biochemical composition of *Nannochloropsis gaditana* and enriched *Artemia* juveniles

The microalga *N. gaditana* was shown to contain nearly 40% protein, 25% lipid and 9% carbohydrate (Table II). The *Artemia* juveniles enriched for 24 h with *N. gaditana* (ANANO) were found to grow very poorly (small individuals grew from an initial length of 1.4±0.1 mm to 1.6±0.2 mm, while juveniles with an initial size of 2.2±0.2 mm grew to 2.3±0.2 mm), in comparison with juveniles enriched with *R. lens*/*I. galbana* (from 1.4±0.1 mm to 2.2±0.2 mm, and from 2.2±0.2 mm to 2.9±0.3 mm). The composition of *Artemia* juveniles varied according to its size (Table I). Small ANANO juveniles (≈ 1.6 mm) contained lower protein levels (49%) than the big size (≈ 2.3 mm) juveniles (65%), whereas lipid levels ranged between 10 and 12% for both sizes and carbohydrate between 8 and 10%. The composition of *Artemia* juveniles from group AR+I was very similar to big size juveniles from ANANO (Table II).

Table I. Fatty acid (FA) composition (% of total FA) and total FA (% of dry weight) of *Octopus vulgaris* hatchlings and of 15-dph and 25-dph paralarvae from the group established in clear water conditions.

Fatty acid	Hatchlings	15-dph paralarvae	25-dph paralarvae
14:0	2.9 ± 0.1	2.2 ± 0.4	2.8 ± 0.4
15:0	0.2 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
16:0	28.0 ± 1.0	23.4 ± 0.5	26.1 ± 0.9
16:1n-7	1.7 ± 0.1	2.4 ± 0.5	3.2 ± 0.6
18:0	11.3 ± 1.1	16.1 ± 0.6	18.1 ± 0.8
18:1n11 + n9	5.6 ± 0.1	4.9 ± 1.0	6.3 ± 0.8
18:1n-7	1.7 ± 0.1	7.6 ± 0.2	7.0 ± 0.7
18:2n-6	0.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
18:3n-3	n.f.	4.7 ± 0.7	5.5 ± 0.3
18:4n-3	n.f.	1.2 ± 0.2	1.2 ± 0.1
20:1n-9	4.4 ± 0.3	2.4 ± 0.2	1.4 ± 0.4
20:2n-6	0.3 ± 0.1	0.5 ± 0.0	0.3 ± 0.1
20:4n-6	4.6 ± 0.2	2.5 ± 0.2	1.2 ± 0.1
20:3n-3	0.7 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
20:4n-3	n.f.	0.3 ± 0.1	0.6 ± 0.1
20:5n-3	16.8 ± 0.7	17.9 ± 0.5	15.0 ± 1.2
22:1	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.1
22:4n-6	0.1 ± 0.0	0.7 ± 0.1	0.4 ± 0.1
22:5n-3	0.2 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
22:6n-3	19.4 ± 1.4	8.5 ± 0.6	7.1 ± 0.7
∑ Saturated	42.3 ± 1.7	42.7 ± 0.3	47.8 ± 0.5
∑ Monoenes	15.1 ± 0.8	18.5 ± 1.0	18.7 ± 1.4
∑ PUFA	42.6 ± 2.1	39.1 ± 0.9	33.5 ± 1.8
∑ n-3	37.2 ± 2.1	33.7 ± 1.1	30.0 ± 1.7
∑ n-6	5.4 ± 0.0	4.9 ± 0.2	3.6 ± 0.2
DHA/EPA	1.2	0.5	0.5
FA (% of DW)	5.1 ± 0.2	4.4 ± 0.2	3.6 ± 0.1

Data are means ± SD (n=3).

Regarding the fatty acid (FA) composition of *N. gaditana*, the saturated palmitic acid (16:0) and the highly unsaturated fatty acid eicosapentaenoic acid (EPA, 20:5n-3) accounted each for 25% of the total FA (Table II). Arachidonic acid (ARA, 20:4n-6) represented 4.4% of total FAs, whereas docosahexaenoic acid (DHA, 22:6n-3) was not found in this microalga. The major FAs found in *Artemia* juveniles from ANANO (for both sizes) were the saturated FAs 16:0 and 18:0, and EPA (Table II).

Table II. Gross composition (% of dry weight) and fatty acid (FA) profile (% of total FA) of *Nannochloropsis gaditana* and *Artemia* juveniles of two different sizes enriched with this microalga (ANANO). Results found for *Artemia* juveniles enriched with *R. lens* and *I. galbana* are also shown for comparisons.

	Nannochloropsis gaditana	ANANO (≈ 1.6 mm)	ANANO (≈ 2.3 mm)	AR+I (≈ 2.9 mm)
Protein (%)	39.9 ± 2.7	48.6 ± 1.4	65.8 ± 2.4	64.6 ± 2.7
Lipid (%)	25.1 ± 1.4	11.7 ± 0.7	10.4 ± 0.8	11.8 ± 0.8
Carbohydrate (%)	8.7 ± 0.5	7.8 ± 0.5	9.6 ± 0.7	9.9 ± 0.5
Fatty acid				
14:0	8.1 ± 0.5	3.7 ± 0.5	3.5 ± 0.1	1.9 ± 0.2
16:0	25.4 ± 0.9	17.7 ± 1.5	17.0 ± 0.4	14.6 ± 0.2
16:1 n-9	22.7 ± 0.6	9.4 ± 0.7	9.5 ± 0.5	2.5 ± 0.1
18:0	1.4 ± 0.2	15.8 ± 1.3	12.9 ± 0.6	10.9 ± 0.2
18:1n-9	4.7 ± 0.2	7.6 ± 1.0	7.2 ± 0.1	3.1 ± 0.1
18:1n-7	1.1 ± 0.2	12.1 ± 0.9	13.1 ± 0.4	9.1 ± 0.2
18:2n-6	4.0 ± 0.0	2.5 ± 0.4	3.4 ± 0.2	1.0 ± 0.2
18:3n-3	0.8 ± 0.1	9.1 ± 1.0	10.3 ± 0.1	23.2 ± 0.5
18:4n-3	0.4 ± 0.1	1.9 ± 0.9	2.5 ± 0.1	13.0 ± 0.3
20:4n-6	4.4 ± 0.1	1.7 ± 0.2	2.1 ± 0.2	0.4 ± 0.2
20:3n-3	n.f.	0.5 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
20:4n-3	n.f.	0.2 ± 0.1	0.1 ± 0.1	2.2 ± 0.1
20:5n-3	25.0 ± 0.6	14.1 ± 0.6	14.9 ± 0.4	13.8 ± 0.6
22:6n-3	n.f.	n.f.	n.f.	1.5 ± 0.1
DHA/EPA	-	-	-	0.1
EPA/ARA	5.7	8.3	7.1	34.5
∑ Saturated	35.0 ± 1.1	38.5 ± 2.0	34.7 ± 1.1	28.1 ± 0.4
∑ Monoenes	29.1 ± 0.4	31.5 ± 1.0	31.2 ± 0.2	15.8 ± 0.3
∑ PUFA	35.2 ± 1.0	30.0 ± 3.0	34.1 ± 1.0	56.1 ± 0.6
∑ n-3	26.2 ± 0.7	25.8 ± 2.4	28.6 ± 0.6	54.7 ± 0.6
∑ n-6	8.9 ± 0.3	4.2 ± 0.6	5.5 ± 0.4	1.4 ± 0.3

The biochemical composition results found for *N. gaditana* are means ± S.D. (n=3 from cultures harvested in three different days). Composition data of *Artemia* juveniles are means ± S.D. (n=3).

As observed for *N. gaditana*, the *Artemia* juveniles enriched with this microalga did not contained DHA, whereas EPA and ARA levels found in both sizes of juveniles corresponded to nearly 14-15% and 2% of the total FAs, respectively. In *Artemia* juveniles from AR+I, EPA levels (13.8%) were slightly lower than in juveniles from ANANO, whereas the content of ARA was much lower (0.4%) than in those juveniles. However, juveniles from AR+I contained 1.5% of DHA. The sum of total PUFAs was substancially lower in juveniles from ANANO, in comparison with juveniles from AR+I, at the expense of increased levels of monoenes and saturated FAs (Table II).

4. Discussion

In this study the DW of 25-dph paralarvae maintained in clear waters (840±150 µg paralarvae^{-1}) was in the same range as values reported by other authors for paralarvae fed enriched *Artemia* alone (580-900 µg paralarvae^{-1}), at similar water temperature (Villanueva *et al*., 2002; Villanueva *et al*., 2004). However, in comparison with the DW of 25-dph paralarvae fed enriched *Artemia* complemented with millicapsules (1210-1360 µg paralarvae^{-1}) (Villanueva *et al*., 2002), or with the DW of paralarvae fed *Artemia* and crustacean zoeae (1.4-3.4 mg paralarvae^{-1}) (Itami *et al*., 1963; Villanueva, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006), lower DW were found in this work. In comparison with the DW of 15-dph paralarvae fed on monodiets of *Artemia* enriched with either DHA-Selco or with *R. lens*/*I. galbana* reported in chapter 2 (442 µg paralarvae^{-1} and circa 500 µg paralarvae^{-1}, respectively) higher values were found in this study (585±96 µg paralarvae^{-1}). As previsouly discussed, these differences could be explained by the different rearing temperatures used, as this parameter is one of the major factors influencing cephalopod growth rates (Leporati *et al*., 2007).

In this work the use of *N. gaditana* to generate green waters was not successful at all, as mass mortality of paralarvae was observed after 12 days of rearing. The causes that led to this unexpected situation remained unclear, as ammonia levels were kept under 0.25 mg l^{-1} and paralarvae displayed normal behaviour and excellent survival rates the days before mass mortality happened. It is unlike that the increase of the cell density in tanks, recorded from 200x10^3 cells ml^{-1} to 600x10^3 cells ml^{-1}, could be responsible for mass mortality which seems to be reasonable as other authors reported cell densities of 1x10^6 cells ml^{-1} in their experiments (Hamazaki *et al*., 1991; Moxica *et al*., 2006). The green-water technique

was previously used by these authors, with *Nannochloropsis* sp., in large scale trials and with good results. Hamazaki *et al*. (1991) attained benthic octopus with a survival of 28% after 25 days, at mean water temperature of 26.9 ºC, when using a 20 m^3 tank to which *Nannochloropsis* sp. was added at a concentration of $1x10^6$ cells ml^{-1}, and supplying *Artemia* (1.5-2 mm) enriched with this same microalga as prey. When comparing the effect of green water condition with that of clear waters, or with a group to which *Artemia* enriched with *Nannochloropsis* sp. was supplied in clear waters, those authors found no differences in the survival rates among groups, but the development of paralarvae was better in the group of green-waters, as quantified by the number of suckers per arm. In Spain, Moxica *et al*. (2006) attained paralarvae in the pre-settlement stage after 70 days of rearing at mean water of 22.4±0.8 ºC and a survival of 0.9%, when using a 1 m^3 tank, to which *Nannochloropsis* sp. was added at the same concentration described by the Japanese authors and supplying juvenile *Artemia* enriched with the same microalga. In the present work, the fact that low-volume tanks (50 l) in closed regimen and without recirculation were used, could be the reason for certain changes in water parameters that affected paralarvae. It is difficult to conclude which causes originated this mass mortality because in such low volume tanks changes can be quick and unperceptible.

Regarding the biochemical composition of *Artemia* enriched with *N. gaditana*, both sizes of juveniles contained protein, lipid and carbohydrate levels similar to values reported for *Artemia* juveniles of nearly the same size enriched with monodiets of other microalgal species (*R. lens*, *I. galbana*, *I. galbana* T-ISO or *Tetraselmis suecica*; see results from chapter 1 or Seixas *et al*., 2008). Similarly, the composition of large size juveniles enriched with *N. gaditana* was about the same as that found for juveniles enriched with the mixed diet of *R. lens*/*I. galbana*. Concerning the FA composition of both *N. gaditana* and *Artemia* juveniles, some differences could be observed in comparison with the FA profiles found for the other microalgal species and enriched juveniles (see chapter 1 or Seixas *et al*., 2008). *N. gaditana* was shown to contain higher levels of EPA (25% of total FA) than the remaining microalgal species, but no DHA was observed in this species. *I. galbana* contained a lower percentage of EPA (19%), but DHA represented 6% of total FA, whereas *R. lens* contained 10% EPA and 7% DHA. The most striking difference among these microalgal species is related with ARA content. Whereas in *N. gaditana* this FA accounted for 4% of the total FA, in the remaining microalgal species mentioned above, ARA represented less than 0.3% of total FA. As for *Artemia* juveniles, despite no DHA was found in individuals enriched with *N. gaditana*, the percentage of EPA observed (14-

15%) was in general higher or at least equal than in juveniles enriched with the other microalgal species (3-15%, see chapter 1). Like observed in microalgae, the major difference in the PUFA profile among *Artemia* juveniles was related with the ARA content, which was much higher in the enrichment with *N. gaditana* (circa 2%) than previously observed in enrichment with the other microalgae (≤0.4% of total FA). Similar percentages of EPA and ARA were reported by Moxica *et al*. (2006) in *Artemia* enriched with *Nannochloropsis* sp. (13% and 3% of total FA, respectively), who also described the presence of minor amounts of DHA in those prey (0.19%). These authors also suggested that *Artemia* could act as a carrier to deliver *Nannochloropsis* sp. to paralarvae, and this would be beneficial to promote paralarval growth and survival. In addition, Moxica *et al*. (2006) also reported the percentages of EPA, DHA and ARA in 30-dph paralarvae fed those prey (24%, 7% and 2.5%, respectively), and in 51-dph paralarvae (24%, 9% and 6%, respectively), and paralarvae displaying pre-settlement behaviour could be observed after 70 days of rearing, at mean water temperature of 22.4±0.8 ºC. In this study, the percentages of DHA and ARA found in 25-dph paralarvae were similar to the values reported by those authors for 30-dph paralarvae, while EPA was lower (15%). The FA composition of 15-dph paralarvae fed the combination of *Artemia* juveniles (present study) was similar to that of 15-dph paralarvae fed on a monodiet of juveniles enriched with *R. lens* and *I. galbana* (see data in chapter 2), though a sligh lower DHA content was observed in this experiment (8.5% in comparison with 10.6% in the former work). However, this could be related with the different rearing temperatures, as lower water temperatures tend to favour higher levels of PUFAs. Other authors (Navarro *et al*., 2000, 2003) had previously reported that the DHA content in reared paralarvae decreased substancially, and suggested that the mortality observed could be related with lipid imbalances. As mentioned above, the most striking difference between *Artemia* juveniles enriched with either *N. gaditana* or the mixed diet of *R. lens*/*I. galbana* was the content of ARA. It would be necessary to investigate the importance of the ratio EPA/ARA in the diet for octopus paralarvae, in order to elucidate whether this parameter is responsible for the reported success obtained when using this microalgal species to enrich *Artemia*, or if other causes are involved. In fact, the fundamental importance of ARA for the correct development and normal pigmentation of some fish larvae was emphasized by Bell *et al*. (2003), besides the importance of DHA and EPA.

Further works using *Nannochloropsis* sp. to enrich *Artemia*, or the use of green water conditions with this microalga, or with other species, in controlled conditions and with

replicates are needed to evaluate and understand the real effects of this procedements on the survival and growth of octopus paralarvae. This would help to elucidate if environmental conditions (light diffusion in tanks, gut flora enhancement, anti-bacterial effects on water) are on the basis of the good results observed, or if nutritional causes are the main reason. It is also necessary to carry out further studies about the effects of the diet on the performance and composition of paralarvae.

High dietary protein:lipid ratio improves growth of *Octopus vulgaris* paralarvae

Capítulo 4 / Chapter 4

Abstract

In this study *Octopus vulgaris* paralarvae were fed on different combinations of enriched *Artemia* juveniles (1.6-2.8 mm), with the aim of analysing the effects of the diet on the growth, survival and biochemical composition of paralarvae. Food was supplied twice a day, with the first meal being common to all groups and consisting of 3-day old *Artemia* enriched with a mixed diet of microalgae (*Rhodomonas lens* and *Isochrysis galbana* in a proportion of 70%:30% dry weight basis). In the second meal paralarvae received *Artemia* enriched as follows: group control (AR+I) was given 3-day old juveniles enriched for another 6 h with the same microalgae; group AGOLD was given juveniles enriched for 6 h with Ori-Gold® (Skretting); and group AGOPEL received *Artemia* enriched for 6 h with a manual prepared diet consisting of grinded pellets for turbot supplemented with 10% of Ori-Gold® (wet weight basis). The gross biochemical composition of wild *Maja brachydactyla* zoeae was also analyzed in order to establish nutritional comparisons with the *Artemia* juveniles. The percentage of docosahexaenoic acid (DHA, 22:6n-3) in *Artemia* (% of total fatty acids) increased as follows: AR+I (1.6%) < AGOPEL (5.7%) < AGOLD (8.0%, $P<0.05$). The protein:lipid (P:L) ratios found in the different *Artemia* juveniles were: 5.4 in AR+I, 4.5 in AGOPEL and 3.9 in AGOLD. Survival of paralarvae ranged between 35 and 53% after 15 days, and between 7 and 20% at day 25. Despite there was a tendency for higher survival rate in group AR+I, no statistically significant differences were found among groups. The dry weight (DW) and total length (TL) of 15-day post-hatch (dph) and 25-dph paralarvae from groups AR+I and AGOPEL was higher than values found for paralarvae from group AGOLD ($P<0.05$). A positive linear correlation was found between dietary P:L ratio and paralarval DW for both 15-dph and 25-dph paralarvae ($P<0.01$), while no correlation could be established between EPA or DHA and any of the growth parameters. Regarding the biochemical composition data, octopus hatchlings contained nearly 68% protein (% of DW), which decreased slightly to 64-66% in 15-dph and 25-dph paralarvae from all groups. Despite no significant differences were found in the lipid content of 15-dph paralarvae, in 25-dph individuals from group AGOLD, lipid was higher (11.8%) than in paralarvae from the remaining groups (10.7-10.9%, $P<0.05$). As for the fatty acid (FA) composition, eicosapentaenoic acid (EPA, 20:5n-3)

increased from an initial value of 13% in hatchlings, to 20-22% in 25-dph paralarvae from all groups. In contrast, a remarkable drop of DHA was found from hatchlings (20% of total FA) to 25-dph paralarvae from all groups, though individuals from groups AGOLD and AGOPEL contained significantly higher levels (nearly 10%) than paralarvae from AR+I (7%, $P<0.05$). Despite this observation, paralarvae from AR+I showed the highest DW and TL, and this group was the only one to attain 35-dph paralarvae. The FA composition of 25-dph and 35-dph paralarvae from group AR+I also revealed that DHA levels stabilized in this period. These results showed that the P:L ratio of *Artemia* was far more important to sustain a good performance of paralarvae than the FA composition of *Artemia* per se.

1. Introduction

Zoeae of some crustacean species have been shown to represent appropriate prey to rear *Octopus vulgaris* paralarvae until the settlement stage, when they become benthic juveniles (Itami *et al*., 1963; Villanueva, 1994, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006). In contrast, the use of *Artemia* (enriched nauplii or juvenile) alone or in co-feeding regime with inert microdiets was not successful in the majority of the studies (Iglesias *et al*., 2000; Navarro and Villanueva, 2000; 2003; Villanueva *et al*., 2002, 2004; Okumura *et al*., 2005). So far, only Hamazaki *et al*. (1991) reported the achievement of benthic *O. vulgaris* when using juvenile *Artemia* (1.5-2 mm) alone, through enrichment with *Nannochloropsis* sp and creating green-water conditions with the same microalgal species. Similarly, Moxica *et al*. (2006) reported good results when trying the same culture conditions, obtaining paralarvae in the pre-settlement stage after 70 days of rearing. Previous works with other cephalopods have also shown that the performance and growth of *Sepia officinalis* hatchlings was better with natural zooplankton dominated by mysid shrimp than with adult *Artemia* (Domingues *et al*., 2001).

Marine zooplankton is known to represent better prey for marine larvae than rotifer or *Artemia* per se, mainly due to differences in key nutritional components (Sargent *et al*., 1999; Bell, *et al*., 2003; Støttrup, 2003). Zooplankton is naturally rich in phospholipids, cholesterol, and in n-3 highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) (Navarro and Villanueva, 2000; Bell *et al*., 2003), which are of major importance as structural components for membrane biogenesis and as precursors of physiological active molecules (Sargent *et al*., 1999; Tocher *et al*., 2008). Furthermore, natural zooplankton was shown to contain higher amounts of free amino acids than nauplii of *Artemia franciscana* (Helland *et al*., 2003). Limitations to the use of copepods and other zooplankton arise principally from the difficulty in providing sufficient live food and for the risks of disease that extensive culture methods imply (Bell *et al*., 2003). Moreover, no control in the production of constant amounts of certain crustacean zoeae can be exerted, and the production of copepods in intensive culture is often unpredictable. Due to these limitations, the improvement of *Artemia* biochemical composition and the development of artificial microdiets have been pointed as key issues in octopus paralarval rearing, to overcome the high mortalities often encountered (reviewed by Iglesias *et al*., 2007a).

Important information about the body composition of early life stages of *O. vulgaris* has been published in the last few years (Navarro and Villanueva, 2000, 2003; Villanueva *et al*., 2004; Villanueva and Bustamante, 2006; Villanueva *et al*., 2009), which allowed to address many questions related with the possible nutrient requirements of this species. In the experiments in which paralarvae were fed *Artemia* enriched with commercial products or in co-feeding regime with microdiets, important lipid changes, both at quantitative and qualitative levels, were observed in the composition of the reared paralarvae in comparison with the profiles found in octopus hatchlings or in wild juveniles (Navarro and Villanueva, 2000, 2003). Those findings lead the authors to suggest that lipid imbalances in the diet composition, especially in the FA profile (levels of HUFAs and ratio DHA/EPA), could be the reason for the poor growth and high mortalities encountered. Recently, we have also described (results from chapter 2) similar changes in the FA composition of paralarvae fed juvenile *Artemia* enriched with either a mixed diet of *Rhodomonas lens* and *Isochrysis galbana*, or with a commercial lipid emulsion rich in DHA, with a remarkable drop of DHA levels being observed in paralarvae with time.

In the present work three feeding regimes, consisting of combinations of enriched *Artemia* juveniles, were tested to rear *O. vulgaris* paralarvae. For the enrichment of *Artemia* juveniles the following diets were used: "premium" quality microalgae (*R. lens* and *I. galbana*) with constant and controlled composition, a commercial product very rich in n-3 HUFAs (Ori-Gold®), or a manually prepared diet rich in protein and in n-3 HUFAs. The purpose of enriching *Artemia* juveniles with different diets was to modulate its biochemical composition, with the aim of delivering different essential nutrient compounds to paralarvae. The gross composition of wild *Maja brachydactyla* zoeae, a prey that has been described as suitable to rear octopus paralarvae (as mentioned in chapter 1), was also assessed to establish nutritional comparisons with the *Artemia* juveniles.

2. Material and methods

2.1 Production and enrichment of *Artemia* sp. juveniles

Artemia nauplii (AF, INVE, Belgium) were initially grown with *Rhodomonas lens* in 12-l plastic tanks at 26.5±0.5 ºC. Two-day old *Artemia* (≈ 1.3 mm) were finally enriched for 24 h with a mixed diet of *Rhodomonas lens* and *Isochrysis galbana* (70%:30% DW basis)

cultured semi-continuously in in nutrient saturated conditions and with a daily renewal rate of 30%, as previously described (see chapter 2 or Seixas *et al*., 2008). These *Artemia* juveniles were supplied to all paralarval treatments as the first meal. In the second daily meal, paralarvae were fed 3-day old *Artemia* (≈ 2.1 mm) enriched for further 6 h with one of the following diets: control group (AR+I) was fed *Artemia* enriched with the same microalgae described above; group AGOLD was fed *Artemia* enriched with a commercial product very rich in DHA (Ori-Gold®, Skretting) according to recommendations of the manufacturer (analysis of the FA composition of Ori-Gold® showed 24% of DHA and 5% of EPA, of the total FA, data not shown); and finally group AGOPEL was fed *Artemia* enriched with grinded commercial pellets for turbot (Sorgal, Portugal) deprived of fish-oil but supplemented with 10% of Ori-Gold® (wet weight basis) to assure a higher DHA level, using 0.4 mg grinded pellets Artemia^{-1}. Samples of *Artemia* juveniles were washed with distilled water and immediately frozen at -18 ºC for later biochemical analysis. The gross composition of *Artemia* juveniles is shown in Table I, whereas the total FA composition is shown in Table II.

2.2 Rearing experiment of *Octopus vulgaris* paralarvae

One-dph paralarvae hatched from two broods of *O. vulgaris* kept in the facilities of the Spanish Oceanographic Institute of Vigo (IEO of Vigo, Spain) were transported to the University of Santiago de Compostela (Spain) in a 35-l container and in darkness. Upon arrival paralarvae were immediately distributed into nine conical fibre glass tanks with 50 cm diameter and white walls, containing 50-l of seawater. The semi-closed water circuit was equipped with particle retention meshes and biological filters, and water was daily renewed at 10% of the total volume. Ammonia levels and pH were daily checked to assure normal water parameters. Before entering the circuit, seawater (34 ppt) was made to cross through 10 µm filter-cartridges and UV lamps. Water temperature was kept at 19.5±0.5 ºC in a stable climate room using thermo-heaters RENA 100W. Light was provided by fluorescent day-light lamps placed 40 cm above water surface, and a photoperiod of 14 h light:10 h dark was established. Paralarvae density was established at 10 individuals l^{-1}. The meals supplied to paralarvae consisted of juvenile *Artemia* (1.6-2.8 mm) enriched with either microalgae or other nutrient supplements and were distributed twice a day (11:00 a.m. and 05:00 p.m.) at equal proportions, in a total amount of 0.05 *Artemia* ml^{-1} day^{-1}.

Three groups were set in triplicate, each receiving a different combination of *Artemia* juveniles. The first meal was equal for all groups and consisted of juveniles enriched with a mixed diet of microalgae (*Rhodomonas lens* and *Isochrysis galbana*), whereas in the second meal paralarvae received one of the following diets: group control (AR+I) was given the same juveniles enriched with microalgae; group AGOLD was given *Artemia* juveniles enriched for 6 h with Ori-Gold®; and group AGOPEL received *Artemia* enriched for 6 h with the manually prepared diet. Paralarvae were measured under a stereoscope using a calibrated ocular micrometer (n=40 for hatchlings, and 12 individuals per replicate on days 15 and 25 of rearing). Total length and mantle length were measured as described by Villanueva (1995). Dry weight (DW) of paralarvae was determined individually (n=10 per replicate) after washing individuals with distilled water and drying in a stove at 100±1 ºC for 24 h. Daily growth index (DGI, % G) was calculated as follows: 100 x [($DW_f^{1/3}$-$DW_i^{1/3}$)/(Tf-Ti)], where DW_f and DW_i are the dry weight of paralarvae at sampling days T_f and T_i. Samples of 1-dph paralarvae (50 mg) and of 15-dph, 25-dph and 35-dph paralarvae (60 paralarvae per replicate) were collected and immediately frozen at -18 ºC for later biochemical analyses.

2.3 Collection of *Maja brachydactyla* zoeae for gross composition analysis

Wild spider-crab zoeae (*Maja brachydactyla*) obtained from female spider-crabs collected in Portugal between Viana do Castelo (41º 41'N; 8º 52'O) and Caminha (41º49'N; 8º53'W), as described in chapter 1, were used to carry out gross composition analysis. Zoeae were briefly washed with distilled water before being frozen at -18 ºC for later biochemical analysis.

2.4 Biochemical composition analysis

C-N-H analyses of *Artemia* and paralarvae were determined by combustion using an autoanalyzer Fisons Model EA 1108, using acetanilide as standard compound. Protein content was derived from the total amount of nitrogen using the conversion formula Nx6.25 (National Research Council, 1993). Total lipid was extracted with chloroform/methanol (2:1 v/v) according to Bligh and Dyer (1959) and calculated gravimetrically, whereas carbohydrate was determined by the phenol/sulphuric acid

method (Kochert, 1978). Ash content was determined in a muffle furnace heated at 550 ºC for 24 h. Fatty acids composition was determined by submitting total lipid extracts to methanolysis (5% HCl in methanol) at 85 ºC during 2.5 h (Sato and Murata, 1988), followed by extraction of the methyl esters with hexane. Identification and quantification of fatty acids was made using a GC-MS (Fisons Instruments, MD-800) equipped with a column Omegawax 250 (Supelco) 30 m x 0.25 mm and using helium as gas carrier. Triheptadecanoin (Sigma, St. Louis, Mo.) was used as internal standard. All biochemical analyses were carried out in triplicate.

2.5 Statistical analysis

Statistical analyses were performed using the software SPSS V 15.0.1 statistical package (SPSS, Inc.). Total length and mantle length of paralarvae were compared by analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05. After log-transformation of dry weight data and arcsine-√ transformation of biochemical composition and survival percentages the same statistical tests were carried out (Zar, 1999). Statistical comparisons of dietary P:L ratios and of DGI among groups were carried out by the non-parametric test of Kruskal-Wallis (Zar, 1999).

3. Results

3.1 Composition of *Artemia* juveniles

The gross biochemical composition of *Artemia* juveniles (% of DW) is shown in table I. Protein content in juvenile *Artemia* was similar among groups and ranged between 63 and 65%, though group AGOPEL had a significantly higher percentage ($P<0.05$). The highest lipid content was observed in juveniles from group AGOLD (16%), followed by AGOPEL 14.6%), while juveniles from AR+I contained a significantly lower lipid content (12%, $P<0.05$). Carbohydrate content ranged between 9 and 10% in groups AR+I and AGOPEL, and was lower in group AGOLD (7%, $P<0.05$). As discussed in chapter 2, since carbohydrate levels in *Artemia* juveniles were roughly similar (even though juveniles from AGOLD had a slight lower content) and represented less that 10% of the prey composition,

the simplified index P:L ratio was used instead of the protein:energy ratio. Therefore, the P:L ratio among the different *Artemia* juveniles supplied to paralarvae were 5.4 (AR+I), 4.5 (AGOPEL) and 3.9 in AGOLD (Table I).

Regarding the fatty acid composition of *Artemia* juveniles (Table II), important differences among *Artemia* juveniles were found, especially in HUFA content. Individuals from group AGOLD contained the highest levels of DHA (8%) and of arachidonic acid (ARA) (1.8%), whereas juveniles from group AGOPEL contained the highest level of eicosapentaenoic acid (EPA) (15.4%). The percentage of saturated FA were nearly the same in all groups (25-28%), whereas monoenes in *Artemia* juveniles from AR+I were about half of the values found in juveniles from AGOLD and AGOPEL. Total PUFA were higher in juveniles from AR+I mainly due to the high levels of 18:3n-3 and 18:4n-3. The highest DHA/EPA ratio was found in *Artemia* from AGOLD, whereas the ratio EPA/ARA was much lower in this same group in comparison with the other groups (Table II).

Table I. Gross biochemical composition of *Artemia* juveniles (1.5-2.8 mm) enriched with either a mixed diet of microalgae or with other nutrient supplements, which were used to feed *Octopus vulgaris* paralarvae.

	AR+I	AGOLD	AGOPEL
Protein (N x 6.25)*	63.8 ± 0.9^{b}	63.4 ± 0.4^{b}	65.5 ± 0.3^{a}
Lipid*	11.8 ± 0.8^{b}	16.2 ± 1.2^{a}	14.6 ± 1.2^{a}
Carbohydrate*	9.9 ± 0.5^{a}	6.8 ± 0.2^{b}	8.9 ± 0.8^{a}
Ash*	16.2 ± 1.0^{a}	$14.6 \pm 1.2^{a,b}$	13.9 ± 1.1^{b}
P:L ratio**	5.4 ± 0.2^{a}	3.9 ± 0.3^{b}	$4.5 \pm 0.2^{a,b}$
C:N ratio**	4.8 ± 0.08^{b}	5.1 ± 0.03^{a}	5.0 ± 0.02^{b}

AR+I: *Artemia* enriched with *Rhodomonas lens* and *Isochrysis galbana*; AGOLD: *Artemia* enriched for 6 h with Ori-Gold®; AGOPEL: *Artemia* enriched for 6 h with grinded pellets supplemented with 10% of Ori-Gold® (wet weight basis). Data are means ± S.D. Different superscript letters within the same line indicate significant differences among groups. *ANOVA followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons ($P<0.05$); **Non-parametric test of Kruskal-Wallis ($P\leq0.05$).

Table II. Fatty acid (FA) composition (% of total FA) and FA content (% of dry weight) of *Artemia* juveniles enriched with either microalgae or other nutrient supplements, which were used to feed *Octopus vulgaris* paralarvae.

	Artemia juveniles (1.5-2.8 mm)		
Fatty acid	AR+I	AGOLD	AGOPEL
14:0	1.9 ± 0.0[a]	1.3 ± 0.1[b]	1.8 ± 0.0[a]
16:0	14.6 ± 0.2[a]	12.7 ± 0.4[b]	12.8 ± 0.3[b]
16:1n-7	2.5 ± 0.0[a]	3.7± 0.9[b]	7.5 ± 0.1[c]
18:0	10.9 ± 0.2[a]	10.1 ± 0.1[b]	9.5 ± 0.1[c]
18:1n-9	3.1 ± 0.1[a]	14.7 ± 0.2[b]	14.2 ± 0.2[b]
18:1n-7	9.1 ± 0.2[a]	8.0 ± 1.1[b]	10.6 ± 0.2[b]
18:2n-6	1.0 ± 0.2[a]	10.1 ± 0.5[b]	8.0 ± 0.1[b]
18:3n-3	23.2 ± 0.2[a]	9.7 ± 1.5[b]	5.1 ± 0.1[c]
18:4n-3	12.9 ± 0.3[a]	4.1 ± 0.2[b]	4.1 ± 0.1[b]
20:1n-9	0.4 ± 0.2[a]	0.6 ± 0.3[b]	1.0 ± 0.1[b]
20:2n-6	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
20:4n-6	0.4 ± 0.1[a]	1.8 ± 0.8[b]	1.4 ± 0.0[b]
20:3n-3	1.0 ± 0.0[a]	0.7 ± 0.0[b]	0.2 ± 0.0[c]
20:4n-3	2.2 ± 0.1[a]	0.9 ± 0.1[b]	1.4 ± 0.1[c]
20:5n-3	13.8 ± 0.6[a]	11.2 ± 0.3[b]	15.4 ± 0.2[c]
22:1	n.f.	n.f.	0.2 ± 0.0
22:5n-6	n.f.	0.5 ± 0.2	n.f.
22:5n-3	n.f.	0.4 ± 0.3	0.3 ± 0.1
22:6n-3	1.6 ± 0.2[a]	8.0 ± 0.7[b]	5.7 ± 0.1[c]
∑Saturated	28.0 ± 0.4[a]	25.2 ± 0.3[b]	24.8 ± 0.4[b]
∑Monoenes	15.8 ± 0.3[c]	27.1 ± 0.2[b]	33.5 ± 0.1[a]
∑PUFA	56.2 ± 0.5[a]	47.8 ± 0.5[b]	41.7 ± 0.4[c]
n-3	54.8 ± 0.7[a]	35.0 ± 1.2[b]	32.2 ± 0.4[c]
n-6	1.4 ± 0.3[c]	12.8 ± 0.8[a]	9.5 ± 0.1[b]
DHA/EPA	0.1	0.7	0.4
EPA/ARA	34.5	6.2	11.0
FA content	2.5 ± 0.2[b]	3.4 ± 0.1[a]	3.1 ± 0.3[a]

∑Saturated include the FA: 15:0 and 22:0

Abbreviations are like in table I. Data are means ± S.D. of triplicate analyses. n.f.: not found. Different superscript letters within the same line indicate significant differences among groups (α=0.05).

3.2 Survival, growth and biochemical composition of paralarvae

Survival rates of 15-dph paralarvae among groups ranged between 35 and 53%, and between 7 and 20% at day 25 (Fig. 1), but despite these differences no statistically significant differences were found among groups, due to high standard deviations in some of the treatments.

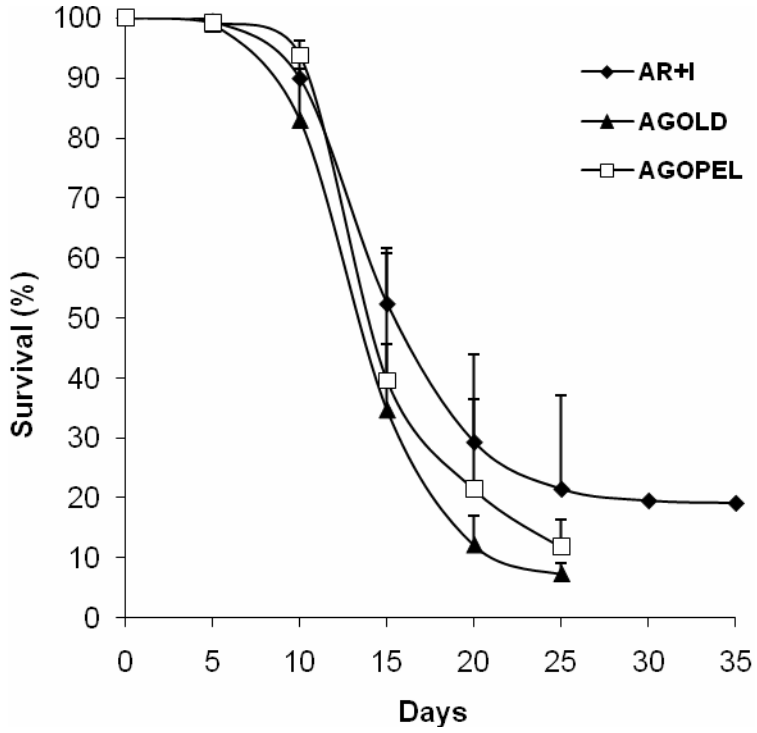

Figure 1 - Survival (%) of paralarvae fed on three different dietary treatments in the course of the experiment. Data are means ± S.D. (n=3). Data of survival on days 30 and 35 correspond to a single tank of group AR+I.

After sampling paralarvae on day 25 for biometric and biochemical composition analyses, only group AR+I remained with individuals and after 35 days a single tank attained a survival of 19%. Regarding the dry weight (DW) of 15-dph paralarvae (Fig. 2), individuals from groups AR+I and AGOPEL showed higher DW than those from group AGOLD, with significance levels of $P<0.001$ and $P<0.01$, respectively. The same trend for higher DW of 25-dph paralarvae from groups AR+I and AGOPEL was observed in comparison with paralarvae from group AGOLD ($P<0.001$ and $P<0.05$, respectively). The DGI (% G) of 15-

dph and of 25-dph paralarvae was higher in groups AR+I and AGOPEL (Table III). The total length (TL) and dorsal mantle length (ML) of paralarvae followed the same tendency of the results found for paralarval DW (Table III). The maximum values of TL and ML were found in paralarvae from group AR+I, and the lowest in paralarvae from group AGOLD, with significant differences being observed among groups at both sampling days (Table III). In general, significant differences in both TL and ML were only observed between paralarvae from groups AR+I and AGOLD, whereas between groups AGOPEL and AGOLD, or between AR+I and AGOPEL, significant differences were only found in the TL of 25-dph paralarvae (Table III).

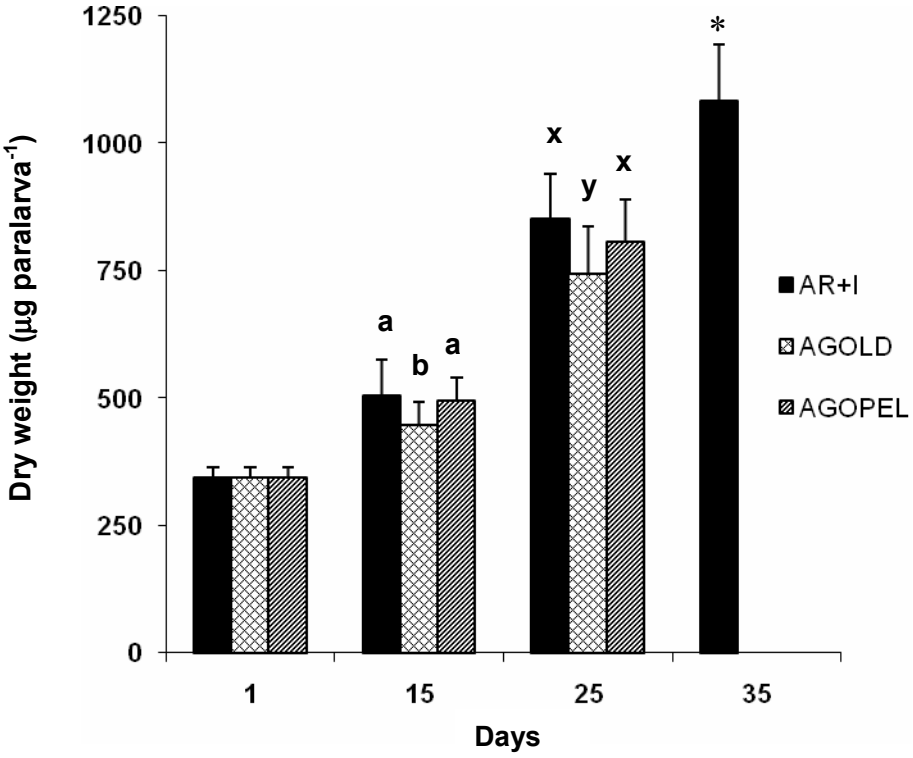

Figure 2 - Dry weight of octopus paralarvae (µg paralarva^{-1}) fed on three different dietary treatments. Data are means ± S.D. (n=3, 10 individuals per replicate were individually weighed). Different superscript letters within the same day indicate significant differences among groups (α=0.05). *Dry weight of 35-dph paralarvae from group AR+I correspond to individuals from a single tank (n=15).

Table III. Total length (TL), dorsal mantle length (ML), daily growth index (DGI x 100, % G) and gross biochemical composition (% of dry weight) of *Octopus vulgaris* hatchlings and of reared paralarvae fed on three different diets.

		15-dph paralarvae			25-dph paralarvae			35-dph
	Hatchlings	AR+I	AGOLD	AGOPEL	AR+I	AGOLD	AGOPEL	AR+I♦
TL (mm)*	2.77 ± 0.17	3.96 ± 0.14^{a}	3.71 ± 0.21^{b}	3.77 ± 0.23^{b}	4.52 ± 0.32^{x}	4.13 ± 0.26^{y}	4.33 ± 0.30^{z}	5.45 ± 0.24
ML (mm)*	2.01 ± 0.12	2.54 ± 0.10^{a}	2.46 ± 0.15^{b}	$2.49 \pm 0.16^{a,b}$	2.96 ± 0.20^{x}	2.81 ± 0.16^{y}	$2.94 \pm 0.16^{x,y}$	3.56 ± 0.13
DGI (% G)**		6.8 ± 0.3	4.5 ± 0.6	6.4 ± 0.5	10.3 ± 0.2^{x}	8.5 ± 0.2^{y}	9.6 ± 0.1^{z}	2.6
Protein (%)*	67.8 ± 2.9	65.2 ± 2.2	64.5 ± 1.5	65.2 ± 0.7	65.8 ± 1.7	66.3 ± 1.1	66.5 ± 0.8	66.2 ± 0.1
Lipid (%)*	14.7 ± 1.1	14.6 ± 1.4	15.1 ± 1.4	13.8 ± 1.8	10.9 ± 0.7^{x}	11.8 ± 0.5^{y}	10.7 ± 0.4^{x}	10.0 ± 0.6
C:N ratio**	4.6 ± 0.04	4.7 ± 0.08	4.8 ± 0.08	4.7 ± 0.06	4.8 ± 0.05^{x}	$4.9 \pm 0.05^{x,y}$	5.0 ± 0.07^{y}	4.7 ± 0.01

AR+I: group fed 100% *Artemia* enriched with *R. lens* and *I. galbana*; AGOLD: group fed 50% *Artemia* enriched with *R. lens* and *I. galbana*, and 50% *Artemia* enriched for 6 h with Ori-Gold®; AGOPEL: group fed 50% *Artemia* enriched with *R. lens* and *I. galbana*, and 50% *Artemia* enriched for 6 h with grinded pellets supplemented with 10% of Ori-Gold® (wet weight basis). –dph: days post hatch. C:N ratio is in (atoms). Data are means ± S.D. Different superscript letters within the same day indicate significant differences among groups. *ANOVA followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons (α=0.05); **Non-parametric test of Kruskal-Wallis ($P \leq 0.05$). ♦Data of 35-dph paralarvae are from a single tank of group AR+I (n=15 for total length and dorsal mantle length, and triplicate analysis for biochemical composition results).

In figure 3 are shown photos under a stereoscope of octopus hatchlings and of 25-dph and 35-dph paralarvae.

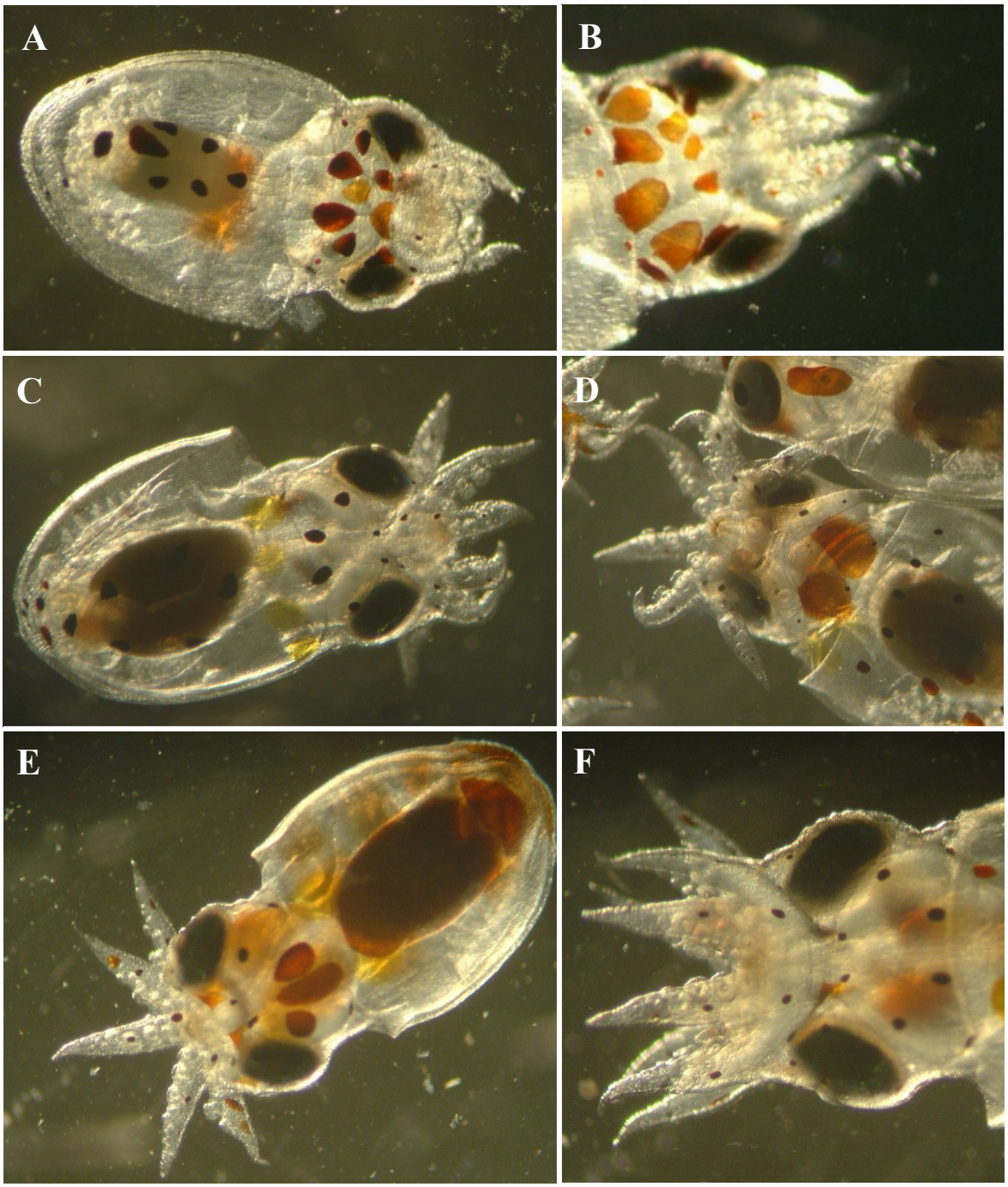

Figure 3 – Photographs of *Octopus vulgaris* hatchlings and of reared paralarvae under a stereoscope. A-B: Newly born hatchlings (total length ≈ 2.8 mm); C-D: 25-dph paralarvae with longer arms but still with 3 suckers per arm (total length ≈ 4.5 mm); E-F: 35-dph paralarvae with more suckers coming up in the arms (total length ≈ 5.4 mm).

When plotting the values of the dietary P:L ratio and the DW of 15-dph and 25-dph paralarvae, positive linear correlations could be found between these two parameters at a significant level of $P<0.01$ (Fig. 4).

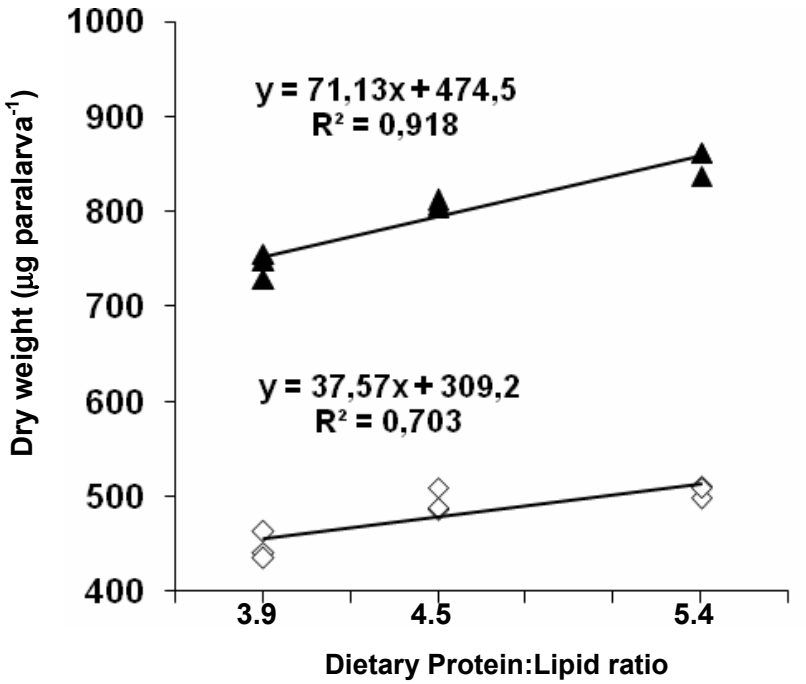

Figure 4 - Correlations between dietary protein:lipid ratios (AGOLD= 3.9; AGOPEL= 4.5; AR+I= 5.4) and the dry weight of 15-dph (◇) and 25-dph (▲) paralarvae from the corresponding groups. The equations given for each of the positive linear slopes were statistically significant at a level of $P<0.01$.

The protein content found in octopus hatchlings was circa 68%, decreasing slightly to 64-66% in 15-dph and 25-dph paralarvae from all groups (Table III). Despite no significant differences were found in the lipid content of 15-dph paralarvae, in 25-dph individuals from group AGOLD a higher lipid content was found in comparison with paralarvae from the remaining groups ($P<0.05$). The C:N ratio found in paralarvae corroborated the results found for the proportion of protein to lipid content in paralarvae (Table III).

Results of the total FA composition of paralarvae are shown in Table IV. The major FA found in octopus hatchlings were DHA and palmitic acid (16:0), each accounting for 20% of total FA, followed by estearic acid (18:0) and EPA (15% and 13%, respectively), as observed in the previous experiments of paralarvae rearing (chapters 2 and 3, though a lower value of 16:0 was found in this study). ARA represented 6.6% of total FA. Total PUFA in hatchlings were circa 48% and remained nearly unchanged in paralarvae along time. In contrast, monoenes increased in paralarvae in comparison with initial values, at the expense of a decrease in saturated FA. The most striking differences between hatchlings and paralarvae were observed at the level of HUFA. Whereas DHA and ARA decreased dramatically with time, a progressively increase of EPA was observed in all groups. Paralarvae from both groups AGOLD and AGOPEL contained significantly higher percentages of DHA than paralarvae from AR+I ($P<0.05$). These lower rates of DHA-decrease found in groups AGOLD and AGOPEL could be related to the higher DHA content found in the diets. However, this remarkable reduction of DHA was not correlated with poor growth or low survival, as no differences were found between paralarvae from groups AR+I and AGOPEL. Moreover, despite containing the lowest levels of DHA, paralarvae from AR+I had higher DW and TL than paralarvae from AGOLD, and this was the only group to attain 35-dph paralarvae. Interestingly, the levels of DHA in group AR+I stabilized between day 25 and 35, whereas the levels of EPA increased slightly.

3.3 Gross composition of *Maja brachydactyla* zoeae

The gross composition (% of DW) of wild spider-crab zoeae revealed a protein content of 55.7 ± 0.3%, whereas lipid comprised 7.9 ± 1.1%, and thus a P:L ratio of 7.1 was observed. Ash represented 34.4 ± 2.3% of the DW, whereas the C:N ratio found in zoeae was 4.2 ± 0.001.

Table IV. Fatty acid (FA) composition (% of total FA) and total FA (% of dry weight) of *Octopus vulgaris* hatchlings and of 15-dph, 25-dph and 35-dph paralarvae from the three dietary treatments.

		15-dph paralarvae		
Fatty acid	Hatchlings	AR+I	AGOLD	AGOPEL
14:0	1.7 ± 0.4	1.1 ± 0.1	1.1 ± 0.2	1.4 ± 0.2
15:0	0.5 ± 0.3	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
16:0	20.3 ± 1.1	14.5 ± 0.6	14.5 ± 0.0	15.6 ± 2.0
16:1n-7	1.2 ± 0.3	1.9 ± 0.2	2.0 ± 0.1	2.2 ± 0.2
18:0	15.5 ± 0.7	15.1 ± 0.7	14.6 ± 0.5	15.0 ± 2.2
18:1n-11	1.8 ± 0.2	n.f.	n.f.	n.f.
18:1n-9	3.0 ± 0.2	7.1 ± 1.0	7.7± 0.1	7.7 ± 0.9
18:1n-7	1.8 ± 0.0	4.9 ± 0.6	4.8 ± 0.2	5.3 ± 0.6
18:2n-6	1.2 ± 0.4	3.3 ± 1.5	4.0 ± 0.1	3.5 ± 0.4
18:3n-3	n.f.	5.2 ± 0.9	4.1 ± 0.2	4.3 ± 0.8
18:4n-3	n.f.	2.6 ± 0.3^{a}	1.7 ± 0.2^{b}	1.8 ± 0.4^{b}
20:0	n.f.	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
20:1n-9	4.2 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
20:2n-6	0.7 ± 0.0	0.6 ± 0.0^{b}	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}
20:4n-6	6.6 ± 0.3	3.1 ± 0.4^{b}	3.8 ± 0.2^{a}	$3.5 \pm 0.1^{a,b}$
20:3n-3	1.8 ± 0.1	1.6 ± 0.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}
20:4n-3	n.f.	1.7 ± 0.4^{a}	$1.2 \pm 0.1^{a,b}$	1.0 ± 0.1^{b}
20:5n-3	13.4 ± 0.5	18.3 ± 1.2	16.3 ± 0.2	16.3 ± 1.1
22:0	n.f.	0.9 ± 0.1	0.8 ± 0.2	0.7 ± 0.0
22:1	1.5 ± 0.1	1.4 ± 0.3	1.1 ± 0.1	1.1 ± 0.3
22:4n-6	1.6 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
22:5n-6	0.7 ± 0.0	0.9 ± 0.2	0.8 ± 0.1	0.7 ± 0.1
22:5n-3	2.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.1 ± 0.1
22:6n-3	20.3 ± 0.1	9.8 ± 1.0^{b}	13.6 ± 0.7^{a}	12.4 ± 0.1^{a}
Saturated	38.1 ± 1.2	32.5 ± 1.2	32.0 ± 0.3	33.6 ± 4.3
Monoenes	13.6 ± 0.6	18.1 ± 0.9	18.3 ± 0.1	18.8 ± 1.7
PUFA	48.4 ± 0.6	49.4 ± 1.2	49.7 ± 0.3	47.6 ± 2.7
n-3	37.7 ± 0.6	40.4 ± 0.9	39.5 ± 0.3	38.3 ± 2.5
n-6	10.7 ± 0.4	$9.0 \pm 1.0^{a,b}$	10.2 ± 0.0^{a}	9.3 ± 0.3^{b}
DHA/EPA	1.5	0.5	0.8	0.8
FA (% of DW)	8.6 ± 0.2	7.3 ± 0.7	8.8 ± 0.6	7.2 ± 0.8

Abbreviations are like in Table III. Data are means ± S.D. (n=3). n.f.: not found. Different superscript letters within the same day indicate significant differences among groups ($P<0.05$).

Table IV. (continued)

	25-dph paralarvae			35-dph paralarvae
Fatty acid	AR+I	AGOLD	AGOPEL	AR+I*
14:0	1.2 ± 0.2	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.0
15:0	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
16:0	$13.3 \pm 0.6^{x,y}$	14.2 ± 0.2^{y}	12.7 ± 0.6^{x}	14.0 ± 0.7
16:1n-7	2.4 ± 0.3	2.2 ± 0.4	2.6 ± 0.2	2.0 ± 0.4
18:0	15.7 ± 1.0^{x}	$15.1 \pm 0.8^{x,y}$	13.6 ± 0.4^{y}	14.5 ± 1.0
18:1n-11	n.f.	n.f.	n.f.	2.2 ± 0.5
18:1n-9	6.7 ± 0.1	7.1 ± 0.7	8.8 ± 0.5	4.9 ± 0.5
18:1n-7	7.3 ± 0.2	6.3 ± 1.4	6.8 ± 0.5	5.6 ± 0.6
18:2n-6	1.4 ± 0.0^{y}	2.8 ± 0.9^{x}	3.1 ± 0.3^{x}	2.3 ± 1.0
18:3n-3	5.9 ± 0.2^{x}	4.8 ± 0.2^{y}	$5.4 \pm 0.6^{x,y}$	5.0 ± 0.3
18:4n-3	3.1 ± 0.1^{x}	2.2 ± 0.1^{y}	$2.6 \pm 0.4^{x,y}$	2.4 ± 0.1
20:0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
20:1n-9	2.7 ± 0.2	2.7 ± 0.1	2.9 ± 0.2	2.9 ± 0.3
20:2n-6	0.6 ± 0.0^{y}	0.8 ± 0.1^{x}	0.8 ± 0.1^{x}	0.8 ± 0.1
20:4n-6	2.7 ± 0.1	3.1 ± 0.2	3.2 ± 0.3	3.2 ± 0.4
20:3n-3	1.6 ± 0.1	1.5 ± 0.2	1.4 ± 0.1	1.6 ± 0.1
20:4n-3	2.1 ± 0.1^{x}	1.5 ± 0.1^{y}	1.6 ± 0.1^{y}	1.7 ± 0.1
20:5n-3	21.8 ± 0.1	20.4 ± 2.3	19.6 ± 0.4	23.8 ± 2.2
22:0	0.5 ± 1.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
22:1	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
22:4n-6	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
22:5n-6	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.2
22:5n-3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
22:6n-3	7.2 ± 0.5^{y}	10.0 ± 0.3^{x}	9.6 ± 0.6^{x}	8.0 ± 0.7
Saturated	31.6 ± 1.9	31.6 ± 1.0	28.5 ± 0.9	30.6 ± 1.4
Monoenes	20.0 ± 0.2	19.3 ± 2.3	22.1 ± 1.0	18.7 ± 1.0
PUFA	48.4 ± 1.9	49.1 ±1.7	49.4 ± 0.2	50.7 ± 0.5
n-3	42.7 ± 1.8	41.4 ± 2.6	41.3 ± 0.4	43.6 ± 1.4
n-6	5.7 ± 0.2^{y}	7.7 ± 1.1^{x}	8.1 ± 0.6^{x}	7.1 ± 1.9
DHA/EPA	0.3	0.5	0.5	0.3
FA (% of DW)	6.8 ± 0.6	7.5 ± 0.2	7.4 ± 0.6	4.9 ± 0.2

Abbreviations are like in Table III. Data are means ± SD (n=3). *Analyses of 35-dph paralarvae are from a single tank of group AR+I (triplicate analysis). n.f.: not found. Different superscript letters within the same day indicate significant differences among groups ($P<0.05$).

Discussion

In the present work the DW of 15-dph and of 25-dph paralarvae was well correlated with the P:L ratio of the supplied *Artemia* juveniles, as higher DW of paralarvae were found with increasing dietary P:L ratio. On the contrary, no correlation could be established between DHA content of the diet and paralarvae growth. Despite no significant differences in paralarvae DW were found between groups AR+I and AGOPEL, the trend of growth followed the increase of P:L ratio in *Artemia*. In other molluscs such as juvenile *Sepia officinalis* and abalone *Haliotis fulgens* maximum dietary protein:energy ratios were also found to promote the best growth rates (Lee, 1994; Gómez-Montes *et al*., 2003). Previous works with marine-fish larvae have also shown that low P:L ratio diets or inadequate quantitative or qualitative lipid levels would promote poor growth, low survival or could interfere with larval digestion and absorption ability (Øie *et al*., 1997; Olsen *et al*., 2000; Morais *et al*., 2005).

The DW of 25-dph paralarvae found in this study (0.74-0.85 mg) was lower than values reported by other authors (1.4-3.4 mg) when using crustacean zoeae alone or *Artemia* combined with zoeae (Itami *et al*., 1963; Villanueva, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006). However, in comparison with other experiments on which paralarvae were fed *Artemia* alone or in co-feeding regime with micro-diets, the reported values are within the same range of those previously reported (Navarro and Villanueva, 2000; Moxica *et al*., 2002; Villanueva *et al*., 2002; Villanueva *et al*., 2004; Okumura *et al*., 2005). The direct comparison of DW values among different works is always risky to undertake, as many other key factors often vary considerably, such as temperature, prey density, tank dimensions and the use of green- or clear-water conditions.

Crustacean zoeae seem to undoubtedly represent better diets for paralarvae, and the reasons behind it are still not fully understood. The fact that zoeae contain higher levels of phospholipids and a better fatty acid composition than *Artemia* was already pointed out (Navarro and Villanueva, 2000), which could be one of the main reasons why zoeae are more beneficial to feed paralarvae and to obtain better growth rates. Bell *et al*. (2003) revised the major differences between *Artemia* and copepods concerning the lipid classes and the localization of HUFA with its implications to the correct development and lipid nutrition of flatfish larvae. These authors have described the importance of providing diets with well balanced DHA/EPA ratios, and emphasized that perhaps is not less important to

achieve correct ratios EPA/ARA to fulfil the nutritional requirements of larvae. Even if the composition analyses revealed that *M. brachydactyla* zoeae contained a lower protein percentage than *Artemia* juveniles, this was basically due to the high ash content of zoeae, corresponding mainly to its exoskeleton. This issue is of major importance when establishing nutritional comparisons between zoeae and *Artemia* in a DW basis, as differences in the behaviour of prey ingestion are also observed. Whereas the content of zoeae is ingested after an external digestion leaving the whole exoskeleton empty (Hernández-García *et al*., 2000), the whole body of *Artemia* is ingested (Iglesias *et al*., 2006). Subsequently, the P:L ratio of preys seems to be more useful to establish nutritional comparisons between zoeae and *Artemia* than composition as percentages of DW. Indeed, the P:L ratio of zoeae was higher than any of the values found in *Artemia* juveniles, and this prey was found by other authors to give some of the best results in the rearing the paralarvae (Iglesias, *et al*., 2004; Carrasco *et al*., 2006).

The protein content observed in *O. vulgaris* hatchlings (68%) was very similar to values reported by Villanueva *et al*. (2004) for hatchlings or wild juveniles (69 to 76%). Other authors reported lower values of protein in paralarvae (40-47%), but used different methodology for protein determination (Moxica *et al*., 2002). In this study, although a slight decrease in the protein content was observed from hatchlings to 15-dph paralarvae (64.5 to 66.5%), values remained unchanged in 25-dph and in 35-dph paralarvae. Lipid levels found in octopus hatchlings (14.7%) were slightly higher than values found in previous experiments (12%, see chapter 2) or than values reported by other authors (10-13%, Navarro and Villanueva, 2000; Okumura *et al*., 2005), remaining stable in 15-dph paralarvae. However, a decrease of lipid content was found in 25-dph paralarvae and in 35-dph paralarvae from group AR+I. The 25-dph paralarvae from group AGOLD contained higher lipid levels than paralarvae from the remaining groups ($P<0.05$), reflecting the higher lipid content of the supplied diet, i.e. *Artemia* enriched with Ori-Gold, a product that contains 85% lipid (data not shown). Navarro and Villanueva (2000, 2003) have also described an increase of lipid levels in reared paralarvae (14-25%) fed high-lipid diets, in comparison with the initial values found in hatchlings (13%). Moreover, these authors pointed out that this tendency was not in agreement with the progressive reduction of lipid levels found in wild octopus juveniles with increasing weight (from 12% to circa 7%). The reasons for the lower lipid levels found in paralarvae in the present work, in comparison with the results described by Navarro and Villanueva (2000, 2003), could be related with the lipid content of the diets. Whereas in this study *Artemia* juveniles contained from 12-

16% lipid, those authors used 24-h enriched *Artemia* containing 21-38% lipid or microdiets with 22-33% lipid to feed paralarvae.

The FA composition of octopus hatchlings found in this work was similar to results observed in previous experiments (see chapters 1, 2 and 3, Seixas *et al*., 2008) or reported by other authors (Navarro and Villanueva, 2000; Okumura *et al*., 2005), with an overall abundance of the saturated FA 16:0 and 18:0, and the HUFAs DHA, EPA and ARA, though some discrepancies in the percentages of certain FAs could be found. Although few changes in the proportion of the FA classes in paralarvae were observed over time, in comparison with the levels found in hatchlings, remarkable differences were observed in the percentages of certain PUFAs. A significant drop of DHA levels was found in 15-dph paralarvae from all groups, until less than 50% of the initial levels found in octopus hatchlings. This remarkable drop in DHA levels had been previously described by Navarro and Villanueva (2000, 2003), and was also observed in the previous experiments of paralarvae rearing (see chapters 2 and 3). The FA composition of 15-dph paralarvae from group AR+I was very similar to values observed in 15-dph paralarvae fed the same diet in previous works (chapter 2), supporting the reproducibility of results when using prey of controlled composition. However, other authors reported stabilization of DHA levels in octopus paralarvae fed enriched *Artemia* nauplii complemented with sandeel flakes (Okumura *et al*., 2005). Irrespective of the different findings, in this work we found that a diet with low DHA-content (*Artemia* from AR+I) is not a limiting issue for octopus paralarvae development, whenever the P:L ratio of the diet is high, as *Artemia* with four-times more DHA (AGOLD) did not induce better growth and survival rates. In experiments carried out with Senegal sole (*Solea senegalensis*) larvae, it has been shown that larvae fed *Artemia* nauplii absent of DHA, but containing other n-3 PUFA, could survive and grow as well as larvae fed nauplii with graded levels of DHA (4.4 to 14.7% of total FA), up to 36 days post-hatch (Villalta *et al*., 2005). These authors suggested that this species may have a low or negligible requirement for DHA when other n-3 PUFA are present, a postulation that had been previously described by Morais *et al*. (2004), who found no evidences that Senegal sole required high dietary DHA or EPA, as no relationship between dietary HUFA concentration and growth or survival was found. Other studies conducted with seabream (*Sparus aurata*) or turbot (*Scophtalmus maximus*) showed no relation between larval survival and n-3 HUFA levels in supplied diets (Koven *et al*., 1990; Rainuzzo *et al*., 1994; Reitan *et al*., 1994). However, in other works with read-sea bream (*Pagrus major*), seabream (*Sparus aurata*) and Atlantic halibut (*Hippoglossus*

hippoglossus) positive relationships between n-3 HUFA and performance of fish have been described (Watanabe, 1993; Salhi *et al*., 1994; Ibeas *et al*., 1994; Hamre and Harboe, 2008). Reaching the accurate n-3 HUFA requirements in early life stages of marine species is a hard task that needs lots of experimental work, due to the complexity of finding the exact quantity of n-3 HUFA to be included in the diet, the best relations of the HUFA in the diet (DHA/EPA, EPA/ARA, etc) or the effects of using different lipid sources in diets, as previously reported for seabream (Ibeas *et al*., 1996, 1997, 2000). The importance of phospholipids, HUFA and cholesterol in diets for paralarvae was suggested by Navarro and Villanueva (2000, 2003), based on information derived from the biochemical composition of octopus early life stages. However, specific works are still needed to find the particular requirements of n-3 HUFA by octopus paralarvae, both at quantitative and qualitative levels, and also to investigate the role and importance of each HUFA. The modulation of cholesterol and phospholipid levels in *Artemia* juveniles may also be an important subject to look for. For example, it has been shown that the enhancement of phospholipids, vitamins and free amino acid levels in live prey such as rotifer and *Artemia* nauplii, is very effective in short-term boosting (Barr *et al*., 2005; Monroig *et al*., 2007). These techniques might be useful to improve the nutritional composition of *Artemia* juveniles for the first feeding of octopus paralarvae, in order to overcome this bottleneck in octopus rearing.

In conclusion, the P:L ratio of the diet supplied to paralarvae was shown to be of major importance to promote paralarval growth and tended to improve their survival as well. In addition, low dietary DHA was not a limiting factor for the growth and survival of paralarvae, as diets with four-times more DHA (juveniles enriched with Ori-Gold®) did not improve growth or survival. The fine tuning of *Artemia* biochemical composition is required to improve growth and survival of paralarvae, and should continue to be a matter of research.

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Effects of supplementing *Artemia* juveniles with free amino acids on the growth and survival rates of *Octopus vulgaris* paralarvae

Capítulo 5 / Chapter 5

Abstract

Cephalopods are carnivorous animals that sustain their high growth rates and energy demand through a vigorous protein and amino acid (AA) metabolism. The difficulty in rearing *O. vulgaris* paralarvae until the benthic stage is well known, as mass mortality of paralarvae is often observed. In the few successful works on which benthic octopuses were achieved, crustacean zoeae alone or complemented with enriched juvenile *Artemia* have been used as food items. It is believed that the lipid composition of zoeae is more suitable to meet the requirements of octopus paralarvae, but additional components other than lipids might be in the origin of this "high" quality prey, such as the AA composition. Additionally, previous results have also shown the importance of the dietary protein/lipid ratio to improve octopus paralarvae growth. In this study three groups of paralarvae, each in triplicate, were fed juvenile *Artemia* (1.6-2.8 mm) enriched with one of the following diets: a combination of the microalgal species *Rhodomonas lens* and *Isochrysis galbana* (70%:30% in a dry weigth basis) - group AR+I; the same combination of microalgae supplemented with essential free L-amino acids (3.4 mM lysine, 2.5 mM arginine, and 1.5 mM methionine) dissolved in the water - group AR+I+AA; or with *Nannochloropsis gaditana* - group ANANO. The gross composition (% of dry weight, DW) of *Artemia* juveniles was almost equal irrespective of the enrichment diet: 65-66% protein, and 10-11% for both lipid and carbohydrate. Survival of paralarvae was not different among groups until day 15, ranging between 51 and 62%. However, from day 20 onward, the survival rate of paralarvae from groups AR+I+AA (8.0±2.0%) and AR+I (7.3±3.1%) was higher than in group ANANO (2.2±0.7%, $P<0.05$). Regarding the DW of paralarvae, a tendency for the positive effects of supplemeting *Artemia* juveniles with free AA was observed, as the highest DW of 25-dph paralarvae was observed in group AR+I+AA (870±81 μg paralarvae^{-1}). However, significant differences were only found in comparison with individuals from group ANANO (798±99 μg paralarvae^{-1}, $P<0.05$), whereas paralarvae from AR+I (834±88 μg paralarvae^{-1}) presented intermediate results. Boosting the content of essential AA in *Artemia* juveniles to feed paralarvae should be further investigated as it may be useful to improve their growth. Exhaustive analysis of the free-AA pool and protein-bound AA in enriched *Artemia* juveniles should be carried out in a

first instance, for comparison with the profiles found in early life stages of *O. vulgaris*. The potential AA deficiencies in *Artemia* can then be adjusted through enrichment with free AA, followed by the evaluation of the practical effects that these actions may have in octopus paralarve rearing.

1. Introduction

The difficulty in rearing *Octopus vulgaris* paralarvae until the benthic stage is well known as mass mortality of paralarvae is often observed (reviewed by Iglesias *et al*., 2007a). In the few successful works on which settled juvenile octopuses have been attained, crustacean zoeae alone or complemented with *Artemia* have been used as food items (Itami *et al*., 1963; Villanueva, 1994, 1995; Iglesias *et al*., 2004; Carrasco *et al*., 2006). Only Hamazaki *et al*. (1991) reported the achievement of benthic juveniles using *Artemia* (1.5-2 mm) as sole prey, through enrichment with *Nannochloropis* sp. and establishing green-waters in a large scale tank. The better results of paralarval rearing observed with zoeae of different crustacean species, in comparison with the use of *Artemia*, have been primarily attributed to the better nutritional composition of zoeae (Iglesias *et al*., 2007a). Furthermore, it has been shown that the lipid composition of wild zooplankton (*Pagurus prideux* zoeae and *Acanthomysis longicornis* mysids), which was previously used with success to rear paralarvae (Villanueva, 1994), is more in accordance with the profile of octopus hatchlings than enriched *Artemia*, mainly due to higher levels of phospholipids and much higher levels the highly unsaturated fatty acids (HUFA) docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) (Navarro and Villanueva, 2000). When comparing the fatty acid profile of wild *Maja brachydactyla* zoeae with that of enriched *Artemia* juveniles, we also observed that zoeae contained substantial higher levels of C_{20} and C_{22} HUFA (see chapter 1). However, additional components other than lipids might be in the origin of this "high" quality prey for paralarvae that need further attention. In previous studies we observed that the protein/lipid (P:L) ratio of *Artemia* juveniles supplied to octopus paralarvae was rather more important to promote growth than the DHA content in *Artemia* juveniles (see results of chapters 2 and 4). In those experiments we found that enriched *Artemia* with a high P:L ratio, even if containing low DHA levels, could induce a better growth of paralarvae than *Artemia* containing 3.5 to 4-times more DHA, but with a lower P:L ratio. In view of these findings, in this chapter it was decided to focus the attention on the importance of protein and amino acids (AA) in the rearing of octopus paralarvae. The effects that AA supplementation in *Artemia* juveniles might have in the growth and survival rates of paralarvae were evaluated. From previous results related with the analysis of the total AA composition of *Artemia* juveniles

enriched with four different microalgae (see chapter 1), which were then compared with the total AA profile of *O. vulgaris* hatchlings (data reported by Villanueva *et al*., 2004), we found that lysine could be the first limiting AA in *Artemia* juveniles enriched with either *R. lens* or *I. galbana*, as lysine content in those juveniles was below the levels found in octopus hatchlings.

As mentioned above, the importance of the lipid composition, especially the FA profile, of live prey for octopus paralarvae has been the main subject of research in some of the most important works published to date (Navarro and Villanueva, 2000, 2003; Okumura et al., 2005), as well as in this thesis. Therefore, an overall introduction on the importance of protein and of AA for marine larvae will be done, but keeping in mind that *O. vulgaris* paralarvae is the main issue of this chapter.

1.1 The nutritional role of protein and amino acids for marine species

Cephalopods are carnivorous animals that sustain their fast growth and energy demand through high ingestion rates and vigorous protein and AA metabolism (Lee, 1994). However, unlike fishes, cephalopods absorbe and utilize poorly lipids (Ballantyne *et al*., 1981; O'Dor *et al*., 1984), and the direct use of protein as energy reserve may account for the lack of major glycogen and lipid reserves in cephalopod tissues (Storey and Storey 1983; O'Dor *et al*. 1984). Previous works in which the body composition of early life stages of *O. vulgaris* were analyzed gave support to this information, as protein represented nearly 70% of its dry weigth (DW) and lipids were mainly composed of structural lipids (Navarro and Villanueva, 2000; Villanueva *et al*., 2004).

Protein is fundamental for the development of the embryo and thus in newly spawned marine-fish eggs this fraction may represent 40 to 60% of the egg DW, from which up to 50% of the total AA may be in the form of free amino acids (FAA) (Rønnestad and Fyhn, 1993; Rønnestad *et al*., 1999). Similarly, Villanueva *et al*. (2004) reported for initial eggs stages of *O. vulgaris* a total AA content of nearly 60% of their DW, which tended to decrease to nearly 40% until the end of egg development. As observed in fish larvae, during the period of yolk resorption the FAA pool is probably depleted as metabolic fuel and for body protein synthesis, reaching low levels at first feeding (Rønnestad *et al*., 1999). Endogeneous energy reserves in cephalopod hatchlings may be exhausted in the first 3 to 4 days after hatching (Vidal *et al*., 2005), but unlike some fish species, that are not ready for exogeneous feeding, cephalopod hatchlings can capture prey as soon as they hatch (Vidal

et al., 2002; Iglesias *et al*., 2006). Therefore, after the onset of first feeding, both endogeneous reserves and exogeneous food contribute for the supply of energy. In marine fish larvae, AA are also important catabolic substrates after the onset of first feeding and may account for 60% or higher of the energy dissipation, and thus a high and balanced dietary AA content is required to maximize growth (Rønnestad *et al*., 1999; Conceição *et al*., 2003; Rønnestad *et al*., 2003). Since growth is primarily an increase in body muscle mass by protein synthesis and accretion a high dietary requirement for AA must exist in marine larvae. *O. vulgaris* paralarvae grow exponentially and show relative daily growth rates of 5.5-11.5% (Villanueva *et al*., 1995), which certainly implies high protein synthesis rates and high AA requirement to sustain this intensive metabolism.

The ideal AA composition of a diet can be defined as the one that will permit an optimal protein growth (Boisen *et al*., 2000; Conceição *et al*., 2003), and hence this profile depends on the AA profile of the proteins being synthesized and the AA that are primarly used for energy or for other metabolic purposes (Conceição *et al*., 1998, 2003). In order to verify to what extent AA requirements are met by the live prey supplied to marine larvae, a first approximation can be done by comparing the AA profiles of both live food and larvae (Conceição *et al*., 2003). Moreover, the indispensable (also called essential) amino acids (EAAs) profile of larval fish (Watanabe and Kiron, 1994) has been proposed as a good index for the EAAs requirements of larval species, and can be used for comparisons with the EAAs profiles of live food or diets used to feed those larvae (Aragão *et al*., 2004b; Saavedra *et al*., 2006). However, direct comparisons alone may not be enough to determine the AA requirements of a target species, as different rates of absorption and catabolism of AA exist throughout the process of digestion and metabolic pathways in the larvae (Conceição *et al*., 2003), that can lead to some imbalances. AA deficiencies in the diets for larvae can cause reduction of growth, decreased protein retention (Tibaldi *et al*., 1994; Luzzana *et al*., 1998) or an increase of other AA oxidation (Fauconneau *et al*., 1992). When supplementing the diet of Senegalese sole (*Solea senegalensis*) post-larvae with potential limiting AA, Aragão *et al*. (2004c) have found that the catabolism of AA decreased at the time AA retention increased.

In previous studies about the total AA composition of *O. vulgaris* hatchlings and wild juveniles, Villanueva *et al*. (2004) found that lysine, leucine and arginine represented half of the total content of EAAs, and glutamate and aspartate represented nearly half of the non-essential amino acids (NEAAs), whereas taurine was the major AA in the FAA pool. Taurine is well known for its role in cell volume regulation (Huxtable, 1992) and is

common in other marine molluscs, being the major AA in the FAA pool of demersal fish eggs as well (Rønnestad *et al*., 1999). Similarly, Rosa *et al*. (2004) described in three tissues (gonad, digestive gland and muscle) of adult *O. vulgaris* individuals from the Portuguese coast in different sexual maturation stages, that the major EAA were also leucine, lysine and arginine, whereas glutamic acid, aspartic acid and alanine comprised the major NEAA. The formulation of artificial diets or the enrichment of live prey to feed early life stages of cephalopods should take into account this information. Previous works with juveniles or adult cuttlefish (*Sepia officinalis*) or octopuses (*Octopus vulgaris* and *Octopus maya*) have shown the possibility of using artificial feeds to promote growth of individuals, despite very poor results are observed in comparison with natural diets (Castro *et al*., 1993; Cerezo *et al*., 2008; Quintana *et al*., 2008; Rosas *et al*., 2008). In experiments with adult cuttlefish, Domingues *et al*. (2005) found that artificial diets supplemented with lysine induced significantly higher growth rates of animals, in comparison with non enriched diets or enriched with lower levels of EAAs (including lysine), which indicated that lysine was deficient in the remaining diets.

Another feature that has been described in early life stages of cephalopods is the capacity for active uptake of nutrients dissolved in the water, such as AA and simple sugars, through the epidermis (Castille and Lawrence, 1978). Lee (1994) suggested that this nutrient uptake pathway could contribute significantly to meeting the metabolic and nutritional requirements of hatchlings. This trait has been recently investigated by Villanueva *et al*. (2004) for *O. vulgaris* paralarvae, who found no clear benefits in adding a solution of EAAs, based in the AA composition of octopus hatchlings, to the rearing tanks on the growth of paralarvae. Nevertheless, these authors confirmed the direct absorption of radiolabelled phenylalanine dissolved in the water by hatchlings of three cephalopod species (including *O. vulgaris* paralarvae).

In the following section some considerations about the composition of live prey for marine larvae are done, with special emphasys on protein and AA composition.

1.2 Protein and amino acid composition of live prey

The protein content of different live preys varies greatly depending on the species, development stage, geographical area and nutritional status of the animal. In rotifers the protein content ranges from 28 to 63% (Lubzens and Zmora, 2003), whereas in the brine shrimp (*Artemia* sp.) its content may vary between 42-62% in nauplii and between 39-67%

in adult (Dhont and Van Stappen, 2003). These authors also pointed out that adult *Artemia* have a slightly higher protein content and more essential AA than nauplii. Marine pelagic copepods have a protein content that ranges from 24 to 82% of its DW (Støttrup, 2003). In wild *Maja brachydactyla* zoeae we found a protein content of 56% (see chapter 4), which was much higher than values reported by other authors (13 to 24% of its DW, Moxica *et al*., 2002; Andrés *et al*., 2004), though these differences can be attributed to the different methodologies used for protein determination. Despite similar protein contents are observed among the different live preys, important differences might be found in their AA composition and availability as protein-bound AA or FAA, which can greatly affect the absorption of protein by larvae. Marine zooplankton contain a pool of FAA that comprise up to 10–20% of their total AA (Fyhn *et al*., 1995; van der Meeren *et al*., 2001), whereas the FAA fraction in *Artemia* nauplii was found to represent between 2 and 6% of its total AA, depending on the species, origin and feeding status (Naess *et al*., 1995; Helland *et al*., 2000; van der Meeren *et al*., 2001; Aragão *et al*., 2004a). After prey ingestion, the rapid release of FAA during digestion may play a significant physiological role during early feeding. The higher assimilation capacity of FAA in comparison with protein AA as a dietary source of AA has been shown in Senegal sole postlarvae (Rønnestad *et al*., 2000).

Taurine may also be essential in early life stages of marine finfish, as demonstrated for the Japanese flounder larvae (Takeuchi, 2001). This author found that not only the growth, but also the normal behavior of the Japanese flounder during its early life development, was affected by the levels of dietary taurine. Additionally, when comparing the content of taurine in natural preys (mysids) of this species with other live prey or microdiets, Takeuchi (2001) found that mysids contained much higher taurine levels (29.0 mg g^{-1}) than *Artemia* (6.9 mg g^{-1}), commercial diets (3.8 mg g^{-1}) or rotifers (0.8–1.8 mg g^{-1}).

Besides modulating the profile of AA in live prey (*Artemia* and rotifers) through the use of different enrichment diets, eventual manipulations of the FAA pool in those preys could have an impact in compensating for AA imbalances. In experiments of *Artemia* metanauplii enriched with either microalgae or commercial products, Aragão *et al*. (2004a) have found that in spite of having similar protein contents, the FAA pool of microalgae-enriched *Artemia* was significantly higher than in *Artemia* enriched with the commercial products. On the other hand, Tonheim *et al*. (2000) have shown that the content of free methionine could be increased by up to 60 times in *Artemia* nauplii using liposomes after 16 h of enrichment, and between 20 to 30 times using a solution of dissolved free methionine at a concentration of 5.3 mM. This capacity of AA enrichment in *Artemia*

could thus be used to deliver EAA to paralarvae, and seems to be more reasonable than the use of dissolved EAA in the water of tanks, as no clear beneficial effects of this practice were previously found in octopus paralarval rearing (as described above). Moreover, in semi-closed or open water circuits it would be difficult to keep dissolved AA concentrations constant, whereas methionine-enriched *Artemia* nauplii were shown to keep high levels of free methionine for further 10 h, after simulating its distribution to rearing tanks maintained at 22 ºC (Tonheim *et al*., 2000).

The aim of this work was to compare the survival and growth rates of *O. vulgaris* paralarvae fed on three different dietary treatments, consisting of *Artemia* juveniles (1.6-2.8 mm) enriched with different microalgae (either a mixed diet of *R. lens* and *I. galbana*, or *N. gaditana*), or receiving a supplement of free EAAs. From previous results related with the total AA composition of *Artemia* juveniles enriched with four different microalgal species (*Tetraselmis suecica*, *Rhodomonas lens*, *Isochrysis* aff. *galbana* T-ISO and *Isochrysis galbana*, see chapter 1), that were then compared with the AA composition of *O. vulgaris* early life stages (data reported by Villanueva *et al*., 2004), we found that lysine was the first limiting AA in *Artemia* juveniles. In addition, when compared with the AA composition results found in wild juvenile and adult octopuses (data reported by Villanueva *et al*., 2004 and Rosa *et al*., 2004), *Artemia* juveniles also contained lower levels of arginine and methionine. Moreover, arginine is vigorously metabolized in cephalopods as energy substrate (Hochachka *et al*., 1983). Therefore, the EAA chosen as a supplement of *R. lens* and *I. galbana* enrichment in *Artemia* juveniles were lysine, arginine and methionine.

2. Material and methods

2.1 Growth and enrichment of *Artemia* juveniles to feed octopus paralarvae

The microalgal species *Rhodomonas lens* CCMP 739, *Isochrysis galbana* Parke and *Nannochloropsis gaditana* CCMP 527 were cultured semi-continuously in 6-l glass flasks in nutrient saturated conditions and with a daily renewal rate of 30% of the volume of cultures, which ensured biomass of constant and controlled biochemical composition. For more details about the culture conditions see material and methods of chapter 3.

Juveniles of *Artemia* (AF, INVE, Belgium) were obtained by feeding nauplii with *Rhodomonas lens* in 12-L plastic tanks for two or three days at 26.5 ± 0.5 ºC. Juveniles were finally enriched for 24 h with either a mixed diet of *R. lens* and *I. galbana* (70%:30% dry weight basis) or with *N. gaditana* (Fig. 1). Before *Artemia* juveniles were supplied to feed octopus paralarvae, they were further enriched for 3 to 9 h with one of the following diets: the same combination of *R. lens* and *I. galbana* (group AR+I); the same diet as group AR+I plus free L-amino acids dissolved in the water to attain the following concentrations: 3.4 mM lysine, 2.5 mM arginine, and 1.5 mM methionine (group AR+I+AA); or *Nannochloropsis gaditana* (group ANANO). Free L-amino acids were all Sigma products with a purity grade ≥ 97%. Samples of *Artemia* juveniles were collected, washed with distilled water and immediately frozen at -18 ºC for later biochemical analysis.

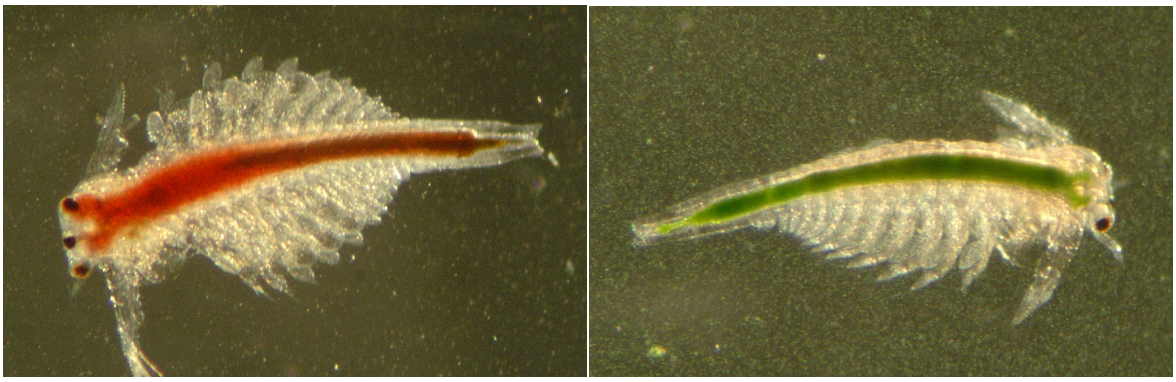

Figure 1 – Photographs under a stereoscope of *Artemia* juveniles (1.6-2.0 mm) enriched with either a combination of the microalgal species *Rhodomonas lens* and *Isochrysis galbana* (on the left) or with *Nannochloropsis gaditana* (on the right).

2.2 Experiment of *Octopus vulgaris* paralarvae rearing

Newly hatched paralarvae from a single egg mass of *O. vulgaris* which was transported to the facilities of the University of Santiago de Compostela (Spain), and that hatched on its way were used to carry out the experiment. Paralarvae were immediately distributed into 50 l-conical fibre glass tanks with 50 cm diameter and white walls. The semi-closed seawater circuit was previously described in detail (see material and methods of chapter 4). A photoperiod of 14 h/10 h light:dark was used with fluorescent day-light lamps placed 40 cm above water surface. Density of paralarvae was established at 10 individuals l^{-1} and

water temperature was kept at 19.5±0.5 ºC in a stable climate room using thermo-heaters RENA 100W. Three groups, each in triplicate, control group (AR+I), group AR+I+AA and group ANANO were fed *Artemia* juveniles (1.6-2.8 mm) enriched as described above. Food was distributed at 12:00 a.m., 04:00 p.m. and 08:00 p.m. in equal rations and the total daily food amount was established as 0.05 *Artemia* ml^{-1} day^{-1}. Mortality of paralarvae was recorded daily by counting the individuals found death in the bottom of tanks. Dry weight (DW) of paralarvae was determined individually (10 individuals per replicate) by washing them with distilled water followed by drying in a stove for 24 h at 100±1 ºC. Daily growth index (DGI) was calculated as follows: $100 \times [(DW_f^{1/3}-DW_i^{1/3})/(T_f-T_i)]$, where DW_f and DW_i are the DW of paralarvae at sampling days T_f and T_i.

2.3 Biochemical composition analyses

C-N-H analyses of *Artemia* were determined by combustion using an autoanalyzer Fisons Model EA 1108. The equipment was calibrated with the standard compound acetanilide, normally recommended when samples with high organic content are to be measured. Protein content was calculated from the total amount of nitrogen using the conversion formula Nx6.25 (National Research Council, 1993). Total lipid was extracted with chloroform/methanol 2:1 v/v (Bligh and Dyer, 1959) after complete disruption of *Artemia* tissues, followed by gravimetric determination. Carbohydrate was determined by the phenol/sulphuric acid method (Kochert, 1978).

2.4 Statistical analysis

Statistical analyses were performed using the software SPSS V 17.0.1 statistical package (SPSS, Inc.). Paralarval dry weight was compared by analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05. Percentages of survival and composition data were arcsine-√ transformed and the same statistical tests were carried out (Zar, 1999). Statistical comparisons of dietary P:L ratios and of DGI among groups were carried out by the non-parametric test of Kruskal-Wallis (Zar, 1999).

3. Results

3.1 Gross composition of *Artemia* juveniles

The gross composition of *Artemia* juveniles was very similar in all groups for protein, lipid and carbohydrate contents (Table I). Protein content ranged between 65 to 66%, whereas both lipid and carbohydrate ranged between 10 and 11%, without any significant differences among groups. Likewise, the P:L ratios were also very similar among *Artemia* juveniles, ranging between 6.1 and 6.3.

Table I. Gross composition of the *Artemia* juveniles (1.6-2.8 mm) used to feed octopus paralarvae. AR+I: *Artemia* juveniles enriched with *Rhodomonas lens* and *Isochrysis galbana*; AR+I+AA: juveniles enriched with the same microalgal diet plus dissolved free amino acids (lysine, arginine and methionine); ANANO: juveniles enriched with *Nannochloropsis gadiatana*.

	AR+I*	AR+I+AA**	ANANO*
Protein (N x 6.25)	64.8 ± 0.4	65.9 ± 1.2	65.5 ± 0.3
Lipid	10.7 ± 1.1	10.7 ± 0.9	10.4 ± 0.8
Carbohydrate	10.6 ± 0.7	9.6 ± 0.5	9.6 ± 0.7
C:N ratio	4.9 ± 0.07	4.8 ± 0.03	4.8 ± 0.02
P:L ratio	6.1 ± 0.3	6.2 ± 0.3	6.3 ± 0.2

* Juveniles previously enriched for 24 h and then further enriched for 3 to 9 h before being supplied to paralarvae. **Juveniles enriched for 24 h with *R. lens* and *I. galbana* and then for further 3 to 9 h with the same microalgal diet plus free L-amino acids dissolved in the water. Data are means ± S.D. of triplicate analyses.

3.2 Survival and growth of paralarvae

Survival of paralarvae fed the different dietary treatments is shown in figure 2. Although no statistically significant differences among groups were observed until day 15, with percentages ranging between 51 and 62%, at day 20 and in the next days group ANANO showed lower survival rate than the remaining groups ($P<0.05$). The individuals remaining in groups AR+I and AR+I+AA after sampling paralarvae on day 25 lived until 33-dph.

Regarding the DW of paralarvae, no statistically significant differences were found among groups after 15 days of rearing, despite individuals from group AR+I+AA showed the highest DW (Fig. 3). In contrast, significant differences were found in the DW of 25-dph paralarvae fed on different diets. Whereas between 25-dph paralarvae from groups AR+I

(834±88 µg paralarva^{-1}) and AR+I+AA (870±81 µg paralarva^{-1}) no significant differences were found, nor between individuals from groups AR+I and ANANO (798±99 µg paralarva^{-1}), the DW of paralarvae from AR+I+AA was significantly higher than the DW of individuals from ANANO ($P<0.01$).

The daily growth index (DGI) of 15-dph paralarvae from the different groups decreased as follows: AR+I+AA (8.3±0.5) > AR+I (7.8±0.4) > ANANO (7.5±0.5). For 25-dph paralarvae the same order was observed: AR+I+AA (11.1±0.2) > AR+I (10.6±0.2) > ANANO (10.0±0.2), supporting the trend for a higher growth of paralarvae in group receiving the supplement of AA (AR+I+AA).

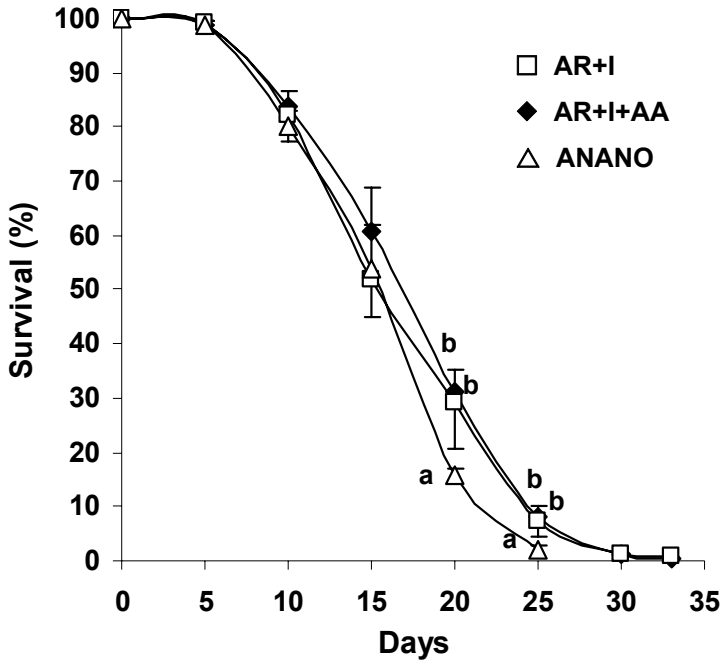

Figure 2 - Survival (%) of paralarvae fed on three different dietary treatments in the course of the experiment. AR+I: *Artemia* juveniles enriched with *Rhodomonas lens* and *Isochrysis galbana*; AR+I+AA: juveniles enriched with the same microalgal diet plus dissolved free amino acids (lysine, arginine and methionine); ANANO: juveniles enriched with *Nannochloropsis gadiatana*. Data are means ± S.D. (n=3). Different superscript letters within the same day indicate significant differences among groups ($P<0.05$).

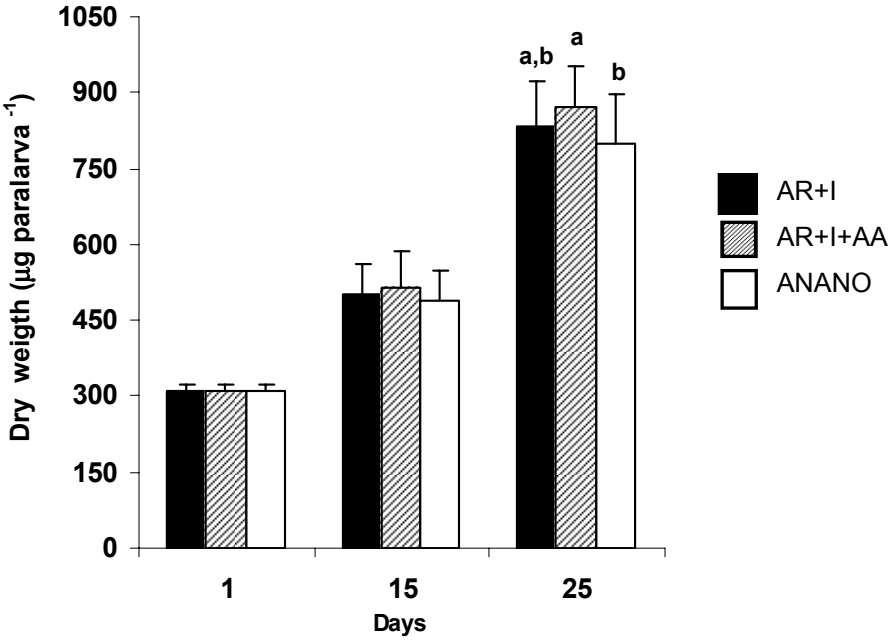

Figure 3 - Dry weight of *O. vulgaris* paralarvae (µg paralarva^{-1}) from the different dietary treatments in the course of the experiment. Abbreviations are like in figura 2. Data are means ± S.D. (n=3, 10 paralarvae individually weighed per replicate). Different superscript letters within the same day indicate significant differences among groups ($P<0.01$).

Discussion

The gross composition of the different *Artemia* juveniles was nearly the same regardeless the enrichment diet used. Protein, lipid and carbohydrate contents of juveniles enriched with either *N. gaditana* or with *R. lens* plus *I. galbana* were similar to values described in juveniles with similar sizes enriched with these same microalgae (see results described in chapters 3 and 4), supporting the idea that the composition of *Artemia* can be modulated through the use of semi-continuous cultures of microalgae in order to achieve prey of constant and controlled composition. The addition of free AA as a supplement to the mixed diet of *R. lens* and *I. galbana* did not increase the protein content in *Artemia* juveniles. Exhaustive AA composition analyses (FAA pool and protein-bound AA) would certainly help to clarify possible differences. Aragão *et al*. (2004a), when analyzing those two fractions of AA pools in *Artemia* metanauplii enriched with either microalgae (*I. galbana* or *N. gaditana*) or commercial products (Super Selco, INVE), found important differences

in the FAA pool among groups and in EAAs profiles. Whereas metanauplii enriched with *I. galbana* had a higher content of EAAs than *N. gaditana*-enriched metanauplii, both groups enriched with microalgae contained significantly higher amounts of FAA than metanauplii enriched with Super-Selco (despite this group showed higher or at least equal protein content than microalgae-enriched *Artemia*). Microalgae are a good source of AA and considerable differences may exist among species. Seixas *et al*. (submitted, Annex I) have found that *R. lens* contained as much as twice total AA than *T. suecica*, despite the proportion of EAA and NEAA was similar between species. It has also been shown that in microalgae the FAA pool may represent a significant proportion of total AA. In common species used in aquaculture, Dortch *et al*. (1984) have found that the FAA could represent between 3 and 18% of the total AA, reporting for a species a FAA content of 39% of total AA. Aragão *et al*. (2004a) for *I. galbana* and *N. gaditana* reported FAA contents of 6.7% and 4.2% of DW, respectively. In contrast, Brown (1991) stated that in sixteen different microalgal species cultured under the same conditions few differences in their AA composition existed. However, its should be kept in mind that even for a single microalgal species considerably changes in its composition are observed depending on the nutrient medium and culture conditions (Otero and Fábregas, 1997).

In this study, despite no differences were found in the gross composition of the *Artemia* juveniles supplied to paralarvae, significant differences in the survival and DW of 25-dph paralarvae were found among groups. Although the survival rate of the different groups was similar until day 15, from day 20 onward paralarvae from groups AR+I and AR+I+AA showed higher survival rates than group fed *Artemia* enriched with *N. gaditana*. Regarding the DW of paralarvae, individuals from group AR+I+AA evidenced a trend for higher growth rate than individuals from the remaining groups. However, significant differences were only found in 25-dph paralarvae, with individuals from AR+I+AA showing a higher DW than those from ANANO. Paralarvae from group AR+I showed similar survival and a slighly lower DW, though not significant, than paralarvae from group AR+I+AA, and the only difference in prey enrichment was the free AA supplement. On the other hand, the DW of 25-dph paralarvae was not different between groups AR+I and ANANO. However, despite the gross composition of prey was similar, other components such as the fatty acid profile were different (as previously found in chapter 3). In view of these findings, we could speculate if in fact the combination of the free AA supplement plus a better lipid composition of *Artemia* juveniles from AR+I could be the reason for the better growth of paralarvae from group AR+I+AA. In disagreement with the hypothesis launched in chapter

3, related with the better EPA/ARA ratio found in *Artemia* juveniles enriched with *N. gaditana* in comparison with juveniles enriched with ther microalgae, this diet did not promoted better growth or survival of paralarvae than the control diet (AR+I). In order to interpret the significance of the results obtained, further trials should be carried out, analyzing the profiles of the FAA and protein-bound AA of both enriched *Artemia* and reared paralarvae, which could help to explain the differences in paralarval growth and provide possible proofs of AA deficiencies.

The DW of 25-dph paralarvae found in the study (798-870 µg paralarva^{-1}), regardless the enrichment diet of *Artemia*, were higher than the DW reported by Villanueva *et al*. (2004) for 25-dph paralarvae (650-718 µg paralarva^{-1}) fed *Artemia* nauplii enriched with either DC Super Selco (INVE), or further supplemented with free L-methionine using a solution of 5.3 mM, or receiving a solution of free EAA in the the rearing water. Moreover, the mean water temperature in this study (19.5±0.5 ºC) was slightly lower than in that study (20.4 ºC). Those authors found that increasing the content of free methionine in nauplii to feed octopus paralarvae did not have any positive effects on the growth or survival of paralarvae. However, cautions must be taken when increasing the content of certain EAA in the diets. Excessive levels of free methionine in diets can produce growth deterioration and imbalances in methionine-cystine interactions, as shown for tilapia *Sarotherodon mossambicus* (Jackson and Capper, 1982).

In recent works with *Octopus bimaculoides* hatchlings, which were fed either enriched *Artemia* juveniles or unenriched juveniles for 20 days, Solorzano *et al*. (2009) observed no differences in the AA profile of 20-dph octopuses from the different treatments, but detected a decrease of isoleucine, leucine and tyrosine in individuals from all groups with respect to the initial AA levels found in hatchlings. However, this could be related with deficient levels of phenylalanine, isoleucine, leucine and valine in the *Artemia* juveniles, as mentioned by the authors after establishing direct comparisons with the total AA composition of octopus hatchlings, which also suggested that higher growth rates could be promoted through the use of more suitable diets.

In future experiments of *O. vulgaris* paralarvae rearing, the EAA profiles of *Artemia* juveniles enriched with monoalgal diets or with mixed diets of microalgae should be assessed and compared with the EAA profile of octopus hatchlings, in order to adress potential AA deficiencies. The addition of crystalline free AA can then be used to supplement possible limiting AA than need to be modulated, in order to reach optimal levels. Additionally, it will be necessary to study the kinetics of FAA increase in *Artemia*

juveniles with enrichment-time and the rates of FAA declining at the rearing temperature of paralarvae. Experiments of *O. vulgaris* paralarvae rearing are then needed in order to evaluate the effects of AA supplementation on the growth, survival and AA composition of paralarve.

Engorde del pulpo (*Octopus vulgaris*) en la ría de Vigo: efectos de la separación por tamaños y por sexos en el crecimiento y la supervivencia

Capítulo 6 / Chapter 6

Resumen

El engorde de pulpo (*Octopus vulgaris*) se basa en la captura de individuos adultos en el medio natural, con un peso mínimo permitido por ley, seguido de su distribución en jaulas flotantes o suspendidas en bateas. Un ciclo de engorde típico comprende un periodo de 3 a 4 meses, al cabo del cual se pueden alcanzar pesos medios de alrededor de 2,5-3,0 kg. Con el fin de evaluar nuevas estrategias para optimizar el proceso de engorde del pulpo a escala industrial, se realizaron dos experimentos en jaulas de engorde suspendidas de una batea, en la ría de Vigo, que consistieron en: a) experimento 1 – engorde de pulpos con separación por tamaños. Se constituyó un grupo homogéneo (HOM), cuya diferencia en el peso inicial (Pi) de los individuos no sobrepasaba los 100 g, y un grupo heterogéneo (HET), que consistió de ejemplares cuya diferencia en el Pi de los individuos sobrepasaba los 500 g (que es lo habitual en el sistema de de cultivo industrial); b) experimento 2 – engorde de pulpos clasificados por sexos: grupo MACHOS, constituido por ejemplares sólo machos con Pi de 953±146 g; grupo HEMBRAS, formado por sólo hembras con Pi de 899±135 g; y grupo MIXTO, formado por machos y hembras con Pi de 899±137 g. Los pulpos se alimentaron a diario (excepto los domingos) con distintas especies de pescado (jurel, bacaladilla, caballa, boga) y mejillón, con una ración que varió entre el 3 y el 6% de la biomasa en jaula. En el primer experimento, la supervivencia apenas varió entre los grupos, siendo del 70,0% en el grupo HOM y del 68,7% en el grupo HET. El peso final (Pf) de los ejemplares en el grupo HET (2188±574 g) fue superior al del grupo HOM (2091±499 g), mientras que la biomasa final del grupo HOM fue mayor debido a la diferencia en la supervivencia. El engorde de pulpos obtenidos del medio natural y seleccionados para formar un grupo homogéneo, no supuso una clara ventaja frente a un ciclo de engorde típico, con individuos de peso heterogéneo. Las pequeñas diferencias observadas en cuanto a biomasa total alcanzada posiblemente no compensarían, económicamente, el esfuerzo en personal necesario para realizar la selección por tamaños muy ajustados. En el segundo experimento, la supervivencia de los grupos MACHOS y MIXTO (ambos con 86,7%) ha sido superior a la del grupo HEMBRAS (73,3%). El Pf de los individuos del grupo MACHOS (2261±428 g) ha sido superior al Pf observado en los grupos HEMBRAS (2015±428 g) y MIXTO (2125±450 g), aunque el Pi de los pulpos del

grupo MACHOS también había sido superior. Los resultados han demostrado que la mortalidad en el grupo MACHOS no ha sido diferente de la del grupo MIXTO. Dados los resultados dispares que se barajan por diversos autores en el engorde con separación de sexos, sería necesario el diseño de experimentos que traten de encontrar las causas que puedan explicar los diferentes comportamientos observados. De cara al futuro, sería interesante realizar nuevos experimentos de engorde con grupos de individuos separados por tamaños "a simple vista", o sea, con rangos de peso más amplios (ej.: 250 g), estableciéndose períodos de engorde más cortos o más largos en función del Pi del grupo. Con estas medidas se podría ahorrar alimento y mejorar la gestión del cultivo, ya que el tiempo de engorde se acortaría en las jaulas con ejemplares más grandes y se prolongaría en aquellas con ejemplares más pequeños.

1. Introducción

El pulpo (*Octopus vulgaris*) es una especie de elevado valor comercial que en países del sur de Europa como España, Italia, Portugal o Grecia se cotiza en lonja a precios medios de entre 3 y 8 euros kg^{-1} (Globefish, 2005). A principios de los años 90, la creciente demanda del pulpo común en países asiáticos y mediterráneos y la ligera disminución de las capturas a nivel mundial (Globefish, 2005), conllevaron un aumento de los precios de compra de este cefalópodo. En este sentido, en Galicia, el Instituto Español de Oceanografía (IEO) de Vigo y la Universidad de Santiago de Compostela se lanzaron a realizar, durante el período 1995-1999, una serie de experimentos de engorde de pulpo con el fin de evaluar la posibilidad de su cultivo e intentar diversificar las especies producidas hasta entonces, totalmente dominadas por el mejillón y el rodaballo. Desde el año 1995 se han realizado numerosas experiencias de engorde, con el objetivo de aplicar a la explotación industrial los conocimientos y avances logrados. Los trabajos experimentales desarrollados, por un lado, por el grupo liderado por José Iglesias, en el IEO de Vigo, y por otro, por Manuel Rey Méndez, en la Universidad de Santiago de Compostela, han demostrado que el engorde del pulpo es una actividad que puede ser bastante rentable debido a las elevadas tasas de crecimiento de la especie y a la corta duración que implica la etapa de engorde de pulpos con un tamaño inicial de 750-1000 g hasta los 3 kg. A continuación se detallarán algunos de los trabajos y resultados más importantes publicados por estos autores entre los años 1995 y la actualidad.

a) Experiencias realizadas en tanques en laboratorio

En el Centro Oceanográfico de Vigo (IEO de Vigo), los trabajos se centraron en evaluar el potencial de crecimiento de ejemplares de pulpo con diferentes tamaños iniciales y durante diferentes tiempos de engorde, establecer la mejor densidad de cultivo y analizar la tasa de crecimiento de ejemplares separados por sexos (Iglesias *et al*., 1996; Sánchez *et al*., 1998; Iglesias *et al*., 2000, 2003). Las condiciones en las que se desarrollaron estas experiencias consistieron en el mantenimiento de los pulpos en tanques de 5 a 10 m^3, en circuito de agua abierto, con un rango de temperatura de 13 a 19,5ºC., salinidad entre 32 y 35 ppt y un flujo de agua variable en función de la carga animal. El alimento consistió en combinaciones de crustáceos (*Polybus henslowi*, *Macropipus corrugatos*, *Carcinus maenas*), peces (*Micromesistius poutasou*) y moluscos (*Mytilus sp*.) congelados. En la

tabla I se presenta un resumen de los resultados obtenidos por estos autores (Iglesias *et al*., 2000, 2003). En estos experimentos, destacaron el enorme potencial de crecimiento de los pulpos mantenidos en tanques, la casi nula mortalidad y las altas tasas de conversión alimentaria (aunque los valores puedan parecer altos, hay que tener en cuenta que el alimento es natural y por lo tanto contiene un alto porcentaje de humedad).

Los autores han comprobado que pulpos con un tamaño inicial de 300 g pueden alcanzar los 2200 g en tan sólo 4 meses. En 8 meses de engorde, los pesos finales pueden llegar a los 5400 g, lo que pone en evidencia el enorme aumento de biomasa y la rentabilidad que esta especie puede tener, cuando es alimentada en buenas condiciones. Este grupo ha conseguido también que ejemplares ya adultos incrementasen su peso en 11 kg en diez meses.

Tabla I. Resumen de los principales trabajos de engorde de pulpo llevados a cabo por el grupo del IEO en Vigo. Engorde de pulpos con diferentes pesos iniciales (Grupos 1, 2 y 3), engorde con separación de sexos (Machos y Hembras) y engorde a diferentes densidades iníciales (10 kg m^{-3} y 20 kg m^{-3}). Iglesias *et al*. (2000, 2003).

Grupo	Tiempo de engorde (meses)	Peso inicial (g)	Peso final (g)	Ración diaria (%)	Índice de Conversión (%)	Mortalidad (%)	Alimento
1	10	1,300	12,300	3	3.0	0	80% crustáceos
2	8	600	5,400	6	3.6	0	15% peces
3	4	300	2,200	10	4.8	3	5% moluscos
Machos	5	637,0	3624,0	3,5 – 7		13	crustáceos y
Hembras	5	657,9	2780,0	3,5 – 7		11	peces
10 kg m^{-3}	3	883,1	2746,5	7		6	80% cangrejo
20 kg m^{-3}	3	872,9	2205,0	7		11	20% lirio

Otros de los resultados obtenidos se refieren a las mejores tasas de crecimiento de los machos frente a las hembras, al cabo de 5 meses de engorde, aunque estas diferencias no se verificaban o eran muy poco evidentes hasta los tres meses, cuando los pulpos tenían casi 2 kg de peso. De los experimentos de engorde con diferentes densidades se concluyó que para optimizar el proceso de crecimiento de los pulpos y reducir la mortalidad, es conveniente iniciar la etapa con cargas de no más de 10 kg m^{-3} ya que al final del proceso la densidad final rondará los 30 kg m^{-3}.

b) Experiencias realizadas en jaulas suspendidos de bateas

Mediante el uso de una batea experimental situada en la ría de Muros-Noia, el grupo liderado por Manuel Rey Méndez realizó diversos experimentos de engorde de pulpo para estudiar el crecimiento y la supervivencia en diferentes condiciones de cultivo, así como tipos de jaulas y refugios que ofreciesen un mejor control y adaptación de los animales confinados. Una parte significativa de esos trabajos se hizo también en colaboración con las empresas que se iniciaban en esta actividad y que contaban con más medios y facilidad de seguimiento de los trabajos en las rías. La puesta a punto de estos objetivos permitiría disponer con facilidad de abundantes ejemplares de procedencia conocida, para realizar experimentos de laboratorio, para conocer mejor algunos aspectos referentes a la especie como comportamiento, genética, fisiología y bioquímica entre otros. Por otra parte, al tratarse de una especie de gran interés comercial, el cultivo en jaulas suspendidas de batea supondría el desarrollo de una tecnología de bajo costo para el cultivo, suponiendo también una posible solución a la necesidad de diversificación de la acuicultura convencional, centrada principalmente en la mitilicultura.

A continuación se describen algunos de los resultados obtenidos por el grupo de la USC en materia de engorde de pulpo en pruebas experimentales o a escala industrial (Rama-Villar *et al*., 1997; Luaces-Canosa y Rey-Méndez, 1999; Tuñon *et al*., 1999, 2000, 2001, 2002, 2003; Rey-Méndez *et al*., 2001).

En estudios de engorde realizados en los años 1997-1998, en colaboración con la empresa Arrecifes del Atlántico S.L., situada en la Ría de Camariñas, se analizó el efecto del diseño de las jaulas y de la época del año sobre el crecimiento de los pulpos (Luaces-Canosa y Rey-Méndez, 1999). En estos trabajos se ha observado que las jaulas de sección circular originaban peores tasas de supervivencia de los individuos en comparación con las jaulas de sección cuadrada, lo que en principio se puede achacar a la peor dinámica de flujos de agua a través de esas jaulas y a las situaciones de estrés generadas dependiendo de la disposición de los refugios (Rey-Méndez *et al*., 2001). Otro dato que resultó evidente en los trabajos realizados fue el efecto de la época del año sobre el crecimiento de los pulpos, con resultados claramente superiores en los meses de verano (época alta) y peores entre noviembre y marzo (época baja). Las causas de estas mejoras se relacionan con la mayor temperatura del agua, que favorece no sólo el crecimiento sino también una mejor eficiencia de conversión alimentaria. También se comprobó que en los meses de época alta, el engorde de pulpos por un tiempo superior a 100 días tiende a ser perjudicial para la

relación Biomasa final/Biomasa inicial, debido al aumento de la mortalidad, mientras que en la época baja la continuación del engorde más allá de los 100 días (hasta los 150 días) tiende a favorecer esta relación, ya que el crecimiento es más lento, pero la mortalidad no aumenta (excepto en casos de cambios bruscos de salinidad). En la figura 1 se puede apreciar el levantamiento de una jaula de engorde de pulpos en la empresa Arrecifes del Atlántico S.L., observándose ejemplares saliendo de los tubos de PVC que sirven de refugio.

El engorde de pulpos con separación de sexos y tamaños había demostrado ser favorable en condiciones de laboratorio (Sánchez *et al*., 1998), pero su aplicación a escala industrial no era de todo clara, ya que el coste de trabajo asociado podría no compensar los beneficios aportados. Así, en un estudio realizado en 1999 en colaboración con la empresa Ameixa de Carril S.L., ubicada en la ría de Arousa, y en la que se utilizaron jaulas de sección rectangular suspendidas de batea y sumergidas a 6 m de profundidad, se constituyeron los siguientes grupos de pulpos: (M1) machos menores de 1kg, con peso medio inicial de 0,80±0,12 kg; (M2) machos mayores de 1 kg, con peso medio inicial de 1,16±0,16 kg; (H1) hembras menores de 1 kg, con peso medio inicial de 0,82±0,10 kg; (H2) hembras mayores de 1 kg, con peso medio inicial de 1,15±0,10 kg (Tuñon *et al.*, 2000, Tabla II). En engorde se realizó con una dieta basada en tres especies de pescado: jurel (*Trachurus trachurus*), caballa (*Scomber scombrus*) y lirio (*Micromesistius poutassou*), suministrados a una razón de un 10% de la biomasa de pulpos en cada grupo cada dos días, entre marzo y julio.

En otro estudio realizado en el año 2000, en colaboración con la misma empresa, se estudió el efecto del tamaño inicial de los pulpos (Tuñon *et al*., 2001). Las condiciones fueron similares a las descritas en el experimento anterior, a diferencia de la dieta que en este trabajo consistió de crustáceos como bogavante (*Homarus gammarus*), buey de mar (*Cancer pagurus*), centolla (*Maja brachydactyla*) y nécora (*Necora puber*). Estos alimentos de "lujo" fueron utilizados debido a la propia actividad de la empresa con otros recursos, que disponiendo de crustáceos moribundos o recién muertos no aptos para comercializar o para consumo humano, los aprovechó para este fin. También se les ofreció jurel y lirio. El alimento se suministró a razón del 10% de la biomasa. Los grupos se constituyeron con igual número de hembras y machos de la siguiente forma: pulpos pequeños - P1 (844±146 g), P2 (838±134 g); pulpos grandes - G (1521±354 g); y un grupo con una mezcla de pulpos grandes y pequeños - GP (1473±280 g y 798±143 g) (Tabla II).

Figura 1 – Levantamiento de una jaula de engorde con sistema de tubos "T" de PVC encajados y que sirven como refugio para los pulpos. (Foto: Manuel Rey Méndez)

En el experimento de engorde con separación por sexos se ha observado que en todos los grupos la biomasa se incrementó considerablemente hasta los 2 meses de engorde, bajando en seguida a causa de la mortalidad. Sin embargo, a pesar del descenso de la biomasa en las jaulas, su valor económico no varió entre los 2 meses y el final del engorde, a raíz del mayor valor comercial de los ejemplares grandes (>2 kg), compensando la pérdida de pulpos por mortalidad. De este trabajo se derivó la conclusión de que estudios económicos más completos y profundos serían necesarios para evaluar el momento idóneo de recogida de los ejemplares, ya que la pérdida de biomasa por mortalidad a partir de determinado momento puede conllevar más gastos de mano de obra, sin que eso se vea compensado por el mayor precio de venta de pulpos grandes. La clasificación por sexos y tamaños demostró que los machos, en general, presentan mayores incrementos de peso que las hembras, pero también mayores tasas de mortalidad (Tabla II). El menor crecimiento de las hembras estaría relacionado con la ocurrencia de puestas, ya que en la época marzo-julio se da el gran pico de reproducción de pulpos en la costa gallega.

De los trabajos realizados en el 2000, se concluyó que los individuos con peso inicial inferior o igual a 1 kg presentan mejor índice de crecimiento que aquellos individuos con peso superior a 1 kg, aunque a nivel económico resultaba más lucrativo iniciar el engorde con ejemplares de alrededor de 1,5 kg, puesto que alcanzaban las tallas comerciales mejor cotizadas en un período menor de cultivo y la mortalidad era más baja (Tabla II).

En la actividad de engorde, el peso recomendable de la recogida de los individuos es de hasta los 3,0 kg, ya que a partir de estos pesos aumenta el índice de mortalidad, lo que podría comprometer seriamente la productividad.

Los datos obtenidos demostraban la viabilidad de utilizar sexos mezclados, puesto que se lograban tallas similares a los estudios precedentes (Tabla II).

Tabla II. Resultados obtenidos en dos años consecutivos de engorde de pulpo en la Ría de Arousa. (Tuñon *et al*., 2000, 2001)

AÑO 1999 – Efecto de la clasificación por sexo y tamaño en el engorde industrial de pulpo

Grupo experimental	Peso medio inicial (g)	Incremento medio de peso (kg)	Incremento medio de peso diario (g)	Nº de días de cultivo	Mortalidad (%)
M1 < 1kg	801±120	1,84	10,10	120	51,5
H1 < 1 kg	816±100	1,20	9,10	120	42,8
M2 > 1 kg	1159±160	1,60	13,48	120	47,0
H2 > 1 kg	1154±100	1,10	9,22	120	34,5

AÑO 2000 – Efecto del tamaño inicial en el engorde industrial de pulpo en batea

P 1 < 1 kg	844±146	1,17	21,3	55	22,0
P 2 < 1 kg	838±134	1,19	21,6	55	25,0
G 1 < 1 kg	1521±354	0,88	20,4	43	16,0
GP < 1 kg y >1 kg	1473±280 y 798±143	0,72	15,6	46	18,0

Posteriormente, en el año 2001, en colaboración con la empresa Arrecifes del Atlántico S.L., se realizó un estudio de engorde de pulpo haciendo un seguimiento individual de los ejemplares metidos en las jaulas, a través de la utilización de microchips subcutáneos (Fig. 2), con el fin de averiguar la existencia de jerarquías entre los individuos o preferencias de utilización de determinados refugios y de relacionar esa información con el crecimiento y la mortalidad observada. Al final de 115 días de engorde, se pesaron e identificaron todos

los pulpos mediante la lectura de los microchips. También se efectuaron mediciones de la localización de los pulpos en los refugios de la jaula en varios días a lo largo del tiempo de engorde.

Figura 2 - Microchip subcutáneo introducido en la parte superior del manto, un poco por encima del punto medio de los ojos. Foto: Pedro Seixas.

En la tabla III se presentan los resultados obtenidos. Tal como había sido observado en trabajos precedentes, el crecimiento de los machos fue mayor que el de las hembras. La mortalidad total fue del 18%, siendo más alta en los machos que en las hembras.

Tabla III. Resultados obtenidos en el experimento de engorde de pulpos marcados individualmente con microchips para evaluación de su comportamiento individual y crecimiento. Tomada de Tuñon *et al*. (2002).

	Nº ind. inicio	Nº ind. final	Peso medio inicial (g)	Peso medio final (g)	Biomasa final (kg)	Mortalidad (%)
Total	90	74	761±123	3074±722	227,5	17,8
Machos	41	30	768±125	3341±739	100,2	26,8
Hembras	49	44	755±122	2893±660	127,3	10,2

Analizando la mortalidad por tallas iníciales de cultivo, distribuidas en tres rangos (<750 g; 750-850 g; > 850 g), los autores han comprobado que los machos de peso inicial menor y mayor se destacaron en cuanto a alta mortalidad, mientras que las hembras de peso inicial menor no presentaron mortalidad. Otro dato interesante se refiere a la buena correlación

entre los pesos pesos iniciales de cada individuo y los pesos finales de los pulpos de ambos sexos (R^2=0,8282 para los machos y un R^2=0,9141 para las hembras). En efecto, los machos de mayor peso inicial fueron los que alcanzaron los pesos finales más elevados, al igual que en las hembras. Los datos obtenidos hicieron que se conjeturase a cerca del probable comportamiento agresivo ligado al sexo y al tamaño, que serían más frecuentes entre los machos de proporciones "colas" o "cabezas".

c) Resultados obtenidos por otros grupos de investigación en otras CC.AA. de España

Los datos de engorde de pulpo divulgados por otros autores en pruebas realizadas en otras CC.AA. de España apuntan a resultados de crecimiento y supervivencia similares a los obtenidos en Galicia. En las Islas Canarias, Socorro *et al*. (2005) realizaron pruebas en una jaula flotante dividida en dos compartimientos, utilizando como único alimento la boga (*Boops boops* L., 1758). Los pulpos tenían pesos medios iniciales de 1528±407 g (G1) y 2337±766 g (G2) y se establecieron a una densidad de 11 kg m^{-3}. En un tiempo de engorde de 57 días, los autores observaron pesos finales de 2643±745 g y 3812±1392 g, en los grupos G1 y G2, respectivamente, con supervivencias del 91% y del 82 %, también respectivamente. Sin embargo, en experimentos posteriores partiendo de ejemplares de tamaño inferior (1031±345 g y 805±280 g), y efectuando el engorde durante 81 días, los pulpos alcanzaron pesos finales medios de 3111±1330 g y 3052±1061 g, con supervivencias bastante reducidas (44 y 50 %, respectivamente).

En la comunidad Valenciana, Oltra *et al*. (2005), estudiaron el engorde de pulpos en una jaula flotante situada en el puerto de Denia (Alicante), con el objetivo de comprobar su rendimiento en aguas de la costa de Levante. Los subadultos fueron capturados mediante arrastre en aguas de Denia y fueron separados por sexos. La alimentación consistió en una dieta variada de peces, crustáceos y moluscos en un porcentaje aproximado de 79:17:4%, respectivamente. El primer engorde tuvo lugar entre octubre de 2004 y enero de 2005 (94 días) y el segundo entre marzo y junio de 2005 (86 días). En el primer experimento, iniciando la etapa de engorde con pulpos de peso medio inicial idéntico para machos y hembras (554 g), se alcanzaron pesos finales de entre 2500-2700 g, pero con supervivencias bastante bajas (52% en el grupo de las hembras y 30% en el grupo de los machos). En el segundo ensayo, comparando el engorde de grupos constituidos por sólo machos, sólo hembras y un grupo mixto, y partiendo de pesos medios iniciales similares,

estos autores han encontrado pesos medios finales de entre 3250-3440 g, sin que se registrasen diferencias significativas entre los grupos, con tasas de superviencia del 30 al 70%.

En la comunidad autónoma de Asturias, Rodríguez *et al*. (2006) han descrito los resultados de engorde de experiencias realizadas en los años 2002 y 2003, utilizando como alimento pescado, cangrejos y mejillón (Tabla IV).

Tabla IV. Resultados de crecimiento y supervivencia de pulpos en experiencias realizadas en los años 2002 y 2003 en la comunidad autónoma de Asturias (tomada de: Rodríguez *et al.*, 2006).

	Year 2002		Year 2003	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Number of individuals	90	47	50	48
Initial biomass (kg)	98.1	43.1	50.4	47.8
Initial density (kg m^{-3})	24.5	10.7	12.6	11.9
Initial mean weight (g)	1090±220.8	917±231.6	1009±242.1	996±237.9
Range (g)	900–1400	600–1600	800–1400	700–1300
Mean temperature (°C)	14.3±1.1	15.5±1.6	15.3±1.2	16.7±1.7
Days of trial	86	96	90	91
Number of individuals	53	39	32	39
Final biomass (kg)	89.8	145.8	53.7	155.3
Final density (kg m^{-3})	22.5	36.5	13.4	38.8
Final mean weight (g)	1694±461.3	3739±889.5	1790±604.5	3982±818.7
Range (g)	700–2800	1500–5500	700–3400	1600–5000
DWI (g day^{-1})	7.02	29.39	8.69	33.18
SGR (%)	0.51	1.46	0.64	1.54
FC	10.5	4.03	9.2	3.8
FE (%)	9.5	24.7	10.8	25.8
Mortality rate (%)	41.1	17.0	36.0	18.7

García-García *et al*. (2007), en la comunidad autónoma de Murcía, han realizado una serie de ensayos en jaulas en mar abierto ubicadas a 6 millas de la costa de San Pedro de Pinatar, para evaluar el efecto de las variables peso inicial de los ejemplares, temperatura (14-26 ºC), carga de cultivo (6-64 kg m^{-3}) y dispersión de tamaños de la población en cultivo (coeficiente de variación) sobre el crecimiento y la mortalidad del pulpo. Estos autores han verificado que el peso inicial y la temperatura fueron las variables que más afectaron al crecimiento y a la mortalidad de los pulpos, pero el coeficiente de variación también influyó sobre la mortalidad. La carga de cultivo no tuvo una influencia significativa sobre los parámetros analizados.

En el marco del proyecto nacional JACUMAR (Optimización del engorde del pulpo *Octopus vulgaris* 2007-2009), nuestro grupo de investigación (Departamento de Bioquímica y Biología Molecular) ha realizado algunos trabajos de engorde de pulpo a lo largo de los años 2007 y 2008, en jaulas suspendidas en batea localizadas en la ría de Vigo. Estos trabajos han incidido sobre el engorde de pulpo mediante separación por tamaños y en la clasificación por sexos. Se ha analizado los efectos que puede tener la selección de pulpos de tamaño muy uniforme (diferencias en el peso inicial de los ejemplares no superiores a 100 g) frente al procedimiento habitual (pulpo con diferencias de peso inicial que pueden superar los 500 g) en una etapa de engorde corriente. Por otro lado, se estudió el engorde de pulpos clasificados por sexos, en la época del verano, complementando así estudios previos realizados por Iglesias *et al.* (2007b) en la misma batea, pero realizados en invierno (época baja del engorde).

2. Material y métodos

2.1 Engorde de pulpos mediante separación por tamaños

Las pruebas de crecimiento se llevaron a cabo en la ría de Vigo, Galicia, en una batea semejante a las utilizadas para cultivar mejillón, pero modificada para que se pudieran colgar jaulas de engorde. Las jaulas de engorde, de hierro galvanizado y con las dimensiones de 1,5 x 1,5 x 3 m de altura, poseen en su interior 8 columnas de tubos "T" de PVC encajados, cada una con 7 refugios, colocadas en lados opuestos dentro de la jaula, que sirven de cobijo a los pulpos.

Los pulpos utilizados en este experimento han sido capturados con nasas entre la zona de las islas Cíes y Ons, los días 5 a 8 de mayo de 2008. Una vez llegados a las jaulas de engorde, los pulpos fueron pesados individualmente en una balanza Kern de precisión ± 1 g, e introducidos en dos jaulas, estableciéndose dos grupos: un grupo homogéneo (HOM) cuya diferencia de peso entre los individuos no sobrepasaba los 100 g (pulpos con ≥800 y ≤900 g), y otro heterogéneo (HET) que consistió de una jaula típica con la que se inicia un ciclo de engorde (o sea, con individuos cuya diferencia de peso puede sobrepasar los 500 g).

El alimento fue distribuído a diario (excepto los domingos) con distintas especies de pescado (jurel, bacaladilla, caballa, boga) y mejillón, con una ración que varió entre el 3% y el 6% de la biomasa en jaula. Una vez a la semana se realizaron operaciones de limpieza

de las jaulas, tanto en la parte exterior de las paredes como dentro de la misma, recogiendo los restos de comida y los ejemplares muertos. Al final del periodo de engorde, se han contabilizado y pesado nuevamente los pulpos de forma individual, analizándose la supervivencia y el crecimiento de los grupos. Se ha seguido igualmente el proceso de engorde de otras tres jaulas "típicas" en dos épocas del año distintas, con el fin de evaluar la supervivencia y el incremento del peso de los pulpos en diferentes condiciones anuales. En la tabla V se puden ver las características iniciales de cada grupo estudiado.

2.2 Engorde de pulpos mediante separación por sexos

Este trabajo se ha llevado a cabo igualmente en la ría de Vigo en las mismas condiciones descritas en el apartado anterior. Los pulpos utilizados en este experimento han sido capturados con nasas entre la zona de las islas Cíes y Ons, los días 12 a 16 de agosto de 2008. Los ejemplares han sido pesados individualmente en una balanza Kern de precisión ± 1 g, estableciéndose los siguientes grupos: grupo 1 (MACHOS) – constituido por ejemplares macho, los cuales se han identificado por la presencia de las grandes ventosas o por el tercer brazo derecho hectocotilizado; grupo 2 (HEMBRAS) – constituido únicamente por hembras; grupo MIXTO – constituido por machos y hembras en proporción aleatoria, tal y como se suele hacer en un ciclo de engorde típico. En la tabla VI se pueden observar las características de los grupos iniciales.

3. Resultados

3.1 Engorde de pulpos mediante separación por tamaños

Los resultados obtenidos se presentan en la tabla V. La supervivencia apenas varió entre los grupos, siendo del 70,0% en el grupo homogéneo y del 68,7% en el grupo heterogéneo. El peso medio final de los pulpos en el grupo heterogéneo (2188±574 g) fue superior al del grupo homogéneo (2091±499 g), mientras que la biomasa final del grupo homogéneo fue mayor, debido a la diferencia en la supervivencia. La supervivencia de los pulpos de otras jaulas heterogéneas que se habían seguido en diferentes épocas del año fue similar a la observada en las jaulas del presente estudio (Tabla V). En cuanto a la biomasa de estos grupos heterogéneos, se puede verificar que ésta varió considerablemente con la época del

año en la que se realizó el engorde. En los meses de la época baja, aunque la supervivencia fue praticamente igual a la de la época alta, el crecimiento de los pulpos fue bastante inferior (grupo heterogéneo 1), aunque el tiempo de engorde fuera superior (Tabla V). En la época de engorde alta (grupos heterogéneos 2 y 3), los pesos medios finales fueron similares o incluso superiores, debido al mayor numero de días de engorde.

Tabla V. Resultados del engorde de pulpos con separación por tamaños. Grupo homogéneo: constituído por ejemplares con un peso inicial de entre 800-900 g; y grupo heterogéneo: ejemplares con diferencias en el peso inicial superiores a 500 g. Se presentan igualmente los resultados obtenidos en otras jaulas heterogéneas, seguidas en otras épocas del año, para comparación.

	Heterogéneo 1	Heterogéneo 2	Heterogéneo 3	**Homogéneo**	**Heterogéneo**
Nº pulpos inicial	110	112	108	**120**	**115**
Nº pulpos final	70	74	72	**84**	**79**
Supervivencia	63,6%	66,1%	66,7%	**70,0%**	**68,7%**
Peso inicial (g)	786 ± 97	895 ± 115	892 ± 118	**853 ± 29**	**894 ± 160**
Peso final (g)	1801 ± 456	2369 ± 733	2039 ± 621	**2091 ± 499**	**2188 ± 574**
Biomasa inicial (kg)	86,5	100,2	93,7	**102,3**	**102,8**
Biomasa final (kg)	126,1	175,3	146,8	**175,7**	**167,3**
Tiempo de engorde	107 días 03/12/2007 – 07/04/2008	107 días 14/04/2008 – 29/07/2008	97 días 21/04/2008 – 29/07/2008	**85 días (7/05/2008 – 30/07/2008)**	

En la figura 3 se puede observar la frecuencia de los pesos finales de los ejemplares. En el grupo homogéneo se verificó una distribución normal bien demarcada en tan sólo tres meses de engorde. En este grupo se observaron once pulpos con peso inferir a 1,5 kg, registrándose únicamente tres individuos con peso superior a 3 kg. A su vez, el grupo heterogéneo tuvo una distribución de pesos asimétrica, observándose trece pulpos con peso inferior a 1,5 kg y siete con peso superior a 3 kg (Fig. 3).

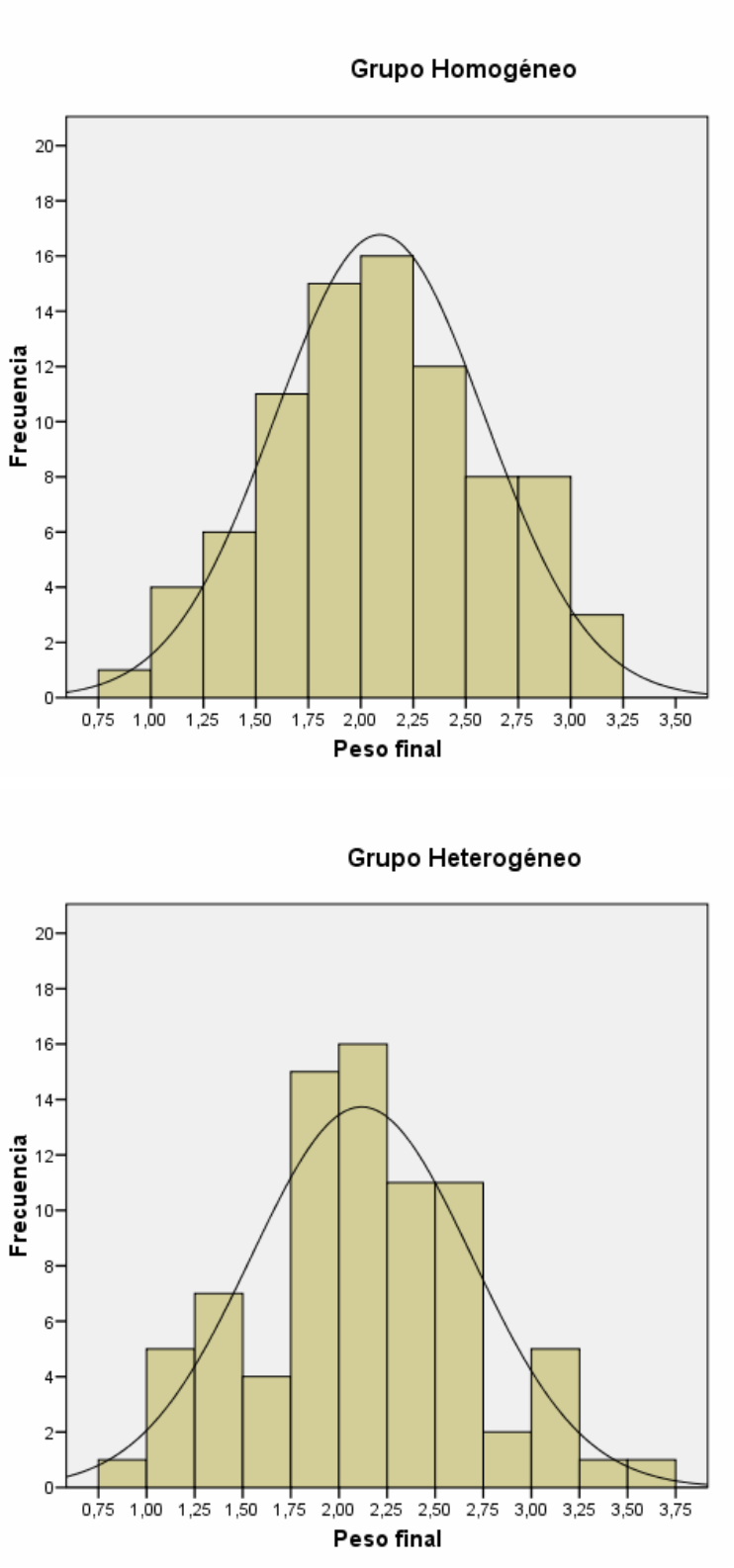

Figura 3 – Frecuencia de los pesos finales de los pulpos en los grupos homogéneo (arriba) y heterogéneo (abajo).

3.2 Engorde de pulpos mediante separación por sexos

Los resultados del estudio del engorde con clasificación por sexos se presentan en la tabla VI. La supervivencia de los grupos MACHOS y MIXTO (ambos con 86,7%) fue superior a la del grupo HEMBRAS (73,3%). Sin embargo, comparando la supervivencia de esta jaula con la supervivencia de la otra jaula mixta que se siguió (Mixta 2 – 71,9%), se puede observar que ambas presentan valores similares (Tabla VI).

Tabla VI. Resultados de supervivencia y crecimiento de los pulpos en los grupos separados por sexos (Machos y Hembras) y en los grupos control (Mixtos). Se presentan igualmente los resultados obtenidos en otra jaula con pulpos mixta que ha sido seguida (Mixto 2).

	MIXTO	MACHOS	HEMBRAS	Mixto 2
Nº pulpos inicial	120	120	120	121
Nº pulpos final	104	104	88	87
Supervivencia	86,7%	86,7%	73,3%	71,9%
Peso medio inicial	899 ± 137[a]	953 ± 146[b]	899 ± 135[a]	921 ± 138
Peso medio final	2125 ± 450[a]	2261 ± 428[b]	2015 ± 428[a]	2328 ± 456
Biomasa inicial	107,8 kg	114,3 kg	107,9 kg	102,3 kg
Biomasa final	221,0 kg	235,2 kg	177,4 kg	175,7 kg
Días de engorde	103 días 15/08/2008 - 25/11/2008	95 días 15/08/2008 - 18/11/2008	103 días 15/08/2008 - 25/11/2008	103 días 08/08/2008 - 18/11/2008

Medias ± desv. est. (Teste no paramétrico de Kruskal Wallis para comparación de los tres grupos, seguido de Mann-Whitney para identificación de los grupos con diferencia significativa P<0,05).

El peso final de los individuos del grupo MACHOS fue superior al de los pulpos de los grupos HEMBRAS y MIXTO, aunque el peso inicial de ejemplares también había sido superior. Por ello se mantuvieron los individuos de las jaulas HEMBRAS y MIXTA una semana más, con el fin de compensar la desigualdad de los pesos iniciales. Entre el grupo HEMBRAS y MIXTO no se encontraron diferencias estadísticamente significativas, a pesar del mayor peso medio del grupo MIXTO. Comparando el crecimiento del grupo MACHOS con el Mixto 2, cuyos pesos iniciales no fueron estadísticamente diferentes (P=0,083, Mann-Whitney), se pudo verificar que tampoco existieron diferencias significativas en el peso final de los ejemplares (P=0,349, Mann-Whitney). En la figura 4

se presentan las frecuencias de los pesos finales de los grupos MACHOS, HEMBRAS y MIXTO.

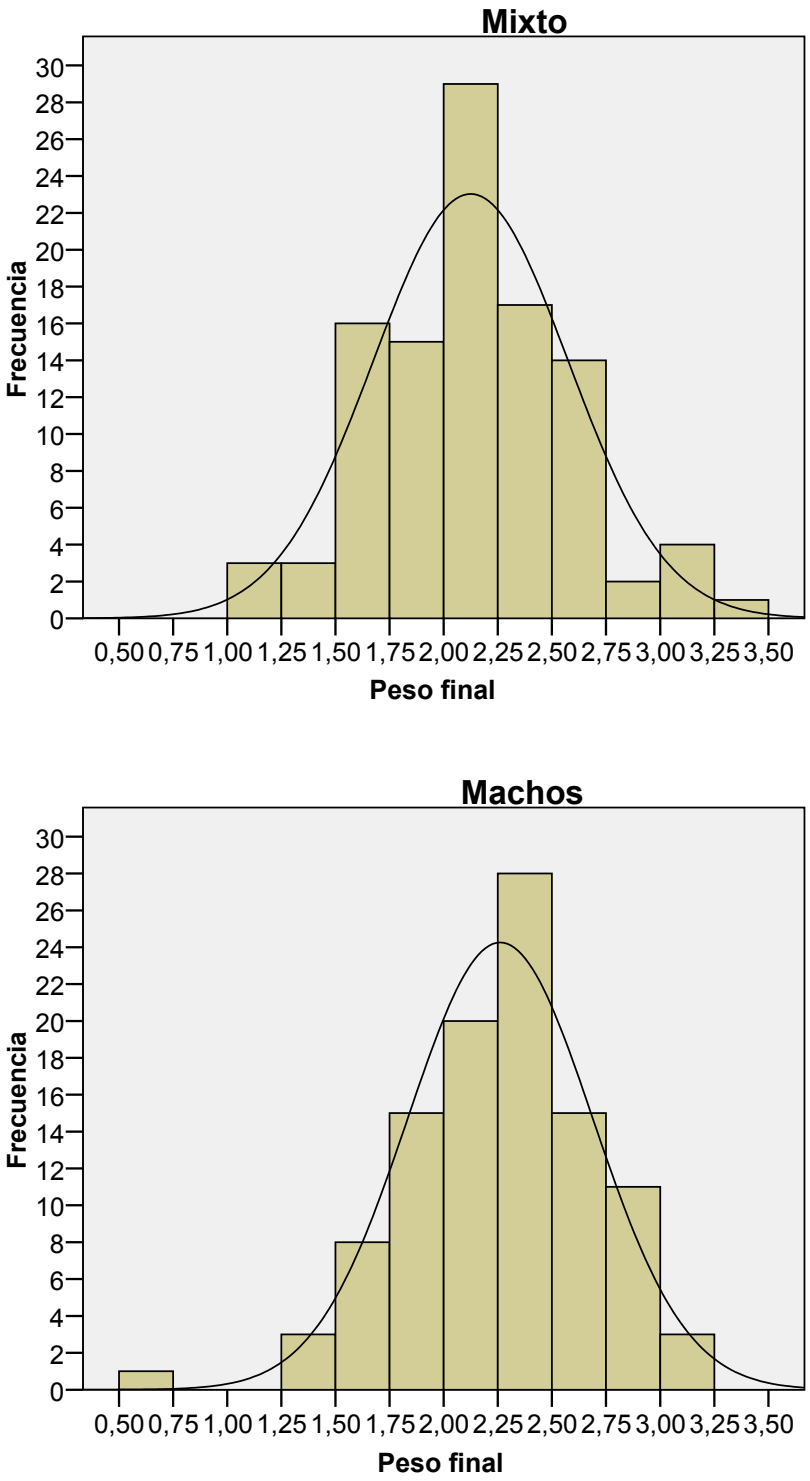

Figura 4 - Frecuencia de los pesos finales de los pulpos en los grupos MIXTO (pulpos machos y hembras en proporción aleatoria), MACHOS (sólo machos) y HEMBRAS (sólo hembras). La figura continua en la página siguiente.

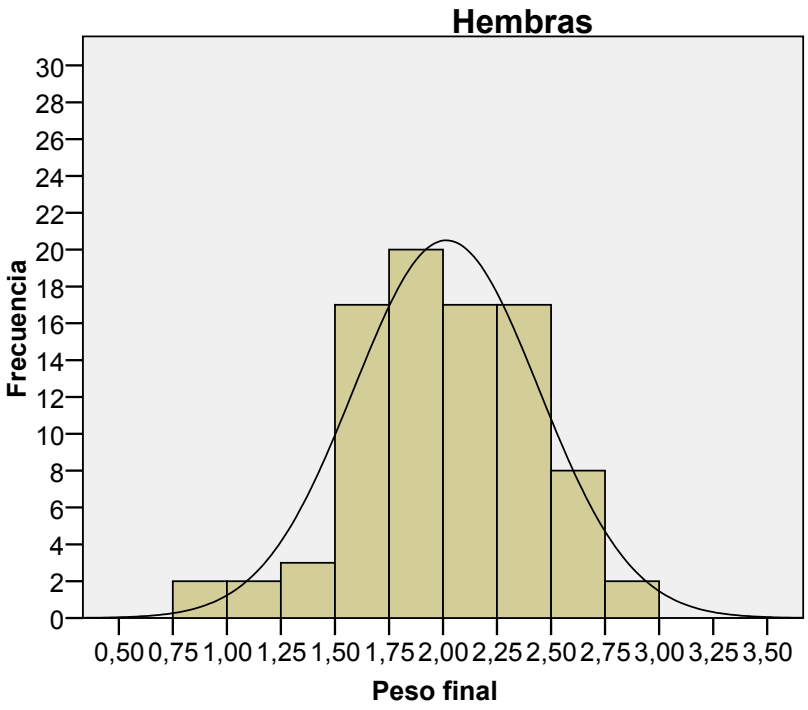

Figura 4. Continuación.

Observando la frecuencia de los pesos finales de los pulpos en los diferentes grupos, se puede constatar que los grupos MIXTO y MACHOS tuvieron más individuos con peso superior a 2 kg que el grupo HEMBRAS. En el grupo HEMBRAS no se encontraron ejemplares de más de 3 kg, mientras que en los grupos MIXTO y MACHOS sí se observaron algunos con más de 3 kg de peso final.

Discusión

Los resultados de crecimiento y supervivencia obtenidos en este estudio se encontraron dentro de los valores normales descritos por otros autores en experimentos de engorde (Tuñon *et al*., 1999, 2000, 2001, 2002, 2003; Rey-Méndez *et al*., 2001; Rodríguez *et al.*, 2006; Iglesias *et al.*, 2007b), aunque en algunos casos los pesos medios finales pudieron ser inferiores. La supervivencia registrada en el experimento de engorde con separación

por tamaños (64-70%) fue, en general, inferior a los valores encontrados en el experimento con clasificación de sexos (72-87%). Sin embargo, los valores observados en los dos experimentos estaban dentro del rango descrito por otros autores (45-90%) en condiciones de engorde similares (Tuñon *et al*., 2000, 2001, 2002; Rodríguez *et al*., 2006; Iglesias *et al*., 2007b).

En el experimento con separación de tamaños, una de las causas que pudo influenciar en la diferencia de los pesos medios finales fue la media de los pesos iniciales, que fue superior en el grupo heterogéneo. Esta diferencia se debió a la introducción de varios ejemplares con peso superior a 1 kg en el grupo heterogéneo. Sin embargo, el objetivo del trabajo era comparar un ciclo de engorde típico frente a un grupo de pulpos de peso homogéneo, por lo que nos limitamos a seleccionar individuos para formar este último grupo, permitiendo que la formación del grupo heterogéneo fuera totalmente aleatoria, tal y como se hace en un proceso habitual. Los resultados del grupo homogéneo han demostrado que los pulpos han crecido de acuerdo con lo que suele ocurrir en la naturaleza, es decir, algunos ejemplares han crecido más de lo normal ("cabezas"), mientras que otros apenas se han desarrollado, lo que correspondería a las "colas". Otra posible explicación es que se haya establecido algún tipo de jerarquía dentro del grupo, de tal forma que algunos pulpos se han desarrollado más debido a diversos factores de comportamiento, como previamente describieron Tuñón *et al*. (1999, 2003).

El engorde de pulpos obtenidos del medio natural y seleccionados para formar un grupo homogéneo no supuso una clara ventaja frente a un ciclo de engorde típico con individuos de diferentes tamaños iniciales. Las pequeñas diferencias observadas en cuanto a biomasa total, posiblemente no compensarían, económicamente, el esfuerzo en personal necesario para realizar la selección por tamaños.

En el experimento con clasificación por sexos, la mortalidad del grupo MACHOS no fue diferente de la del grupo MIXTO. La mayor mortalidad de pulpos en el grupo HEMBRAS podría estar relacionada con la ocurrencia de puestas, ya que al final del período de incubación de los huevos las hembras suelen morir al cabo de pocos días. Sin embargo, en este estudio no se encontró un gran número de puestas (tan sólo cuatro), por lo que en este caso se podría descartar este factor. Los resultados de supervivencia encontrados en este trabajo en los grupos MACHOS y HEMBRAS son similares a los descritos por otros autores (Chapela *et al*., 2006; Iglesias *et al*., 2007b), pero contrarios a otros estudios anteriores (Tuñon *et al*., 2000, 2002), por lo sería necesario profundizar este tema y encontrar las causas que expliquen los diferentes resultados encontrados.

Aunque se observó una clara tendencia de mayor crecimiento de los individuos del grupo MACHOS frente a los del grupo HEMBRAS, estas diferencias no fueron claras en comparación con los grupos MIXTOS. Estos resultados corroboran la evidencia de que los machos crecen más que las hembras, ya que éstas a partir de determinado peso empiezan a desarrollar y a madurar el ovario para realizar la puesta. Esta inversión de energía para el desarrollo de la gónada se traduce en un menor crecimiento somático, problema que no afecta a los machos. Teniendo en cuenta que no se encontraron grandes diferencias en la supervivencia entre los grupos clasificados por sexos y en los mixtos, esta separación no ofrece una ventaja clara a la hora de engordar pulpos.

En experimentos previos de engorde de pulpo sin clasificación por sexos, entre los meses de abril y julio, en la comunidad autónoma de Asturias, Rodríguez *et al*. (2006) describieron pesos medios finales de 1694±461,3 g y 1790±604,5 g al cabo de 3 meses de engorde, e iniciando la etapa con ejemplares de cerca de 1 kg, siendo estos pesos inferiores a los encontrados en el presente estudio. Los autores encontraron tasas de supervivencia del 59 al 64%. Sin embargo, en etapas de engorde realizadas entre los meses de agosto y octubre, estos autores encontraron pesos medios finales de 3739±889,5 g y 3982±818,7 g, con supervivencias de alrededor del 82%.

En trabajos con clasificación por sexos realizados por Iglesias *et al*. (2007b) en la misma batea del presente estudio, en la Ría de Vigo, en invierno, estos autores describieron pesos medios finales de 2316±596 g en el grupo de los machos, y de 2600±545 g en el grupo de las hembras (sin que estas diferencias fuesen estadísticamente significativas), al cabo de 4 meses de engorde, y tasas de supervivencia del 82 al 85%. En otros estudios llevados a cabo también en la Ría de Vigo, Chapela *et al*. (2006) describieron igualmente un ligero mejor crecimiento de las hembras en la época del invierno, aunque en este caso los autores habían iniciado el engorde con diferencias en el peso medio inicial de los grupos (0,81 kg en las hembras frente a 0,79 kg en los machos). Sin embargo, en los meses de verano, estos autores han observado mejores tasas de crecimiento en los machos que en las hembras, y una mayor mortalidad de las hembras (24%) frente a los machos (14%), que pudo ser explicada por el gran número de puestas encontradas.

Comparando la rentabilidad del engorde entre la época alta (verano) y baja (invierno), Iglesias *et al*. (2007b) observaron que aunque los pesos medios finales en verano (3376±873 g) fuesen claramente superiores a los obtenidos en invierno (1862±328 g), la biomasa final alcanzada apenas variaba entre las dos etapas, a raíz de la gran diferencia en la supervivencia (en torno a un 75% en invierno y a un 50% en verano). Los pesos medios

finales obtenidos en este trabajo en los grupos separados por sexos o mixtos, fueron más bajos que los descritos por Iglesias *et al*. (2007b) en grupos mixtos engordados en verano. Sin embargo, debido a la mayor tasa de supervivencia de los pulpos en este estudio, la biomasa total ganada fue superior a la observada por aquellos autores. Las diferencias en los pesos medios finales también se podrían explicar por el tiempo de engorde más reducido en el presente trabajo (95-103 días), en comparación con el tiempo de engorde realizado por aquellos autores (4 meses). La realización de ciclos de engorde más cortos en los meses de verano había sido ya sugerida por Iglesias *et al*. (2007b), con el fín de abaratar los costes de producción y reducir la mortalidad de los individuos, que a menudo se incrementa en el último mes de engorde. Tal y como se ha observado en el estudio, aquellos autores no encontraron beneficios claros en la clasificación de sexos a la hora de mejorar la etapa de engorde del pulpo.

De cara al futuro, sería interesante realizar nuevos experimentos de engorde con grupos de individuos separados por tamaños, pero con rangos de peso más amplios (ej.: 250 g) que se puden separar "a simple vista", estableciéndose períodos de engorde más cortos o más largos en función del peso inicial del grupo. Con estas medidas se podría ahorrar en la alimentación y mejorar la gestión del cultivo, ya que el tiempo de engorde de acortaría en las jaulas con ejemplares más grandes y se prolongaría en aquellas con ejemplares más pequeños, evitando además le elevada dispersión en los pesos finales de los pulpo, tal y como observado en este trabajo. Una vez resuelto el problema del cultivo larvario y disponiendo de juveniles con la misma edad y de peso similar, sería necesario estudiar el desarrollo de los individuos y establecer comparaciones con los resultados observados en el presente estudio.

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Conclusiones / Conclusions

-1.

El enriquecimiento de juveniles de *Artemia* con distintas especies de microalgas cultivadas en régimen semicontinuo, ha generado diferencias considerables en su composición bioquímica, especialmente en el perfil de ácidos grasos, que se mantuvieron estables en los distintos experimentos de cultivo de paralarvas. Los juveniles enriquecidos con *Rhodomonas lens* presentaron la mejor composición bioquímica, en relación a los posibles requerimientos nutricionales de las paralarvas de *Octopus vulgaris*, debido a la mayor relación proteína/energía y al perfil de ácidos grasos poliinsaturados (PUFAs). Sin embargo, el mayor contenido de la suma de los ácidos grasos 22:6n-3 (DHA) y 20:5n-3 (EPA) se ha encontrado en los juveniles enriquecidos con *Isochrysis galbana*.

Considerable differences in the biochemical composition of *Artemia* juveniles enriched with different microalgal species were found, especially in its fatty acid profile, which remained stable in the different experiments of paralarvae rearing, due to the use of semicontinuous cultures of microalgae. Taking into consideration the possible nutritional requirements of *Octopus vulgaris* paralarvae, the enrichment of *Artemia* juveniles with *Rhodomonas lens* provided the best results among monoalgal enrichments, due to the highest protein/energy ratio and to its polyunsaturated fatty acid (PUFAs) profile. However, the highest sum of the fatty acids 22:6n-3 (DHA) and 20:5n-3 (EPA) was found in *Artemia* juveniles enriched with *Isochrysis galbana*.

-2.

El contenido de los ácidos grasos 22:6n-3 (DHA) y 20:4n-6 (ARA) de los juveniles de *Artemia* enriquecidos con las distintas especies de microalgas fue muy inferior al encontrado en paralarvas de *O. vulgaris* recién eclosionadas, o en zoeas de centolla (*Maja brachydactyla*), mientras que el contenido de EPA fue en general inferior, o en algunos casos igual (en el caso de los juveniles enriquecidos con *I. galbana* o *N. gaditana*), al observado en las paralarvas. Estos resultados sugieren la existencia de un déficit de PUFAs en los juveniles de *Artemia* enriquecidos con microalgas.

The content of the fatty acids 22:6n-3 (DHA) and 20:4n-6 (ARA) found in *Artemia* juveniles enriched with the different microalgal species, was considerably lower than the

levels found in both *O. vulgaris* hatchlings and spider-crab (*Maja brachydactyla*) zoeae, whereas eicosapentaenoic acid (20:5n-3, EPA) was in general lower, or in some cases equal (in the case of juveniles enriched with either *I. galbana* or *N. gaditana*), to the levels found in octopus hatchlings. These results suggest a deficit of PUFAs in the *Artemia* juveniles enriched with microalgae.

-3.

El crecimiento de las paralarvas de pulpo alimentadas con juveniles de *Artemia* enriquecidos con microalgas (mezcla de *Rhodomonas lens* e *Isochrysis galbana*, 70%:30% en peso seco), ha sido superior al de las paralarvas alimentadas con juveniles de *Artemia* enriquecidos con productos comerciales lipídicos muy ricos en DHA (22:6n-3).

The growth of paralarvae fed *Artemia* juveniles enriched with a mixed diet of the microalgal species *Rhodomonas lens*/*Isochrysis galbana* (70%:30% in a dry weight basis), was higher than that of paralarvae fed *Artemia* juveniles enriched with commercial lipid products rich in DHA (22:6n-3).

-4.

El bajo contenido del ácido graso DHA en la dieta de las paralarvas no fue un factor limitante para su desarrollo y crecimiento, ni originó una mayor mortalidad de los individuos. El suministro de juveniles de *Artemia* enriquecidos con DHA-Selco® (con un contenido en DHA tres veces superior al obtenido en el enriquecimiento con *Rhodomonas lens* e *Isochrysis galbana*), no produjo ningún beneficio directo en la supervivencia y crecimiento de las paralarvas, aunque en éstas se observa un contenido corporal en DHA superior al de las paralarvas alimentadas con *Artemia* enriquecida con *R. lens* e *I. galbana*.

A low content of DHA in the diet for octopus paralarvae was not a limiting factor for their growth and development, or promoted higher mortality of individuals. The utilization of *Artemia* juveniles enriched with DHA-Selco® (which contained three-times more DHA than *Artemia* enriched with *Rhodomonas lens*/*Isochrysis galbana*) to feed octopus paralarvae, did not produced any beneficial effects on its growth and survival rates, even though these paralarvae contained higher DHA levels in its composition, in comparison with paralarvae fed *Artemia* juveniles enriched with *Rhodomonas lens*/*Isochrysis galbana*.

-5.

El suministro a las paralarvas de una dieta combinada de juveniles de *Artemia* enriquecidos con *Rhodomonas lens* e *Isochrysis galbana* (AR+I) y juveniles de *Artemia* enriquecidos con productos comerciales muy ricos en DHA (Ori-Gold®) (1:1), tampoco supuso una ventaja frente a la utilización de una monodieta basada en juveniles AR+I. Esta monodieta, a pesar de su bajo contenido en DHA, produjo mayor supervivencia y mejor crecimiento de las paralarvas, y ha sido la única que permitió que se alcanzasen paralarvas con 35 días de vida.

Feeding octopus paralarvae with a combination of *Artemia* juveniles (1:1) enriched with either *Rhodomonas lens*/*Isochrysis galbana* (AR+I), or with a commercial product very rich in DHA (Ori-Gold®), did not improved paralarval performance, in comparison with individuals fed on a monodiet of AR+I. Despite the low content of DHA in *Artemia* juveniles enriched with *Rhodomonas lens*/*Isochrysis galbana*, this diet was shown to produce the best survival and growth of paralarvae, and was the only one that allowed the achievement of 35-days post hatch paralarvae.

-6.

La relación proteína:lípido (P:L) en la dieta influyó más sobre el crecimiento y la supervivencia de las paralarvas que el contenido del DHA. Se ha encontrado una correlación lineal, positiva y significativa, entre el aumento del cociente P:L de la dieta suministrada y el peso seco de las paralarvas, a los 15 y 25 días de cultivo, mientras que no fue posible establecer una relación entre el contenido del DHA y la supervivencia y crecimiento.

The protein:lipid ratio (P:L) of the diet was found to be more important to promote a good growth and survival of paralarvae, than its content in DHA. A significant positive linear correlation was found between the augment of the P:L ratio in the diet and the dry weight of paralarvae, at 15 and 25 days of rearing, whereas no correlations could be established between dietary DHA content and growth or survival of paralarvae.

-7.

El cultivo de paralarvas de pulpo con juveniles de *Artemia* enriquecidos con *Nannochloropsis gaditana*, previamente utilizados por otros autores y descritas como adecuadas, ha generado peores resultados que la utilización de juveniles enriquecidos con *Rhodomonas lens* e *Isochrysis galbana* (AR+I). Aunque en este trabajo la utilización de aguas verdes con *N. gaditana,* en sistemas de pequeño volumen y sin recirculación, no fue viable, los efectos de la utilización de aguas verdes con *Nannochloropsis* sp. u otras especies de microalgas deberá ser investigado con mayor intensidad.

The rearing of octopus paralarvae with *Artemia* juveniles enriched with *Nannochloropsis gaditana*, previously described by other authors to offer good results, was shown to provide worst results than the use of *Artemia* juveniles enriched with *Rhodomonas lens*/*Isochrysis galbana*. Despite the use of green waters with *N. gaditana* in low-volume tanks without recirculation was not successful in the present study, the effects of using green waters with *Nannochloropsis* sp. or other microalgal species should be further investigated.

-8.

El enriquecimiento de juveniles de *Artemia* con las microalgas *R. lens*/*I. galbana,* y además suplementados con aminoácidos esenciales (lisina, arginina y metionina) disueltos en el agua, ha originado mejoras en el peso seco de las paralarvas, por lo que sería interesante profundizar en esta nueva línea de investigación.

The enrichment of *Artemia* juveniles with *Rhodomonas lens*/*Isochrysis galbana* and further boosted with free essential amino acids (lysine, arginine and methionine) dissolved in the water, was shown to improve the dry weight of octopus paralarvae, and hence it would be interesting to further investigate this subject in the future.

-9.

El engorde de pulpos obtenidos del medio natural y seleccionados para formar un grupo de individuos con peso medio muy similar (grupo homogéneo), no supuso una clara ventaja frente a un ciclo de engorde típico con individuos de peso medio heterogéneo. La

ligera mejora en la supervivencia del grupo homogéneo, en comparación con el grupo heterogéneo, y las pequeñas diferencias observadas en cuanto a biomasa total alcanzada, posiblemente no compensarían económicamente el trabajo necesario para realizar la selección de los pulpos por tamaños.

The on-growing of adult octopuses caught in nature and selected to establish a homogeneous group, i.e. with similar individual initial weights, did not produced any clear benefits in comparison with a typical rearing-cycle group, i.e. octopuses with a wide range of initial weights (> 500 g, heterogeneous group). The slightly higher survival rate and the sligh higher biomass gain observed in the homogeneous group, in comparison with the heterogenous group, probably would not compensate economically the efforts to establish homogeneous groups.

-10.

El engorde de pulpos con separación de sexos en la época alta de engorde (verano), tampoco originó diferencias claras de crecimiento y supervivencia frente a grupos de engorde típicos. La mortalidad en el grupo constituido sólo por machos no fue diferente de los valores observados en los grupos mixtos o de sólo hembras. Dados los resultados dispares que se barajan por diversos autores en el engorde con separación de sexos, sería necesario el diseño de experimentos que traten de encontrar las causas que puedan explicar los diferentes comportamientos observados.

Similarly, the on-growing of octopuses separated by sexes in the high rearing-season (summer) did not produced any improvements in comparison with typical rearing groups, i.e. random mix of octopuses. The mortality rate observed for the group established only with male octopuses was not different from values found for the other groups. Since controversial information exists concerning this issue, proper rearing experiments should be designed in order to clarify the different results observed until now.

Resumen

El pulpo común (*Octopus vulgaris* Cuvier, 1797) es una especie de gran interés para la acuicultura, debido a su elevada demanda en varios países de Asia y Europa, y a su elevado valor comercial (2-8 euros kg^{-1}). Presenta, además, algunas características biológicas muy interesantes que lo convierten en un serio candidato para la diversificación en acuicultura: ciclo de vida corto (1-2 años), altas tasas de crecimiento (del 1,0 al 11,5% peso corporal dia^{-1} a lo largo de toda su vida), elevada tasa de conversión alimentaria (30-60%), fácil adaptación y comportamiento reproductivo en cautividad, elevada fertilidad (100.000-500.000 huevos $hembra^{-1}$), aceptación de alimentos de bajo valor comercial, y prácticamente ausencia de patologías.

En los últimos 15 años, se han realizado numerosos trabajos de engorde de pulpo, en varios países de Europa con fuerte tradición en su consumo, con el fin de evaluar su potencial para la acuicultura. En España, un ciclo de engorde típico comprende la captura de individuos adultos en el medio natural, con un peso mínimo permitido por ley (750-1000 g), seguido de su distribución en jaulas flotantes o suspendidas de bateas, donde permanecerán de 3 a 4 meses, siendo alimentados hasta que alcancen pesos medios de alrededor de 2,5 -3,0 kg.

A pesar del enorme potencial de *O. vulgaris* para la acuicultura, existe todavía un importante cuello de botella en su cultivo: la obtención de juveniles bentónicos resultantes del cultivo de las paralarvas planctónicas. Hasta hoy día, han sido muy pocos los grupos de investigadores que han logrado cultivar paralarvas hasta la fase bentónica, ya que en general se observan mortalidades casi totales de éstas a las pocas semanas de vida. En los trabajos que fueron exitosos, la dieta consistió de zoeas de diferentes crustáceos (Itami *et al*., 1963; Villanueva, 1994, 1995), o de *Artemia* (1-4 mm) enriquecida con microalgas, complementada con zoeas de centolla (*Maja brachydactyla*) (Iglesias *et al*., 2004; Carrasco *et al*., 2006). El cultivo de paralarvas hasta la fase bentónica con una dieta exclusiva de *Artemia* sólo ha sido descrito por Hamazaki *et al*. (1991), a través del enriquecimiento de la *Artemia* (1,5-2 mm) con *Nannochloropsis* sp. y estableciendo condiciones de aguas verdes con esta misma microlga. Aunque las zoeas se consideren presas más adecuadas que la *Artemia* para el cultivo de paralarvas, debido a su mejor composición bioquímica, el continuo aporte de zoeas resulta inviable más allá de la escala experimental. Por ello, la mejora de la composición bioquímica de *Artemia*, la búsqueda de presas alternativas, y la formulación de microdietas inertes, han sido señalados como prioritarios para solucionar el problema del cultivo larvario del pulpo (Iglesias *et al*., 2007a).

En cuanto a la mejora de la composición del alimento vivo, se ha demostrado que el cultivo de microalgas en régimen continuo representa una poderosa herramienta a la hora de controlar y modular su composición nutricional (Scott, 1980; Taub, 1980; Otero and Fábregas, 1997; Otero *et al*., 2002), de forma que su utilización para la alimentación y enriquecimiento de *Artemia* sp. conlleva mejoras significativas en el crecimiento, la supervivencia y en la composición bioquímica de los individuos (Fábregas *et al*., 1996b, Fábregas *et al*., 2001), pudiendo ser aplicadas en la mejora de este alimento para las paralarvas de pulpo.

Los principales objetivos del presente trabajo fueron:
1) La mejora del crecimiento y la supervivencia de paralarvas de pulpo (*Octopus vulgaris*) utilizando distintas dietas, que fueron moduladas o formuladas teniendo en cuenta la composición bioquímica de estadios iniciales de *O. vulgaris*, y que consistieron en: a) juveniles de *Artemia* enriquecidos con microalgas de composición optimizada y controlada, o con otros suplementos nutricionales tales como emulsiones lipídicas comerciales, compuestos purificados, etc.; b) microdietas artificiales formuladas específicamente para las paralarvas de pulpo.
2) La evaluación de nuevas estrategias para mejorar el cultivo de pulpo en jaulas flotantes a escala industrial.

En el primer capítulo se analizó la composición bioquímica (proteína total, lípidos totales, carbohidratos y perfil de ácidos grasos) de juveniles de *Artemia* de dos tamaños diferentes (1,5-2,0 mm y 3,0-3,5 mm), adecuados para las paralarvas de *O. vulgaris*, enriquecidos con cuatro microalgas diferentes: *Tetraselmis suecica*, *Isochrysis galbana*, *Isochrysis* aff. *galbana* (T-ISO) y *Rhodomonas lens*, con el fin de evaluar su valor nutricional en relación con los posibles requerimientos de las paralarvas. Las microalgas fueron cultivadas en régimen semicontinuo, en saturación de nutrientes y con una tasa de renovación diaria del 30% del volumen total, con el fin de obtener biomasa de composición estable y optimizada. Se analizó igualmente la composición en ácidos grasos (AGs) de paralarvas de pulpo (*O. vulgaris*) recién eclosionadas, y de zoeas de centolla (*Maja brachydactyla*), ya que esta presa fue descrita como adecuada para cultivar paralarvas de pulpo, con el fin de establecer comparaciones con los perfiles de AGs de los juveniles de *Artemia* enriquecidos. El contenido de proteínas en *R. lens* (62 % del peso seco) fue más elevado que en las restantes microalgas (42-44%, $P<0,001$), mientras que el mayor contenido de

lípidos y de carbohidratos se observó en las especies T-ISO e *I. galbana* (20-21% y 17-19%, respectivamente) (P <0,05). Los juveniles de *Artemia* de menor tamaño (1,5-2,0 mm) enriquecidos con las distintas microalgas presentaron un contenido en proteínas de alrededor del 51%, excepto los del grupo enriquecido con *I. galbana* (AISO), que presentaron un contenido inferior (41%, P <0,01). En estos juveniles, los porcentajes de lípidos más elevados se observaron en los enriquecimientos con T-ISO (grupo AT-ISO) o con *R. lens* (ARHO), ambos con alrededor del 16% (P<0,05), mientras que los niveles más altos de carbohidratos se encontraron en los juveniles de los grupos AISO y AT-ISO (11%, P<0,05). Los juveniles de *Artemia* de mayor tamaño (3,0-3,5 mm) presentaron niveles de proteína más altos (64-68%) que los juveniles pequeños. La fracción lipídica de los juveniles aumentó del siguiente modo: ARHO (10%) < ATET = AT-ISO (16%) < AISO (18%). El porcentaje más bajo de carbohidratos se observó en el grupo ARHO (6%, P<0,01). La relación proteína/energía fue máxima en los juveniles de *Artemia* de gran tamaño del grupo ARHO (P/E=31,7). Los análisis de AGs revelaron que en los juveniles pequeños, el mayor porcentaje del ácido eicosapentaenóico (EPA, 20:5n-3) se encontró en los grupos AISO y ARHO (9% del total de AGs), mientras que en los juveniles de gran tamaño el valor máximo se halló en el grupo AISO (14,6%, P<0,05). En cuanto al ácido graso docosahexaenóico (DHA, 22:6n-3), los valores más elevados en los juveniles de pequeño tamaño se encontraron en los grupos AT-ISO y AISO (1,9% y 1,5%, respectivamente), seguido del grupo ARHO (1%, P<0,05), mientras que en los juveniles grandes el mayor valor se observó en el grupo AT-ISO (3,9%, P<0,05). Los juveniles de *Artemia* enriquecidos con *T. suecica* no presentaron DHA en su composición. El perfil de AGs de las paralarvas de pulpo recién eclosionadas reveló niveles de DHA (19,7% del total de AGs) y de ácido araquidónico (ARA, 20:4n-6) (3,4%), muy superiores a los observados en los juveniles de *Artemia*, y, en general, también de EPA (14,7%). En las zoeas de centolla (*M. brachydactyla*) los niveles encontrados fueron de 8,7% (DHA), 7,8% (ARA) y 24,3% (EPA). Estos resultados sugieren la existencia de un déficit de ácidos grasos poliinsaturados (PUFAs) en los juveniles de *Artemia*. Los juveniles enriquecidos con *R. lens* presentaron la mejor composición bioquímica, en relación a los posibles requerimientos nutricionales de las paralarvas de *O. vulgaris*, debido a la mayor relación proteína/energía y al perfil de PUFAs. Sin embargo, el mayor contenido de EPA más DHA se encontró en los juveniles enriquecidos con *I. galbana*. Por ello, en los trabajos de cultivo de paralarvas, se optó por enriquecer los juveniles de *Artemia* con una mezcla de *R. lens* e *I. galbana*, en la proporción de 70%:30% (peso seco). En los diversos experimentos de

cultivo de paralarvas de pulpo se observó una alta reproducibilidad de la composición bioquímica de los juveniles de *Artemia*, derivada de la utilización de cultivos contínuos de microlgas.

En el capítulo dos se analizó el efecto de tres dietas distintas sobre la supervivencia, el crecimiento y la composición en ácidos grasos de paralarvas de *O. vulgaris*. El grupo ADHA recibió juveniles de *Artemia* enriquecidos con una emulsión comercial rica en DHA (DHA-Selco®, INVE), mientras que el grupo AR+I recibió juveniles enriquecidos con la mencionada mezcla de microalgas, cultivadas en régimen semicontinuo. El grupo P+AR+I fue alimentado con la misma dieta del grupo AR+I, complementada con pellets de alrededor de 1 mm. La supervivencia de las paralarvas al cabo de 15 días de cultivo tendió a ser superior en los grupos AR+I (19±8%) y P+AR+I (17±4%), en comparación con el grupo ADHA (13±5%), a pesar de que estadísticamente no hubo diferencias significativas. El incremento de peso seco (PS) de las paralarvas a los 15 días de cultivo fue de casi el 60% en los grupos AR+I y P+AR+I, y de alrededor del 40% en el grupo ADHA. A los 10 días de cultivo, ya existían diferencias significativas en el PS de las paralarvas, siendo superiores en los individuos de los grupos AR+I y P+AR+I, frente a los del grupo ADHA ($P<0,05$). A pesar de observarse esta misma tendencia a los 15 días de cultivo, sólo se han encontrado diferencias significativas entre el PS de las paralarvas de los grupos P+AR+I y ADHA ($P<0,05$). Los resultados del tamaño total (TT) y del manto (TM) siguieron la misma tendencia del PS, siendo mayores en las paralarvas de los grupos AR+I y P+AR+I, que en las del grupo ADHA ($P<0,05$). Los análisis de AGs revelaron una disminución acentuada del DHA en las paralarvas de todos los grupos ($P<0,05$), frente a los valores encontrados en paralarvas recién eclosionadas (19,2% del total de AG). Sin embargo, las paralarvas del grupo ADHA presentaron un contenido de DHA (12,5%) superior al observado en las de los grupos AR+I y P+AR+I (ambas con niveles de alrededor del 10%, $P<0,05$). En cuanto al EPA, mientras que los niveles se mantuvieron estables en las paralarvas del grupo ADHA (14,5%), en comparación con las recién eclosionadas (14,3%), en las de los grupos AR+I y P+AR+I el EPA se incrementó (16,1% y 17,2%, respectivamente) ($P<0,05$). Aunque los juveniles de *Artemia* enriquecidos con DHA-Selco® tenían un contenido de DHA tres veces superior al obtenido en el enriquecimiento con la mezcla de *R. lens* e *I. galbana*, no se observó ningún beneficio directo en la supervivencia y crecimiento de las paralarvas. Los resultados encontrados en este trabajo sugieren, además, que la relación proteína:lipido (P:L) de la dieta se relaciona con el crecimiento de las paralarvas.

Debido a los resultados obtenidos en el capítulo anterior, en el capítulo tres se estudió el crecimiento de las paralarvas con una dieta que consistió en juveniles de *Artemia* (en una proporción de 7:3) enriquecidos con *R. lens* e *I. galbana* o con DHA-Selco®, respectivamente. Suministrando la misma dieta a paralarvas de pulpo, se analizó el efecto de las aguas verdes, frente a las aguas claras, en su cultivo. Se seleccionó la microalga *Nannochloropsis gaditana*, ya que otros autores habían descrito buenos resultados al establecer aguas verdes con especies del género *Nannochloropsis*, en experimentos a gran escala (Hamazaki *et al*., 1991; Moxica *et al*., 2006). Así, se establecieron dos grupos de paralarvas, en tanques de 50 l de volumen, añadiendo al de las aguas verdes *N. gaditana* a una concentración de $200x10^3$ cel. ml^{-1}. Además, se analizó la composición bioquímica de juveniles de *Artemia* enriquecidos con *N. gaditana*, con el fin de evaluar qué propiedades nutricionales podrían estar detrás de los buenos resultados descritos por aquellos autores. Se observó una mortalidad masiva súbita de las paralarvas del grupo de las aguas verdes entre los días 9 y 11 de cultivo, aunque éstas demostrasen los días anteriores muy buena supervivencia, comportamiento saludable y captura activa de presas. La supervivencia de las paralarvas en aguas claras fue del 38%, al cabo de 15 días de cultivo, disminuyendo hasta el 3% a los 25 días. El peso seco (PS) de las paralarvas recién eclosionadas fue de 330±20 μg $paralarva^{-1}$, alcanzando las paralarvas del grupo de aguas claras un PS de 840±150 μg $paralarva^{-1}$ a los 25 días de cultivo. Los resultados de la composición bioquímica de los juveniles de *Artemia* enriquecidos con *N. gaditana* revelaron niveles de proteína (49-66% del PS), de lípidos (10-12%) y de carbohidratos (8-10%), similares a los valores encontrados en los juveniles de pequeño y gran tamaño (citados en el capítulo uno) enriquecidos con otras monodietas microalgales. El perfil de AG de los juveniles de *Artemia* demostró altos niveles de EPA (14-15% del total de AG), ausencia de DHA, y un contenido de ARA (≈ 2%), bastante superior al observado en los juveniles enriquecidos con las otras microalgas (≤0,4%), por lo que sería interesante averiguar la posible influencia positiva de la relación EPA/ARA en la dieta de las paralarvas.

En el capítulo cuatro, con el fin de seguir mejorando la composición de *Artemia* y de suministrar nutrientes esenciales a las paralarvas, se probaron diferentes combinaciones de juveniles de *Artemia* (1,6-2,8 mm) enriquecidos con distintas dietas. La primera toma de alimento fue igual para todos los grupos, y consistió de juveniles de *Artemia* enriquecidos con la mezcla de *R. lens* e *I. galbana*, mientras que en la segunda toma del día, las paralarvas se alimentaron con juveniles enriquecidos durante 6 h con una de las siguientes dietas: *R. lens* e *I. galbana* (grupo control - AR+I); un producto comercial lipídico muy

rico en DHA (Ori-Gold®, Skretting) - grupo AGOLD; un triturado de pellets para rodaballo (Sorgal) rico en proteína y sin aceite de pescado añadido, pero suplementado con Ori-Gold® al 10% (peso húmedo) para incrementar el nivel de DHA - grupo AGOPEL. El contenido de DHA en los juveniles de *Artemia* (% del total de AG) se incrementó en el orden: AR+I (1,6%) < AGOPEL (5,7%) < AGOLD (8%), mientras que la relación P:L disminuyó en el sentido: AR+I (5,4) > AGOPEL (4,5) > AGOLD (3,9). La supervivencia de las paralarvas varió entre el 35 y el 53% a los 15 días de cultivo, y entre el 7 y el 20% a los 25 días, observándose los valores más altos en el grupo AR+I, pero sin que existiesen diferencias estadísticamente significativas entre los grupos. El PS y el TT de las paralarvas de los grupos AR+I y AGOPEL fue superior al de las paralarvas del grupo AGOLD, tanto a día 15 como a día 25 de cultivo, siendo máximos en el grupo AR+I. Se ha encontrado una relación lineal, positiva y significativa ($P<0,01$), entre el aumento del cociente P:L en la dieta y el PS de las paralarvas, tanto a día 15 como a 25. En contraste, no se observó ninguna relación entre los niveles de EPA o DHA de la dieta, y cualquiera de los parámetros de crecimiento de las paralarvas. En relación a la composición bioquímica de las paralarvas, los niveles de proteína fueron iguales en todos los grupos (64-66%), mientras que el contenido de lípidos en las paralarvas del grupo AGOLD (11,8%) fue superior al de los demás individuos (10,7-10,9%, $P<0,05$), a día 25 de cultivo, lo que pudo estar relacionado con el contenido lipídico de las respectivas dietas. La composición de AG de las paralarvas reveló un fuerte descenso de DHA en todos los grupos, frente a los valores iníciales (20%), aunque en las de los grupos AGOLD y AGOPEL los valores de DHA fueron superiores (≈10%) al observado en las paralarvas del grupo AR+I (7%, $P<0,05$). La dieta AR+I, a pesar de su bajo contenido en DHA, produjo mayor supervivencia y mejor crecimiento de las paralarvas, y ha sido la única que permitió que se alcanzasen paralarvas con 35 días de vida. Estos resultados demostraron que la relación P:L en la dieta, influyó más sobre el crecimiento y la supervivencia de las paralarvas que el contenido del DHA.

Teniendo en cuenta que el perfil de AGs no fue tan determinante para el crecimiento de las paralarvas, se ha abordado otro aspecto de la composición de las presas: su contenido proteico y relación P:L, y el perfil de aminoácidos (AA). Los análisis de la composición de AA totales de juveniles de *Artemia* (3,0-3,5 mm) enriquecidos con distintas microalgas (*T. suecica*, *I. galbana*, *I. galbana* (T-ISO) y *R. lens*), demostraron que el contenido de lisina en los juveniles de *Artemia* estaban por debajo de los valores encontrados en las paralarvas

descritos con anterioridad por Villanueva *et al*. (2004). Además, comparando el perfil de AA de los juveniles de *Artemia* con otros datos de AA totales de juveniles y adultos de pulpos salvajes, los AA arginina y metionina también podrían ser deficitarios. Por ello, en el capítulo cinco, se establecieron tres grupos de paralarvas que fueron alimentadas con juveniles de *Artemia* (1,6-2,8 mm) enriquecidos con las siguientes dietas: *R. lens* e *I. galbana* (AR+I); la misma mezcla de microalgas y además con una solución de AA libres en el agua, en las siguientes concentraciones (3,4 mM de lisina, 2,5 mM de arginina, 1,5 mM de metionina) - grupo AR+I+AA; o con *N. gaditana* (ANANO). La composición bioquímica de los juveniles de *Artemia* fue muy similar y varió entre el 65-66% de proteína, y el 10-11% para lípidos y carbohidratos. La supervivencia de las paralarvas varió entre el 51 y el 62% a día 15 de cultivo en todos los grupos. A día 25, los grupos AR+I+AA y AR+I presentaron mayores supervivencias (7-8%, $P<0,05$) y PS (870±81 µg paralarva^{-1} y 834±88 µg paralarva^{-1}, respectivamente), que el grupo ANANO (2% supervivencia y 798±99 µg paralarva^{-1} de PS), aunque estadísticamente sólo se encontraron diferencias de PS entre los grupos AR+I+AA y ANANO ($P<0,05$). El enriquecimiento de juveniles de *Artemia* con AA esenciales disueltos en el agua tuvo efectos positivos sobre el PS de las paralarvas, por lo que sería interesante profundizar en esta nueva línea de investigación.

En el capítulo seis, con el fin de evaluar nuevas estrategias para optimizar el proceso de engorde del pulpo a escala industrial, se realizaron dos experimentos en jaulas de engorde suspendidas de una batea, en la ría de Vigo, que consistieron en: a) experimento 1 – engorde de pulpos con separación por tamaños. Se constituyó un grupo grupo homogéneo (HOM), cuya diferencia en el peso inicial (Pi) de los individuos no sobrepasaba los 100 g, y un grupo heterogéneo (HET), que consistió de ejemplares cuya diferencia en el Pi de los individuos sobrepasaba los 500 g (que es lo habitual en el sistema de cultivo industrial); b) experimento 2 – engorde de pulpos clasificados por sexos: grupo MACHOS, constituido por ejemplares sólo machos con Pi de 953±146 g; grupo HEMBRAS, formado por sólo hembras con Pi de 899±135 g; y grupo MIXTO, formado por machos y hembras con Pi de 899±137 g. En el primer experimento, la supervivencia apenas varió entre los grupos, siendo del 70,0% en el grupo HOM y del 68,7% en el grupo HET. El peso final (Pf) de los ejemplares en el grupo HET (2188±574 g) fue superior al del grupo HOM (2091±499 g), mientras que la biomasa final del grupo HOM fue mayor debido a la diferencia en la supervivencia. El engorde de pulpos obtenidos del medio natural y seleccionados para

formar un grupo homogéneo no supuso una clara ventaja frente a un ciclo de engorde típico, con individuos de peso heterogéneo. La ligera mejora en la supervivencia del grupo homogéneo, en comparación con el grupo heterogéneo, y las pequeñas diferencias observadas en cuanto a biomasa total alcanzada, posiblemente no compensarían el trabajo necesario para la selección por tamaños. En el segundo experimento, la supervivencia de los grupos MACHOS y MIXTO (ambos con 86,7%) fue superior a la del grupo HEMBRAS (73,3%). El Pf de los individuos del grupo MACHOS (2261±428 g) fue superior al Pf observado en los grupos HEMBRAS (2015±428 g) y MIXTO (2125±450 g), aunque el Pi de los pulpos del grupo MACHOS también había sido superior. Los resultados demostraron que la mortalidad en el grupo MACHOS no fue diferente de la del grupo MIXTO. Sería necesario el diseño de experimentos que tratasen de encontrar las causas que puedan explicar los diferentes comportamientos observados, por lo que en el futuro, sería interesante establecer grupos clasificados por tamaños "a simple vista", o sea, con rangos de peso más amplios (ej.: 250 g). Con estas medidas se podría mejorar la gestión del cultivo y obtener una menor dispersión en el tamaño de los pulpos de cada jaula.

Abstract

The common octopus (*Octopus vulgaris* Cuvier, 1797) is a species of great interest for aquaculture due to its high demand in Asian and European countries and to its high market price (2-8 euros kg^{-1}). *O. vulgaris* also presents interesting biological characteristics to be considered a serious candidate for aquaculture diversification, such as: a short life cycle (1-2 years), growth rates of 1.0-11.5% body weight day^{-1} through its whole life cycle, high food conversion rates (30-60%), easy reproduction behaviour and high fecundity (100.000-500.000 eggs $female^{-1}$), easy adaptation to captivity conditions, acceptance of low value natural foods and almost no diseases.

In the last 15 years several experiments of octopus rearing have been carried out in European countries with strong tradition in its consumption (Spain, Italy, Portugal and Greece), with the aim of evaluating the potential of this species for aquaculture. In Spain, a typical rearing (or fattening) cycle consists in the capture of adult octopuses in nature, with the minimal legal size (750-1000 g), followed by their distribution into individual floating cages or suspended from rafts. This cycle usually lasts from 3 to 4 months, after which octopuses attain an average weight of 2.5 to 3.0 kg.

Although *O. vulgaris* shows a huge potential to be considered a candidate for aquaculture, there is still a major bottleneck to be solved: the rearing of its early life stage, termed paralarvae, until they settle in the bottom becoming benthic juveniles. So far, few researchers have succeeded in rearing paralarvae until the benthic stage. Zoeae of different crustacean species supplied as single prey (Itami *et al*., 1963; Villanueva, 1994, 1995); or *Artemia* (1-4 mm) enriched with microalgae complemented with spider-crab (*Maja brachydactyla*) zoeae in moments of its availability (Iglesias *et al*., 2004; Carrasco *et al*., 2006) were used as food items. Only one group from Japan has reported the achievement of benthic octopuses using *Artemia* (1.5-2 mm) as sole prey (Hamazaki *et al*., 1991), through enrichment of *Artemia* juveniles with *Nannochloropsis* sp. and establishing gree-waters with this same microalga. Yet, the constant supply of decapod zoeae to feed paralarvae would hardly be realistic beyond the experimental scale. Therefore, the improvement of the biochemical composition of *Artemia*, the search for alternative live prey, and development of microdiets have all been pointed out as key issues to solve the problem of octopus paralarvae rearing (Iglesias *et al*., 2007a).

Regarding the improvement of the live prey, it has been shown that the cultura of microalgae in continuos regimen represents a powerful tool to modulate and control their

biochemical composition (Scott, 1980; Taub, 1980; Otero and Fábregas, 1997; Otero *et al*., 2002). Moreover, it has been shown that the growth, survival and the biochemical composition of *Artemia* sp. could be markedly improved through the use of microalgae cultured in continuos cultures (Fábregas *et al*., 1996b, Fábregas *et al*., 2001), which can be used to improve and modulate the composition of *Artemia* juveniles for the first feeding of *O. vulgaris* paralarvae.

The main objectives of this work were:

1) The improvement of *Octopus vulgaris* paralarvae growth and survival rates through the use of different dietary regimes (live prey or complemented with microdiets), which were modulated or formulated taking into consideration the biochemical composition of *O. vulgaris* early life stages, consisting of: a) *Artemia* juveniles enriched with either microalgae of optimal and controlled composition, or other nutrient supplements such as commercial lipid emulsions, purified compounds, etc.; or b) artificial pellets formulated specifically for paralarvae.

2) The evaluation of new strategies to improve the rearing conditions of adult octopuses at an industrial scale in floating cages.

In chapter one the gross composition and the total fatty acid (FA) profile of *Artemia* juveniles of two different sizes (1.5–2.0 mm and 3.0–3.5 mm), appropriate to feed *O. vulgaris* paralarvae, enriched with four different marine microalgal species (*Tetraselmis suecica*, *Isochrysis galbana*, *Isochrysis* aff. *galbana* (T-ISO) and *Rhodomonas lens*) was assessed in order to evaluate their nutritional value for octopus paralarvae. Microalgae were cultured semi-continuously in nutrient saturated conditions and with a daily renewal rate of 30% of the volume of cultures, in order to achieve biomass of constant and optimal biochemical composition. The FA composition of newly hatched *O. vulgaris* paralarvae and of wild *Maja brachydactyla* zoeae, a prey that has been described as suitable to rear paralarvae, were also analysed with the aim of establishing comparisons of FA profiles. The total amino acid (AA) composition of big *Artemia* juveniles (3.0-3.5 mm) was also analyzed and compared with data previously published by other authors concerning the total AA composition of octopus hatchlings. The protein content of *R. lens* (62% of dry weight) was considerably higher than that of the remaining microalgae (42-44%, $P<0.001$),

whereas lipid and carbohydrate were significantly higher in both T-ISO and *I. galbana* (20-21% and 17-19%, respectively) (P<0.05). Small juvenile *Artemia* (1.5–2.0 mm) contained nearly 51% protein regardless the enrichment diet used, with the exception of individuals enriched with *I. galbana* (group AISO) which contained a lower protein content (41%, P<0.01). In these juveniles, lipid percentages were higher when enriched with T-ISO (AT-ISO) or with *R. lens* (ARHO), both with circa 16% (P<0.05); whereas carbohydrate was higher in juveniles from groups AISO or AT-ISO (11%, P<0.05). Large juvenile *Artemia* (3.0–3.5 mm) contained higher protein levels than small juveniles with values ranging between 64 and 68% for all treatments, whereas the lipid fraction among groups increased in the order: ARHO (10%) < ATET = AT-ISO (16%) < AISO (18%) (P<0.05). The lowest percentage of carbohydrate was found in group ARHO (6%, P<0.01). Maximum protein/energy ratio was observed in 5-day old juveniles from group ARHO (P/E ratio=31). The highest percentage (% total FA) of eicosapentaenoic acid (EPA, 20:5n-3) in small juvenile *Artemia* was found in individuals from groups AISO or ARHO (circa 9%), whereas in 5-day old juveniles the highest value was found in group AISO (14.6%, P<0.05). Regarding docosahexaenoic acid (DHA, 22:6n-3), small juveniles from groups AT-ISO or AISO had higher values (1.9 and 1.5%, respectively) than juveniles from group ARHO (1.0%, P<0.05), whereas in 5-day old *Artemia* maximum percentage of DHA was found in group AT-ISO (3.9%, P<0.05). DHA was absent in *Artemia* juveniles enriched with *T. suecica*. The FA composition of *O. vulgaris* paralarvae revealed much higher percentages of DHA (19.7%) and arachidonic acid (ARA, 20:4n-6) (3.4%), and in general also of EPA (14.7%) than values found in *Artemia* juveniles. In *M. brachydactyla* zoeae, the percentages of those FA were, in the same order: 8.7%, 7.8% and 24.3%. These results suggest that *Artemia* may have a deficit of highly unsaturated fatty acids (HUFA) to cover paralarvae needs. In this study we found that lysine could be a limiting AA in *Artemia* juveniles, but this observation needs further studies based in more replicate analysis and appropriate experimental trials of octopus paralarvae, to evaluate the possible effects of lysine supplementation. If the general composition of *Artemia* juveniles (gross composition and FA profiles of both *Artemia* sizes) is taken into consideration, the enrichment with *R. lens* provided the best results among groups, though the highest sum of EPA and DHA was found in *Artemia* juveniles enriched with *I. galbana*.

In chapter two, we examined the effects of three dietary treatments on the survival and growth rates of *O. vulgaris* paralarvae, as well as on its fatty acid composition. Group ADHA was fed juvenile *Artemia* enriched with a commercial lipid emulsion (DHA-

Selco®) rich in docosahexaenoic acid (DHA, 22:6n-3); group AR+I was fed juvenile *Artemia* enriched with a mixed diet of microalgae (70%:30% of *Rhodomonas lens* and *Isochrysis galbana*, respectively, in a dry weight basis) produced semi-continuously to achieve biomass of optimal and controlled composition; and group P+AR+I received the same *Artemia* as group AR+I complemented with artificial pellets. The survival rates of 15-days post hatch (-dph) paralarvae from groups AR+I (19±8%) and P+AR+I (17±4%) tended to be higher than in group ADHA (13±5%), though these differences were not significantly different. The increase in the dry weight (DW) from hatchlings to 15-dph paralarvae was almost 60% in groups AR+I and P+AR+I and nearly 40% in group ADHA. At day 10, significant differences in the DW of paralarvae could already be noticed, with individuals from groups AR+I and P+AR+I showing higher values than paralarvae from ADHA ($P<0.05$). However, significant differences in the DW of 15-dph paralarvae were only found between groups P+AR+I and ADHA, being higher in group P+AR+I ($P<0.05$), despite the trend of higher DW also observed in paralarvae from AR+I. Regarding the total length (TL) and mantle length (ML) of paralarvae, higher values of TL and ML were found in 10-dph and 15-dph paralarvae from groups AR+I and P+AR+I, in comparison with individuals from ADHA ($P<0.05$). Analysis of the fatty acid (FA) composition of paralarvae showed a remarkable drop of DHA from hatchlings (19.2% of total FA) to 15-dph paralarvae in all groups ($P<0.05$). However, paralarvae from group ADHA contained higher levels of DHA (12.5%) than paralarvae from groups AR+I and P+AR+I (both with circa 10%, $P<0.05$). As for eicosapentaenoic acid (EPA, 20:5n-3), the percentage found in hatchlings (14.3% of total FA) did not changed with time in paralarvae from group ADHA (14.5% in 15-dph paralarvae), but increased significantly in paralarvae from groups AR+I and P+AR+I (16.1% and 17.2%, respectively) ($P<0.05$). Even though paralarvae from group ADHA displayed a FA composition more closely related with the values found in hatchlings, the growth of these individuals was worst than paralarvae from groups AR+I and P+AR+I. Moreover, despite *Artemia* enriched with DHA-Selco® contained three-times more DHA than *Artemia* enriched with microalgae, we did not observe any clear positive effects over the growth and survival of paralarvae. Results also suggest that the protein:lipid ratio of the diet is an important factor to improve the growth of *O. vulgaris* paralarvae, but efforts to obtain an optimal balance of FA in *Artemia* should continue with the aim of avoiding such FA changes in octopus paralarvae.

In chapter three the effects of green water conditions was tested in octopus paralarvae rearing. The species *Nannochloropsis gaditana* was used since a species of the same

microalgal genera was previously used at large scale by other authors with good results (Hamazaki *et al*., 1991; Moxica *et al*., 2006). We also analysed the gross biochemical composition and the FA profile of *Artemia* juveniles (1.6 - 2.3 mm) enriched with *N. gaditana,* in order to address its nutritional composition and evaluate which properties may be behind the positive effects that this microalgal genera produces in paralarvae rearing. Two groups of paralarvae were set, each in triplicate: one group was maintained in clear water conditions, whereas the other was established in green waters conditions, adding *N. gaditana* to tanks at an initial concentration of $200x10^3$ cells ml^{-1}. The diet consisted of a combination of *Artemia* juveniles enriched with either a mixed diet of microalgae (*Rhodomonas lens* and *Isochrysis galbana*, in a proportion of 70%:30% dry weight basis), or with DHA-Selco® (INVE). In this work the gross biochemical composition and the fatty acid profiles of *N. gaditana* and of two sizes of *Artemia* juveniles (1.6 mm and 2.3 mm) enriched with *N. gaditana* were also addressed, as few data about their composition was previously reported, to evaluate which properties may be behind the positive effects that this genera of microalga produces in paralarvae rearing. In clear water tanks survival rate at day 15 was 38%, decreasing to nearly 3% at day 25. Unexpectedly and due to unknown reasons, a sudden mass mortality of paralarvae was observed in the green water tanks between days 9 and 11, even if paralarvae displayed healthy behaviour, good survival and normal capture of prey in the previous days. The initial dry weight (DW) of hatchlings was 330±20 µg $paralarva^{-1}$, whereas 25-day post-hatch paralarvae maintained in clear waters attained a DW of 840±150 µg $paralarva^{-1}$. In this study the utilization of green water conditions in low-volume tanks (50-l) in closed systems without re-circulation was not successful, but further experiments using green water conditions with this microalga, or with other microalgal species, should be carried out in order to elucidate if environmental conditions (light diffusion in tanks, gut flora enhancement, anti-bacterial effects on water) and/or nutritional issues promote higher growth and survival of paralarvae. Regarding the biochemical composition of the small *Artemia* juveniles enriched with *N. gaditana* (≈ 1.6 mm), protein was found to represent 49% of its DW, whereas in big size juveniles (≈ 2.3 mm) protein accounted for 65% of DW. Lipid levels ranged between 10 and 12% in both *Artemia* juveniles, whereas carbohydrates ranged between 8 and 10%. The major fatty acids (FAs) found in *N. gaditana* were the saturated palmitic acid (16:0) and eicosapentaenoic acid (EPA, 20:5n-3), each accounting 25% of the total FA. Arachidonic acid (ARA, 20:4n-6) represented 4.4% of total FAs, whereas docosahexaenoic acid (DHA, 22:6n-3) was not found in this microalga. EPA and ARA levels found in both sizes of

Artemia juveniles represented 14-15% and 2%, respectively, of the total FAs. As observed for *N. gaditana*, no DHA was found in *Artemia* juveniles. The major differences between *Artemia* juveniles enriched with *N. gaditana* or enriched with *R. lens* and *I. galbana* were related with the lower content of ARA in this last group (0.4% of total FAs).

In chapter four, paralarvae were fed on different combinations of enriched *Artemia* juveniles (1.6-2.8 mm), with the aim of analysing the effects of the diet on the growth, survival and biochemical composition of paralarvae. Food was supplied twice a day, with the first meal being common to all groups and consisting of 3-day old *Artemia* enriched with a mixed diet of microalgae (*Rhodomonas lens* and *Isochrysis galbana* in a proportion of 70%:30% dry weight basis). In the second meal paralarvae received *Artemia* enriched as follows: group control (AR+I) was given 3-day old juveniles enriched with the same microalgae for another 6 h; group AGOLD was given juveniles enriched for 6 h with Ori-Gold®; and group AGOPEL received *Artemia* enriched for 6 h with a manual prepared diet consisting of grinded pellets for turbot supplemented with 10% of Ori-Gold® (wet weight basis). The percentage of docosahexaenoic acid (DHA, 22:6n-3) in *Artemia* (% of total fatty acids) increased as follows: AR+I (1.6%) < AGOPEL (5.7%) < AGOLD (8.0%) ($P<0.05$); whereas the protein:lipid (P:L) ratios of *Artemia* juveniles decreased in the sense: AR+I (5.4) > AGOPEL (4.5) > AGOLD (3.9). Survival of paralarvae ranged between 35 and 53% at the end of 15 days, and between 7 and 20% at day 25, but no significant differences were found among groups. The dry weight (DW) and total length (TL) of 15-day post-hatch (dph) and 25-dph paralarvae from groups AR+I and AGOPEL was higher than values found for paralarvae from group AGOLD ($P<0.05$). A positive linear correlation was found between dietary P:L ratio and paralarval DW for both 15-dph and 25-dph paralarvae ($P<0.01$), while no correlation could be established between EPA or DHA with any of the growth parameters. Regarding the biochemical composition data, octopus hatchlings contained nearly 68% protein (% of DW), which decreased slightly to 64-66% in 15-dph and 25-dph paralarvae from all groups. Despite no significant differences were found in the lipid content of 15-dph paralarvae, in 25-dph individuals from group AGOLD the lipid content (11.8%) was higher than in paralarvae from the remaining groups (10.7-10.9%, $P<0.05$). As for the fatty acid (FA) composition, a remarkable drop of DHA was found from hatchlings (20%) to paralarvae from all groups over time, though individuals from groups AGOLD and AGOPEL contained significantly higher levels of DHA than paralarvae from AR+I ($P<0.05$). Despite this observation, paralarvae from AR+I showed the highest DW and TL, and this group was the only one to

attain 35-dph paralarvae. In contrast, eicosapentaenoic acid (EPA) increased from an initial value of 13% in hatchlings to 20-22% in 25-dph paralarvae from all groups. These results suggest that the P:L ratio of *Artemia* is rather more important to sustain a good performance of paralarvae than *Artemia* FA composition per se.

In chapter 5, the effects of boosting the content of essential AA (EAA) in *Artemia* juveniles on the growth and survival of paralarvae was analyzed. It is believed that the lipid composition of zoeae is more suitable to meet the requirements of octopus paralarvae, but additional components other than lipids might be in the origin of this "high" quality prey, such as the AA composition. Additionally, previous results have also shown the importance of the dietary protein/lipid ratio to improve octopus paralarvae growth. In this study three groups of paralarvae, each in triplicate, were fed juvenile *Artemia* (1.6-2.8 mm) enriched with one of the following diets: a combination of the microalgal species *Rhodomonas lens* and *Isochrysis galbana* (70%:30% in a dry weigth basis) - group AR+I; the same combination of microalgae supplemented with essential free L-amino acids (3.4 mM lysine, 2.5 mM arginine, and 1.5 mM methionine) dissolved in the water - group AR+I+AA; or with *Nannochloropsis gaditana* - group ANANO. The gross composition (% of dry weight, DW) of *Artemia* juveniles was almost equal irrespective of the dietary enrichment: 65-66% protein, and 10-11% for both lipid and carbohydrate. Survival of paralarvae was not different among groups until day 15, ranging between 51 and 62%. However, from day 20 onward, the survival rate of paralarvae from groups AR+I+AA (8.0±2.0%) and AR+I (7.3±3.1%) was higher than in group ANANO (2.2±0.7%, $P<0.05$). Regarding the DW of paralarvae, a tendency for the positive effects of supplemeting *Artemia* juveniles with free AA was observed, as the highest DW of 25-dph paralarvae was observed in group AR+I+AA (870±81 μg paralarvae^{-1}). However, significant differences were only found in comparison with individuals from group ANANO (798±99 μg paralarvae^{-1}, $P<0.05$), whereas paralarvae from AR+I (834±88 μg paralarvae^{-1}) had no significant differences with the other groups. Boosting the content of essential AA in *Artemia* juveniles to feed paralarvae should be further investigated as it may be useful to improve their growth. Exhaustive analysis of the free-AA pool and protein-bound AA in enriched *Artemia* juveniles should be carried out as a first instance, for comparison with the profiles found in early life stages of *O. vulgaris*. The adjustment of potential AA deficiencies in *Artemia* can then be carried out through enrichment with free AA followed by the evaluation of the practical effects that these actions may have in octopus paralarve rearing.

In chapter 6, in order to evaluate new strategies to optimize the on-growing process octopuses at an industrial scale, two experiments were carried out in cages suspended from a raft, located in the "ría" of Vigo: a) experiment 1 – octopuses with initial weight (IW) not exceeding 100 g were chosen to create an homogeneous group (HOM), which was compared with an heterogeneous group (HET), consisting of individuals with differences in the IW that could exceed 500 g (similar to the process currently done in the companies); b) experiment 2 - octopuses were separated by sexes. Group 1 (MALES), was constituted only by males with an IW of 953±146 g; group 2 (FEMALES), only by females with IW of 899±135 g; and group MIXED by males and females in random proportion (IW of 899±137 g). Octopuses were fed different species of fishes (horse-mackerel, mackerel) and mussel, and the daily ration ranged between 3-6 % of the total biomass. In the first experiment, the survival rate was almost equal between groups (70.0% and 68.7% in HOM and HET, respectively). The final weight (FW) of octopuses in the HET group (2188±574 g) was superior to that of HOM group (2091±499 g), whereas the total biomass in group HOM was slightly higher due to the difference in the survival rate. The on-growing of octopuses caught in nature with similar sizes and selected to form a HOM group did not suppose a clear advantage in comparison with a typical on-growing cycle (HET). The small differences achieved in the final biomass would not compensate the additional work in hand work to separate the octopuses in very exact sizes. In the second experiment, the survival rate in groups MALES and MIXED (both with 86.7 %) was superior to that of group FEMALES (73.3 %). The FW of octopuses from group MALES (2261±428 g) was higher than that of groups FEMALES (2015±428 g) and MIXED (2125±450 g). However, the IW of individuals from group MALES had also been higher. These results have shown that the mortality in group MALES was not different from group MIXED. In the future, it would be interesting to carry out new rearing experiments with groups of octopuses separated in a "rough way", i.e. groups with IW not exceeding 250 g, which would allow to prolong or to short the on-growing period according to the IW of individuals.

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Annex I

Nutritional value of the cryptophyte *Rhodomonas lens* for *Artemia* sp.

Pedro Seixas, Paula Coutinho, Martiña Ferreira and Ana Otero

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Abstract

Juvenile or adult *Artemia* sp. are often used as live prey for the rearing of early life stages of some marine species including crustaceans, fishes and cephalopods. The improvements of *Artemia* growth as well as its biochemical composition are key issues for its suitable use in many rearing processes. In this study we evaluated the growth and survival rates of *Artemia* fed on different microalgal species of controlled and optimized composition, including the prasinophyte *Tetraselmis suecica*, the prymnesiophyte *Isochrysis galbana* Parke, the eustigmatophyte *Nannochloropsis gaditana* and the cryptophyte *Rhodomonas lens*. Microalgae were cultured semicontinuously under nutrient-saturated conditions with a daily renewal rate of 30% of the volume of cultures. Considerable differences in *Artemia* growth were observed, with final lengths at day 8 decreasing as follows: *Artemia* fed *R. lens* (4.9±0.6 mm) > *Artemia* fed *T. suecica* (4.2±0.7 mm) > *Artemia* fed *I. galbana* (3.6±0.7 mm) > *Artemia* fed *N. gaditana* (1.5±0.2 mm) ($P<0.001$). Survival rates were also significantly different among groups, being much lower in group fed *N. gaditana* (18±3%, $P<0.001$) than in the remaining groups (69 to 88%). These results could not be explained only because of differences in the nutritional composition of microalgae, but perhaps also because of different digestability factors. Another trial was carried out to investigate differences in *Artemia* growth and on its biochemical composition when fed *R. lens* or *T. suecica*. The fatty acid profile and total amino acid composition of both microalgal species was also assessed. After 5 days the survival of *Artemia* was nearly the same in both groups, but as reported for the first experiment, individuals fed *R. lens* (group ARHO) grew faster than those fed *T. suecica* (group ATET). Whereas juveniles from group ARHO had a length of 3.6±0.3 mm, those from ATET had 3.2±0.4 mm ($P<0.001$). These differences

could be related with the higher protein and total amino acids content found in *R. lens* in comparison with *T. suecica*. Protein levels in *Artemia* juveniles were very similar in both groups and ranged between 64-68%, whereas carbohydrate ranged between 8 to 10%. Lipid was slightly lower in ARHO (12%) than in ATET (15%, $P<0.01$). Regarding the fatty acid composition, juveniles from group ARHO contained more eicosapentaenoic acid (EPA, 20:5n-3) (6.2%) than juveniles from ATET (4.1%, $P<0.01$), whereas docosahexaenoic acid (DHA, 22:6n-3) was only found in juveniles from ARHO (1.1%). Considering that the productivity of *R. lens* cultures was higher or at least equal than that of the remaining microalgal species, this cryptophyte is confirmed as an excellent diet to optimize the growth of *Artemia* and to improve its biochemical composition as well.

Key words: *Artemia*, growth, microalgae, *Rhodomonas*, Cryptophyte, *Tetraselmis*, composition, productivity.

1. Introduction

Microalgae are the basis of the food chain in many aquaculture processes. They are used to directly feed all life stages of filtering molluscs (Enright *et al*., 1986; Brown *et al*., 1997) and larval or juvenile stages of some fishes and crustacean species (Reitan *et al*., 1997; Piña *et al*., 2006); or indirectly to feed/enrich copepods (Støttrup and Jensen, 1990; Støttrup, 2003), rotifers and *Artemia* which in turn are commonly used as major live feed for the rearing of many marine larval species (Dhert *et al*., 2001; Sorgeloos *et al*., 2001; Aragão *et al*., 2004). Despite newly hatched nauplii and/or 24 h-enriched nauplii are the most common form of *Artemia* used in aquaculture (Sorgeloos *et al*., 2001), juvenile or adult *Artemia* are also utilized to rear early life stages of some species of crustaceans (Dhert *et al*., 1993; Conklin, 1995; Ritar *et al*., 2003; Tlusty *et al*., 2005), fishes (Lim *et al*., 2001; Woods, 2003) and cephalopods (Domingues *et al*., 2001; Iglesias *et al*., 2007). Different kind of diets are frequently used for the on-growing of *Artemia* such as live microalgae, dried algae, bacteria and/or yeast and waste products from food industry, but best yields are undoubtedly obtained with live microalgae (Dhont and Lavens, 1996). The microalgal species selected is a crucial issue for the improvement of *Artemia* growth, modifying both, its growth rate and biochemical composition. Despite the difficulties in the direct comparison of growth results due to the diversity of culture conditions of both the microalgae and *Artemia,* a summary of some of the results reported by different authors with several microalgal species in shown in Table I. *Tetraselmis suecica* has been repeatedly reported as the best species for the on-growing of *Artemia* (Table I). Nevertheless an undefined species of *Cryptomonas* showed best growth of *Artemia* nauplii in only 24 h and after 7 days of rearing, when compared to other standard microalgae such as *Tetraselmis* sp., T-ISO or *Chaetoceros* sp (Thinh *et al*., 1999), but the nutritional potential of Cryptophyte species has not been thoroughly investigated. We have recently shown that another Cryptophyte, *Rhodomonas lens*, produces excellent results in the growth and composition of *Artemia* metanauplii enriched for 26 h, in comparison with other microalgal species such as *T. suecica*, *Isochrysis galbana* Parke and *Isochrysis* aff. *galbana* T-ISO (Seixas *et al*., 2008).

Table 1. Summary of some works reporting *Artemia* sp growth with different microalgal species. Data of *Artemia* sp lenght at different days is shown according to information reported by authors and with relevance to the present work.

Authors	Microalgae species used to feed *Artemia* sp	Culture method and nutrient concentration	Temperature and days of rearing	Best microalgal diet	Length of *Artemia* sp
Sick (1976)	Five species tested: *Chlamydomonas sphagnicola, Dunaliella viridis, Chlorella conductrix, Platymonas elliptica, Nitzschia closterium*	Batch, harvest of 120 h-old algae No information about nutrient medium	25 ºC 16 days	*C. sphagnicola* *D. viridis*	1.8 mm (day 6) 8.9 mm (day 16) 2.9 mm (day 6) 5.7 mm (day 16)
Abre-Grobois et al. (1991)	*Dunaliella tertiolecta*	Batch, harvest of cells with no more than 5 days. No information about nutrient medium	28 ºC 14 days		10.0 mm (day 14)
Gamallo (1992)	Six species: *Tetraselmis suecica, Phaeodactylum tricornutum, Dunaliella tertiolecta, Isochrysis galbana* Parke, *Nannochloris atomus, Nitzschia acicularis*	Batch Medium ALGAL 2 mM N l-1	25 ºC 23 days	*T. suecica* *D. tertiolecta*	4.6 mm (day 8) 4.4 mm (day 8)
Fábregas et al. (1996)	*Tetraselmis suecica*	Semi-continuous with daily renewal rate of 50%. Algal 8 mmol N l^{-1}	25 ºC 19 days		8.3 mm (day 19)
Evjemo and Olsen (1999)	*Isochrysis galbana* T-ISO	Semi-continuous culture f/2 medium	26-28 ºC 12 days		3.3 mm (day 6) 5.9 mm (day 12)
García-Ulloa et al. (1999)	*Tetraselmis suecica* *Chaetoceros calcitrans* *Spirulina* sp (dried)	Semi-continuous Daily harvest of a partial volume f/2 medium	25.5 ± 2.5 ºC 10 days	*T. suecica* *Spirulina* sp (dried)	2.1 mm (day 6) 4.9 mm (day 10) 3.2 mm (day 6) 4.7 mm (day 10)
Naegel (1999)	*Chaetoceros* sp	Not reported	25.0 ± 0.2 ºC 11 days		2.0 mm (day 6) 4.6 mm (day 11)
Thinh et al. (1999)	Thirteen species of tropical Autralian microalgae (benthic and planktonic)	Batch, harvest at the end of log-phase f medium	25 ºC 7 days	*Cryptomonas* sp *Chaetoceros* sp *Tetraselmis* sp *Cryptomonas* sp *Chaetoceros* sp	0.92 (day 1) 0.89 (day 1) 0.88 (day 1) 6.5 mm (day 7) 5.5 mm (day 7)
Godínez et al. (2004)	*Tetraselmis suecica* *Chaetoceros muelleri*	Semi-continuous with daily harvest of a partial volume. f/2 medium	25 ± 1 ºC 10 days	*T. suecica* *C. muelleri*	4.5 mm (day 10) 3.7 mm (day 10)
Lora-Vilchis et al. (2004)	*Chaetoceros muelleri* *Isochrysis galbana* T-ISO	Semi-continuous with daily renewal rate of 25%. f medium	27.5 ± 0.5 ºC 7 days	*C. muelleri* *I. galbana* T-ISO	6.0 mm (day 7) 4.2 mm (day 7)
Marques et al. (2004)	*Dunaliella tertiolecta* (two strains: 19/6B; 19/27) *Tetraselmis suecica* (two strains: 66/4; 66/22A)	Batch, harvest in the middle of exponential growth or in stationary (both conditions) Walne medium	28 ºC 6 days	*T. suecica* 66/4 *T. suecica* 66/22A *D. tertiolecta* 19/6B	3.5 mm (day 6) 4.0 mm (day 6) 3.2 mm (day 6)
Present work (two experiments)	*Tetraselmis suecica, Rhodomonas lens, Nannochloropis gaditana, Isochrysis galbana* Parke	Semi-continuous with daily renewal rate of 30%. Nutrient saturated (2 or 4 mM N l^{-1})	26.5 ± 0.5 ºC 5 and 8 days	*R. lens*	3.6 mm (day 5) 4.9 mm (dia 8)

Rhodomonas sp. has also been previously shown to constitute a high-quality diet to rear calanoid copepods (Koski *et al*., 1998), and was recently confirmed as an excellent microalgal diet for the development of the copepod *Acartia sinjiensis* (Knuckey *et al*., 2005). It is therefore interesting to test the nutritional value of this species for *Artemia* sp. in comparison with other species under controlled conditions.

One of the main problems encountered in the interpretation of results from culture experiments comparing the nutritional value of different microalgal species is the lack of control of the biochemical composition of the microalgal biomass. Most feeding experiments have been carried out using microalgal batch cultures in which the biochemical composition of the microalgae is not stable. Moreover, differences in the growth rate of the microalgal species make direct comparison of their nutritional value difficult. Batch-cultures, often used in aquaculture, offer low productivity, and cultures are harvested near the stationary phase, when microalgae contain lower protein and polyunsaturated fatty acids and the level of contaminant bacteria is higher (Otero *et al*., 2002). The N-deprived characterizing stationary-phase of microalgal batch cultures has been also associated to poor nutritional value, and was shown to produce growth population cessation of the dinoflagellate *Oxyrrhis marina* (Flynn *et al*., 1996). On the contrary, continuous and semi-continuous cultures produce microalgal biomass of constant and controlled biochemical composition, and have long been demonstrated to noticeably improve the growth and development of filter-feeders (Scott, 1980; Taub, 1980, Fábregas *et al*. 1996; 1998) and to boost their nutritional value even in short enrichment periods (Fábregas *et al*., 2001; Ferreira *et al*., 2008). Therefore, they constitute an exceptional tool for the evaluation of the nutritional value of different microalgal species. In the case of *Artemia*, previous works showed that growth and survival of *Artemia* was superior in groups receiving *T. suecica* cultured semi-continuously in nutrient saturated conditions (Fábregas *et al*., 1996). Moreover, important changes in the gross biochemical composition of adult *Artemia* were found when feeding individuals with *T. suecica* cultured semi-continuously at different daily renewal rates (Fábregas *et al*., 2001).

In the present study we compared the growth of *Artemia* fed either *Rhodomonas lens* or other microalgal species widely used in aquaculture: *T. suecica, Isochrysis galbana* Parke and *Nannochloropsis gaditana,* all cultured semi-continuously under nutrient saturated conditions. Since the nutritional composition of *Artemia* is also a main concern when feeding marine larval species, as these ones do not have the capacity, for example, to biosynthesize highly unsaturated fatty acids (HUFAs) from lower chain unsaturated fatty

acids (Bell *et al*., 2003), we also analysed the biochemical composition of the microalgal diets producing best growth results, namely *R. lens* and *T. suecica* and of the *Artemia* juveniles obtained.

2. Material and methods

2.1 Microalgae cultures

The marine microalgae *Tetraselmis suecica* Kylin, *Isochrysis galbana* Parke (both isolated from Ría de Arousa, Spain), *Rhodomonas lens* Pascher et Ruttner CCMP 739 and *Nannochloropsis gaditana* CCMP 527, were grown semi-continuously in 6-l glass flat-bottom flasks containing 5 l of autoclaved seawater (salinity of 35 ppt), being submitted to 12 h:12 h light/dark cycle periods and an irradiance of 197 µmol photon m^{-2} s^{-1} in the rear and of 166 µmol photon m^{-2} s^{-1} from below. Irradiance was measured with a luxmeter Neurtex HD8366 followed by conversion according to the formula proposed by Ginzburg (1987). The pH of cultures was kept below 8.0 through the injection of CO_2 during the light period. Cultures were started in batch mode with a nutrient concentration of 4 mM N l^{-1} (Fábregas *et al*., 1984), and daily dilutions at a renewal rate of 30% of the volume of cultures were carried out once cultures approached late-logarithmic growth, using seawater enriched with the same nutrient concentration for *T. suecica* and *R. lens* cultures, and 2 mM N l^{-1} for *I. galbana* and *N. gaditana*. These nutrient concentrations ensured nutrient-saturation for the conditions applied, which was confirmed by determination of the NO_3^{-2} concentration (Clesceri *et al*., 1989) left in the harvested cultures. Once the steady-state was achieved, as assessed by cell density, the daily harvested cultures were used to feed *Artemia* nauplii. Cell density was measured by means of a Neubauer haemacytometer. Microalgae dry weight was determined by filtering 2 ml of the harvested biomass through carbonised Whatman GF/C glass fibre filters (Whatman, Brentford, UK). Filters were washed twice with ammonium formiate 0.5 M before being dried at 80 ºC for 24 h. Microalgal biomass was harvested in different days during the steady state, centrifuged and immediately frozen at -18 ºC for biochemical analysis.

2.2 Experiments of Artemia sp. growth

Artemia cysts (AF, INVE, Dendermonde, Belgium) were hatched in seawater adjusted to a salinity of 30 ppt, constant aeration and water temperature of 28±1 ºC. On day 0 newly hatched nauplii were rinsed with sterilized sea water, concentrated in a glass beaker and after estimating density they were transferred by triplicate to glass-flasks containing 700 ml of sterilized seawater (30 ppt). Nauplii rearing conditions were as follow: initial density of 2.0 nauplii ml^{-1}, constant aeration provided by capillary tubes, temperature of 26.5±0.5 ºC and dim light for 24 h. Initial food ration was established as 25 µg dry weight (DW) of microalgae per nauplii, equivalent to the following doses: $125x10^3$ cells of *T. suecica*, assuming a cellular DW of 200 pg $cell^{-1}$; $250x10^3$ cells of *R. lens* (DW of 100 pg $cell^{-1}$); $1.0x10^6$ cells of *I. galbana* (DW of 25 pg $cell^{-1}$); and $3.1x10^6$ cells of *N. gaditana* (DW of 8.0 pg $cell^{-1}$). The amount of food was increased gradually as *Artemia* individuals were growing, depending on the transparency of the culture media, so that almost all food supplied was ingested (Sorgeloos *et al*., 1986). Water was completely renewed every 2 days to remove *Artemia* faeces and old microalgae cells that could remain in the water.

In a first experiment *Artemia* nauplii were grown for 8 days for the comparison between *R. lens* (ARHO) and three other species commonly used in aquaculture: *T. suecica* (ATET), *I. galbana* (AISO) and *N. gaditana* (ANANO). In a second experiment *Artemia* nauplii were grown for 5 days only with *T. suecica* or *R. lens*, in order to find out further differences in the biochemical composition of these microalgal species that could explain the reasons behind the good results obtained in *Artemia* growth, and to address the nutritional composition of *Artemia* juveniles as well. On the fifth day of culture, food was supplied for further 4 h to ensure maximum enrichment of *Artemia* prior to its sampling for biochemical composition analysis. The total length of *Artemia* individuals was measured under a stereoscope using a calibrated ocular micrometer (25 *Artemia* per replicate), whereas final dry weight was calculated by weighing samples of 20 individuals (n=5) per replicate, which were washed with distilled water and dried at 101±1 ºC for 24 h. Feed conversion rate (FCR) was calculated as follows: total food supplied (dry weight of microlgae)/biomass of *Artemia* obtained (dry weight). Survival was recorded at day 2 and at the end of the experiments.

2.3 Biochemical composition analysis

Protein content was determined by the Folin-phenol method (Lowry *et al*., 1951), after hydrolysis with NaOH 1.0 M at 95 ºC; carbohydrates by the phenol/sulphuric acid method (Kochert *et al*., 1978) and lipids were quantified by the charring method (Marsh and Weinstein, 1966) after extraction of total lipids (Bligh and Dyer, 1959). Phycoerythrin was extracted from *R. lens* by re-suspending 3 ml culture-samples previously centrifuged in distilled water, followed by frosting at -20 ºC to disrupt cells. Determination of phycoerythrin was carried out using the formulas proposed by Bennett and Bogorad (1973) after reading pigment concentrations in a spectrophotometer at wavelengths of 565 nm, 620 nm and 650 nm (Bryant *et al*., 1979). C-N-H of microalgae was determined with an elemental autoanalyser (Fisons 1108) on freeze-dried samples. Total fatty acids were identified and quantified using a gas chromatograph-mass spectrograph (GC-MS Fisons Instruments, MD-800, Beverly, Mass.), equipped with an Omegawax^{TM} 250 column 30m x 0.25mm (Supelco, Inc.), after methanolysis of the lipid extracts with 5% HCl in methanol at 85 ºC during 2:30 h, and extracted with hexane (Sato and Murata, 1988). Triheptadecanoin (Sigma®, St. Louis, Mo.) was used as internal standard. Caloric values were calculated using the conversion factors proposed by the National Research Council (1993) for protein (5.64), lipid (9.44) and carbohydrate (4.11). Total amino acids of *T. suecica* and *R. lens* were determined in 17.5 mg of centrifuged cultures harvested in different days along the steady state period. Samples were hydrolysed in 25 ml of HCl 6.0 M at 105 ºC for 24 h. The obtained hydrolyzed solutions and AA standards (Waters, standards WAT 088122) were derivatized using AccQ-Tag® System for amino acid analysis (Water, Milford, MA) and run on a modification of the reversed-phase HPLC system (Waters Associates). To separate the different amino acids, a reverse-phase column (AccQ Tag, 150 mm long, 3.9 mm internal diameter) was used. Samples of 10 µl were injected by autosampler, and the eluting products were measured with a fluorescent detector at excitation wavelengths of 250 and 395 nm. Chromatograms were recorded using the software program Breeze (Waters, USA). Results for tryptophan are not reported since this AA is destroyed by acid hydrolysis. All the analyses were carried out in triplicate, except for total amino acids for which a single analysis (double injection in HPLC) was done.

2.4 Statistical analysis

Statistical analyses were done using the software SPSS V 15.0.1 statistical package (SPSS, Inc.). Data of *Artemia* length was checked for requirements of normality (Kolmogorov-Smirnov test). For comparisons of dry weight and *Artemia* length among groups, analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons were carried out (α=0.05). Comparisons of data between groups of experiment two were carried out by student's t-test. Percentages of survival and biochemical composition were arcsine-√ transformed before statistical analysis (Zar, 1999).

3. Results

3.1 Productivity and gross biochemical composition of microalgae

The steady state densities (cells ml^{-1}) attained by the different microalgal species cultured semi-continuously with a daily renewal rate of 30% and in nutrient saturated conditions increased as follows: *T. suecica* (2.0±0.2 x 10^6) < *R. lens* (3.7±0.2 x 10^6) < *I. galbana* (15.4±0.9 x 10^6) < *N. gaditana* (47.7±2.0 x 10^6) ($p<0.01$). On the other hand, the cellular dry weigth (pg $cell^{-1}$) of each species increased in the inverse sense of steady state densities: *N. gaditana* (8.0±0.3) < *I. galbana* (22.3±2.8) < *R. lens* (120.8±6.4) < *T. suecica* (220.7±11.5) ($p<0.001$). Regarding the daily productivity, defined as the dry weight (DW) of harvested culture per liter of culture per day (mg l^{-1} day^{-1}), similar values were found in *R. lens* and *T. suecica* cultures (133±5 and 129±9 mg l^{-1} day^{-1}, respectively), being lower in *N. gaditana* and *I. galbana* cultures (116±4 and 103±6 mg l^{-1} $day^{-1,}$ respectively).

The gross biochemical composition (% DW) of the microalgal species is shown in figure 1. Protein content was higher in *R. lens* (55%) than in the remaining species ($p<0.05$), whereas maximum lipid content was found in both *I. galbana* and *N. gaditana* (25-27%) ($p<0.001$). Carbohydrate was higher in *T. suecica* (16%), followed by *R. lens* (11%), and was nearly the same in both *I. galbana* and *N. gaditana* (circa 9%). The C:N ratio increased among species in the sense: *R. lens* (4.5±0.2) < *T. suecica* (5.2±0.2) < *N. gaditana* (7.5±0.0) < *I. galbana* (8.8±0.2) ($p<0.05$), supporting the observed gross

biochemical composition results, concerning the proportion of protein to lipid and carbohydrate. *R. lens* contained 7.8±0.3 pg cell^{-1} of the pigment phycoerythrin, which corresponded to circa 12% of its total protein content. Maximal productivity of protein per day was achieved in *R. lens* culture (72.2 mg protein l^{-1} day^{-1}), followed by *T. suecica* (57.5 mg protein l^{-1} day^{-1}), while lower values were found in *I. galbana* and *N. gaditana* cultures (45.8 and 47.6 protein l^{-1} day^{-1}, respectively).

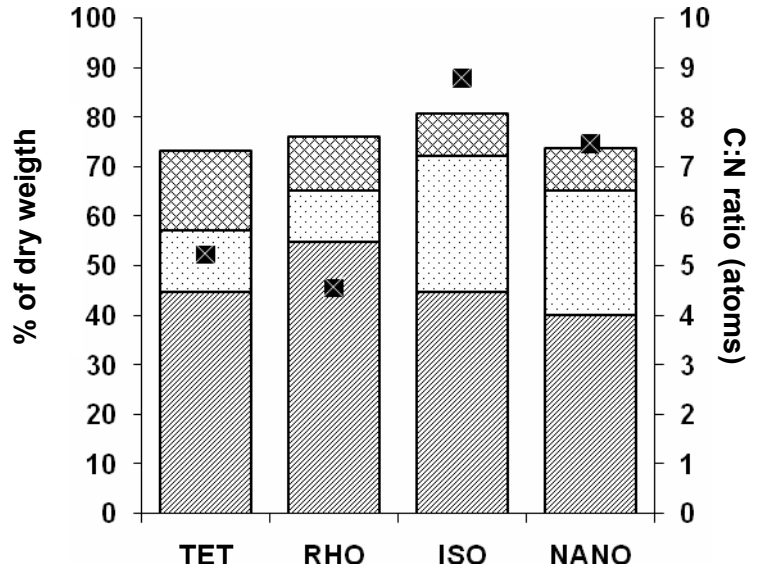

Figure 1 - Gross biochemical composition (% of dry weight) and C:N ratio (atoms) of the different microalgal species cultured semi-continuously with a daily renewal rate of 30% of the volume of cultures and in saturated nutrient conditions. TET (*T. suecica*), RHO (*R. lens*), ISO (*I. galbana*), NANO (*N. gaditana*). Protein, Lipid, Carbohydrate, C:N ratio. Data are mean values (n=3). S.D. were all below 10% and are not shown.

In the second experiment the steady state densities observed in *R. lens* and *T. suecica* cultures were highly reproducible and similar to values found for the first experiment.

3.2 Fatty acid and amino acid composition of *T. suecica* and *R. lens*

Important differences in the total fatty acid (FA) composition were found between *T. suecica* and *R. lens* (Table II). The major FA found in *T. suecica* was the saturated palmitic acid 16:0 (37%), whereas in *R. lens* it was the polyunsaturated linolenic acid 18:3n-3 (26% of total FA).

Table II. Fatty acid composition (% of total fatty acids) of *Tetraselmis suecica* and *Rhodomonas lens* cultured semi-continuously with a daily renewal rate of 30% of the volume of cultures and of 5 day-old *Artemia* fed those microalgal species.

	Microalgal species		5-day old *Artemia*	
Fatty acid	*T. suecica*	*R. lens*	ATET	ARHO
14:0	6.5 ± 0.2^{a}	7.6 ± 0.7^{a}	0.7 ± 0.1^{x}	1.3 ± 0.6^{x}
16:0	37.1 ± 2.4^{a}	21.2 ± 1.0^{b}	29.8 ± 2.1^{x}	24.2 ± 1.3^{y}
16:1n-9	1.5 ± 0.1^{a}	1.1 ± 0.2^{b}	1.3 ± 0.2^{x}	1.2 ± 0.3^{x}
16:1n-7	2.2 ± 0.1^{a}	2.6 ± 0.7^{b}	0.3 ± 0.1^{x}	1.4 ± 0.6^{y}
16:4n-3	14.6 ± 1.1^{a}	n.f.	3.8 ± 0.6	n.f.
18:0	0.0	0.8 ± 0.0	4.7 ± 1.2^{x}	12.0 ± 0.9^{y}
18:1n-9	9.9 ± 0.9^{a}	0.7 ± 0.0^{b}	20.1 ± 1.2^{x}	3.3 ± 1.1^{y}
18:1n-7	1.1 ± 0.1^{a}	2.8 ± 0.4^{b}	6.0 ± 0.6^{x}	20.9 ± 1.8^{y}
18:2n-6	1.4 ± 0.1^{a}	0.4 ± 0.1^{b}	1.9 ± 0.1^{x}	0.3 ± 0.1^{y}
18:3n-3	11.8 ± 1.4^{a}	26.4 ± 1.5^{b}	15.8 ± 0.7^{x}	20.5 ± 1.3^{y}
18:4n-3	7.3 ± 0.8^{a}	18.3 ± 1.5^{b}	9.8 ± 0.9^{x}	6.0 ± 1.5^{y}
20:1n-9	1.2 ± 0.1	n.f.	0.4 ± 0.1^{x}	0.1 ± 0.1^{y}
20:4n-6	0.2 ± 0.0	0.0	0.1 ± 0.0^{x}	0.4 ± 0.1^{y}
20:4n-3	0.4 ± 0.1^{a}	1.1 ± 0.2^{b}	0.4 ± 0.1^{x}	0.7 ± 0.1^{x}
20:5n-3	4.7 ± 0.8^{a}	8.4 ± 0.6^{b}	4.1 ± 0.6^{x}	6.2 ± 0.5^{y}
22:6n-3	n.f.	6.9 ± 0.7	n.f.	1.1 ± 0.2
Others	0.1	1.7	0.8	0.4
Saturated	43.6	29.6	35.3	37.5
Monoenes	14.7	7.2	28.1	26.8
PUFAs	40.4	61.5	35.2	35.2
n-3	38.8	61.1	33.9	34.5
n-6	1.6	0.4	2.1	0.7
DHA/EPA	-	0.8	-	0.2

ATET: *Artemia* fed *T. suecica*; ARHO: *Artemia* fed *R. lens*. PUFAs: polyunsaturated fatty acid. Values are means ± S.D., n=3; values pointed out as 0.0 are below 0.05. n.f.: not found. Different superscript letters indicate significant differences between groups (α=0.05)

The sum of saturated FA was nearly 30% in *R. lens* and 43% in *T. suecica,* whereas monoenes represented 7 and 15%, respectively (Table II). Polyunsaturated fatty acids (PUFAs) were clearly higher in *R. lens* (circa 62%) than in *T. suecica* (40%). Eicosapentaenoic acid (EPA, 20:5n-3) was higher in *R. lens* (8.4%) than in *T. suecica* (4.7%), while docosahexaenoic acid (DHA, 22:6n-3) was only found in *R. lens* (6.9%).
Regarding the total amino acid (AA) composition of *T. suecica* and *R. lens* (Table III), major differences between species were more quantitative than qualitative. The amount of AA per single cell was 49.2 pg cell^{-1} in *R. lens*, which corresponded to nearly 40% of its DW, whereas *T. suecica* contained 42.5 pg AA cell^{-1}, representing about 20% of its DW.

Table III. Total amino acid (AA) composition (in pg cell^{-1} and in % of total AA) of *Tetraselmis suecica* and *Rhodomonas lens* cultured semi-continuously with a daily renewal rate of 30% of the volume of cultures.

	T. suecica		*R. lens*			*T. suecica*		*R. lens*	
EAA	pg cell^{-1}	%	pg cell^{-1}	%	NEAA	pg cell^{-1}	%	pg cell^{-1}	%
Arginine	3.4	8.0	4.0	8.2	*Alanine*	2.3	5.3	3.9	7.9
Histidine	1.4	3.3	1.1	2.2	*Asp+Asn*	2.5	5.8	5.3	10.7
Isoleucine	1.9	4.4	2.4	4.8	*Cystine*	0.0*	0.1	0.3	0.6
Leucine	4.0	9.5	4.2	8.5	*Glu+Gln*	3.5	8.3	6.8	13.9
Valine	2.6	6.2	3.2	6.5	*Glycine*	3.7	8.7	3.2	6.5
Lysine	1.0	2.4	2.7	5.5	*Proline*	2.2	5.2	2.0	4.0
Phenylalanine	4.3	10.2	2.7	5.5	*Serine*	1.9	4.4	2.0	4.0
Methionine	1.5	3.4	1.4	2.9	*Tyrosine*	2.9	6.8	2.1	4.3
Threonine	3.3	7.8	1.9	3.9					
TOTAL	23.5	55.3	23.6	48.0	TOTAL	19.0	44.7	25.5	52.0

EAA: essential amino acids, NEAA: non essential amino acids. Asp + Asn: aspartic acid plus asparagine; Glu + Gln: glutamic acid plus glutamine. Values are from a single analysis. * 0.0 corresponds to 0.04.

3.3 Growth and survival of *Artemia* sp.

Survival of *Artemia* at the end of 8 days was significantly higher in groups ARHO and ATET (88±4% and 83±8%, respectively), followed by group AISO (69±4%) ($p<0.01$), and group ANANO (18±3%) ($p<0.001$). However, the mortality of *Artemia* individuals in groups ARHO, ATET and AISO ocurred mainly in the first two days of the experiment, as survival did not change until the end ($p<0.05$), whereas in group ANANO it happened gradually along the experiment (survival was 81±8% at day 2). Growth of individuals was markedly faster in group ARHO than in the remaining groups (Fig. 2), with the final length

of 8-day old *Artemia* decreasing as follows: ARHO (4.9±0.6 mm) > ATET (4.2±0.7 mm), AISO (3.6±0.7 mm) > ANANO (1.5±0.2 mm) ($p<0.001$). The same trend was observed for the DW of individuals, which decreased in the sense: ARHO (111.2±8.6 µg *Artemia*$^{-1}$) > ATET (96.6±5.2 µg *Artemia*$^{-1}$) > AISO (81.3±7.9 µg *Artemia*$^{-1}$) > ANANO (25.7±3.6 µg *Artemia*$^{-1}$) ($p<0.001$).

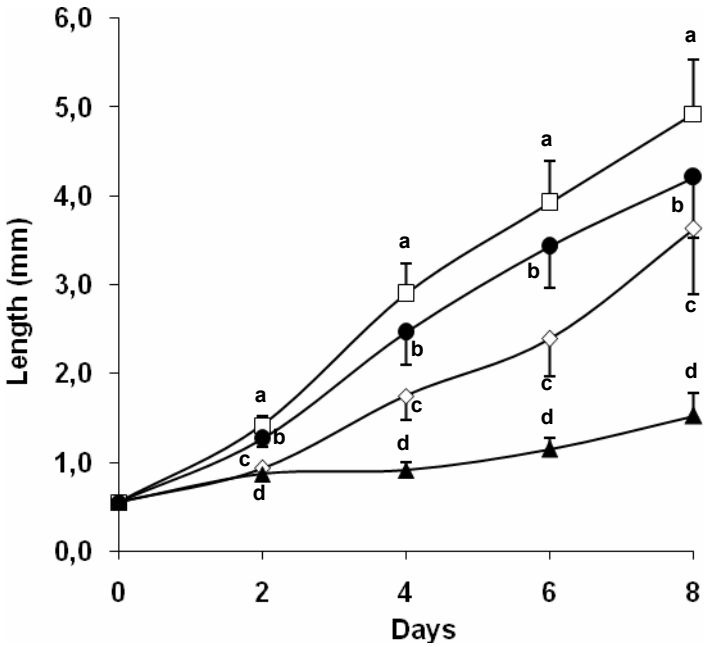

Figure 2 - Growth of *Artemia* fed on four different microalgal species for 8 days. Squares (ARHO - group fed *Rhodomonas lens*); circles (ATET - group fed *Tetraselmis suecica*); diamonds (AISO - group fed *Isochrysis galbana*); triangles (ANANO - group fed *Nannochloropsis gaditana*). Data are means±S.D. Different superscript letters within the same day indicate significant differences among groups ($P<0.001$).

In the second experiment survival of individuals from groups ARHO and ATET was in the same range as in the first experiment, and again no significant difference between groups were found ($p<0.05$). Differences in *Artemia* length could already be observed from day 2 onward (Fig. 3) and at the end of 5 days juveniles from ARHO had a higher length (3.6±0.3 mm) than those from ATET (3.2±0.4 mm) ($p<0.001$). Differences in the DW of 5-day old *Artemia* were also observed (Table IV), with juveniles from ARHO weighing more that those from ATET ($p<0.01$). Feed conversion rate (FCR) was slightly higher in group ARHO than in ATET (6.5 and 6.2, respectively).

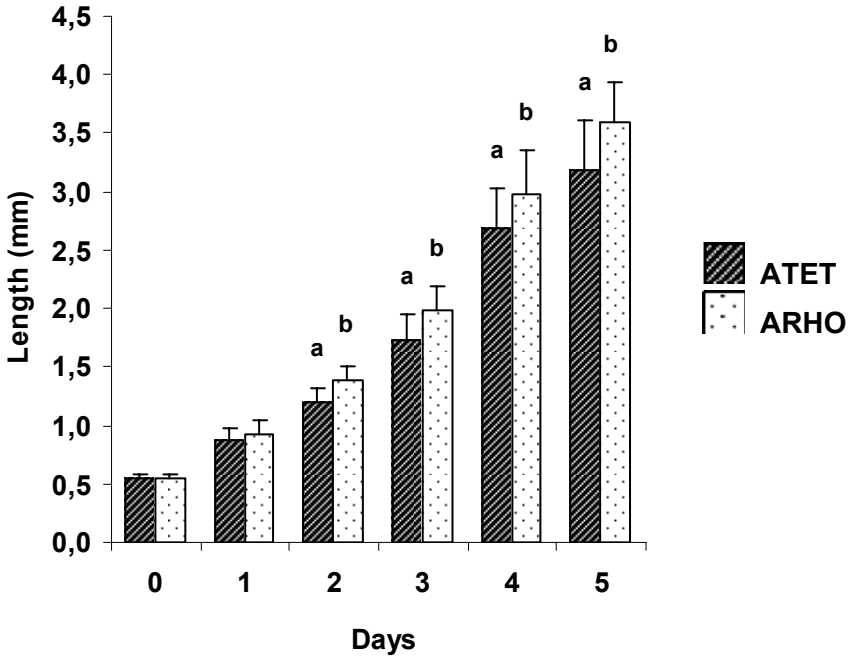

Figure 3 - Growth of *Artemia* along 5 days (experiment 2) when fed either *Tetraselmis suecica* (ATET) or *Rhodomonas lens* (ARHO). ATET - stripped bars, ARHO - dotted bars. Data are means±S.D. (n=3, 25 *Artemia* measured per replicate). Different superscript letters within the same day indicate significant differences between groups ($P<0.001$).

3.3 Biochemical composition of *Artemia* juveniles fed *R. lens* or *T. suecica*

Differences in the biochemical composition of *Artemia* juveniles from groups ATET and ARHO (Table IV) were lower than in the supplied microalgae. No significant differences were found in protein and carbohydrate content ($p>0.05$), whereas lipid content was higher in juveniles from ATET (15.2%) ($p<0.01$). The inorganic fraction, calculated as the difference between the total dry weight and the organic fraction, was nearly the same in both groups (Table IV). The ingestion of the different microalgal species modulated into a certain extent the FA composition of *Artemia* juveniles (Table II). The sum of saturated FA was nearly the same in both groups (35 to 38%), as well as the proportion of monoenes (27-28%) and PUFAs (both with 35%). The saturated palmitic acid (16:0) was the major FA found in both groups. Only juveniles from group ATET contained 16:4n-3 in its composition (3.8%), while DHA was only found in ARHO (1.1%). Arachidonic acid

(20:4n-6) was found in very small amounts in both groups (≤0.4%), whereas EPA percentage was higher in group ARHO (6.2%) than in ATET (4.2%, $p<0.05$).

Table IV. Dry weight (µg *Artemia*$^{-1}$), feed conversion rate (FCR), biochemical composition (% of dry weight) and caloric value (in J *Artemia*$^{-1}$) of 5-day old *Artemia* fed *T. suecica* (ATET) or *R. lens* (ARHO) produced semi-continuously with a daily renewal rate of 30% of the volume of cultures.

	ATET	ARHO
Dry weight	50.9 ± 4.3^{b}	60.5 ± 3.3^{a}
Protein	63.6 ± 2.6	67.7 ± 1.2
Lipid	15.2 ± 0.6^{a}	12.3 ± 0.6^{b}
Carbohydrate	10.0 ± 1.1	8.3 ± 0.4
Inorganic fraction	11.2	11.7
Feed conversion rate	6.2	6.5

Inorganic fraction calculated as 100% - ∑Organic fraction. FCR = total supplied food (dry weight)/biomass of *Artemia* attained (dry weight). Data are means ± S.D., n=3 except for dry weight (n=5). Different superscript letters indicate significant differences between groups ($p<0.01$).

4. Discussion

4.1 Microalgae cultures

Cell productivities found in 5-l cultures of *I. galbana*, *T. suecica*, *R. lens* and *N. gaditana* ($4.6x10^9$, $0.6x10^9$, $1.1x10^9$ and $14.3x10^9$ cells l^{-1} day^{-1}, respectively) were in general much lower than values reported previously in 80 ml cultures (30 mm diameter glass tubes), when using the same conditions of nutrient concentration and daily renewal rate. Otero *et al*. (1997) and Otero and Fábregas (1997) described for *I. galbana* and *T. suecia* cell productivities of $9.0x10^9$ and $1.8x10^9$ cells l^{-1} day^{-1}, respectively; whereas Ferreira *et al*. (2009) reported for *N. gaditana* $30x10^9$ cells l^{-1} day^{-1}. Coutinho (2008) described for *R. lens* a productivity of $2.0x10^9$ cells l^{-1} day^{-1} at a renewal rate of 30%, but using 8 mM of nutrient concentration. Irradiance and nutrient availability can both affect the productivity of cultures (Fábregas *et al*., 1995; 2004), and were certainly the cause of the discrepancies observed in the productivities between our 5-l cultures and 80 ml cultures. Results demonstrate de importance of the use of efficient culture systems that maximize irradiance availability. Although other authors pointed out the instability and unpredictable growth of

Rhodomonas sp in batch cultures and suggested a 30% exchange every three days as convenient to maintain cultures in exponential growth (Knuckey *et al*., 2005), no problem was encountered for the establishment of steady semicontinuous cultures of *Rhodomonas lens* with a daily renewal rate of 30% of the total volume, being highly reproducible and stable along time. The better results obtained in the present work may be due to the high N concentration used (4 mM N l^{-1}), in comparison to the *f* medium used by other authors (Knuckey *et al*., 2005). In fact, the steady-state density attained in this study for *R. lens* (3.7 x 10^6 cells ml^{-1}) was 2 to 10-fold higher than densities reported by other authors for *Rhodomonas* sp in log, late-log or stationary phase using lower nutrient concentrations (Renaud *et al*., 1999, 2002; Dunstan *et al*., 2005; Lafagarta-De La Cruz *et al*., 2006). Nevertheless, it should be emphasized that previous experiments of *R. lens* culture carried out in our laboratory also showed this species to be more sensitive than other microalgae usually used in aquaculture, being more sensitive to high light conditions and dying faster than other species once nitrogen is depleted from culture media (Coutinho, 2008).

The daily productivity of *R. lens* (DW l^{-1} day^{-1}) was higher or at least equal than that of the remaining cultures, but the protein productivity was by far the best among cultures (25 to 55% higher than the remaining species). The high protein content of *R. lens* can be partially attributed to the accumulation of phycoerithrin (which represented nearly 12% of the total protein of *R. lens*). Phycoerithrin is a proteic pigment located inside the thylakoids of *R. lens* (Ludwig and Gibbs, 1989) that functions as light-harvesting and energy-transfer pigment in photosynthesis. Bartual *et al*. (2002) reported maximum values of phycoerythrin of 4.9 pg $cell^{-1}$ in dense cultures of *Rhodomonas salina*, cultured under different irradiance levels but using $f/_2$ medium as nutrient concentration. Phycoerythrin content seems to be strongly dependent on effective light intensity and nutrient availability. However, this result was obtained at the expense of using twice as much nutrient as in *I. galbana* or *N. gaditana* cultures to achieve N saturation conditions. In order to optimize cost productions it would be necessary to adjust nutrient concentration at the exact turning point, from limitation to saturation conditions.

In this study the protein content of *T. suecica* (45% of DW) was considerably higher than values reported by other authors (16 to 31%) for the same species cultured under different nutrient mediums (Brown, 1991; D'Souza and Kelly, 2000), but similar to values described by Otero and Fábregas (1997) when using similar semi-continuous culture conditions in 30 mm tubes. *R. lens* was shown to contain circa 55% protein, which is higher or similar to percentages found by other authors or *Rhodomonas* sp (Renaud *et al*., 1999; Renaud *et al*.,

2002; Dunstan *et al*. 2005). Other authors reported for *Rhodomonas salina* protein percentages between 48 to 59% (Brown *et al*., 1998; McCausland *et al*., 1999). Results demonstrate the effectiveness of using daily renewal rates and nutrient saturation conditions to reach maximal protein contents in the microalgae.

The C:N ratios found in *R. lens* and *T. suecica* were lower than the values predicted by the Redfield ratio (C:N=6.6) for microalgae under non-limited nutrient conditions. However, this same observation had previously been reported by Otero *et al*. (1998) in *Phaeodactylum tricornutum* cultures maintained under light-dark photoperiods and saturated nutrient conditions, suggesting that carbon respiratory losses during the dark period could be the reason for the lower C:N ratios obtained when harvesting the microalgae at the beginning of the light period.

Regarding the FA composition of *R. lens*, EPA and DHA percentages found in here (8.4 and 6.9%, respectively) were very similar to values reported by Renaud *et al*. (1999) and Dunstan *et al*. (2005) for *Rhodomonas* sp. These authors also reported the presence of high levels of 18:3n-3 and 18:4n-3 in *Rhodomonas* sp., as observed for *R. lens* in this study (26.4 and 18.3%, respectively).

Total amino acid (AA) percentages found in *T. suecica* were similar to those reported by Brown (1991) except for arginine and lysine, which were lower in the present work (8 and 2.4%, respectively), compared to 13.2 and 6.0% reported by that author. The percentages of total AA found in *R. lens* were also closely related to those described by Dunstan *et al*. (2005) for *Rhodomonas* sp. The amount of intracellular AA in microalgae increases strikingly with increasing growth rate in cultures which are not deprived of N (Flynn, 1990). When analysing the AA content of about 40 species of microalgae from seven algal classes, Brown *et al*. (1997) found that they were all very similar in composition (weight % of total AA), with few exceptions being observed (as the very high content of arginine in *Tetraselmis* sp). However, in the present work *R. lens* was found to contain much higher amounts of total AA (41% of DW) in comparison with *T. suecica* (19%). This observation may be a result from the intrinsic presence of phycoerythrin in *R. lens* and to and hypothetic accumulation of higher amounts of intracellular free amino acids to build up this pigment. The lower amount of total amino acids in both microalgal species, compared to total protein, is probably the result of missing values of amino acids which were not quantified (e.g. tryptophan, taurine) or to the possible overestimation of protein by the Folin-phenol method, as previously described for other microalgae (Berges *et al*., 1993).

4.2 Artemia *growth and biochemical composition*

Growth of *Artemia* sp. up to juvenile or adult stages is strongly influenced by the availability and quality of food and by temperature as well (Dhont and Lavens, 1996). Establishing comparisons of *Artemia* growth among different works is always difficult and trouble, due to differences in many rearing parameters, such as temperature, *Artemia* strains, handling conditions, food diets, etc. Even when the same microalgal species is used, culture conditions strongly affect the nutritional value of microalgae to be harvested (Otero and Fábregas, 1997; D'Souza and Kelly, 2000) and therefore the use of continuous or semi-continuous cultures is advisable.

After examining the data reported by several authors (Table I) about *Artemia* rearing conditions and type of microalgal diets, and taking also into consideration the present results, it seems reasonable to state that feeding *Artemia* with *R. lens* originates a faster growth of individuals than with other microalgal diets previously used by those authors. Among the works examined, only Thinh *et al*. (1999) described higher lengths of *Artemia* after feeding individuals for 7 days with *Cryptomonas* sp or *Chaetoceros* sp (6.5 and 5.5 mm, respectively). Our results also corroborate previous observations found for the optimal development of the calanoid *Acartia sinjiensis* fed on *Rhodomonas* sp (Knuckey *et al*., 2005).These findings lead to the assumption that Cryptophytes may in fact represent excellent diets to improve the growth of live prey and other filter-feeders. Previous works with filtering molluscs have shown that *Rhodomonas salina* constitutes a high-quality food for Pacific oyster *Crassostrea gigas* spat (Brown *et al*., 1998), and improves metamorphosis rate and culture productivity of *Pecten maximus* when used as a supplement food of "standard" microalgae used in hatcheries (Tremblay *et al*., 2007).

Another interesting feature concerning *Artemia* growth is related with the size of microalgal species. When analysing our results and data from Table 1 we could see that there is a certain tendency for *Artemia* to grow better with "big size" microalgae. Taking as examples the sizes described by Brown *et al*. (1997) for *Tetraselmis* sp (15 x 9 μm), *Rhodomonas* sp (10 x 12 μm), *Dunaliella tertiolecta* (10 x 12 μm), *Isochrysis galbana* (3 x 5 μm) and *Nannochloropsis* sp (3 μm), we can observe that bigger microalgae gave, in general, better results of *Artemia* growth even when the protein content is similar between

species (e.g. *T. suecica* and *I. galbana*). Even if *Artemia* can filter particles ranging in size from 1 to 50 μm (D'Agostino, 1980; Van Stappen, 1996), other studies have shown that ranges between 7 and 28 μm are preferable and an optimum size of 16.0 μm has been pointed out (Fernández, 2001). The poor growth and low survival of *Artemia* fed *N. gaditana* could be explained by both factors difficult digestibility of their cell walls and small size of cells, as the gross biochemical composition of *N. gaditana* was similar to *I. galbana* and this last species generated better growth of *Artemia*. Indeed, not all microalgal species are suitable to feed *Artemia* as previously described (Sick, 1976; reviewed by Dhont and Lavens, 1996). For example, *Chlorella* and *Stichococcus* have a thick cell wall that cannot be digested by *Artemia* and the genera *Coccochloris* produce gelatinous substances that interfere with food uptake during the filtering process. Differences in the growth of *Artemia* between groups fed *R. lens*, *I. galbana* and *N. gaditana* could be due to the higher protein content of *R. lens* and bigger cell size. Between *T. suecica* and *R. lens*, the same reason of higher protein content in *R. lens* content could explain the faster growth of *Artemia* individuals.

Another interesting feature that we observed in this work and in previous experiments, is that the growth of *Artemia* with *Isochrisis* sp can be as fast as with other "good" microalgal diets as soon as *Artemia* have attained a size of about 2.0-2.5 mm, as found in a previous work where *T. suecica*, *Isochrysis* aff. *galbana* T-ISO, *R. lens* and *I. galbana* were used to enrich *Artemia* for 26 h (Seixas *et al*., 2008). Growth rate of *Artemia* with *I. galbana* between days 6 and 8 was even higher than with *R. lens* (see growth rate in Fig. 2). Evjemo and Olsen (1999) also shown a slow development of *A. franciscana* fed *I. galbana* T-ISO in the first days of development, and a considerable increase in the growth rate of juveniles after they reached a size of 2.4 mm.

Differences in the biochemical composition of *Artemia* juveniles fed *R. lens* or *T. suecica* were also found, especially in their fatty acid profile. Juveniles fed *R. lens* contained 50% more EPA than those fed *T. suecica* and a small percentage of DHA (1.1%), reflecting the ingested diet. Similar percentages of DHA were also found in juvenile *Artemia* enriched with *Isochrysis galbana* T-ISO (1.2% in 1.5 mm *Artemia* and 3.4% in 2.5 mm *Artemia*) or with *C. muelleri* (≤0.8%) (Ritar *et al*., 2003, 2004). It is well known that HUFAs are essential in diets for crustacean and other marine larvae (Sorgeloos *et al*., 1998; Bell *et al*., 2003), and as for nauplii, the lipid composition of *Artemia* biomass might be modulated by the ingested diet, with the additional advantage of having a higher filtration capacity

(Dhont and Van Stappen, 2003). Regarding the gross biochemical composition, protein content in juveniles from both groups (64-68%) was within the highest values described for adult *Artemia* sp (39 to 67%) (Dhont and Van Stappen, 2003), whereas lipid and carbohydrate content (4-31% and 4-20%, respectively) were in the normal ranges pointed out by those authors.

Further studies of the total and free amino acid profiles of *Artemia* nauplii/juveniles enriched with *R. lens* would be useful to confirm the high nutritional value of this microalgal diet, taking into account the high amounts of total amino acids found in this study in *R. lens*. Helland *et al*. (2000) showed that the free AA pool of *Artemia franciscana* nauplii was considerable higher in nauplii enriched for 12 h or 24 h with *T. suecica* or *C. gracilis* than in unenriched nauplii, especially in essential AA and in taurine. Moreover, the content of protein increased by 50% after 24 h enrichment with microalgae when compared to starved nauplii, which did not showed a significant decrease in protein. Studies with other live prey such as rotifers would also be useful, as short and long term enrichment of *Brachionus plicatilis* with other "premium" microalgae have shown to strongly modulate their composition (Ferreira *et al*., 2008).

In conclusion, the Cryptophyte *Rhodomonas lens* was confirmed as an excellent monodiet to optimize the growth of *Artemia* sp and to improve the nutritional composition of *Artemia* juveniles, probably due to the high content of protein and total amino acids, together with a very good profile of PUFA (especially the 18:3n-3, 18:4n-3, 20:5n-3 and 22:6n-3). However, as shown for the copepod *Acartia tonsa* when fed mixed algal diets of diatoms and dinoflagellates (Jones and Flynn, 2005), further studies using combinations of *R. lens* and other microalgae would be useful, for further improvements of growth and nutritional composition of different development stages of *Artemia*,

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Annex II

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Producing juvenile *Artemia* as prey for *Octopus vulgaris* paralarvae with different microalgal species of controlled biochemical composition

Pedro Seixas [a,b], Manuel Rey-Méndez [b], Luísa M.P. Valente [c], Ana Otero [a,*]

[a] *Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, Spain*
[b] *Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, A Coruña, Spain*
[c] *CIMAR/CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental and ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua dos Bragas, 177, 4050-123 Porto, Portugal*

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ABSTRACT

The major bottleneck of *Octopus vulgaris* culture is the rearing of its paralarval life stage, being the obtainment of adequate live prey to feed paralarvae one of the key issues for the success of the culture of this valuable species. *Artemia* has been widely used as a single prey or in combination with crustacean zoeae as food items for paralarvae, but few works have reported the biochemical composition of these prey. The gross biochemical composition and fatty acid profile of *Artemia* juveniles enriched with four marine microalgae of controlled biochemical composition was assessed, as well as the fatty acid composition of newly hatched *O. vulgaris* paralarvae, in order to estimate which prey would be more suitable to meet the nutritional requirements of *O. vulgaris* paralarvae. Microalgae were cultured semi-continuously with a daily renewal rate of 30% of the volume of the cultures in nutrient saturated conditions, in order to achieve biomass of constant and optimal biochemical composition. *Artemia* juveniles of two different sizes (1.5-2.0 mm and 3.0–3.5 mm), appropriate to feed *O. vulgaris* paralarvae, were obtained by growing *Artemia* nauplii with *Tetraselmis suecica* for 2 and 4 days and then enriched for 26 h with four marine microalgae: *T. suecica*, *Isochrysis galbana*, *Isochrysis* aff. *galbana* (T-ISO) and *Rhodomonas lens*. The protein content of *R. lens* (62% of dry weight) was considerably higher than that of the remaining microalgae (42–44%) ($P<0.001$), whereas lipid and carbohydrate were significantly higher in both T-ISO and *I. galbana* ($P<0.05$). Small juvenile *Artemia* (3-day old, 1.5-2.0 mm) contained nearly 51% protein (of dry weight) regardless the enrichment diet used, with the exception of individuals enriched with *I. galbana* (AISO) (41%) ($P<0.01$). In these juveniles, lipid percentages were higher when enriched with T-ISO (group AT-ISO) or *R. lens* (ARHO), both with circa 16% ($P<0.05$); whereas carbohydrate was higher in juveniles from groups AISO or AT-ISO (11%) ($P<0.05$). Large juvenile *Artemia* (5-day old, 3.0-3.5 mm) had higher protein percentages than small juveniles with values ranging between 64 and 68% for all treatments, whereas the lipid fraction among groups increased in the order: ARHO (10%) < ATET = AT-ISO (16%) < AISO (18%) ($P<0.05$). The lowest percentage of carbohydrate was found in group ARHO (6%) ($P<0.01$). Maximum protein/energy ratio was observed in 5-day old juveniles from group ARHO (P/E ratio = 31). The highest percentage (% total fatty acids) of eicosapentaenoic acid (20:5n−3) in small juvenile *Artemia* was found in individuals from groups AISO or ARHO (circa 9%), whereas in 5-day old juveniles the highest value was found in group AISO (14.6%) ($P<0.05$). Regarding docosahexaenoic acid (22:6n−3), small juveniles from groups AT-ISO or AISO had higher values (1.9 and 1.5%, respectively) than juveniles from group ARHO (1.0%) ($P<0.05$), whereas in 5-day old *Artemia* maximum percentage of 22:6n−3 was found in group AT-ISO (3.9%) ($P<0.05$). None of the *Artemia* juveniles enriched with *T. suecica* contained 22:6n−3. The fatty acid composition of *O. vulgaris* paralarvae revealed a much higher percentage of 22:6n−3 (18.3%) than the values found in the enriched *Artemia* juveniles, suggesting a deficit of this fatty acid in *Artemia*. Even though the highest sum of 20:5n−3 and 22:6n−3 was found in *Artemia* juveniles enriched with *I. galbana*, if the general biochemical composition of *Artemia* juveniles is taken into consideration, the enrichment with *R. lens* would provide the best composition to meet the possible nutritional requirements of *O. vulgaris* paralarvae.

1. Introduction

The control of *Octopus vulgaris* life cycle in captivity for rearing purposes has long been a subject of research, with first experiments being carried out in the 60s in Japan (Itami et al., 1963). Yet, it was only

* Corresponding author. Tel.: +34 981563100x16913; fax: +34 981596904.
E-mail address: anamaria.otero@usc.es (A. Otero).

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in the middle of the 90s that intensive experiments to evaluate the potential of this species as a new candidate for aquaculture started to be carried out in countries worldwide. Due to some interesting biological characteristics and to high market demand and price, this species was latter considered as a strong candidate for aquaculture diversification (Iglesias et al., 2000; Navarro and Villanueva, 2000; Vaz-Pires et al., 2004). However, and despite the various attempts to rear the early planktonic life stage of *O. vulgaris*, which is the bottleneck for aquaculture development of this species, researchers still have not succeeded in overcoming the high mortality encountered during paralarvae rearing (reviewed by Iglesias et al., 2007).

Field studies on the feeding habits of early life stages of cephalopods have shown that small planktonic crustaceans constitute major diets for these organisms (Vecchione, 1987; Passarella and Hopkins, 1991) and indeed, most of the successful rearing experiments of squid and cuttlefish hatchlings relied on the harvesting of natural zooplankton to provide as main prey (Yang et al., 1986; Forsythe et al., 1994; Domingues et al., 2004). Few researchers have succeeded in achieving settled *O. vulgaris* juveniles in captivity (reviewed by Iglesias et al., 2007), and in all cases zoeae of different crustacean alone or combined with enriched *Artemia* (of different size according to authors) were used as food items. As far as we know, only one research group has reported the success of rearing octopus paralarvae on *Artemia* as single prey (Hamazaki et al., 1991). In general, it is recognized that *O. vulgaris* paralarvae may have a requirement for diets rich in phospholipids, cholesterol, and in the highly unsaturated fatty acids (HUFAs) 20:5n−3 and 22:6n−3; and also very rich in protein and essential amino acids (Navarro and Villanueva, 2000, 2003; Villanueva et al., 2004). Analysis of the biochemical composition of decapod zoeae have shown that their lipid classes and fatty acid composition are undoubtedly more in accordance to *O. vulgaris* hatchlings and wild juveniles than *Artemia per se*, due to higher levels of phospholipids and the HUFAs 20:5n−3 and 22:6n−3 (Navarro and Villanueva, 2000, 2003). However, the use of parallel cultures of crustaceans to obtain newly hatched zoeae as live prey would be impractical beyond the experimental scale as cost effectiveness and risks implied would be limiting issues (Navarro and Villanueva, 2000).

The enrichment of *Artemia* (1–4 mm) with microalgae for first feeding of *O. vulgaris* or the addition of microalgae to tanks to keep prey fully enriched is a common practice so far, and microalgae species like *Nannochloropsis* sp, *Isochrysis galbana*, *Chaetoceros* sp, *Chlorella* sp, and *Tetraselmis suecica* have previously been used (Hamazaki et al., 1991; Moxica et al., 2002, Iglesias et al., 2002, 2004; Okumura et al., 2005; Carrasco et al., 2006). Commercial lipid emulsions, cereal mixes, and fish egg powder were also tried to enrich *Artemia* nauplii or juveniles to feed paralarvae (Navarro and Villanueva, 2000, 2003; Villanueva et al., 2002; Iglesias et al., 2004; Okumura et al., 2005). In spite of the common utilization of enriched *Artemia* (nauplii or juveniles) in the rearing of *O. vulgaris* paralarvae, few authors (Navarro and Villanueva, 2000, 2003; Villanueva et al., 2004; Okumura et al., 2005) have reported biochemical composition data of these prey, even if this information is crucial to understand possible nutritional deficiencies in paralarvae rearing.

Dramatic changes in the biochemical composition of microalgae can be induced through the use of continuous culture techniques, which have been used to produce microalgal biomass of constant and controlled composition, which in turn lead to improvement in the growth and survival of filter-feeder species used in aquaculture (Scott, 1980; Taub, 1980; Fábregas et al., 1996a, 2001). Semi-continuous cultures are a variant of continuous cultures in which cultures are maintained under light:dark cycles and culture medium is renewed at a fixed rate every 24 h, and have been demonstrated to be as efficient as standard continuous cultures in controlling the nutritional value of microalgae (Fábregas et al., 1996a; Otero and Fábregas, 1997; Otero et al., 1997, 2002). Remarkable improvements in *Artemia* length, dry weight and survival were observed when *T. suecica* supplied to *Artemia* was cultured under nutrient saturated conditions (Fábregas et al., 1996b) or high renewal rates (Fábregas et al., 2001). Besides improvement of growth and survival, important changes in the gross biochemical composition of *Artemia* fed *T. suecica* cultured through semi-continuous culture at different renewal rates have been demonstrated (Fábregas et al., 2001). The standardization of culture conditions to control the biochemical composition of the microalgae used in feeding and/or enrichment experiments is therefore crucial in order to make results obtained with different species comparable and repeatable.

With the aim of evaluating which microalgal species would be more suitable to improve the biochemical composition of juvenile *Artemia* as prey for *O. vulgaris* paralarvae, we analysed the biochemical composition of juvenile *Artemia* of two different sizes, commonly used in paralarvae rearing (1.5-2.0 and 3.0-3.5 mm), enriched for 26 h with four different marine microalgae cultured semi-continuously with a high daily renewal rate and in nutrient saturated conditions. Two microalgal species were selected due to either high protein content (*Rhodomonas lens*) or high content of HUFA (*Isochrysis* aff. *galbana* T-ISO) and the others (*I. galbana* Parke and *T. suecica*) because they have been previously used by different authors and are hereby compared. Analysis of the fatty acid composition of *O. vulgaris* hatchlings were also carried out to establish comparisons with the results found for *Artemia* juveniles.

2. Materials and methods

2.1. Microalgae cultures

Monoalgal cultures of *T. suecica* Kylin (strain isolated from Ría de Arousa, Spain), *I. galbana* Parke, *Isochrysis* aff. *galbana* (T-ISO) CCMP 1324, and *R. lens* Pascher et Ruttner CCMP 739, were carried out in 1 l glass bottles with sterilized sea water adjusted to a salinity of 35‰ and enriched with nutrients at a final concentration of 4 mM $NaNO_3$ (Fábregas et al., 1986), which ensured saturation of nutrients. Cultures were provided with constant aeration through capillary tubes and submitted to 12 h:12 h light/dark photoperiod with an irradiance of 148 μmol photon m^{-2} s^{-1} under and 141 μmol photon m^{-2} s^{-1} beneath cultures, provided by day-light fluorescent lamps (OSRAM L36 W). Irradiance was measured with a luxmeter Neurtex HD8366 followed by conversion according to the formula proposed by Ginzburg (1987). The pH of all cultures was kept between 7.5 and 8.3 through pulses of CO_2 during the light period. The temperature of the culture chamber was 21.0±1.5 °C. Once cultures reached early stationary phase, daily renewal rates of 30% of the volume of cultures were carried out during the first hour of the light period with sterilized seawater and the same nutrient concentration. When cellular density attained the steady state, the harvested cultures were used to enrich different *Artemia* groups. Cell density was calculated in the harvested cultures by microscope counting using an improved Neubauer haemacytometer. Microalgae dry weight was determined by filtering 2 ml of cultures through carbonised Whatman GF/C glass fibre filters (Whatman, Brentford, UK). Filters were washed twice with ammonium formiate (0.5 M) and dried at 80 °C until constant weight. In order to assess the stability of the biochemical composition of each microalgal species along the steady state, samples (8 ml each) of the different cultures of microalgae were obtained on 3 different days, centrifuged and immediately frozen at −18 °C for later biochemical analysis.

2.2. Artemia sp growth and enrichment

Artemia sp cysts (AF, INVE, Dendermonde, Belgium) were incubated in seawater adjusted to a salinity of 30‰, with constant aeration, temperature of 28±1 °C, and exposed to an irradiance of 39 μmol photon m^{-2} s^{-1}. Newly hatched nauplii were transferred into glass-

flasks containing 700 ml of filtered and sterilized seawater also adjusted to 30‰ salinity and placed in the following conditions: initial density of 2.0 nauplii ml^{-1}, water temperature of 26.5 ± 0.5 °C, constant aeration provided by capillary tubes, and dim light for 24 h. Nauplii were initially fed *T. suecica* with a food ration established at 25 µg dry weight of microalgae per nauplii (equivalent to 125×10^3 cells $nauplii^{-1}$), increasing the amount of supplied food along the course of the experiment, depending on the transparency of the culture media so that almost all food supplied was ingested (Sorgeloos et al., 1986). At days 2 and 4 of *Artemia* growth (at day 2 individuals had a total length of 1.2 ± 0.1 mm and a survival of 76 ± 2%, and at day 4 2.7 ± 0.4 mm and a survival of 74 ± 4%), different groups of *Artemia* in triplicate were enriched with different marine microalgal species for 26 h. Four hours before the end of the enrichment process, a second dose of microalgae was supplied in order to ensure a complete enrichment. Group AISO was enriched with *I. galbana* Parke, group AT-ISO with *Isochrysis* aff. *galbana* T-ISO, group ARHO with *R. lens*, and group ATET continued to receive *T. suecica*. Before carrying out the enrichment process, water was completely renewed (100%) in order to remove any remaining food and *Artemia* faeces. The same amount of food was provided to all groups and was calculated according to the dry weight of each microalgal species (obtained from previous experiments in semi-continuous culture regime).

The length of *Artemia* juveniles was measured under an ocular micrometer (25 individuals per replicate). Dry weight was determined by washing *Artemia* juveniles with distilled water (samples of 10 individuals placed in fibre-glass filters, $n = 5$), followed by 24 h drying at 100 °C. Survival was calculated to verify possible effects of each enrichment treatment. Samples of *Artemia* juveniles for biochemical analysis (60 juveniles per sample were counted individually on day 3, and 50 on day 5) were rinsed with distilled water and immediately frozen at −18 °C.

2.3. Hatchlings of *O. vulgaris* for fatty acid analysis

A female *O. vulgaris* with eggs was transported to the facilities of the University of Santiago de Compostela (Spain) and placed in a tank of a recirculation water circuit with mean water temperature of 17.5 ± 1.0 °C. When paralarvae started to hatch, newly born paralarvae found in the tank on the fifth day of hatching where collected, briefly washed with distilled water and immediately frozen at −18 °C for fatty acid analysis.

2.4. Biochemical composition analysis

Analysis of the biochemical composition of microalgae and *Artemia* juveniles included determination of total protein, lipid, carbohydrate and total fatty acids, whereas for *O. vulgaris* hatchlings only total fatty acids were analysed. Protein content was determined by the method of Lowry (Lowry et al., 1951), after hydrolysis with NaOH 1.0 M at 95 °C. Carbohydrate was determined by the phenol/sulphuric acid method (Kochert, 1978), and lipid was quantified by carbonization at 200 °C (Marsh and Weinstein, 1966) after extraction of total lipid (Bligh and Dyer, 1959). Fatty acids were identified and quantified using a gas chromatograph-mass spectrograph (GC-MS Fisons Instruments, MD-800, Beverly, MA.) equipped with a Omegawax™ 250 column 30 m × 0.25 mm (Supelco, Inc.), using helium as gas carrier, after methanolysis of the lipid extracts with 5% HCl in methanol at 85 °C during 2:30 h and recovery of the methyl esters with hexane (Sato and Murata, 1988). Triheptadecanoin (Sigma, St. Louis, MO.) was used as internal standard. Determination of C–H–N in the different microalgal species was carried out on freeze-dried samples ($n = 3$) using an autoanalyzer (Carlo Erba EA 1108, Rodano, Italy). Caloric values were calculated using the conversion formulas proposed by the National Research Council (1977) for protein, lipid and carbohydrate. All biochemical analyses were carried out in triplicate.

2.5. Statistical analysis

Statistical analyses were performed using the software SPSS V 14.0.1 statistical package (SPSS, Inc.). After verifying that data of *Artemia* length met the requirements of normality (Kolmogorov–Smirnov test) comparisons among groups were performed by an analysis of variance (ANOVA) followed by Tukey–Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05. The same statistical tests were carried out to compare the productivities of the different microalgal species. After log-transformation of dry weight data, and arcsine-$\sqrt{}$ transformation of survival and biochemical composition percentages, ANOVA and Tukey–Kramer HSD tests for post-hoc multiple comparisons ($\alpha = 0.05$) were also performed (Zar, 1999). The percentages of 20:5n−3 and 22:6n−3 found in the enrichment diets and in *Artemia* juveniles were correlated using a linear regression model (Zar, 1999).

3. Results

3.1. Biochemical composition of microalgae

Steady state cell densities and daily productivities (as mg dry weight l^{-1} of culture day^{-1}) obtained in the different microalgal semi-continuous cultures are shown in Table 1. *I. galbana* and T-ISO cultures attained overall 30% higher productivities than *R. lens* and *T. suecica* ($P < 0.01$). Since microalgae used to enrich juvenile *Artemia* were considerably different in size, no direct comparisons of the content in protein, lipid and carbohydrate per single cell could be established, and thus comparisons were done as percentages of dry weight (Fig. 1). *R. lens* contained a considerable higher protein percentage (62%) ($P < 0.001$) than the remaining microalgal species (42–44%). Lipid percentages were nearly the same in *R. lens* and in *T. suecica* (12 and 13%, respectively), but significantly higher in both T-ISO and *I. galbana* (20–21%) ($P < 0.001$); whereas carbohydrate was significantly different in all species and increased in the order: *R. lens* (11%)<*T. suecica* (16%)<T-ISO (17%)<*I. galbana* (19%) ($P < 0.05$). The biochemical composition of microalgae did not change along the course of the experiment, supporting the statement that the harvested biomass was stable in composition and was cultured in controlled conditions. The C:N ratios found in microalgal species confirmed the gross biochemical composition data, as this ratio reflects the proportion of protein to lipid and carbohydrate (Fig. 1). Significant differences were observed among all species, with the C:N ratio increasing in the order: *R. lens* (4.4)<*T. suecica* (5.2)<T-ISO (7.0)<*I. galbana* (8.0) ($P < 0.05$). The high protein and low lipid and carbohydrate percentages found in *R. lens* reflected the lowest C:N ratio found in this species. The higher C:N ratios in both T-ISO and *I. galbana* are also in agreement with the higher percentages of carbohydrate and lipid found in these two species.

The fatty acid (FA) composition (% of total FA) of the different microalgae used to enrich juvenile *Artemia* revealed several

Table 1
Steady state cell density (x 10^6 ml^{-1}), cell dry weight (pg $cell^{-1}$), organic weight (OW as sum of protein, lipid and carbohydrate, pg $cell^{-1}$) and productivity (mg dry weight l^{-1} of culture per day) of the different microalgal species used to enrich juvenile *Artemia*, produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures

	TET	T-ISO	ISO	RHO
Steady state cell density	2.1 ± 0.4^a	20.8 ± 2.3^b	22.8 ± 1.8^b	3.5 ± 0.4^a
Cell dry weight	220.7 ± 11.5^a	30.0 ± 2.8^b	26.8 ± 1.1^b	133.5 ± 5.4^c
OW	157.7 ± 5.1^a	24.3 ± 0.8^b	22.1 ± 0.5^b	112.5 ± 2.0^c
Daily productivity	139 ± 23^a	187 ± 21^b	183 ± 14^b	140 ± 14^a

TET (*Tetraselmis suecica*), T-ISO (*Isochrysis* aff. *galbana* T-ISO), ISO (*Isochrysis galbana*), RHO (*Rhodomonas lens*). Data are means ± S.D., different superscript letters within the same line indicate significant differences among microalgal species ($\alpha = 0.05$).

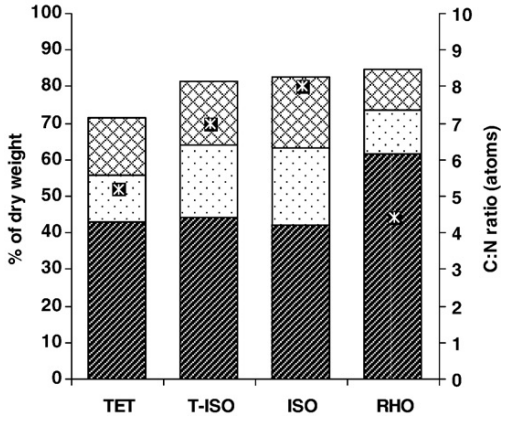

Fig. 1. Gross biochemical composition (% of dry weight) and C:N ratio of the different microalgal species used to enrich juvenile *Artemia*. ▨ Protein, ⊡ Lipid, ⊠ Carbohydrate, ▩ C:N ratio. TET: *Tetraselmis suecica*, T-ISO: *Isochrysis* aff. *galbana* T-ISO, ISO: *Isochrysis galbana*, RHO: *Rhodomonas lens*. Data are mean values (n=3); standard deviations were all below 10%.

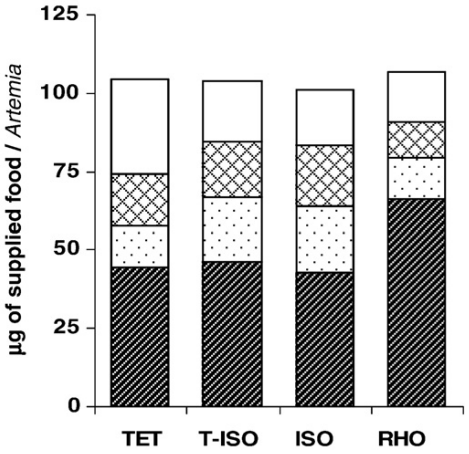

Fig. 2. Composition and total amount of food (µg dry weight microalgal per *Artemia*) supplied to 2-day old juvenile *Artemia* for 26 h during the enrichment process. ▨ Protein, ⊡ Lipid, ⊠ Carbohydrate, □ Total amount of food as dry weight of microalgae. Abbreviations are the same as in Fig. 1.

differences at both quantitative and qualitative levels (Table 2). All microalgae had a predominant saturated FA in its composition, except *R. lens* which had the polyunsaturated fatty acid (PUFA) 18:3n−3 (32%) as the major FA. Both *T. suecica* and *I. galbana* contained 16:0 as the main FA (35 and 28%, respectively), whereas 14:0 was the major FA found in T-ISO (25%). The total saturated FA was clearly lower in *R. lens* (21%) than in the remaining species (42–47%). Due to the high percentages of the PUFA 18:3n−3 and 18:4n−3 in *R. lens* (32 and 20%, respectively) this microalgal doubled the proportion of PUFA when compared to the values found in *I. galbana* and T-ISO (34–36%). *T. suecica* contained 42% of PUFA, but the FA 16:4n−3 accounted for 15% of total FA. The n−6 class of FA represented less than 2% in all microalgae, except in T-ISO which had a considerable higher proportion (7%). The highest percentage of the HUFA 22:6n−3 (DHA) was found in T-ISO (10.7%) (P<0.001), followed by *R. lens* (7.4%) and *I. galbana* (6.4%), whereas no 22:6n−3 was found in *T. suecica* (Table 2). The percentage of 20:5n−3 (EPA) was significantly different among all species (P<0.001), with the maximum value being found in *I. galbana* (19%), followed by *R. lens* (9.7%), *T. suecica* (5.5%) and T-ISO (0.7%). The maximum DHA/EPA ratio was found in T-ISO (15.3). Reflecting the higher lipid content found in both *I. galbana* and T-ISO, the proportion of total FA (% dry weight) was higher in these two species than in *R. lens* or *T. suecica* (Table 2).

Table 2
Fatty acid composition (% of total fatty acid) and total fatty acid (% dry weight) of the different microalgae used to enrich *Artemia*, produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures

Fatty acid	TET	T-ISO	ISO	RHO
14:0	6.7±0.2^a	24.8±1.3^b	19.2±1.2^b	7.4±0.4^a
16:0	35.2±3.3^a	16.3±0.2^b	27.6±1.2^c	12.8±0.2^d
16:1n−9	1.6±0.1^a	6.8±0.3^b	17.7±0.7^c	1.0±0.1^d
16:1n−7	2.1±0.1^a	0.0	n.f.	2.2±0.1^a
16:4n−3	15.2±1.8	n.f.	n.f.	n.f.
18:0	0.0	2.6±0.1^a	0.1±0.1^b	0.4±0.0^c
18:1n−9	9.8±0.6^a	12.0±1.2^b	0.2±0.0^c	0.3±0.0^c
18:1n−7	0.9±0.3^a	0.7±0.2^a	0.4±0.0^a	3.3±0.1^b
18:2n−6	1.3±0.5^a	6.3±0.2^b	0.6±0.0^c	1.7±0.1^a
18:3n−3	11.7±1.1^a	10.1±0.9^a	1.1±0.0^b	31.7±0.5^c
18:4n−3	7.9±0.7^a	7.1±0.4^a	5.9±0.1^a	20.3±0.6^b
20:1n−9	1.2±0.1^a	0.5±0.1^b	n.f.	n.f.
20:4n−6	0.3±0.0^a	0.2±0.1^a	0.2±0.0^a	0.0
20:4n−3	0.5±0.1^a	n.f.	n.f.	1.0±0.1^b
20:5n−3	5.5±0.1^a	0.7±0.2^b	19.0±0.3^c	9.7±0.2^d
22:5n−6	n.f.	0.9±0.4^a	1.2±0.0^a	0.0
22:6n−3	n.f.	10.7±0.3^a	6.4±0.4^b	7.4±0.3^c
Others	0.0	0.0	0.0	0.1
Saturated	41.9	43.7	46.9	20.6
Monoenes	15.6	20.0	18.3	6.8
PUFA	42.4	36.0	34.4	71.8
n−3	40.8	28.6	32.4	70.1
n−6	1.6	7.4	2.0	1.7
DHA/EPA	0.0	15.3	0.3	0.8
Total fatty acid	4.6±0.4	11.1±0.7	13.8±0.3	7.3±0.1

Abbreviations TET, T-ISO, ISO and RHO are the same as in Table 1. Data are means±S.D., n=3. n.f.: not found. Values referred as 0.0 are below 0.05. Different superscript letters within the same line indicate significant differences among microalgal species (α=0.05).

The total amount and composition of the food (expressed as dry weight) supplied to 2-day old *Artemia* for 26 h is shown in Fig. 2. The slight differences observed in the total food supplied (µg of food per *Artemia*) to each group were due to differences in the dry weight of the microalgae obtained in the present work and the theoretical dry weights of each microalgal species that were considered when carrying out the calculations for food distribution. Even so, it should be kept in mind that the amount of food supplied for enrichment was in excess in all cases, and enough to guarantee the correct enrichment of *Artemia* juveniles. The total amount of 20:5n−3 and 22:6n−3 in the enrichment diets (corresponding to the different microalgal species) supplied to 2-day old juvenile *Artemia* for 26 h is shown in Fig. 3. Group enriched with *I. galbana* received considerable higher amounts of 20:5n−3 in the diet than the remaining groups, whereas group

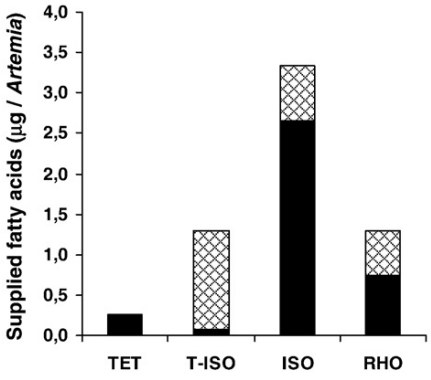

Fig. 3. Total amount of the highly unsaturated fatty acids 20:5n−3 (EPA, ■) and 22:6n−3 (DHA, ⊠) supplied to 2-day old juvenile *Artemia* for 26 h for the enrichment with the different microalgal species.

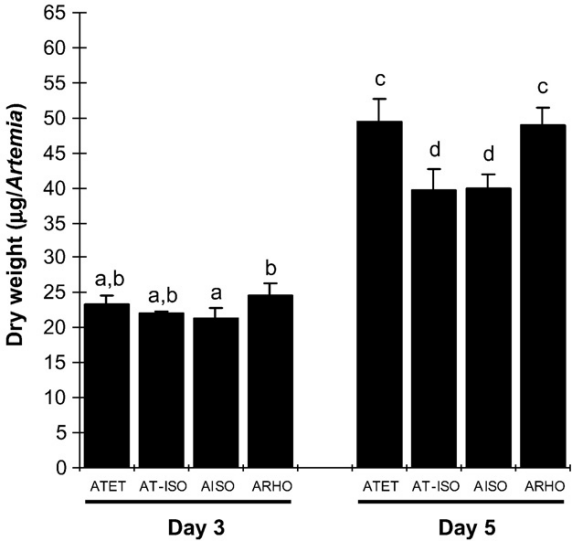

Fig. 4. Dry weight (µg *Artemia*$^{-1}$) of 3 and 5-day old juvenile *Artemia* enriched with different microalgal species for 26 h. ATET: group enriched with *T. suecica*; AT-ISO: group enriched with *I. galbana* T-ISO; AISO: group enriched with *I. galbana*; ARHO: group enriched with R. lens. Data are means ± S.D. Different letters within the same day indicate significant differences among groups ($P<0.05$).

enriched with T-ISO received much more 22:6n-3 than the other groups. Four-day old juvenile *Artemia* received 15% more food than 2-day old juveniles since individuals were bigger.

3.2. Growth and survival of enriched Artemia juveniles

The enrichment of *Artemia* with the different microalgal species for 26 h, at days 2 and 4, did not cause any significant difference in the survival of individuals and was higher than 95% in all treatments. On the other hand, significant differences in the dry weight of *Artemia* juveniles could be observed (Fig. 4) with only 26 h of enrichment period, with differences being clearer among 5-day old juveniles. Regarding the total length of *Artemia* juveniles, significant differences among groups were observed only for 3-day old juveniles (Table 3). Individuals enriched with *R. lens* (group ARHO) or with *T. suecica* (ATET) were larger than those enriched with T-ISO (AT-ISO) or with *I. galbana* (AISO) ($P<0.001$ for comparisons with group ARHO; $P<0.01$ for group ATET).

3.3. Biochemical composition of Artemia juveniles

The enrichment process modified the biochemical composition of *Artemia* juveniles and important differences were observed among groups (Table 3). In 3-day old juvenile *Artemia*, protein content was roughly the same in all groups (51% of dry weight), with the exception of individuals from group AISO (41%) ($P<0.01$). The highest lipid percentages were found in juveniles from groups AT-ISO and ARHO (circa 16%), whereas groups AISO and ATET had 10 and 13%, respectively (Table 3). Juveniles from groups AISO and AT-ISO had almost the same percentage of carbohydrate (11%), which was higher than the values found in juveniles from groups ARHO or ATET ($P<0.05$).

The protein percentage in 5-day old juvenile *Artemia* ranged between 64 and 68%, and in contrast to 3-day old juvenile, no significant differences were observed among treatments. As for lipid, juveniles from group AT-ISO had 17.5%, followed by groups ATET and AISO (both with circa 15.5%) ($P<0.05$), whereas juveniles from group ARHO had the lowest lipid content (10.1%) ($P<0.001$). The lowest carbohydrate percentage was found in individuals from group ARHO (6.1%) ($P<0.01$), whereas juveniles from groups AISO, AT-ISO and ATET had considerable more carbohydrate (Table 3). The protein/energy ratio (P/E, expressed in mg protein kJ^{-1}) in 3-day old juveniles varied between 25 and 27, and increased to 27–31 in 5-day old juveniles (Table 3). Maximum P/E ratio was observed in juveniles enriched with *R. lens* (31.4).

The total FA composition of 3 and 5-day old juvenile *Artemia* reflected to a certain extent the FA composition of the ingested microalgae (see Tables 2 and 4). In small juveniles (3-day old), palmitic acid (16:0) was the major FA found in groups ATET, AT-ISO and AISO (23–33%), whereas group ARHO had 18:3n−3 as main FA (27%). The sum of saturated FA found in small juveniles (40–48%) was similar to values found in *O. vulgaris* hatchlings (42%), except in juveniles from group ARHO which had a lower value (33%) (Table 4). In these juveniles only group ARHO had similar percentages of total monoenes as in hatchlings (14.3%), whereas the other groups roughly doubled this percentage. The higher percentage of PUFAs found in group ARHO (50%) compared to the other groups was mainly due to the presence of 18:3n−3 in its composition (as observed in *R. lens*). Arachidonic acid (20:4n−6) was present in very low percentages in small juveniles (≤0.4%) compared to octopus hatchlings (3.2%). Important differences in the percentages of 20:5n−3 and 22:6n−3 were found among small juvenile *Artemia*. Juveniles from groups AISO and ARHO had significantly higher values of 20:5n−3 (nearly 9%) than groups ATET (4.3%) and AT-ISO (2.6%) (Table 4). The highest percentage of 22:6n−3 was found in groups AT-ISO and AISO (1.9% and 1.5%, respectively), followed by group ARHO (1.0%) ($P<0.05$). No 22:6n−3 was found in juveniles from group ATET. When comparing these values with the percentages of 20:5n−3 and 22:6n−3 found in *O. vulgaris* hatchlings, remarkable differences could be observed. Hatchlings contained 14.5% of 20:5n−3, which was higher than the values found in small *Artemia* juveniles, whereas the percentage of 22:6n−3 was ten-times higher than the best percentage found in small juveniles (group AT-ISO). The ratio DHA/EPA in small *Artemia* juveniles also reflected the imbalance that exists compared to octopus hatchlings. The amount of total FA (expressed as % of the dry weight) was quite similar between hatchlings (4%) and *Artemia* juveniles, except in group AT-ISO which had a higher value (6.6%) (Table 4).

Five-day old *Artemia* (large juveniles) showed some differences in their FA composition when compared with 3-day old juveniles. The saturated FA 16:0 was clearly the major FA found in all *Artemia* groups,

Table 3
Gross biochemical composition (% of dry weight), energy (J *Artemia*$^{-1}$), protein/energy ratio (P/E, mg protein kJ^{-1}) and length (mm) of juvenile *Artemia* enriched with different microalgae produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures

Group	ATET	AT-ISO	AISO	ARHO
3-day old Artemia				
Total length (mm)	1.7 ± 0.2^{a}	1.6 ± 0.2^{b}	1.5 ± 0.2^{b}	1.8 ± 0.2^{a}
% Protein	50.4 ± 1.2^{a}	50.7 ± 0.4^{a}	41.3 ± 1.4^{b}	50.7 ± 1.7^{a}
% Lipid	12.8 ± 1.2^{a}	16.1 ± 1.0^{b}	10.1 ± 0.7^{c}	15.9 ± 0.4^{b}
% Carbohydrate	9.3 ± 0.2^{a}	10.7 ± 0.3^{b}	11.0 ± 0.9^{b}	8.4 ± 0.4^{a}
Energy	0.44	0.45	0.34	0.49
P/E ratio	26.9	24.9	26.2	25.5
5-day old Artemia				
Total length (mm)	3.2 ± 0.3^{a}	3.1 ± 0.4^{a}	3.2 ± 0.4^{a}	3.1 ± 0.3^{a}
% Protein	63.6 ± 2.6^{a}	64.7 ± 2.7^{a}	67.7 ± 0.5^{a}	63.7 ± 1.9^{a}
% Lipid	15.6 ± 0.7^{a}	17.5 ± 1.4^{b}	15.5 ± 0.7^{a}	10.1 ± 0.5^{c}
% Carbohydrate	10.3 ± 1.2^{a,b}	10.9 ± 0.3^{b}	9.0 ± 0.6^{a}	6.1 ± 0.4^{c}
Energy	1.17	0.96	0.95	0.99
P/E ratio	27.7	26.6	28.5	31.4

ATET: *Artemia* enriched with *T. suecica*; AT-ISO: enriched with T-ISO; AISO: enriched with *I. galbana*; ARHO: enriched with *R. lens*. Data are means ± S.D., $n=3$. Different superscript letters within the same line and for the same day indicate statistical differences among treatments ($\alpha=0.05$).

Table 4
Fatty acid (FA) composition (% of total FA) and total FA (% of dry weight) of 3 and 5-day old juvenile *Artemia* enriched with different microalgae produced in semi-continuous cultures with a daily renewal of 30% of the volume of cultures and of *Octopus vulgaris* hatchlings

Fatty acid	3-day old *Artemia* (1.5–2.0 mm)				5-day old *Artemia* (3.0–3.5 mm)				*Octopus vulgaris* hatchlings
	ATET	AT-ISO	AISO	ARHO	ATET	AT-ISO	AISO	ARHO	
14:0	0.8±0.1^{a}	11.9±0.4^{b}	2.9±1.0^{c}	1.4±0.6^{a,c}	0.6±0.1^{x}	16.0±1.3^{y}	6.2±1.1^{z}	2.7±0.8^{w}	2.9±0.3
15:0	0.4±0.1^{a}	0.3±0.0^{a}	0.5±0.3^{a}	0.3±0.1^{a}	0.3±0.0^{x}	0.6±0.1^{y}	0.3±0.0^{x}	0.5±0.2^{x,y}	0.3±0.1
16:0	33.1±0.4^{a}	23.4±0.7^{b}	31.3±2.3^{a}	21.6±1.6^{b}	30.4±1.4^{x}	33.5±3.0^{x}	33.9±3.1^{x}	34.5±1.9^{x}	27.6±1.2
16:1n−9	1.8±0.1^{a}	9.2±0.4^{b}	12.4±1.4^{c}	1.9±0.1^{a}	1.3±0.3^{x}	5.2±1.1^{y}	15.7±1.6^{z}	1.6±0.3^{x}	0.8±0.0
16:1n−7	0.9±0.1^{a}	0.1±0.0^{b}	0.3±0.0^{c}	1.3±0.2^{d}	0.3±0.1^{x}	0.3±0.0^{x}	0.2±0.0^{x}	1.2±0.2^{y}	1.1±0.4
16:3n−3	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	3.8±0.3
16:4n−3	2.5±0.4	n.f.	n.f.	n.f.	3.7±1.0	n.f.	n.f.	n.f.	n.f.
18:0	8.2±0.4^{a}	3.9±0.4^{b}	13.0±2.6^{c}	10.2±1.1^{a,c}	4.7±1.2^{x}	5.0±0.6^{x}	6.3±1.3^{x}	10.3±1.2^{y}	11.6±0.3
18:1n−11	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	1.0±0.1
18:1n−9	17.8±0.5^{a}	16.1±0.4^{a}	10.8±1.3^{b}	6.7±0.4^{c}	20.1±1.1^{x}	13.3±1.2^{y}	7.3±1.7^{z}	10.2±1.3^{y,z}	3.2±0.1
18:1n−7	3.0±0.4^{a}	3.5±0.1^{a}	7.0±0.6^{b}	6.4±0.2^{b}	5.9±0.6^{x,z}	3.8±0.1^{y}	5.2±1.0^{y,z}	7.4±0.6^{x}	1.7±0.2
18:2n−6	2.9±0.2^{a}	7.6±0.1^{b}	2.3±0.9^{a}	2.2±0.1^{a}	1.9±0.1^{x}	3.0±0.2^{y}	0.9±0.1^{z}	1.0±0.2^{z}	0.6±0.0
18:3n−3	14.8±0.2^{a}	12.6±0.3^{b}	4.3±0.8^{c}	26.7±0.4^{d}	15.8±1.1^{x}	5.9±0.7^{y}	2.6±0.4^{z}	14.7±1.1^{x}	n.f.
18:4n−3	7.5±0.5^{a}	5.3±0.1^{b}	2.6±0.3^{c}	8.9±0.8^{a}	9.8±1.0^{x}	4.3±0.4^{y}	2.5±0.3^{z}	4.5±0.5^{y}	n.f.
20:1n−9	0.4±0.0^{a}	0.1±0.0^{b}	0.3±0.1^{c}	0.2±0.0^{c}	0.4±0.1^{x}	0.1±0.0^{y}	0.2±0.0^{y}	0.2±0.0^{y}	5.2±0.5
20:3n−6	0.3±0.1^{a}	0.5±0.0^{b}	0.5±0.1^{b}	0.4±0.1^{a,b}	0.2±0.1^{x}	0.5±0.1^{y}	0.3±0.1^{x,y}	0.3±0.0^{x}	n.f.
20:4n−6	0.1±0.0^{a}	0.0	0.0	0.4±0.0^{b}	0.1±0.0^{x}	0.0	0.0	0.3±0.0^{y}	3.2±0.1
20:3n−3	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	1.4±0.0
20:4n−3	0.4±0.0^{a}	0.3±0.0^{a}	0.4±0.2^{a}	1.0±0.1^{b}	0.4±0.1^{x}	0.3±0.0^{y}	0.2±0.0^{y}	0.6±0.0^{x}	n.f.
20:5n−3	4.3±0.3^{a}	2.6±0.1^{b}	9.3±0.2^{c}	8.7±0.9^{c}	3.9±0.7^{x}	2.9±0.5^{x}	14.6±0.7^{y}	7.0±0.9^{z}	14.5±0.3
22:1	0.2±0.0^{a,b}	0.1±0.0^{a}	0.4±0.2^{b}	0.3±0.0^{a,b}	0.1±0.0^{x}	0.3±0.0^{x,y}	0.3±0.1^{y}	0.4±0.1^{y}	1.3±0.3
22:5n−6	n.f.	0.4±0.0^{a}	0.1±0.0^{b}	n.f.	n.f.	0.7±0.3^{x}	0.3±0.0^{y}	n.f.	0.0
22:6n−3	n.f.	1.9±0.1^{a}	1.5±0.3^{a}	1.0±0.1^{b}	n.f.	3.9±0.5^{x}	2.9±0.2^{y}	1.9±0.2^{z}	18.3±0.8
Others	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.8
Saturated	42.5	39.5	47.7	33.5	36.0	55.1	46.7	48.0	42.4
Monoenes	24.1	29.0	31.2	16.8	28.1	23.0	28.7	21.0	14.3
PUFA	30.3	31.2	21.0	49.7	32.1	21.5	24.3	30.3	38.0
n−3	29.5	22.7	18.1	46.7	33.6	21.5	22.8	28.7	34.2
n−6	3.3	8.5	2.9	3.0	2.2	4.2	1.5	1.6	3.8
DHA/EPA	0.0	0.7	0.2	0.1	0.0	1.3	0.2	0.3	1.3
FA (% of DW)	3.9	6.6	3.7	4.1	5.6	4.2	4.1	3.3	4.0

Abbreviations of ATET, AISO, AT-ISO and ARHO are the same as in Table 3. Data are means±S.D. (n=3). n.f.: not found. Values referred as 0.0 are below 0.05. Different superscript letters within the same line and for the same age of juvenile *Artemia* indicate statistical differences among *Artemia* groups (α=0.05).

with values ranging between 30 and 34%. The percentage of PUFA found in groups ARHO and AT-ISO was lower than the values observed in small juveniles, mainly due to a drop in the percentage of 18:3n−3 found in large juveniles. In groups ATET and AISO the sum of PUFA remained roughly the same. Like observed for small *Artemia*, large juveniles contained only minor percentages of 20:4n−6 (≤0.3%) when compared to octopus hatchlings. The percentage of 20:5n−3 found in juveniles from group AISO (14.6%) was similar to values observed in octopus hatchlings and unlike observed for small juveniles, individuals from group ARHO had a lower percentage of 20:5n−3 (7%) than group AISO (P<0.001). As previously observed for small juvenile, no 22:6n−3 was found in group ATET, while group AT-ISO had the highest percentage (3.9%) among groups (P<0.05). The DHA/EPA ratio in juveniles from group AT-ISO was the same as in octopus hatchlings, but it should be kept in mind that the percentages of each HUFA were very different. The amount of total FA present in large juvenile from groups AISO and AT-ISO was within the same range as hatchlings, while groups ARHO and ATET had lower and higher values, respectively (Table 4). When plotting the percentages of 20:5n−3 and 22:6n−3 found in the diet (i.e., microalgal species supplied) and in 3 and 5-day old *Artemia* juveniles, positive linear correlations could be found (Fig. 5).

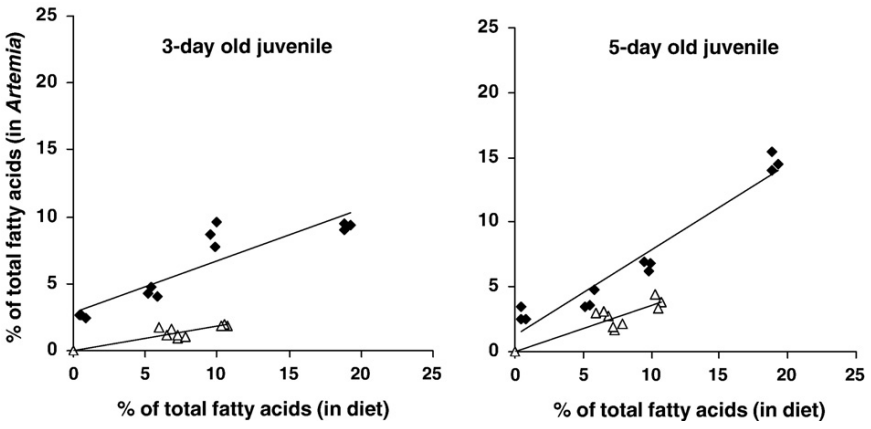

Fig. 5. Relationships between the percentages of the highly unsaturated fatty acids 20:5n−3 (EPA, ♦) and 22:6n−3 (DHA, △) found in the enrichment diets and in *Artemia* juveniles.

For 3-day old juvenile *Artemia* the following equations were found:

$$\%\ \text{EPA} = 0.3834x + 2.8839\ (R^2 = 0.807)$$

$$\%\ \text{DHA} = 0.1748x + 0.0516\ (R^2 = 0.854).$$

For 5-day old juvenile *Artemia*:

$$\%\ \text{EPA} = 0.6659x + 1.2236\ (R^2 = 0.941)$$

$$\%\ \text{DHA} = 0.3499x + 0.0441\ (R^2 = 0.854).$$

It can be observed that both percentages of 20:5n−3 and 22:6n−3 found in *Artemia* juveniles were well correlated with those found in the diets provided for enrichment.

4. Discussion

The production of *Artemia* biomass depends to a great extent on the quantity and quality of the food supplied and on rearing conditions such as temperature among others factors (Sick, 1976; Dhont and Lavens, 1996). In the present work significant differences in the dry weight of 3 and 5-day old juveniles, and in the length of 3-day old juvenile *Artemia*, were observed with different microalgae with only 26 h of enrichment period. Lower growth of *Artemia* juveniles was observed in groups enriched with T-ISO or *I. galbana* when compared to those fed *T. suecica* or *R. lens*. T-ISO was already reported to produce lower growth when compared with *Chaetoceros* sp for the feeding of *Artemia franciscana* (Lora-Vilchis et al., 2004). The total length (TL) and dry weight (DW) of 3 and 5-day old juveniles found in the present work were superior to values reported by Evjemo and Olsen (1999) for *A. franciscana* fed a monodiet of T-ISO: 1.3 mm TL and 3.5 μg individual^{-1} DW for 3-day old *Artemia*; and 2.4 mm TL and 15.9 μg individual^{-1} DW for 5-day old *Artemia*. Naegel (1999) obtained a TL of 1.97 mm for 6-day old *Artemia* fed *Chaetoceros* sp at 25 °C rearing temperature. Growth results in the same range than those reported in the present work were obtained by Lora-Vilchis et al. (2004) for *A. franciscana* fed *Chaetoceros* sp or T-ISO, but cultures were maintained at a slightly higher temperature (27.5±0.5 °C).

Only limited information exists concerning the nutritional requirements of cephalopods' early life stages, though it is generally recognized that diets provided to these fast-growing carnivores must be rich in protein and in essential amino acids (particularly lysine, leucine and arginine), in phospholipids, cholesterol, and particularly in the HUFAs 22:6n−3 and 20:5n−3, and in copper as well (Navarro and Villanueva, 2000, 2003; Villanueva et al., 2004; Villanueva and Bustamante, 2006). Protein percentage in *O. vulgaris* hatchlings and in small wild juveniles was found to be nearly 70% (Villanueva et al., 2004), which suggests the importance of high protein levels in diets for these animals. In fact, the high requirement that cephalopods show for protein is due not only to there rapid growth along all life cycle (3–10% body weigh day^{-1}), but also because they mainly rely on amino acids as source of energy, even for routine metabolism (Lee, 1994). This extraordinary capacity of growing is further explained by their high efficiency of protein retention (up to 90%) as shown for *O. vulgaris* (Houlihan et al., 1990).

In the present work the protein content observed in 3-day old *Artemia* juveniles was circa 51% (except AISO with 41%), which is less than the above mentioned 70% protein found in hatchlings and wild juvenile octopus. However, protein percentages found in 5-day old *Artemia* juveniles ranged between 64 and 68% and seem to be more in accordance with the needs of octopus hatchlings. Andrés et al. (2007) reported a protein content of 13–24% in *Maja brachydactyla* zoeae reared in laboratory, a prey that has given good results for the rearing of octopus paralarvae, but these authors used a methodology specific for determination of soluble protein. Three and 5-day old *Artemia* juveniles enriched with *R. lens* contained the lowest percentages of carbohydrate, reflecting the biochemical composition of the ingested microalga. Since carbohydrate is of minor importance for cephalopods and represents less than 1% of their composition (Lee, 1994), it may be important to minimize this source of energy in diets for paralarvae to avoid an end of food ingestion before the protein requirement is fulfilled.

The content of lipid in 3-day old *Artemia* ranged between 10 and 16%, and similar values were found for 5-day old juveniles (10–17%). Lipid content in *O. vulgaris* hatchlings was found to be nearly 13% of the dry weight (Navarro and Villanueva, 2000) and tended to decrease in small wild juveniles from roughly 12.5 to 6.6% as body weight increased (Navarro and Villanueva, 2003). Total lipid in *Artemia* juveniles was within the same range or in some cases it was superior to values described for octopus hatchlings. The enrichment of *Artemia* with T-ISO gave the highest percentage of lipid in both sizes of juveniles (16 to 17.5%), which may be in excess for the requirements of paralarvae. In the remaining groups the percentage of lipid varied randomly between 10 and 16% in both sizes of *Artemia* juveniles. The optimum protein/energy ratio (P/E, mg protein kJ energy^{-1}) for fishes and aquatic crustaceans has been reported to be 20–30 (reviewed by Lee, 1994), which is much higher than that of homoeothermic vertebrates (10–15), but far from the best P/E ratio of 50 found by that author to achieve maximum growth of the cuttlefish *Sepia officinalis*. The P/E ratio in 3-day old juveniles ranged between 25 and 27, whereas in 5-day old juveniles it increased to 27–29, attaining a maximum ratio of 31.4 in juveniles enriched with *R. lens*.

The FA composition of *O. vulgaris* hatchlings found in the present work was similar to data previously reported by other authors, even though some differences were found. The following percentages of the FAs 16:0 (27.6%), 20:5n−3 (14.5%) and 22:6n−3 (18.3%) were observed in the present work, in comparison to the percentages described by Navarro and Villanueva (2000) (17.5, 12.6, and 21.2%, by same order of FAs); or by Okumura et al. (2005) (18.6, 17.7, and 27.0%, respectively). The percentages of 20:5n−3 and 22:6n−3 found in 3 and in 5-day old juvenile *Artemia* enriched with *I. galbana* and *R. lens*, were similar or even higher than the values described by Navarro and Villanueva (2000) for 1–3 mm *Artemia* enriched with commercial lipid emulsions (20:5n−3≈7 to 8% and 22:6n−3≈2%). Other authors found similar percentages of 20:5n−3 (15%) and 22:6n−3 (0.4%) in 1.6–1.8 mm *Artemia* juveniles enriched for 24 h with *Chaetoceros muelleri* (Ritar et al., 2004). Five-day old juveniles enriched with T-ISO contained the maximum percentage of 22:6n−3 (3.9%) found in the present work, which is similar to values described by Ritar et al. (2003) in 1.5 mm and 2.5 mm *Artemia* enriched with the same microalgal species (1.2 to 3.4%). The evidence that both size of juvenile *Artemia* enriched with T-ISO contained such high percentage of 20:5n−3 (circa 3%) compared to the percentage of 20:5n−3 found in the correspondent microalgal ingested (0.7%) could be partially explained by the capacity of *Artemia* sp to retroconvert 22:6n−3 to 20:5n−3, as described by Navarro et al. (1999). Higher percentages of 22:6n−3 and 20:5n−3 have been obtained by enriching *Artemia* nauplii with commercial lipid emulsions or marine oils (Smith et al., 2002; Bell et al., 2003); however, these products are often quite low in protein and much more susceptible to peroxidation and rancidity during the enrichment period, in addition to generating high levels of contaminant bacteria in *Artemia* (McEvoy et al., 1995; Ritar et al., 2004). Moreover, the use of commercial lipid emulsions designed for nauplii for the enrichment of *Artemia* juveniles for 24 h caused high mortality (>90%, unpublished results), as also described by other authors (Ritar et al., 2004), indicating the need for the development of specific enrichment emulsions for this purpose. Enrichment of juvenile *Artemia* with those products should therefore be considered as useful to enrich only a proportion of the juvenile *Artemia* supplied to paralarvae, to provide

the necessary amount of n−3 HUFA, but it is advisable to carry out short time enrichment periods (i.e.: 6–12 h) in order to avoid peroxidation processes (McEvoy et al., 1995) and also mortality of juvenile *Artemia*. Sargent et al. (1999) suggested the enrichment of nauplii for early feeding of marine fish larvae with highly purified preparations of 22:6n−3 and 20:5n−3 or the use of single cell triacylglycerol oils (e.g. from the heterotrophic dinoflagellate *Crypthecodinium cohnii*, or others) as sources of single HUFA, rather than using commercial fish oils which contain only moderate levels of n−3 HUFA.

The concept of "enrichment" is generally associated with a period of exposure (e.g. 12–24 h) to a certain diet that is supposed to modify principally the gut content of the live prey. Due to the high filtration capacity of *Artemia* juveniles, this type of enrichment can be achieved in much shorter periods (1–4 h) than for the enrichment of nauplii (Dhont and Lavens, 1996). Longer enrichment periods of juveniles will allow the incorporation of the diet compounds to the body tissue as well, producing a more stable enrichment, even though some degradation/conversion of essential nutrients can be produced (Navarro et al., 1999; Dhont and Lavens, 1996). In our case, as demonstrated by differences in the dry weight of the *Artemia* juveniles depending on the algal diet during the 26 h enrichment period, the "enrichment" process included both gut content and changes in the body tissues of the *Artemia*. A similar result was obtained for the enrichment of the rotifer *Brachionus plicatilis*, since changes in the body composition of rotifers enriched for 24 h could hardly be explained by the concept of rotifers acting as simple "carriers" of ingested microalgae (Ferreira et al., 2008).

In conclusion, the fatty acid composition of juvenile *Artemia* enriched with microalgae was far from the adequate fatty acid requirements of *O. vulgaris* paralarvae, mainly due to the very low percentages of 22:6n−3 observed in *Artemia* juveniles. However, since *Artemia* will continue to play an important role as prey in the rearing of octopus paralarvae, while efforts to develop inert micro-diets are done, the variability generated in the biochemical composition through the use of different microalgal species highlights the importance of optimising its biochemical composition, in particularly the P/E ratio and its HUFA composition. Juveniles enriched with *R. lens* contained the best general composition as prey for paralarvae, due to high protein content, low carbohydrate and moderate lipid levels, as well as high levels of HUFA among the juveniles analysed. Yet, the highest sum of the HUFA 20:5n−3 and 22:6n−3 was found in *Artemia* juveniles enriched with *I. galbana*. The identification of alternative live prey, the formulation of suitable and digestible micro-diets for octopus paralarvae and the improvement of the biochemical composition of *Artemia* have all been reported as key issues for the future development of *O. vulgaris* early life-stage rearing (Iglesias et al., 2007), together with further studies on zootechnical conditions. The improvements obtained in the biochemical composition of *Artemia* through the use of selected microalgal species produced under controlled conditions will provide an understanding tool for the study of nutritional requirements of *O. vulgaris* paralarvae.

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