UNIVERSIDADE DO ALGARVE

FORMULATION OF FEEDS FOR Octopus vulgaris SUB-ADULTS IN LAND-BASED CONDITIONS

Tania Rodríguez González

Dissertation to obtain the degree in

Masters in Aquaculture and Fisheries

Speciality in Aquaculture

Thesis coordinated by:

António de Vilhena Andrade Ferreira Sykes, PhD

Jesús Cerezo Valverde, PhD

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(Tania Rodríguez González)

Acknowledgments

“El futuro tiene muchos nombres.

Para los débiles es lo inalcanzable.

Para los temerosos, lo desconocido.

Para los valientes es la oportunidad”

(V.M. Hugo)

La oportunidad…

Estas líneas van dirigidas a todos aquellos que me han dado “la oportunidad” y me han ayudado, de una forma u otra, a realizar este trabajo:

- En primer lugar, al Dr. António de Vilhena Andrade Ferreira Sykes, coordinador interno de esta tesis, por su ayuda constante desde que entré en su despacho por primera vez. Mil gracias por la paciencia, la orientación, el interés y el seguimiento de mi trabajo a lo largo de estos meses.
- Al Dr. Jesús Cerezo Valverde, coordinador externo de esta tesis, por hacer posible mi incorporación en su equipo; por enseñarme a hacer frente a la parte experimental y analítica de este trabajo; así como por su orientación cuando “estaba más perdida que un pulpo en un garaje”.
- Al Dr. Benjamín García García, director del IMIDA, por permitir mi incorporación temporal en el centro y dedicarme diariamente un “hola amiga” acompañado de una sonrisa.
- A los que fueron mis compañeros durante mi estancia en el IMIDA. Irene, Ángel P. y Ángel gracias por amenizarme las mañanas y hacer escandalosamente divertidas las rutinas diarias. Piedad, Ángel H. y Mar gracias por ayudarme a sobrellevar la saturación mental que por momentos se apoderaba de mí. Mari Carmen y Cristino gracias por guiarme por el laboratorio. Pepe gracias por tu tranquilidad y serenidad todos y cada uno de los días.
- A mis padres y mi tía por creer en mis objetivos. En especial a mi madre por su dedicación, apoyo y confianza incondicional.

- A mis amigos y compañeros de toda la vida por seguir mi rumbo. A los de la Universidad de Vigo por haber compartido conmigo los primeros años, especialmente a Manoli. A los de la Universidad de Baja California: en especial a Mundo, Vincent y Roberto por haberme metido “el gusano” de la acuicultura en el cuerpo. A mis compañeros del Máster: Luis, Calisto, Tânia, Dani y Mârcia por estos dos últimos años de convivencia y vivencias, dentro y fuera de la Universidad. Y en especial a Elena y a mis “meninas portuguesas” por darme ánimos, sufrir conmigo e intentar sacarme una sonrisa de desahogo en todo momento.
- Y ya para terminar, le dedico este trabajo a mis Gordos. A Breogán, mi novio; al que debo agradecerle su comprensión, su apoyo, su cariño y su saber esperar todos estos años solo para verme feliz cumpliendo mis objetivos. Y como no, a Foski, mi gato y fiel compañero, por la infinidad de horas que ha pasado encima de mí mientras escribía este texto.

A todos, muchas gracias por acompañarme en el camino.

This thesis was funded by:

- "Aplicación de los nuevos avances en nutrición, desarrollo de piensos y diseño de instalaciones al engorde de pulpo (*Octopus vulgaris*) en tierra y mar abierto." Nº Project: RTA2012-00072-00-00. Entity: Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario.
- SEPIATECH – Development of cuttlefish, *Sepia officinalis*, aquaculture production technology - feed and husbandry refinements. DGPA – PROMAR. 31.03.05.FEP.002
- SEPIABREED - Improving the reproduction of the European cuttlefish, *Sepia officinalis* (Linnaeus, 1758) in captivity: a multidisciplinary approach - PTDC/MAR/120876/2010.

Resumo

A aquacultura mediterrânica necessita diversificação e o polvo comum, *Octopus vulgaris*, é visto como uma alternativa viável e interessante devido ao seu valor de mercado e de consumo elevado não só nesta região mas também na Ásia e Oceânia. Além disso, possui um ciclo de vida curto (12-18 meses); crescimento rápido com taxas que rondam cerca de 5% do seu peso por dia bem como cerca de 13% de incremento de peso por dia em juvenis; elevadas taxas de conversão nas quais se verificam incorporações do alimento ingerido em peso na ordem dos 30-60%; fecundidade elevada (100.000-500.000 ovos por fêmea); elevado conteúdo proteico (70-90% do seu peso seco); boa adaptação a condições de cativeiro; boa aceitação de alimentos inertes; e resistência a manuseamento e transporte.

O polvo é já produzido em Espanha (principalmente na Galiza) desde a década de 1990, através de engorda de juvenis capturados na natureza. No entanto o cultivo completo da espécie enfrenta ainda alguns problemas que impedem um concretizar efectivo deste potencial, dos quais a existência de uma dieta inerte que diminua a logística e os custos de operação que viabilize deste modo o seu cultivo industrial. Parte deste problema deve-se a um conhecimento restrito da fisiologia do polvo e de outros cefalópodes bem como à capacidade cognitiva do próprio animal. Apesar de se terem realizados vários ensaios experimentais no sentido de resolver esta questão desde a década de 1990, não existe ao momento uma dieta para qualquer cefalópode que seja aceite e permita crescimentos pelo menos de cerca de metade do verificado com alimento natural. Nos últimos anos, tem sido feito um esforço adicional no sentido de desenvolver esta dieta, utilizando-se para tal espécies de peixe resultantes de rejeição de pesca e de aquacultura como matéria-prima. Um exemplo é a boga, *Boops boop*s, que surgiu como dieta alternativa utilizada para engordar o polvo. No entanto, a utilização desta e outras matérias-primas numa preparação tradicional de formulação das dietas, que inclui processamento térmico de cozimento, parece influir com a aceitação e crescimento do polvo devido à desnaturação proteica que ocorre nesse processo. Para mitigar este problema, foram recentemente testadas dietas que foram liofilizadas. No entanto, o processo de liofilização implica logística e custos associados que são elevados para serem implementados num futuro processo de preparação de dietas para polvo e, eventualmente, outros cefalópodes. Nesse sentido, o objectivo desta tese foi testar o uso de duas dietas semi-húmidas, que possuem como base farinhas liofilizadas de lula e caranguejo, testando a variável processamento térmico na preparação de farinha de boga (liofilizado - FDb vs. desidratação a temperatura inferior a 60ºC - Mb) no cultivo de juvenis

de polvo comum, *O. vulgaris*. Foi objectivo secundário da tese, verificar a adequabilidade de realizar um protocolo de alimentação semanal que inclua dias não consecutivos sem alimentação (2 vs. 3) com a dieta FDb.

Na experiência 1, testou-se o efeito do uso de boga liofilizada (FDb) ou boga desidratada a menos de 60ºC (Mb), através da inclusão de uma ou outra na preparação de uma dieta semi-húmida. Além de um destes, esta dieta semi-húmida incluía as seguintes matérias-primas: lula (*Todarodes sagittatus*) e caranguejo (*Carcinus mediterranus*) liofilizados; óleo de peixe, glucose, gema de ovo, e gelatina e amido como ligantes. Foi verificado o crescimento, eficiência da dieta, digestibilidade e condição. Ambas as dietas tiveram valores similares de composição proximal e de desagregação ($P>0.05$); sendo que apenas foram marginalmente diferentes em termos de conteúdo proteico (66.14±0.01% e 69.98±0.31% para FDb e Mb, respectivamente) e de cinza (5.77±0.02% e 6.20±0.04% para FDb e Mb, respectivamente). Os polvos aceitaram ambas as dietas, produziram fezes e nenhuma mortalidade foi registada. O crescimento específico (SGR) foi semelhante em ambos os grupos (0.78±0.19 e 0.85±0.09 $\%BWday^{-1}$ para FDb e Mb, respectivamente) bem como a eficiência da dieta (48.31±9.70% e 39.22±2.92% para FDb e Mb, respectivamente). Não foram também encontradas diferenças relativamente aos valores de rácio de conversão da dieta (FCR) e valores produtivos de proteína (PPV) e de lípido (LPV). No entanto, foram verificados diferenças significativas ($P<0.05$) ao nível da taxa absoluta e específica de ingestão (AFR e SFR) e no índice da glândula digestiva (DGI). Nomeadamente, foram verificadas AFRs e SFRs superiores bem como uma DGI superior no grupo Mb. A composição proximal das fezes também apresentou diferenças ao nível do conteúdo de proteína, lípido e mineral ($P<0.05$), que se reflectiram em diferenças verificadas ao nível do coeficiente aparente de digestibilidade (ADC) dos diversos nutrientes. No entanto, não se verificaram qualquer tipo de diferenças na composição proximal dos tecidos estudados (musculo, glândula digestiva e do animal inteiro). Os resultados desta experiência revelam que não existe perda de qualidade da proteína quando se faz desidratação a valores de temperatura inferiores a 60ºC, já que os resultados obtidos foram na generalidade idênticos. No entanto, o processamento térmico que não inclui liofilização poderá eventualmente provocar oxidação lipídica o que poderá explicar as poucas diferenças observadas.

Na experiência 2, testou-se o efeito de realizar dois tipos de protocolos de alimentação distintos: o primeiro (2FDb) incluía dois dias não contínuos em que não se alimentavam os polvos e o segundo 3 dias não contínuos (3FDb). Ambos os protocolos incluíam alimentação com a dieta FDb da experiência anterior. Tanto a ingestão semanal como o crescimento foram similares entre os grupos (P>0.05), apresentando valores médios de SGR inferiores a 1%. Verificaram-se diferenças na eficiência da dieta (FE), sendo a FE e o PPV superiores no grupo 3FDb. No entanto, a digestibilidade da dieta e a composição proximal das fezes e dos tecidos analisados registaram valores idênticos em ambos grupos. Estes resultados sugerem que a inclusão de 3 dias não consecutivos de jejum no protocolo de alimentação poderá ser uma opção interessante no cultivo industrial do polvo já que os valores de eficiência da dieta (FE) foram superiores e de conversão (FCR) estiveram próximos de ser estatisticamente inferiores no grupo 3FDb. Além disso, os resultados parecem apontar para a eventual capacidade da espécie em compensar os dias de jejum através de um incremento na ingestão no dia subsequente. Este protocolo poderá assim promover um uso de menor quantidade de comida e dos custos de alimentação associados.

Palavras-chave: *Octopus vulgaris*; Crescimento; Dietas semi-húmidas; Digestibilidade; Eficiência de dieta; Jejum de curta duração.

Abstract

In the present study growth, feed efficiency, digestibility and condition of *O. vulgaris* fed two different diets (FDb and Mb); and the effects of two (2FDb) or three starvation days (3FDb) per week feeding with FDb diet were analyzed. The diets were formulated using freeze-dried ingredients and only differed on bogue preparation; freeze-dried (diet FDb) or meal prepared under 60˚C (Mb). Both diets were accepted, promoted growth and faeces production with 100% of survival. No significant differences were found in growth (SGR of 0.78±0.19 %BWday^{-1} for FDb and 0.85±0.09 %BWday^{-1} for Mb) and feed efficiency (48.31±9.70% and 39.22±2.92% for FDb and Mb, respectively) or PPV and LPV. Despite the similarity on FCR ($P>0.05$), a better mean value was obtained by FDb (2.15±0.47) compared to Mb (2.56±0.19). However, statistical differences were identified on ingestion and DGI. In this sense, a higher ingestion were found on Mb group regarding to AFR ($P<0.01$), APFR and ALFR ($P<0.01$) and SFR ($P<0.05$). The animals of Mb showed the highest DGI (6.75±1.00 %). Faeces proximate composition differed between groups in protein ($P<0.05$), lipid ($P<0.05$) and mineral content ($P<0.01$), which were reflected on differences on ADC of nutrients ($P<0.01$). Nonetheless, the proximate composition of tissues were similar between both groups ($P>0.05$). Growth and ingestion did not differ with starvation protocols ($P>0.05$). FE displayed differences ($P>0.05$); the better FE and PPV were performed by 3FDb (58.65±6.47% and 23.90±2.61%, respectively). Digestibility and proximate composition of faeces and tissues were also similar ($P>0.05$). The results revealed no loss on diet quality when dehydration is performed under 60˚C which point out the suitability of bogue-meal for *O. vulgaris* feeds. It seems that growth and, survival and proximate composition were not influenced by the applied starvation/feeding protocols. It also seems that O. vulgaris has the ability to compensate starvation through an increase in food intake on the subsequent day. The application of feeding protocols that include starvation days might be an interesting option for industrial application since FE and FCR were enhanced. This will promote a reduction in operational costs, such in manpower and food.

Keywords: *Octopus vulgaris*; Semi-moist diets; Growth; Feed efficiency; Digestibility; Short-term Starvation.

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Materials and methods

Results

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Abbreviations

ADC_N	Apparent Digestibility Coefficient for nutrients
AIA	Acid Insoluble Ash
AFR	Absolute Feeding Rate
AyFR	Absolute protein/lipid Feeding Rate
AGR	Absolute Growth Rate
BW_{est}	Body Weight estimation
BW_{est4}	Body Weight estimation for the 4th day of the week (Thursday)
C	Carcass
CH	Carbohydrates
CIMA	Consellería de Pesca e Asuntos Marítimos
CIFAP	Centro de Investigación y formación Acuícola y Pesquera
CSIC	Consejo Superior de Investigaciones Científicas
DAH	Days After Hatching
DG	Digestive Gland
DGI	Digestive Gland Index
DG_w	Digestive Gland weight
DHA	Docosahexaenoic acid
DW_i	Initial Dry Weight
DW_f	Final Dry Weight
EPA	Eicosapentaenoic acid
EU	European Union

F	Correction factor
FAO	Food and Agriculture Organization of the United Nations
FCR	Food Conversion Ratio
FDb	Freeze-dried bogue based diet
FE	Feed Efficiency
FRA	France
GER	Germany
I.E.O	Instituto Español de Oceanografía
IF	Ingested food
IFAPA	Instituto Andaluz de Investigación y Formación Agraria y Pesquera, Alimentaria y de la Producción Ecológica
IFR	Instantaneous Feeding Rate
IMIDA	Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario
IRTA	Institut de Recerca i Tecnología Agroalimentàries
M	Muscle
Mb	Meal bogue based diet
NET	Netherlands
NFE	Nitrogen-free extract
POL	Poland
P/E	Protein-Energy ratio
SFR	Specific Feeding Rate
SGR	Specific Growth Rate
SPA	Spain

UK	United Kingdom
USD	United States Dollar
W_a	Average weight
W_i	Initial weight
W_f	Final Weight
WFR	Weekly Feeding Rate
Wg	Weight gain
WSI	Water Stability Index / Disaggregation
yPP	Lipid/protein Productive Value
2FDb	Two day starvation protocol fed with freeze-dried bogue based diet
3FDb	Three day starvation protocol fed with freeze-dried bogue based diet

1. Introduction

1.1. Current status of world aquaculture

Aquaculture is a millennia practice apparently originated in ancient China and Mesopotamia 3,000 to 4,000 years ago. It can be defined as farming of aquatic organisms – including fish, molluscs, crustaceans and aquatic plants - under controlled or semi-controlled conditions which imply some practices – stocking, feeding, protection against predation, harvesting - in order to enhance growth, development and production (Barnabé, 1991; FAO, 1997; Stickney, 2005).

At present, global fishery captures are stabilized around 90 million tons per year (FAO, 2012). This is a trend verified since 2000 (Fig. 1.1.1), due to the decline suffered by fisheries stocks at worldwide level. Indeed, the latest FAO report highlights the poor state of fisheries, being 87% of world's fish stocks fully exploited, overexploited or depleted.

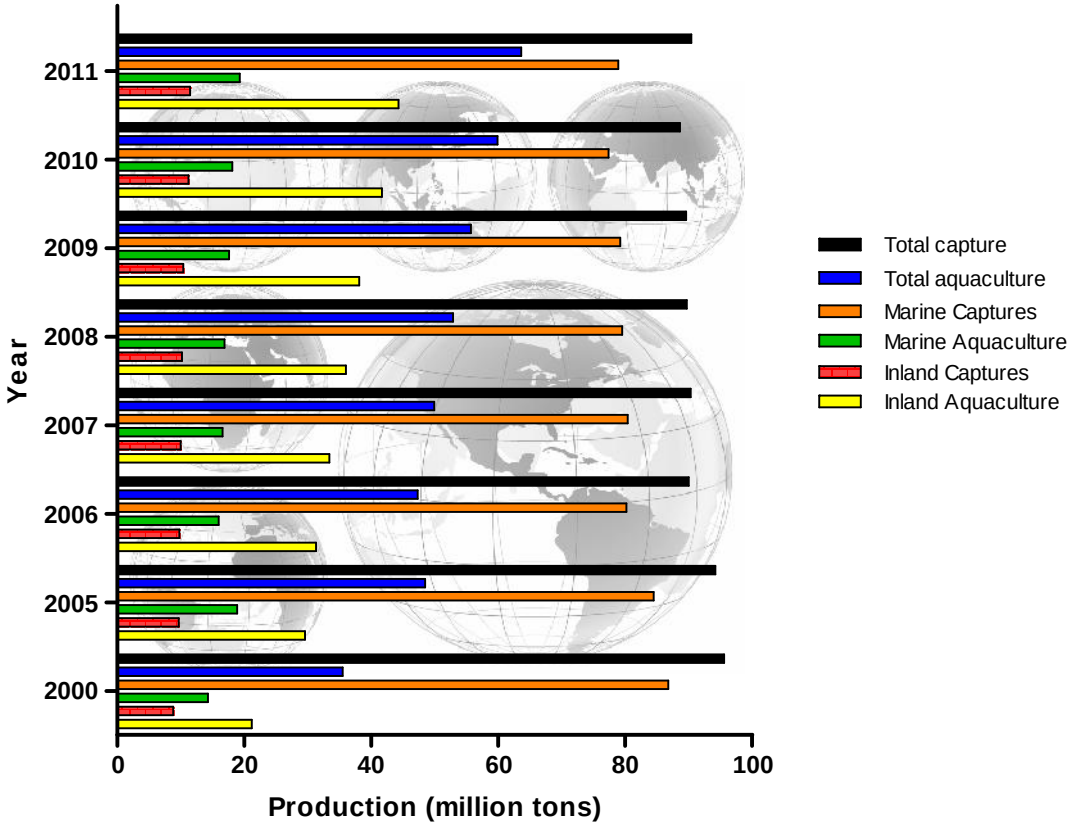


Figure 1.6.1. World Fisheries and Aquaculture Production Statistics, (FAO, 2004; 2009; 2012).

Nonetheless, according to FAO (2009), world food production will need to increase by 70% between 2005/07 and 2050 to support the demands created by the increasing world population, globalization and the connection between markets. Since fisheries production has reached its maximum, the only way of meeting these demands is by aquaculture. Undeniably, aquaculture should be considered not only as an aid to the sustainability of fisheries by reducing the pressure of overfishing on wild stocks but also as a proposal as farming activity with the greatest potential (FAO, 2012; Kura et al., 2004). In fact, the importance of aquaculture can be summarized in the following sentences: "*Give a man a fish and he will have food for a day, teach a man how to fish and he will have food for a lifetime*" (Chinese proverb); so, analogously: "*Teach a man how to grow fish and he can feed the world*".

At present, around 50% of consumed seafood products come from aquaculture but FAO expects an increase above 65% by 2030. Aquaculture contribution to global fisheries production has been increasing gradually since the beginning of this activity: 9% in 1980, 20.9% in 1995, 32.4% in 2005, and 40.3% in 2010 (FAO, 2004; 2012). Since 1970, aquaculture has expanded, diversified its production and improved in technology to occupy the production position of fisheries. Overall aquaculture's year production has doubled in the last decade (2000-2010) to 41.30% of total world production. Recent data (2011) point to a 55.8% increase in total aquaculture production compared with reported data for 2000 (Fig. 1.1.1). During the same period, inland aquaculture production has doubled while marine aquaculture has increased gradually by 25.0%, representing 12.5% of total world production in 2011 (Fig. 1.1.1). Nowadays, aquaculture production per person is higher than that of fisheries, namely 3.6 and 2.3 tons per person per year, respectively (FAO, 2012).

Nearly the entire aquaculture production is for human consumption. Edible fish production has increased 12 times at an annual rate of 8.8% in the last thirty years; while the overall aquaculture production grew more slowly. In 2010, aquaculture production attained an historical maximum production of 59.9 million tons of edible fish, valued as 119,400 million USD. If aquatic plants and non-edible products were included, the total amount would sums up to 79 million tons and 125,000 million USD. Global aquaculture volume production is focused on fresh water species (33.7 million tons), molluscs (14,2 million tons), crustaceans (5.7 million tons), diadromous fish (3.6 million tons), marine fish (1.8 million tons) and others (814,300 tons) [Fig. 1.1.2 (FAO, 2012)]. In 2010, global production importance, in terms of value, was 58.1% freshwater aquaculture, followed by 29.2% of marine, and 12.8%

of brackish water located exploitations (FAO, 2012).

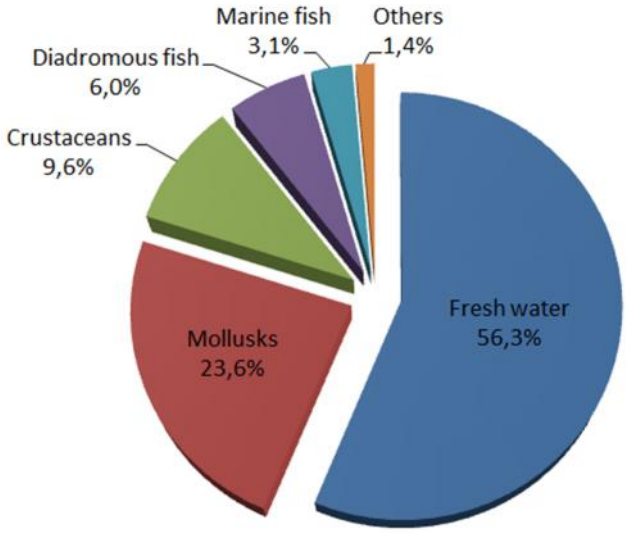


Figure 1.1.7. Global aquaculture volume production (FAO, 2012).

The per capita world consumption of seafood has shown a growing trend in the last half century –from 9.0 kg, in 1961, to 18.5 kg, estimated for 2009. In that same year, European per capita consumption attained 21.9kg, being higher than the world average. (FAO, 2013b).

World aquaculture production is concentrated in Asian countries (91% of world production in 2011), being China the largest contributor with 60% of total world production (47% of global commercial value, which accounts for 64,269 million USD) in the same year, followed by America, Europe, Africa and Oceania (FAO, 2010-2013).

The largest contributors to the European aquaculture production in 2010 (2.5 million tons) was Norway (39.95%), followed by Spain (10.00%), France (8.89%), United Kingdom (7.97%) and Italy (6.08%) (FAO, 2012). In 2011, production raised to 157,817 tons (corresponding to a net value of 11,195 million USD) and represented 3.20% of total global production (FAO, 2010-2013). Marine European aquaculture, was the mainly contributor (82.00% of European production in 2011); which increased 620,177 tons from reported data since 2000. On the other hand, the inland outcomes remained stabilized around 460,000 tons (FAO, 2010-2013). Mariculture and brackish water products increased from 55.6% in 1990 to 81.5% in 2010. From those, three quarters corresponded to finfish production and the

remaining quarter to molluscs; crustaceans and algae aquaculture is reduced (APROMAR, 2012; FAO, 2012).

During the last twenty years aquaculture has developed considerably. The recognition as an alternative to extractive fishery led to the enforcement of several basic and applied researches to increase and diversify the number of exploitable species worldwide. Market oversaturation of certain species being produced, the fast decline of the worldwide fishery stocks and the technological advances over the last 20 years can be identified as other triggers for production diversification (Sarrasquete, 2012). In fact, species diversification, market expansion and the reduction of production costs are strategies that allow greater flexibility to changes in market demand. Produced products, as food and feeds, are well integrated in the current market and global fish trade has grown remarkably. Indeed, trade of fishery goods has increased fourteen times since 1976 to 2010, to an astonishing 109 billion USD. If aquatic plants, non-edible fish fractions, or others are considered, the market grew more 1,300 million USD. The 71% of global exports of fish or fishery products were directly used for human consumption in 2010, highlighting the relevance of world-trade on global feeding. Since 2002, China as attained the position of first worldwide exporter (13,268 and 17,100 million USD in 2010 and 2011, respectively), representing nearly 12% of global trade, followed by Norway with around 4,500 million USD less export value. World imports of fish and fishery products raise 86% from 2000 to 2010, when a maximum of 111,800 million USD was registered. In 2010, the main importing countries of fishery products in the world were the United States of America (USA) and Japan (15,496 and 14,973 million USD, respectively), followed by Spain (6,637 million USD), China and other European countries, such as France, Italy, Germany, United Kingdom and Sweden. In 2011, the USA and Japan, raised their imports up to 17,500 and 17,400 million USD, respectively, followed by China as third, with 7,600 million USD (FAO, 2012).

A consumption increase in the European Union (EU) has converted this region in the largest single market for imported fishery products and fish (FAO, 2012). Interestingly, the EU has traditionally exported much more than it imports. In 2012, exports doubled its volume and almost tripled its economic value registered in 2000. Comparatively, the imports growth rate was less marked over the same period in volume, but grew 60% in value [Fig. 1.13 (Administration, 2013)].

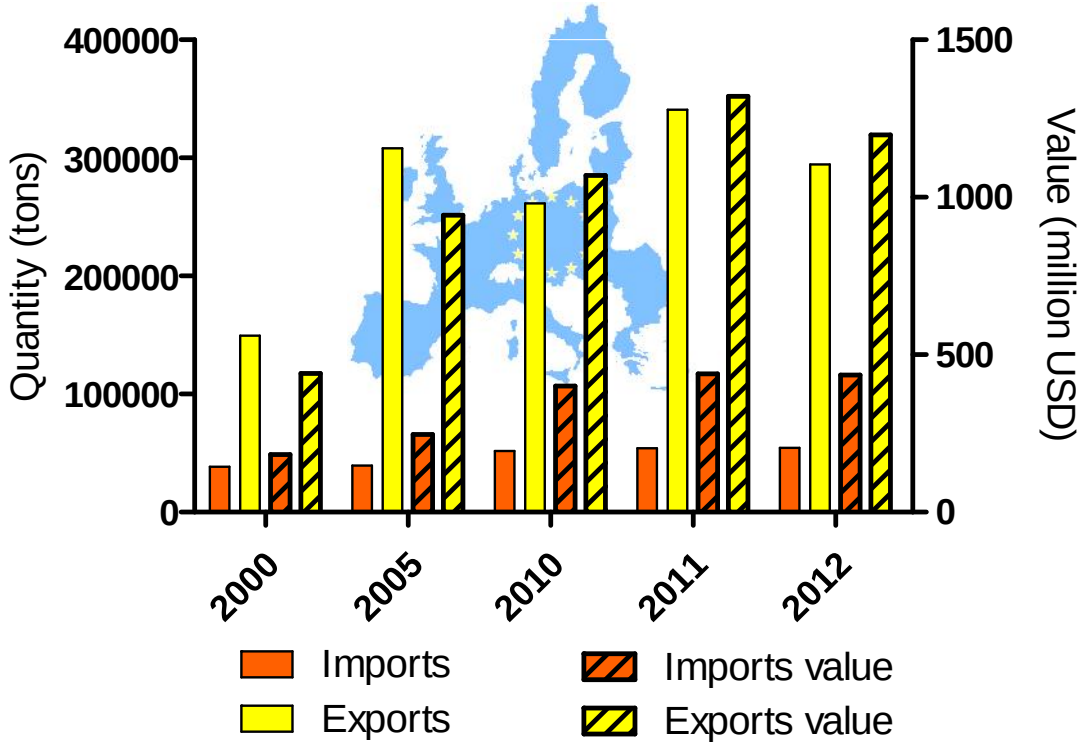


Figure 1.1.8. European Union imports, exports and related values (Administration, 2013).

In 2010, the EU accounted for 40% of total world imports of fishery products in value (44,600 million USD), including community trade. Nevertheless, from those, 23,700 million USD imports were attributed to extra-EU countries – in descending value order of import: Asia, South America, Africa, North America and Oceania -, representing 26% of total world imports. Analogously, in 2011 imports reached 50,000 and 26,500 million USD, including and excluding intra-community trade, respectively (FAO, 2012). In 2010-2011 the United Kingdom (UK), Poland (POL), Spain (SPA) and France (FRA) were the main importer countries of fishery products, corresponding to around 35%, 13% and 12%, respectively, of total EU imports (54,479 tons and 1,198 million USD; Fig. 1.1.4). In 2012, all of these main importers declined, except SPA, which increased up to 16% and attained the second position, only surpassed by UK [32%; Fig. 1.1.4 (Administration, 2013)].

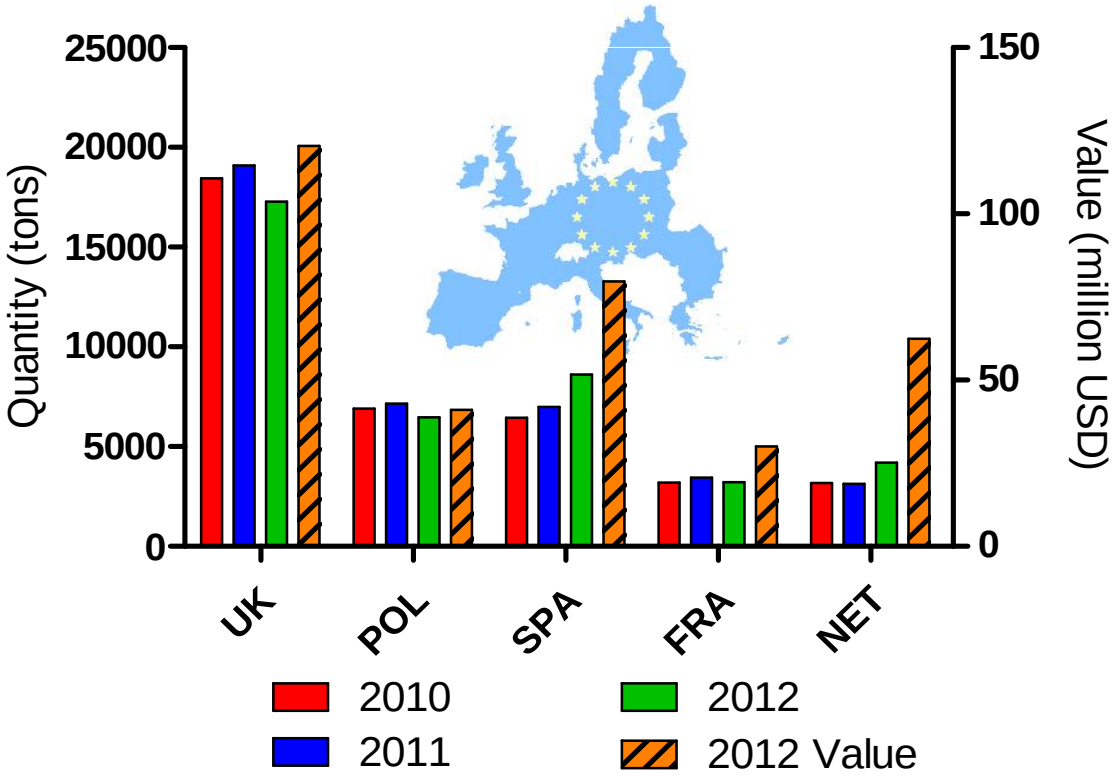


Figure 1.1.9. European Union main import countries, and related value: United Kingdom (UK), Poland (POL), Spain (SPA), France (FRA) and Netherlands (NET); (Administration, 2013)..

It is also interesting that the most valuable imports for 2012 came from the UK (28%), SPA (18%) and NET (14%), Fig. 1.1.4. Analogously, Germany (24%; GER), Netherlands (16%; NET), FRA (13%), SPA (11%) and UK (11%) congregated the economic value of EU exports [Fig. 1.1.5; (Administration, 2013)]. GER (29%) and NET (19%) volume contribution was massively higher than that of other countries, which represented less than 10% of the EU exports volume. Nevertheless, a growing trend was observed, with an EU export maximums being observed in 2011 [Fig. 1.1.5; (Administration, 2013)].

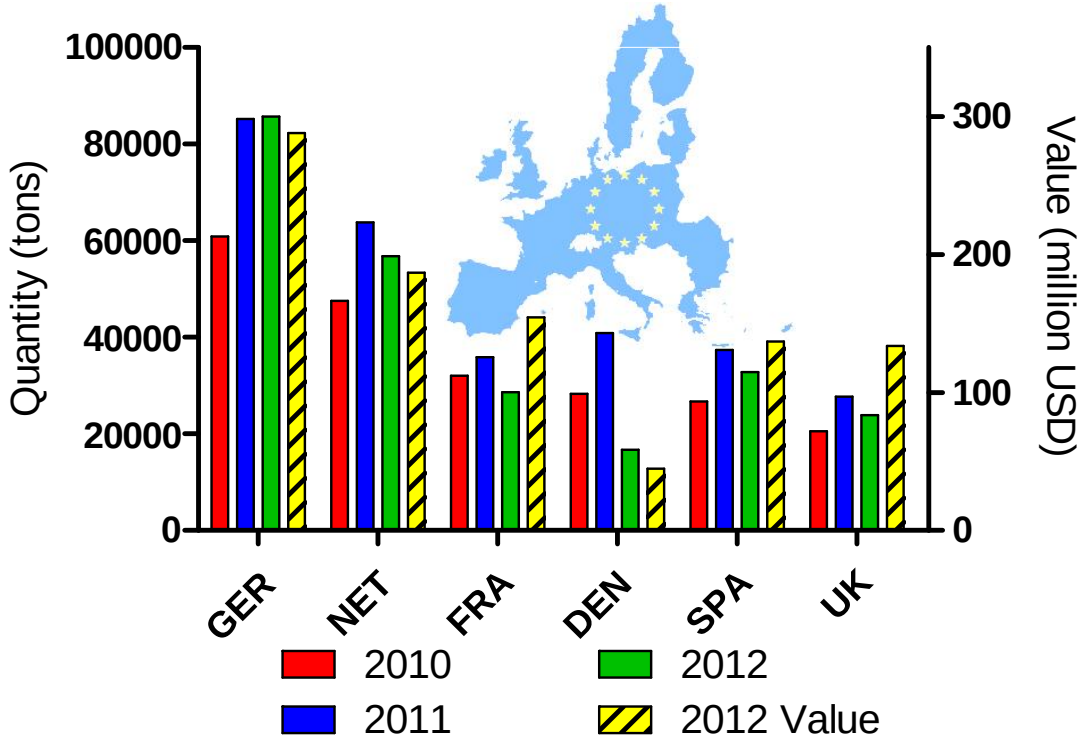


Figure 1.1.10. European Union main export countries and related value: Germany (GER), Netherlands (NET), France (FRA), Denmark (DEN), Spain (SPA) and United Kingdom (UK); (Administration, 2013)

1.2. Aquaculture in Spain

Spain is a peninsular nation, with a large shoreline of almost 8,000 Km that has established a strait association between population, sea and its products. Spanish per capita consumption was the double of World and European average values for 2009 [42.9kg, 18.5kg and 21.9kg per year, respectively (FAO, 2013b)]. Martín (2010) reported a 27.6kg per capita and per year for Spain; where 15.3kg were finfish, 8.3kg seafood, molluscs and crustaceans, and 4.0kg were preserved seafood. According to APROMAR (2012), these corresponded to a preference in consumption of hake, cephalopods and sardine / anchovy, respectively.

Spain is considered a traditionally fishery country with a specific orography and climatology, which translates into physicochemical and environmental advantages for marine aquaculture.

The beginning of Spain's modern marine aquaculture occurs in the 1970's, with the establishment of two private companies (Finisterre Mar and Tinamenor, S.A.). These

companies used to grow molluscs in Galicia (northwest of the country). They started as pilot-scale investments in the 1980's, which were driven by scientific research, until a maturity of the industry was reached in the 1990's, with development of new technologies and industrialization of the sector (FAO, 2005-2013).

At the present, Spanish aquaculture is focused on fish and molluscs species, [Fig. 1.2.1 (MAGRAMA, 2013)]. Therefore, nine species embody the 93% of total production volume (Fig. 1.2.2). Almost all are saltwater species.

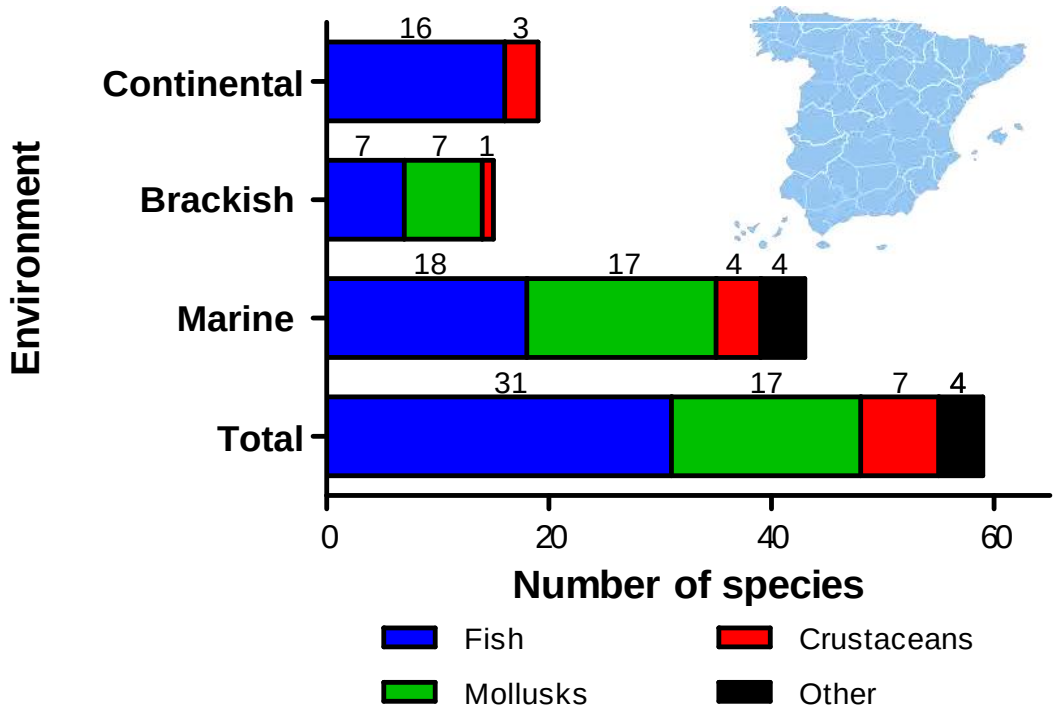


Figure 1.2.1. Number of produced species by culture environment in Spain (2011) (MAGRAMA, 2013).

Mussels (*Mytilus galloprovincialis*) culture volume corresponds to more than ¾ of total aquaculture production (Fig. 1.2.2). The remaining species exceed a million ton per year production (Fig. 1.2.2).

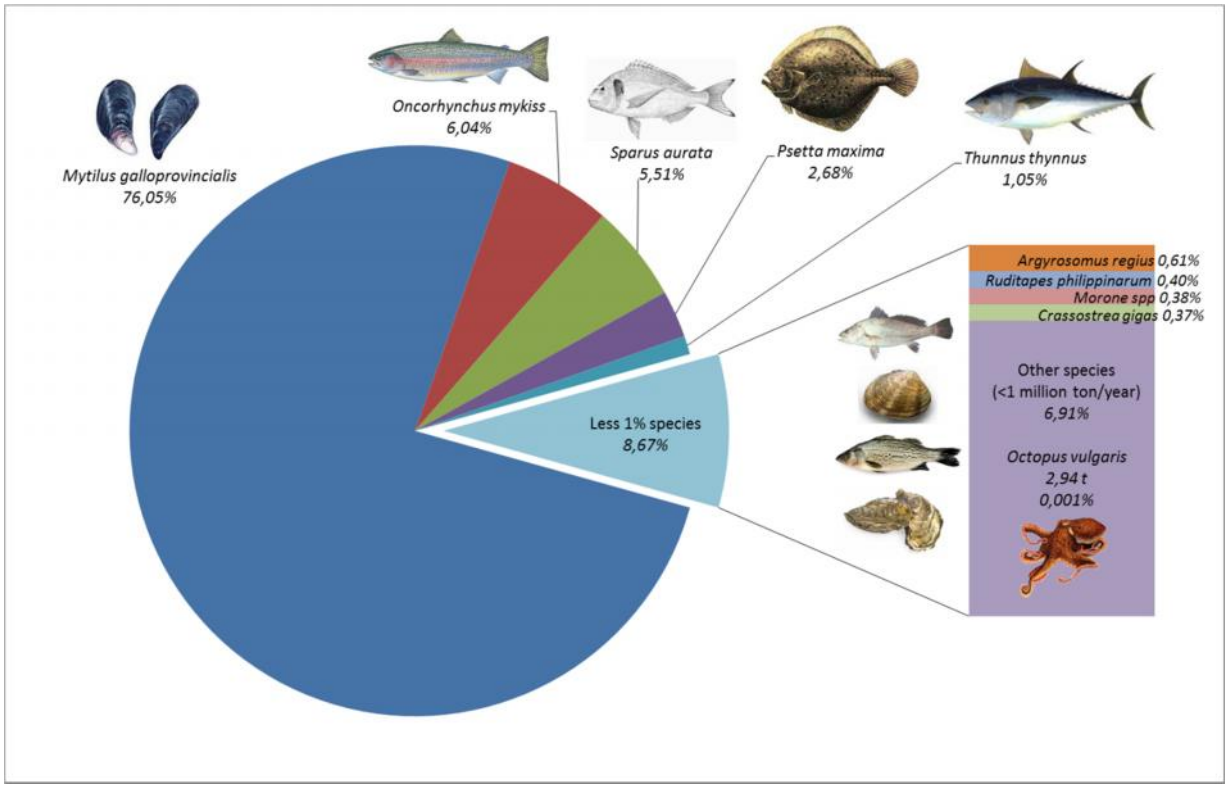
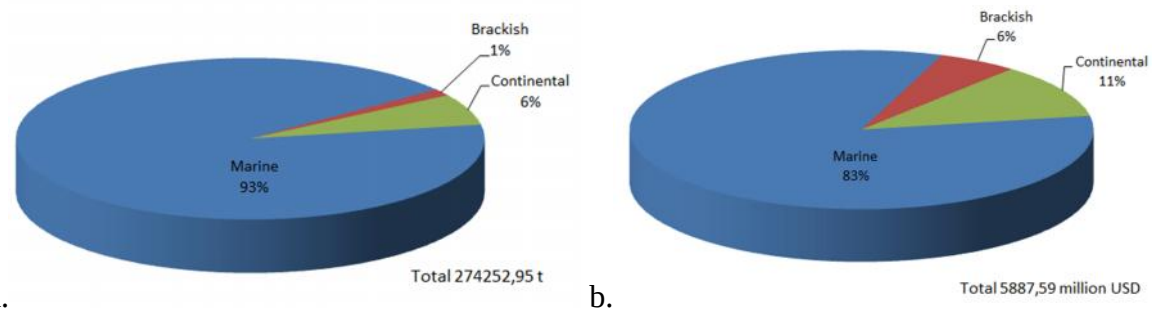


Figure 1.2.2. Main aquaculture species in Spain in 2011; data is only referred to the on-growing phase (MAGRAMA, 2013).

Seventy three per cent (253,354.3 tons which accounts for 4,917.2 million USD) of all the species grown in Spain are cultured in marine environments (Fig. 1.2.3). Freshwater species (approximately 90) are traditionally cultured in continental environments, accounting for 6% and 11% of total Spanish production and economic inputs (Figs. 1.2.1, 1.2.2 and 1.2.3).



a. b.

Figure 1.2.3. Spanish aquaculture: tonnage (a.) and value (b.) in 2011 (MAGRAMA, 2013).

In Spain, the centralized production of few species (mainly sea bream, sea bass and trout) triggered market saturation and, consequently, lower selling prices. For instance, in 1999, the sea bream price was very similar to its production cost (González-Laxe, 2000). The difficulty of selling the production and the little profit margins has made aquaculture less attractive, which led to limited investment in these species. To counteract this situation, the Spanish Government promoted a series of Mariculture Plans, focused on applied research regarding on-growing and diversification of marine species. Diversification can be seen as a tool to improve the sustainability and competitiveness of the industry as well as a strategy to increase production and ensure that aquatic animal protein is obtained from different sources. Nonetheless, industrial farming success requires the control over the biological cycle of the species because, in production terms, any life stage may limit profitability (Sarrasquete, 2012).

In this sense, according to Pillay (2005), the selection of potential species for aquaculture must consider inherent characteristics of the organisms as biological traits, growth rate, size, age of sexual maturity, adaptability to captivity, ease of reproduction in captivity, fecundity and spawning frequency, food habits and acceptability of artificial food, feed conversion efficiency, resistance to unfavourable environmental conditions and density of establishment. In addition, it should also consider market demands, product availability, consumption, and consumers' acceptance.

Currently, Spain is betting on market diversification through an effort in research on species such as the amberjack (*Seriola dumerili*), red porgy (*Pagrus pagrus*) or striped mullet (*Mullus sp.*) and cephalopods, such as the common octopus (*Octopus vulgaris*) (APROMAR, 2012).

1.3. Cephalopods Production

Cephalopods (Schneider, 1784; from Greek *kephalé,* "head" and *podós,* "foot", means "foot in the head") are invertebrate organisms belonging to the Mollusca phylum. The Cephalopoda class includes the Nautiloidea, Ammonoidea and Coleoidea subclasses. The Coleoidea subclass includes cuttlefish, squids and octopus species in various orders (Guerra, 1992). In general terms, cephalopods are bilaterally symmetrical species characterized by the presence of tentacles or arms with suckers; the reduction, migration to internal cavities

or disappearance of the shell; pigment cells in the mantle that allow them to change colour rapidly; developed eyes and low frequency hearing to locate preys; a complex nervous system; body covered by a muscular sac enclosing organs and conferring it flexibility; the ability to eject toxic ink as a defence and the presence of a radula into the mouth connected to the oesophagus (Nesis, 1982).

While ground fish landings have suffered a worldwide generalized decline, cephalopod landings have increased showing the potential of this natural production. According to Caddy, Rodhouse (1998), the markedly increase on cephalopods abundance was possibly caused by an ecosystem response to the heavy fishery pressure. Hence, cephalopods fishery might be recognized as a manner to diversify and reduce fishery effort during the second half of the twentieth century, when these species were considered foreign resources (Vaz-Pires et al., 2004).

Traditionally, cephalopod fisheries were located mainly in southern Europe. However, the depletion of traditional fisheries resources and the high abundance of cephalopod species have caused the spreading of capture by North-European coastlines. Commercial and small-scale cephalopod fishery is done inshore and offshore, especially in Portugal, Spain, Italy and Greece, where human consumption is more relevant. The recent development of more efficient fishery gears, such as plastic pots or fykenets, has resulted on higher catches (Pierce et al., 2010). The Spanish cephalopod trawl fleet fishes mainly in Mauritania and Guinea Bissau, subjected to a communitarian license, and have their base at La Luz harbour in Las Palmas de Gran Canaria (Canary Islands). Fishery is performed during periods of 1-2 months with closures during the breeding season (September and October) in both areas. The nearly 100 fishery trips performed per year, results in a total cephalopod annual captures of 6,100 tonnes (Rafel et al., 2010).

Cephalopods farming for human consumption have never had great relevance due to their abundance from fishery captures (Fig. 1.5.1). Nonetheless, these species are attractive for industrial aquaculture (García García, García García, 2011; Iglesias, Sánchez, 2007; Vaz-Pires et al., 2004) due to its high market value and consumption in the Mediterranean and Asian regions, an observed increasing consumption trend in Oceania [Fig. 1.3.2 (FAO, 2013b)], increasing world consumption predictions and the new open market represented by America (Sykes et al., 2006).

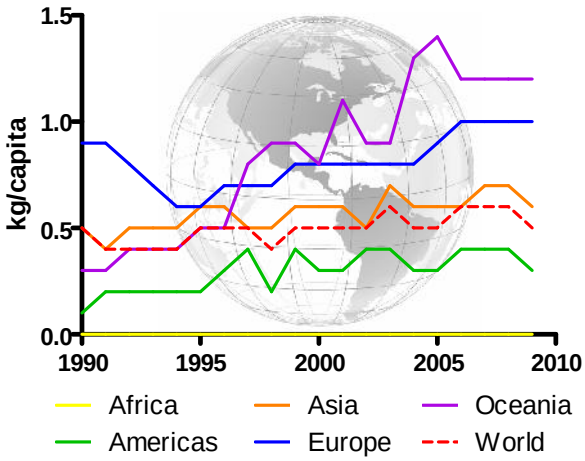


Figure 1.3.1. Cephalopods consumption by continent (FAO, 2013b).

According to FAO (2013b), an increasing consumption trend was verified since 1990's. This was mainly due to contributions from Oceania and Europe (Fig. 1.3.1). In contrast, a higher intake (kg/per capita/year) was observed in Asiatic and European countries compared to Oceania (Fig. 1.3.2). In 2009, New Zealand (3.20kg/capita) - the only Oceania country included into the "top ten cephalopod consumer countries in 2009" - was in seventh position, after Japan (4.90kg/capita), Spain (4.50kg/capita), Portugal (4.10kg/capita), Republic of Korea (4.00kg/capita), Greece (3.70kg/capita) and Italy (3.60kg/capita) (Fig. 1.3.2).

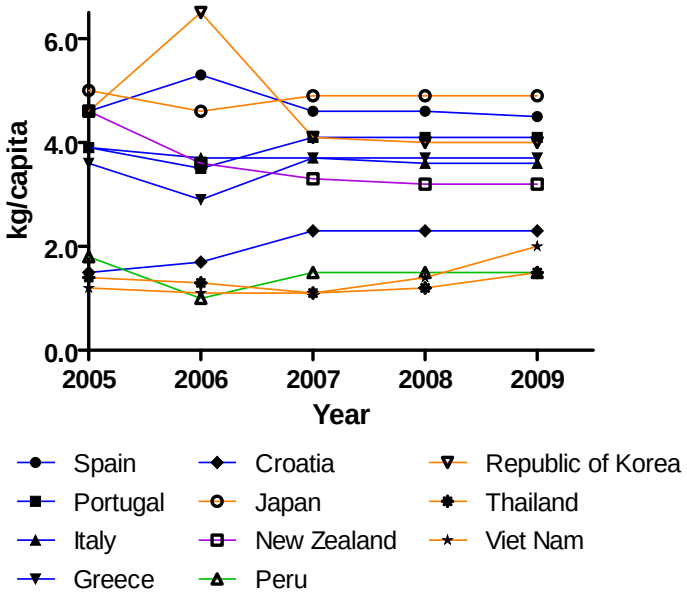


Figure 1.3.2. Main cephalopod consumer countries (FAO, 2013b).

1.4. Cephalopods aquaculture

Currently, there are around 700 known cephalopod species. From those, 10% have been kept in captivity for maintenance (term referred when accommodation is done with individuals of the same age), rearing (when individuals are grown during a period without reaching the second generation), or culture (when the complete vital cycle is achieved in captivity, from hatching to second generation) for different purposes as behaviour and predator-prey relation research or just for obtaining live animals for aquarium display (Boletzky, Hanlon, 1983; Iglesias, Sánchez, 2007).

Culture tests were carried out in a dozen species without obtaining generally satisfactory results for application to industrial scale. Hence, cephalopod commercially viable culture is still in development. The main bottlenecks are the poor growth and high mortality rates of paralarvae (they are carnivorous and require live prey as food), which hinders the development of a suitable food (Iglesias, Sánchez, 2007; Pierce et al., 2010).

Interest on cephalopod maintenance in captivity dates back to the early twentieth century when Grime, in 1928, made an accurate report translated as “Maintenance, handling and breeding cephalopods for zoological and physiological purposes” as referred in Boletzky, Hanlon (1983). Since then, several works were performed by various researchers concerning

biological, ecological, pathological, nutritional and behavioural points of view in various cephalopod species (Boletzky, Hanlon, 1983).

Initial studies made by Japanese and Korean researchers on Sepiidae species (cuttlefish: *Sepia esculenta, Sepia subaculeata, Sepiella maindroni,* and squid: *Sepioteuthis lossoniana, Euprymna berryi*) noted large quantities of fry supply and good rearing results under favourable conditions and appropriated food supply, making them interesting in terms of culture (Choe, 1966; Choe, Ohshima, 1963). Nowadays the most studied species is *Sepia officinalis* (Iglesias, Sánchez, 2007). A culture related research review was published by Sykes et al. (2006) noting lower fertility and fecundity in captivity, a semelparous life, live food requirements by hatchlings, probable intensive culture difficulties due to the species basic immunological system and the absence of an inert food as bottlenecks for culture development (Iglesias, Sánchez, 2007; Pierce et al., 2010).

Within the Octopodidae family, several species are currently being studied in different locations of the world. For instance, the Mexican red octopus (Fig. 1.4.1), *Octopus maya* (Voss, Solis Ramirez, 1966), is an endemic species of the Yucatan Peninsula (Mexico), with great potential as a laboratory model in biomedical research – immunology, behaviour, neurobiology, endocrinology and aging (Van Heukelem, 1977; 1983). It is a holobenthic species, hence new-borns are benthonic and similar to adults. The absence of a paralarvae stage seems to be an advantage for culture purposes. Nonetheless, high mortality, non-appreciable growth and pronounced cannibalism were observed when individuals were fed with dried or prepared diets (Iglesias, Sánchez, 2007; Van Heukelem, 1977). The current research is focused on the development of an artificial diet, testing various ingredients and binders (Águila et al., 2007; Quintana et al., 2011; Rosas et al., 2007; Rosas et al., 2008), feeding strategies (George-Zamora et al., 2011), and temperature related physiological mechanisms (Noyola et al., 2013). According to Rosas et al. (2013) the inexistence of a suitable artificial diet for this species is caused by changes in protein structure (which affects protein digestibility) during cooking and by alterations on nutritional characteristics during ingredient process (which affects the nutritional composition of diet).

Figure 1.4.1. *Octopus maya* [adapted from GrupoGarzaLimón (2012)].

Another South America species, *Octopus mimus* (Fig. 1.4.2) is also being studied. The species inhabits from north Peru to San Vicente bay in Chile. Although the species biology is known and was described by Cardoso et al. (2004), *O. mimus* displays a regional interest for aquaculture production but the obtained results from reproductive and nutritional experiences are still at laboratory level (Uriarte et al., 2012; Uriarte et al., 2011; Zuñiga et al., 1995). Acceptable growth rates and low mortality were observed by supplying natural food on juveniles and sub-adults for extended periods (Baltazar et al., 2000; Carrasco, Guisado, 2010). In contrast, the lack of a proper prey for paralarval feeding represents a bottleneck for juvenile production but, analogously to the previous species, grow-out has been tested and some success was achieved by using formulated diets with various ingredients and binders (Uriarte et al., 2011).

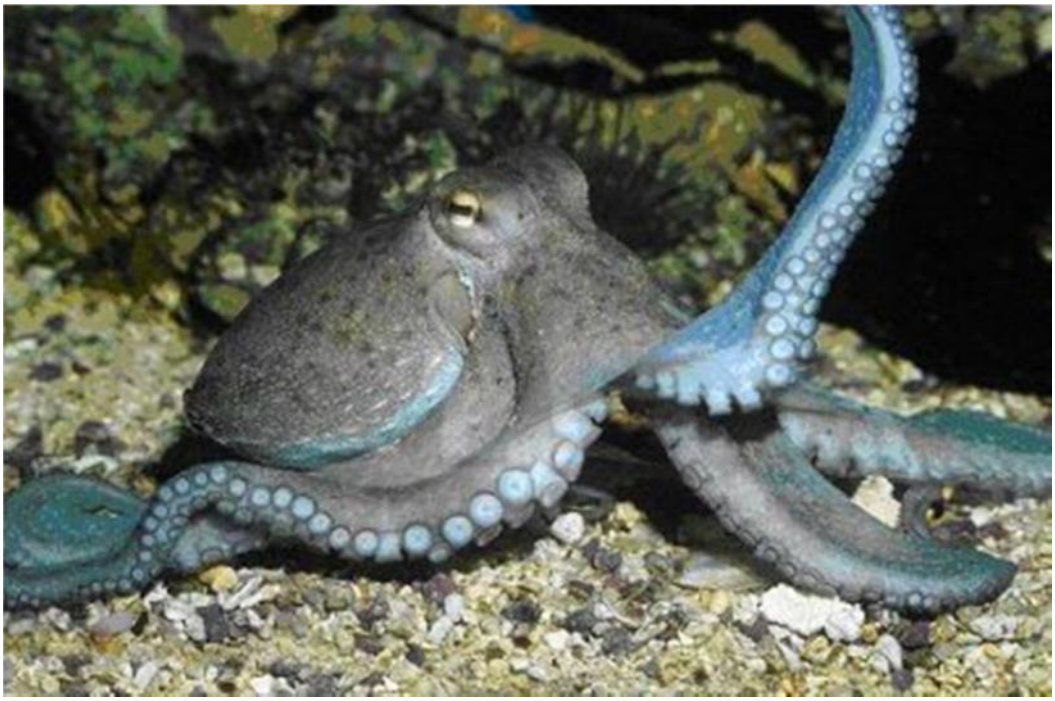


Figure 1.4.2. *Octopus mimus* [adapted from RadioPaladar (2011)].

Several other cephalopod species were and are been studied worldwide; but the most studied species is undoubtedly the common octopus, *Octopus vulgaris* (Fig. 1.4.3).

Figure 1.4.3. *Octopus vulgaris* [adapted from Biopix (2013)].

1.5. *Octopus vulgaris* as a Potential Species in Spain

O. vulgaris, is a benthic-neritic non-shelled cephalopod, worldwide distributed in warm waters (temperate and tropical) with unascertained geographical limits, although their inactivity below 7°C is well-known. This cosmopolitan species inhabits coastal and continental shelf waters - from 0 to 200 meters - in a wide variety of habitats. In nature, the species display a seasonal migration pattern, which is slightly different in time, depending on their location. In early spring, the western Mediterranean population initiates their migration. At this time, maturing and mature octopuses migrate to shallow waters and return to deeper waters by August/September. Immature octopuses show the same pattern with a delay of around two months. *O. vulgaris* has two reproduction peaks, the more relevant in terms of recruitment coinciding with the migration pattern in the Mediterranean (April/May), and the second in October (FAO, 2013a).

Spain has an important *O. vulgaris* fishery fleet. The Spanish fishery production of this species from 1996 to 2012 corresponded to ≈⅓ of global *Octopus* production, and half of the European production (Fig. 1.5.1). In addition, Spain produces this species through aquaculture, but nowadays the achieved production volume is not significant (FAO, 2010-2013).

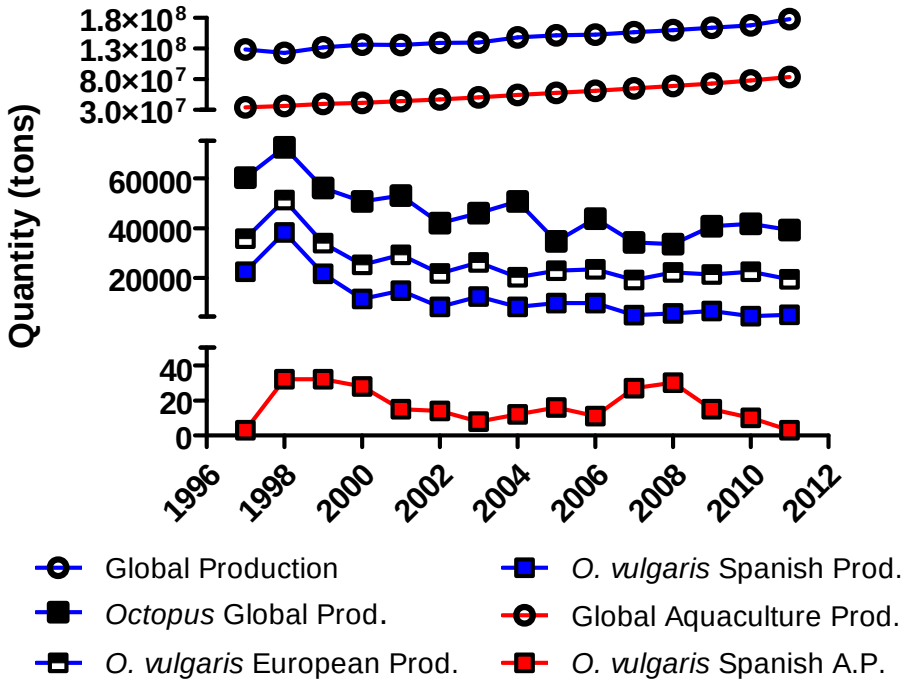


Figure 1.5.1. Global production (including fisheries and aquaculture volumes), global aquaculture production, *Octopus* global production, European and Spanish fisheries production, and Spanish aquaculture volumes for *Octopus vulgaris* (FAO, 2010-2013).

The common octopus has aroused great interest in recent years as a new product for aquaculture because of its great appreciation by consumers, a broad international market, an attractive selling price, its potential for marine aquaculture and industrial scale exploitation (García García, García García, 2011; Vaz-Pires et al., 2004); conferred by: its short life cycle (12-18 months); rapid growth rates up to 5% of their body weight per day and by 13% body weight increase per day on sub-adults; high feed conversion rates with the incorporation of 30-60% of ingested food to their weight (depending on temperature and diet might be 15-43%); high fecundity (100,000-500,000 eggs per female); high protein content (70-90% of dry body weight); fast and good adaptation to captivity; good acceptance of inert food and high resistance to handling and transport (Aguado Giménez, García García, 2002; Iglesias, Sánchez, 2007; Iglesias et al., 1997; Iglesias et al., 2004; Lee, 1994; Mangold, 1983b; Mangold, Boletzky, 1973; Navarro, Villanueva, 2003; Nixon, 1969; Vaz-Pires et al., 2004).

The first feeding and behaviour experiments were performed by Instituto de Ciencias Marinas de Vigo and the Instituto Español de Oceanografía (Guerra, 1978; Guerra, Nixon, 1987). Octopus juveniles were grown in tanks and floating cages obtaining promising results. Since then, many Spanish research centres have shown interest in this species production development, such as Ciencias Marinas del Mar of CSIC, Barcelona (Villanueva, 1995), Centro Costero of I.E.O, Vigo (Iglesias et al., 1997) and Departamento de Bioquímica y Biología Molecular of Universidad de Santiago (Rama-Villar et al., 1997).

A joint initiative by both companies and scientists allowed the optimization of the production systems and the identification of relevant factors at the beginning (García García et al., 2004). The variability on growth, food conversion rates and survival were attributed to culture parameters (temperature, stock density and supplied feed).

Between 2000 and 2004 a National Plan (JACUMAR, "Cultivo de Pulpo" – "Octopus rearing") was implemented by the Spanish Government. Galician, Asturian, Catalonian, Valencian, Murcian, Andalucian, Balear and Canarian research groups started working together on the species aquaculture development. The main targets were paralarvae rearing technology development and juvenile on-growing optimization, through optimization of culture systems and improvements in feeds. Despite these efforts, the closing of the life cycle, extensive juvenile production and the development of a formulated diet were not achieved. Further projects, financed by the JACUMAR National Plan, were developed more recently. For instance, in 2007-2009, "Optimización del engorde de pulpo (*O. vulgaris*) - "On-growing common octopus (*O. vulgaris*) optimization" was performed by research centers of Galicia (CIMA, Consellería de Pesca e Asuntos Marítimos), Asturias (Centro de Experimentación Pesquera, Consejería de Medio Rural y Pesca), Catalonia (Centro de Acuicultura, Institut de Recerca i Tecnología Agroalimentàries (IRTA)), Valencia (Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Valencia), Murcia (Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Consejería de Agruicultura y Agua de la Región de Murcia), Andalucía (Centro de Investigación y Formación Acuícola y Pesquera (CIFAP), Instituto Andaluz de Investigación y Formación Agraria y Pesquera, Alimentaria y de la Producción Ecológica (IFAPA)), and Canary Islands (Instituto Canario de Ciencias Marinas, Gobierno de Canarias). This project was focused on artificial feeding development (semi-moist or lumpy-gelatinous, ensilage or extruded), on-growing optimization in tanks and sea cages, industrial on-growing application and sustainability practices. A more recent project, which run from

2010 to 2013, was called “Nutrición y alimentación de paralarvas y subadultos del pulpo de roca (*O. vulgaris*) - “Paralarvae and sub-adults feeding and nutrition of common octopus (*O. vulgaris*)”. The objectives were to develop a culture methodology for paralarvae and manufactured diets with good yields, defining its nutritional requirements.

The obtained results in cage design development were applied by the industry in Galicia (García García et al., 2004; Iglesias, Sánchez, 2007). This application resulted in on-growing productions reported from five concessions in Galicia of 12.0, 32.3 and 34.0 tons per year for 1997, 1998 and 1999 (García García et al., 2004; Iglesias et al., 2002). After 2000, the octopus on-growing production decreased below 15 tons per year to a minimum of ≈10 tons per year, verified in 2003 and 2006 (Conselheria-do-Medio-Rural-e-do-Mar, 2009). The production increased once again in 2007 and 2008 to 28.5 tons (Iglesias, Sánchez, 2007) just to abruptly decrease in 2009 to 8.8 tons [Figs. 1.5.2 and 1.5.3 (Conselheria-do-Medio-Rural-e-do-Mar, 2009; FAO, 2010-2013)].

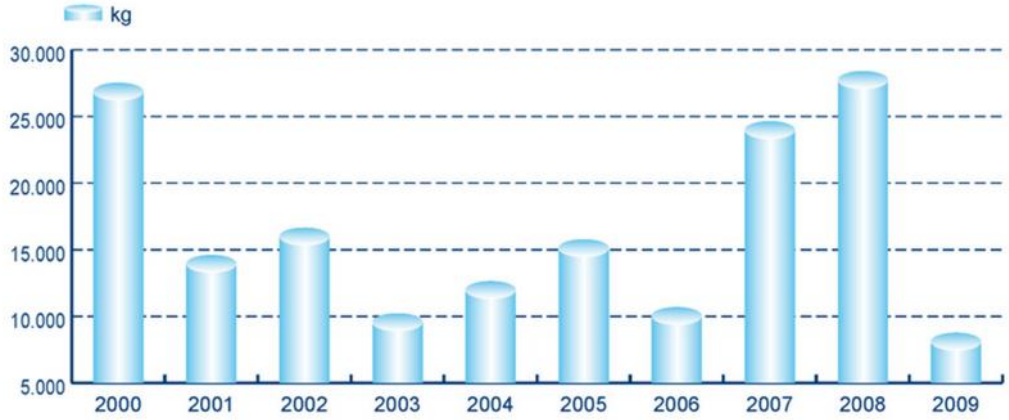


Figure 1.5.2. *O. vulgaris* aquaculture production in five concessions located in Galicia, Spain. [adapted from Conselheria-do-Medio-Rural-e-do-Mar (2009)]..

The commercial value of cultured octopus in Galician market displays a wide variation with on-growing production years, having a minimum of ≈4.0€/Kg and a maximum of 6.6€/Kg [Fig. 1.5.3 (Conselheria-do-Medio-Rural-e-do-Mar, 2009; Iglesias et al., 1999)].

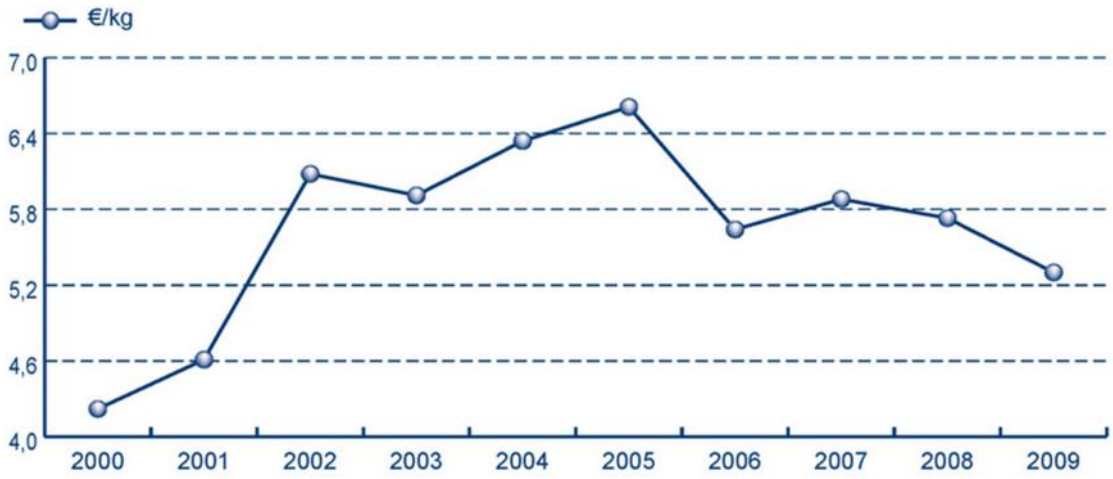


Figure 1.5.3. *O. vulgaris* aquaculture production value of five concessions located in Galicia, Spain [adapted from Conselheria-do-Medio-Rural-e-do-Mar (2009)].

In nutritional terms, this species is interesting for its high protein content. Octopus flesh composition is composed by 80% of water, 16.6% of protein, around 1.0% of carbohydrates and less than 2.0% of lipids (Domingues et al., 2006). Compared to fish, octopus flesh contains more protein (20%) but less lipid (50-90%) and carbohydrate (50-90%) (Domingues et al., 2006). Additionally, the non-edible part of these species (around 30%) might provide an extra income, as it can be used for fish meal or baits (Sykes et al., 2006). Furthermore, cultured cephalopods could also be used for scientific or neuro-physiological purposes, as ornamental species or for restocking natural populations (Boucaud-Camou, 1989).

1.6. *O. vulgaris* aquaculture research

1.6.1. Reproduction and paralarvae rearing

O. vulgaris is a dioeciously species. The reproductive system of males consist of unpaired testis ("tes" in Fig. 1.6.1.1) where spermatozoids are generated and packed into spermatophores ("sp" in Fig. 1.6.1.1.c.), a duct (which contains a pair of spermatophoric glands for spermatozoid agglutination and membrane development) and the Needham's sac (for mature spermatophores storage, "Ns" in Fig. 1.6.1.1.c.) connected to outer duct and the penis. After courtship, mating (Fig. 1.6.1.2) occurs and spermatophores are transferred to the

mantle cavity and placed at the opening of female oviducts by the hectocotylized arm (Guerra, 1992; Mangold, 1983b; 1987; Wells, 1978b). Hectocotylus (the right third arm, Fig. 1.6.1.3) differentiation begins in 50-70 g males (Mangold, 1983b). This modified arm is also used to remove previous stored spermatophores, from other males, from the females' mantle (Quinteiro et al., 2011) .

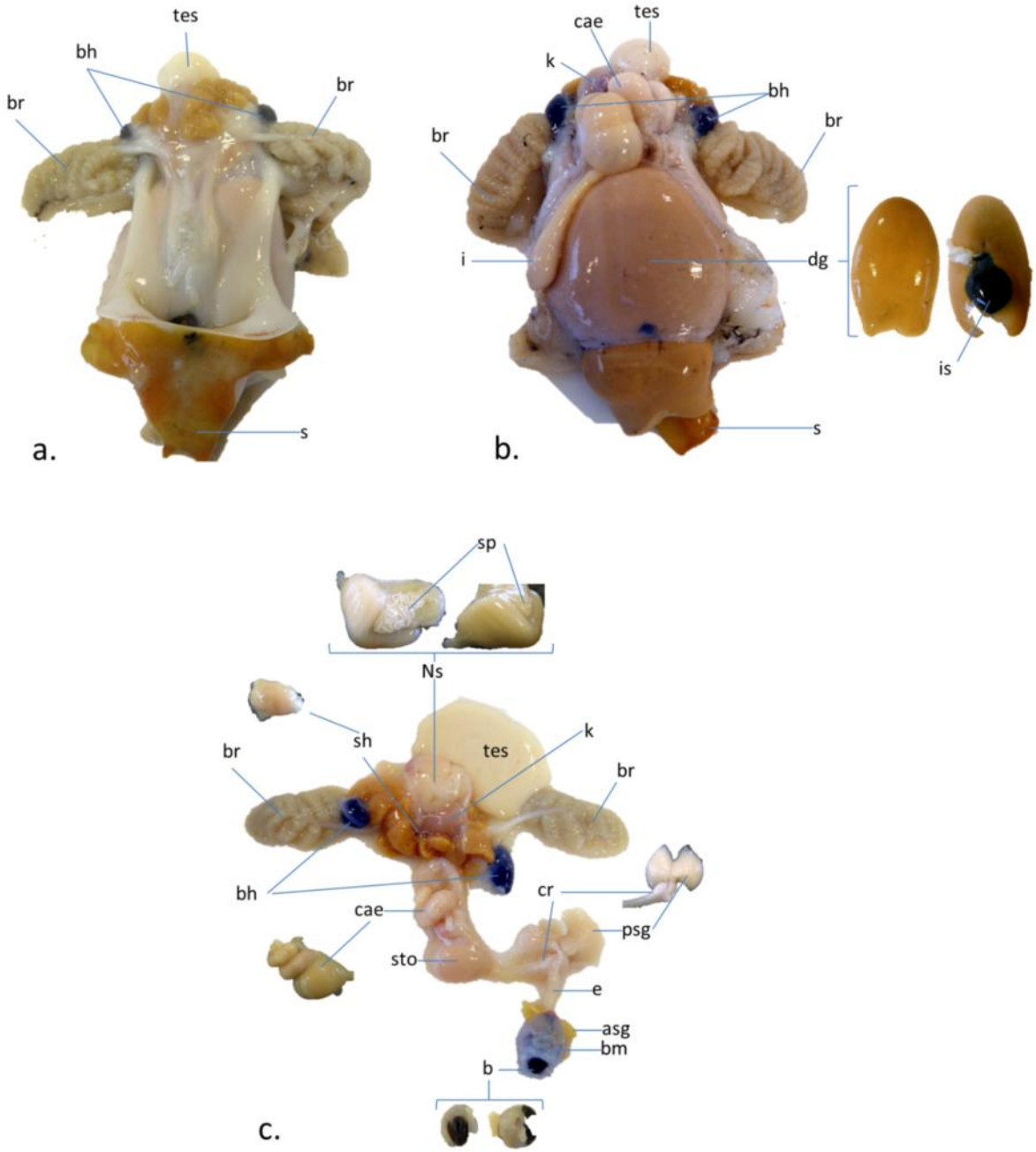


Figure 1.6.1.1 Internal view of *Octopus vulgaris* (a., b., c.). *asg*- anterior salivary glands; *b*- beak; *bh*- branquial hearts; *bm*- buccal area; *br*- branchial arcs; *cae*- caecum; *cr*- crop; *dg*- digestive gland; *e*- esophagus; *i*- intestine; *is*- ink sac; *k*- kidney; *Ns*- Needham's sac; *psg*- posterior salivary glands; *s*- siphon; *sh*- systemic heart; *sp*- spermatophores; *sto*- stomach and *tes*- testicles.

Females have a single ovary (where oocites are formed) connected to a pair of oviducts each one with an oviductal gland (responsible for generating the surrounding egg envelopes after fertilization) opened laterally throughout the mantle cavity (Guerra, 1992; Wells, 1978b).

Figure 1.6.1.2 *Octopus vulgaris* mating [adapted from Dirscheri (2013)].

Sexual maturation in both sexes is controlled by the endocrine system. The ability of the optical glands to produce hormones is heavily influenced by light and temperature (Guerra, 1992). After courtship, mating occurs. The species is polygamous, so there is no pairing, and mating can occur for several hours with more than one male (Mangold, 1983b). This behaviour results in multiple paternal clusters, which are laid by the female (Quinteiro et al., 2011).

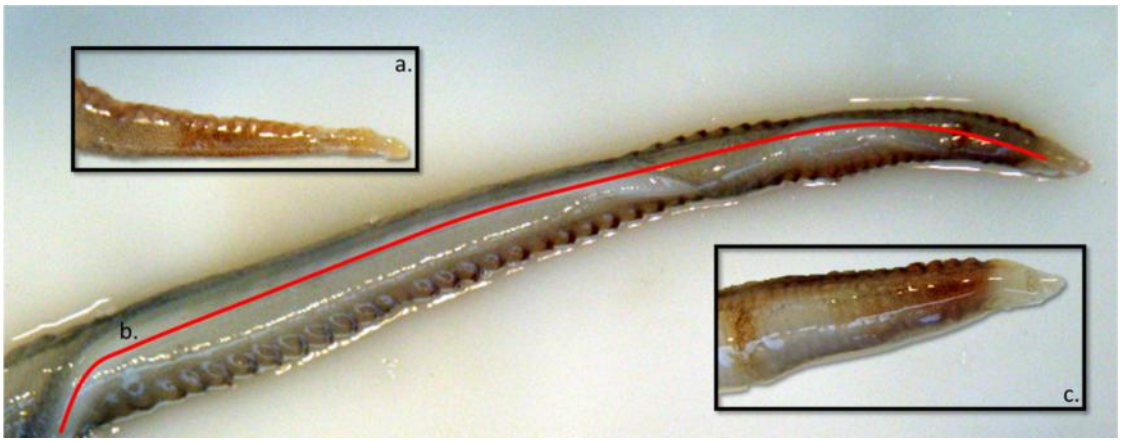


Figure 1.6.1.3 Hectocotylus. a. Octopus arm, b. duct, c. hectocotylus.

Males' maturation coincides with the spermatophore capsule loss (Guerra, 1992). On the other hand, mating often occurs with immature females who can store functional spermatozoa for about one year (Mangold, 1987). Mature eggs are fertilized when they leave the oviducts and run through the oviductal glands. Here, mucosal secretions are ejected to protect and condense them as an egg cluster (Mangold, 1983b). Spawning is common at night and may last as much as 4-6 weeks at 22-23˚C (Wells, 1978b). After spawning, females manipulate the egg clusters (sticking then together in protected areas; Fig. 1.6.1.4), cease feeding and take care of the eggs (aerating, cleaning and protecting them against predators) until hatching (Mangold, 1983b). During this period females lose between 30 and 65% of their body weight and die afterwards (Iglesias et al., 1999). Compared to other octopod species, *O. vulgaris* produce very small (1 x 3mm) but many eggs (Wells, 1978b). An estimation made by Mangold (1983b) amounts to 100,000-500,000 while, under experimental conditions, Iglesias et al. (1997) obtained 605,000 eggs laid by a single female.

Figure 1.6.1.4. *Octopus vulgaris* egg cluster [adapted from Castelló (2013)].

Embryonic development was briefly reviewed by Wells (1978b). Duration is temperature dependent, between 20 days to 4-5 months (Boletzky, 1989; Mangold, 1983b): 34 days at 20±1˚C were reported in the Mediterranean (Villanueva, 1995); in contrast to 80-135 days between 13-20˚C (Iglesias et al., 1999) and 47 days at 17-19˚C (Iglesias et al., 2004).

After hatching, octopuses (with 2 mm mantle length) are planktonic and referred as paralarvae [Fig. 1.6.1.5 (Young, Harman, 1988)]. They subsequently undergo a pre-settlement stage [period mainly planktonic with intermittent contact with the bottom,

(Villanueva, 1995)] from 42 days after hatching (DAH). Paralarvae become juveniles (Fig. 1.6.1.6) after they shift from planktonic to benthonic, which normally occurs after 60 DAH (Carrasco et al., 2003). According to Mangold (1983b), the settlement age varies between 35 and 91 DAH, when octopi are 0.2 g in dry weight and 12 mm in mantle length (FAO, 2013a).

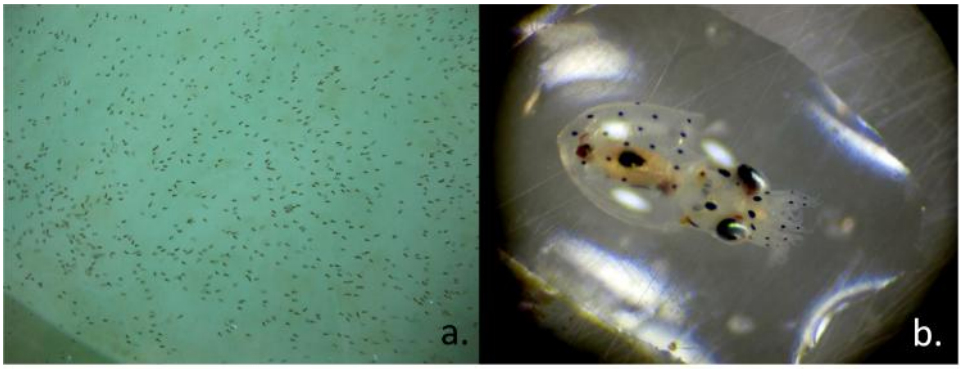


Figure 1.6.1.5. *Octopus vulgaris* paralarvae. a. paralarvae at rearing tank, b. paralarvae (Cerezo-Valverde, personal photos).

Post-embryonic nutrition seems to be more limitative at the paralarvae stage than in juveniles or adults. During the planktonic life, hatchlings first feed on their yolk reserves and gradually start to ingest smooth crustaceans (copepods and decapod larvae) as reserves are exhausted. After settling, juveniles prey on small benthic crustaceans (Boucher-Rodoni et al., 1987). These authors also reported the need for several meals per day and a very efficient and short digestion at this life stage.

Figure 1.6.1.6. *Octopus vulgaris* juveniles. a. Two juveniles fighting, b. common octopus juvenile (Cerezo-Valverde, personal photos).

Several studies have been performed regarding paralarvae nutrition, which tested different preys. Carrasco et al. (2003) reported survivals of 89.6% and 93.5% at day 20 in two experiments alternating spider crab zoea, *Maja squinado*, and *Artemia* spp.. After 28-30 days of rearing, reports of low survival were reported by several authors. For instance, 9% survival using *Palaemon serrifer* larvae (Itami et al., 1963); 34.6% survival when feeding with crab zoea, *Liocarcinus depurator* and *Pagurus prideaux* (Villanueva, 1995); 0.8-4.6% survival when feeding with *Artemia* spp. (Navarro, Villanueva, 2000; Villanueva et al., 2002); 12.5% survival when feeding with enriched *Artemia* spp. (Villanueva et al., 2004); 51.0% survival when feeding with large type *Artemia* spp. and Pacific sandeel, *Ammodytes personatus* (Okumura et al., 2005); and 27% survival with *Grapsus grapsus* zoeae at day 28 (Iglesias et al., 2007b). Survival keeps droping afterwards. For instance, Carrasco et al. (2003) reported a 3.4% survival after 60 DAH.

Iglesias et al. (2004a) observed a clear preference for zoeae of *Maja squinado* by paralarvae. According to Carrasco et al. (2006), a co-feeding on *Maja squinado* and *Artemia* spp. are ideal during the first 5-6 weeks but success is also highly dependent on maintenance procedures and increasing prey density with paralarvae age (Carrasco et al., 2006).

According to Iglesias et al. (2006), *Artemia* spp. metanauplii is an adequate prey for octopus. Co-feeding techniques, always including enriched *Artemia* spp., have been used in paralarvae nutrition experiments (Iglesias et al., 2007b; Villanueva et al., 2004). Co-feeding with live prey should be performed with caution since, as prey grow, prey might lose this status and gain the status of predator, resulting in high paralarvae mortality (Roo et al., 2003). In this sense, paralarvae survival might be enhanced balancing protein and amino acids (Villanueva et al., 2004), essential and non-essential constituents (Villanueva, Bustamante, 2006), vitamins (Villanueva et al., 2009) and lipid (Navarro, Villanueva, 2000; 2003a) levels by the supplied food items. Enrichment protocols were developed to solve the poor lipid profile displayed by *Artemia* spp. (Han et al., 2000; Navarro et al., 1999). However, these strategies were optimized in 2013. In this way, *Artemia* spp. metanauplii enrichment with marine lecithin LC60 displayed good results as enrichment time is reduced (≈2h), and a simultaneous increase of phospholipids and essential high unsaturated fatty acids (HUFA) content on enriched prey is achieved (Guinot et al., 2013). Furthermore, different microalgae were tested as enrichments to improve prey biochemical composition.

The better performances were achieved with an *Artemia* spp. enrichment on *Rhodomonas lens* and *Isochrysis galbana* by Seixas et al. (2010).

According to Iglesias et al. (2004a), the next bottleneck in *O. vulgaris* culture is weaning. In fact, a gradual increase in mortality is registered during this period (15 days at 22.5˚C) and only 10% of 130 tested octopuses survived.

In 2001, the life cycle of the common octopus under captive conditions (from hatchling until death after spawning) was closed through feeding paralarvae with *Maja squinado* and *Artemia* spp. A 31.5% survival rate and 9.5±1.9g dry weight was verified at day 40. Six months later, the animals weighed 0.5-0.6kg. Spawning was obtained two months after maturation, when octopi weighed 1.4-1.8kg (Iglesias, Sánchez, 2007; Iglesias et al., 2004). One year later, similar results of producing captive populations of *O. vulgaris*, with a co-feeding of spider crab zoea and *Artemia* spp., were obtained (Iglesias, Sánchez, 2007).

Although these were pioneer studies, they did not represent a great progress since live spider crab zoeae availability is restricted and its culture logistics are not applicable at industrial scale (Iglesias, Sánchez, 2007).

The generalized low survival rates define paralarvae rearing as “the limiting step in the culture of this species” (Vaz-Pires et al., 2004). The limitation comes from the unavailability of a satisfactory size-nutritive composition live diet, the lack of a standard rearing method and the unknown biology and nutritional needs of this species at early stages (Moxica et al., 2002). Accordingly, there is the need for research focused on prey availability and diversification, temperature and nutritional-metabolic requirements (Forsythe, 1993; Lee, 1994; Navarro, Villanueva, 2003; Villanueva, 1994; 1995). This will avoid relying on wild juveniles captures to perform commercial fattening (Iglesias et al., 1999).

1.6.2. On-growing status and origin

After reaching the benthic stage, this species is easy adaptable to captivity. Therefore, on-growing is seen as the life stage with the highest potential in industrial terms (Vaz-Pires et al., 2004).

In general terms, the existing production system is based on wild juveniles, which are

reared in cages (floating or suspended from rafts), and fed with low priced discarded species until the allowed commercial weight is reached (Chapela et al., 2006; Garcia et al., 2009a; García García, García García, 2011; García García et al., 2004; Rodriguez et al., 2006). This activity was also recognized by Boyle, Rodhouse (2005) as another reproduction opportunity to wild females; which could be traduced on a recruitment increase.

Numerous on-growing studies have been performed in protected areas, around the Spanish coastline, namely: Galicia (Chapela et al., 2006; Iglesias et al., 2007a; Rama-Villar et al., 1997; Tuñon et al., 2001; 2002), Asturias (Rodriguez et al., 2006; Rodríguez et al., 2003), in the Mediterranean coast (Delgado et al., 2007; Oltra et al., 2005) and also in the Canary Islands (Estefanell et al., 2009b; Socorro et al., 2005).

In Galicia, juveniles (800 g) were fed with crabs (*Carcinus* sp.), Atlantic horse mackerel (*Trachurus trachurus*), blue whiting (*Micromesistius poutassou*), and other discarded species from the trawl fleet (until 2.5-3kg). This feeding scheme resulted in conversion feeding rates of 5.8 and 78% survival (García García et al., 2004). An economic study for this kind of exploitations, performed by García García et al. (2004), revealed low profits and high risk, due to high variability on juvenile and food price. Moreover, these authors reported that the feeding expenses were between 25-60% of total production costs.

In the Mediterranean, production is restricted in time (by water temperature) and location (by environmental and coastline-related factors). These reasons have limited octopus production, confining it to offshore facilities. Nonetheless, octopus on-growing is also economically viable (Garcia et al., 2009a; García García, García García, 2011; Iglesias, Sánchez, 2007). Garcia et al. (2009a) proposed two on-growing strategies for facilities placed in protected areas. The feeding was based on local fishery species: crab (*Carcinus mediterraneus)* and bogue (*Boops boops*). The first strategy is performing a 5-month rearing per year (from November-December to April-May). Through its application, octopuses weighing up to 3.5kg are obtained. The second is performing two 3.5-month rearing cycles per year (from October to January, and from February to June), which results in individuals with 2.5kg for each production. Despite both have high survival (80%), the second strategy attains a 33% higher final biomass. On the other hand, the first strategy is more favourable in economic terms. García García et al. (2004) determined that the main costs related to feed and juveniles were approximately 40% and 20%, respectively (García García, García García, 2011).

In order to diversify the location of on-growing sites, Muñoz Pérez (2003) performed an study in a river estuary, using 1 m^3 fibre tanks (1 x 2 x 0.5 m) and 1 m^3 cylindrical floating cages. Juveniles of approximately 1Kg grew to commercial size (3.5kg) in three months, attaining a final density of 40kg/m^3. This researcher recommended production in these sites for 6-9 months per year (when water temperature is between 15-20˚C), avoiding the hottest months to minimize heat shocks, evaporation and salinity disturbances. According to Garcia et al. (2009a) and García García, García García (2011), focused research on non-protected areas was recently performed in the Mediterranean with the objective of increasing the final biomass and density. In addition, information regarding temperature and hydrodynamic influence on mortality and growth of the common octopus was collected.

Behavioural studies have facilitated the development and the optimization of culture methodology. Hence, attending to the reclusive behaviour and negative photo-taxis exhibited by this species (Villanueva, 1995), the placement of artificial opaque refuges has resulted in higher growth rates (Anderson et al., 1999; Mather, 1994). At industrial scale, cannibalism (mainly generated by territorialism) might cause significant mortality. The latter could be minimized by placing dens (T shaped PVC tubes), oriented in opposite directions, and by using rectangular instead of circular cages (Rey-Méndez et al., 2001). Additionally, at the on-growing stage, sex separation is recommended to avoid a decrease in growth due to an energetic investment for reproductive development on females (Aguado Giménez, García García, 2002; Sánchez et al., 1998). These researchers also reported higher feeding rates in females and quicker growth than males, preceding maturation. Contrariwise, Aguado Giménez, García García (2002a) did not identify any sex influence on growth and food intake. In contrast, Rey-Mendez et al. (2003) suggested better rearing results through the application of mixed cultures, which do not seem to result in worst performances. They reported higher final biomass and high mortality in males up to 850 g that also displayed higher aggressive behaviour.

1.6.3. Feeding and digestion

O. vulgaris, as any other cephalopod, is carnivorous, opportunistic and dim-light feeders (Boucher-Rodoni et al., 1987; Nixon, 1987; Wells, 1978a). Boucaud-Camou, Boucher-Rodoni (1983) reported that *O. vulgaris* is more activated at night. Prey items varies according to life stage (depending on size and prey availability in the wild) but, in general

terms, their diet consists of bivalves, crustaceans and bony fish (Boucher-Rodoni et al., 1987; FAO, 2013a; Nixon, 1987). In the north of the Mediterranean sea, Guerra (1978) determined a diet based on 80% crustaceans, 12-30% fish and 8% of other cephalopods, through the analysis of stomach contents (Mangold, 1983b; Nixon, 1987). In western Mediterranean, the more important prey are the bivalves *Pitaria chione, Venus verrucosa* and abalone, *Haliotus tuberculata* (Nixon, 1987); while in Atlantic waters, octopus diet is based on shelled molluscs (Mangold, 1983a).

Searching and prey apprehension is primarily started by visual stimuli and movement (Boucaud-Camou, Boucher-Rodoni, 1983; Lee, 1994) or through chemoreceptors, located in the suckers of the arms (Graziadei, 1964). When octopus perceives the perturbation, it raises its head and proceeds with an approach manoeuvre, followed by a jet-propelled movement to catch the prey with the arms. Then, it covers the prey with the inter-branchial web and directs it to the buccal area ("bm" in Fig. 1.6.1.1.c.). Here, a chitinous beak (associated with strong muscles), "b" in Fig. 1.6.1.1, and a radula are used to scrape it. When necessary, it poisons the prey with a specific toxin, before eating (Boucaud-Camou, Boucher-Rodoni, 1983; Nixon, 1987). The cephalotoxin is secreted by salivary glands and inhibits the synapsis of crustaceans and some fishes (Boucaud-Camou, Boucher-Rodoni, 1983). Hard structures are usually broken and the meat extracted by pressure. When this is not possible, it can drill a hole through the calcareous shell for introducing mucus to kill the animal (although this last practice is more localized in Atlantic and Pacific individuals). Muscle attachments are dissolved, by external digestion, ingested and transported through the digestive system, until it reaches the stomach, where digestion is completed (Boucaud-Camou, Boucher-Rodoni, 1983; Nixon, 1987). The ingestion of crustaceans flesh (avoiding the exosqueleton), is identified as a mechanism for metabolic waste reduction (Nixon, 1987).

The digestive system and digestion process of *O. vulgaris* were described in detail by various authors (Bidder, 1966; Boucaud-Camou, Boucher-Rodoni, 1983; Mangold, Bidder, 1989). Despite octopuses show a voracious feeding pattern they are able to starve for a long time when food is limited. This species can ingest a new meal before the digestion of the previous is finished, but digestion does not stop until the crop ("cr" in Fig. 1.6.1.1.c.) is empty. This, in addition to a sequenced digestion process (which makes possible the simultaneous digestion of two meals), increases the effectiveness of digestion (Boucaud-Camou, Boucher-Rodoni, 1983; Nixon, 1987). Nonetheless, Boucher-Rodoni, Mangold

(1977) reported that digestion is influenced by temperature, sex and sexual maturation, e.g. the lower the temperature the higher the digestion's duration.

The rapid growth of *O. vulgaris* could be justified by an efficient digestive system, which is translated in high food conversion rates [between 25-70% of inclusion were reported by Nixon (1987), and the feeding pattern shown by the species. Nevertheless, digestion efficiency is limited by the dual role of the digestive gland ("dg" in Figs. 1.6.1.1.b.), since this organ is responsible for enzyme secretion and absorption (Boucher-Rodoni et al., 1987).

Lee (1994) reported an amino acid based metabolism for this species. Proteins are required for all vital functions and growth. The high growth rates displayed by *O.vulgaris* are explained by the high rates of protein synthesis and retention, added to little protein degradation. Houlihan et al. (1990) reported that up to 90% of synthetized proteins are used for growth. In contrast, Mangold (1983a) reported values of approximately 50%. These changes are probably due to differences in seawater temperature of animals sampled.

1.6.4. Diet development for octopus on-growing

1.6.4.1. Nutritional requirements

Nutrition is a very important in the process of maximizing growth and survival for mass culture in captivity. Growth patterns of captive animals differ from wild ones due to the different ecosystems. Therefore, an increase in mantle muscle tissue is a combined effect between existing muscle fibres growth and new-fibres production (Pecl, Moltschaniwskyj, 1999). These researchers suggested that the lower growth typically displayed by captive animals is caused by an alteration of cellular growth mechanisms and physiological growth rates, which results in a reduction of new-fibre generation.

Natural feeding of wild *O. vulgaris* is based on bivalves, crustaceans and bony fish (Boucher-Rodoni et al., 1987; FAO, 2013a; Nixon, 1987). Under experimental conditions they prefer live food, although they will accept non-living diets (Boucaud-Camou, Boucher-Rodoni, 1983). Nonetheless, body composition might reveal nutrient requirement and utilization (Lee, 1994).

The first nutritional requirements knowledge was based on natural feeds testing; through marked substrates, starving experiences and biochemical analyses (Castro et al., 1992; García García, Cerezo Valverde, 2006; O'Dor et al., 1984). Nowadays, these are being estimated through the use of formulated diets with known composition (Cerezo Valverde et al., 2008; Estefanell et al., 2011; Garcia-Garrido et al., 2011; Quintana et al., 2008).

The obtained information identified proteins as the main macronutrient required by cephalopods. García García, Cerezo Valverde (2006) reported between 15.5-16.6% of protein content (dry weight) for whole *O. vulgaris.* The main free amino acids (>100mg/100g tissue) observed in tissues were octopine, proline and arginine; while protein-based amino acids (<0,1g/100g tissue) were identified as being glutamate, aspartate, leucine, alanine, lysine and isoleucine (Cerezo Valverde et al., 2012d; Iwasaki, Harada, 1985; Jhaveri et al., 1984; Suyama, Kobayashi, 1980). Recently, Cerezo Valverde et al. (2013) identified the most essential amino acids (arginine, leucine and lysine) and the non-essential amino acids (glutamate and aspartate) for cephalopods. According to Zlatanos et al. (2006), proteins are indispensable to satisfy energy needs and promote growth. Lee (1994) had already reported that proteins are used for locomotion (actine and myosin), structural support (collagen), oxygen transport (hemocyanin), energy source (amino acid catabolism) and osmoregulation (free amino acid and proteins in the hemolymph).

Lipids represents less than 2% of total cephalopods body weight; e.g. the common octopus lipid content is between 0.2-0.6% of dry weight (García García, Cerezo Valverde, 2006). They have a structural function (Moltschaniwskyj, Johnston, 2006; O'Dor et al., 1984) and are stored mainly in the digestive gland, but can be mobilized at fasting times (Morillo-Velarde et al., 2012a).

The main lipid classes of common octopus muscle are phospholipids (mostly phosphatidylcholine and phosphatidylethanolamine), cholesterol, free fatty acids and mono-glycerides. In contrast, neutral lipids are more abundant in the digestive gland, where a high triglycerides and free fatty acid content is found (Morillo-Velarde et al., 2012a). Despite cephalopods digestive tract show lipase activity (Boucher-Rodoni, 1982; Moltschaniwskyj, Johnston, 2006), digestibility and catabolism of lipids is reported as poor (Lee, 1994; O'Dor et al., 1984). Nonetheless, lipids seem to be more relevant for the nutrition of early stages (Navarro, Villanueva, 2000; 2003b; Okumura et al., 2005; Seixas et al., 2008).

Carbohydrates (CH) are present at ≈1% in octopus tissue (Cerezo Valverde et al., 2008; García García, Cerezo Valverde, 2006). Nevertheless, Rosa et al. (2005) observed a significant content in *O. vulgaris* tissues (9% in the gonad, 5% in the muscle and 4% in the digestive gland). It is generally accepted that CH do not represent a specific requirement for cephalopod diets (Lee, 1994). However, Wells, Clarke (1996) reported digestion, storage and use of CH by cephalopods. Despite proteins and amino acids contribute more significantly to energetic purposes (Lee, 1994), carbohydrates are effectively catabolized when energy is rapidly required (Morillo-Velarde et al., 2011a; O'Dor et al., 1984; Wells, Clarke, 1996). Its use specially occurs when energy is needed for explosive activities (as prey capture or quick escape) or during starvation periods, when energy is obtained through anaerobic metabolism (O'Dor et al., 1984; Wells, Clarke, 1996). After a meal, the non-catabolized portion of the ingested CHs is stored in the muscles, as glycogen (O'Dor et al., 1984).

Cephalopods have great accumulation capacity for essential and non-essential elements, in tissues and digestive gland, with non-dependence of surrounding pollution levels (Sykes, Pers. Comm). Essential and non-essential minerals in muscle tissue of the common octopus were reported by Seixas et al. (2005) and (Cerezo Valverde et al., 2009a). Strontium is required for estatolith development during incubation (Hanlon et al., 1989). Villanueva, Bustamante (2006) reported the relevance of copper and sulphur in cephalopods diet. Copper is used for haemocyanin production and adequate oxygen transport (Miller, 1995), and seems to be incorporated directly from seawater by the pancreas (Wells, Wells, 1989). Sulphur has to be provided in excess to maintain muscular protein formation (Lee, 1994; Villanueva et al., 2004).

In nature, the vitamin requirements are obtained from the diet but, under culture conditions, they might be limiting factors. Vitamin content on cephalopod flesh were measured and reported by Sidwell, Service (1981). Vitamin A levels present in octopus muscle are similar to other cephalopods. Vitamin E content is high in ovaries, eggs and juveniles; probably due to high PUFA content at early stages. Both vitamins are important, as they influence growth, reproduction and embryonic development (Villanueva et al., 2009).

1.6.4.2. On-growing diets for the common octopus

On-growing and maintenance of juveniles and adults, under rearing conditions, using inert food (fresh, frozen or prepared food) has been performed in various cephalopod species (Castro, 1991; Castro et al., 1993; DeRusha et al., 1989; Dimarco et al., 1993; Lee et al., 1991). Feeding based on these non-living but natural diets (e.g. crabs) promote highly variable growth and conversion rate results, depending on the used species (Aguado Giménez, García García, 2002; Cagnetta, 2000; Cagnetta, Sublimi, 2000; García García, Aguado Giménez, 2002). The different composition of prey items determines whether they are suitable or not as food (Cerezo Valverde et al., 2009b). Hence, its identification could be a greater advance on cephalopod nutrition for optimizing nutrient content on artificial feeds. With this aim, lipid (Cerezo Valverde et al., 2012b) and protein (Cerezo Valverde et al., 2013) quantitative analysis was recently performed.

Natural mono-diet feeding has been thoroughly tested. The best performances were obtained with crustacean species (Aguado Giménez, García García, 2002; Cagnetta, Sublimi, 2000; Cerezo Valverde et al., 2008) followed by other cephalopods, as tattler (*Illex coindetii*) (Cagnetta, Sublimi, 2000), fish species (Aguado Giménez, García García, 2002; Cagnetta, 2000; Cagnetta, Sublimi, 2000; García García, Aguado Giménez, 2002; López et al., 2009) and bivalves (López et al., 2009).

Generally, the use of crustacean species implies high costs, producing an increase on the price of produced octopus. However, small pelagic fish species represent an alternative to crustaceans. The species used for feeding are classified as blue or white fish, differing basically in lipid content. The first species group (e.g. sardine -*Sardina pilchardus*- and giltsardine *Sardinella aurita*) are fatter than the second (e.g. bogue -*Boops boops*- and mediterranean scad -*Trachurus mediterranus*). García García, Aguado Giménez (2002) reported better specific growth (SGR) but worse feeding rates (SFR) on *Boops boops* (0.78±0.12% and 2.055±0.27%, respectively) fed individuals compared to *Sardina pilchardus* (0.69±0.08% and 1.675±0.17%, respectively). A mixture of fish species (*Sardina pilchardus, Boops boops* and *Trachurus trachurus*) resulted in 1.91% SGR and between 2.34-2.98 food conversion (Cagnetta, 2000). The increased food intake, when diets have higher lipid content, might be caused by an attempt to compensate for the low protein present in the diet (García García, Aguado Giménez, 2002). As poor growth is achieved by wholly fish diets, and crustaceans' limits profitability, mixed diets might be considered an

alternative. In this sense, García García, Cerezo Valverde (2006) reported acceptable growth rates (1.98±0.30%) and feeding efficiency (39.35±4.40%) feeding a mixed diet (*Carcinus mediterranus,* and *Boops boops*) on alternate days. However, the recommended protocol to enhance economic viability was one day crab supply followed by three days bogue resulting in an associated 3kg of food being used to produce 1Kg of octopus.

García García, García García (2011); García García et al. (2004) established feeding costs around 40% of total production costs by using crustaceans and fish based diets. Profitability increases as crustacean proportion on diet decreases (García García, Cerezo Valverde, 2006). Hence, natural diets replacement for prepared diets (decreasing natural prey dependence) might be an interesting option for reducing costs up to 80% (Quintana et al., 2008), and the way to eliminate the existing food bottleneck (Domingues et al., 2007). In fact, the development of formulated diets determined production success in other species (Cho, Bureau, 2001). Focused research on inert diets for cephalopods began in the nineties. Efforts increased during the last decade but, until today, no satisfactory results have been obtained for any cephalopod species (Domingues et al., 2007; Iglesias, Sánchez, 2007). In addition to the nutritional component of diets, palatability is also affected by moisture and therefore acceptability and diet ingestion are influenced (Cerezo Valverde et al., 2008). These researchers, highlighted the "presentation" importance for octopus feeds formulation: particles must be small and firmly cohesive but not granulated as animals manipulate the feed; feed must be water-stable as intake is normally performed at various times; and attractive to avoid starvation.

Moist diets, based on natural diet pastes mixed with some binder, are those that have provided better performances until now. The use of gelatine as binder resulted in acceptable palatability and no-negative effects on diet quality promoting growth and conversion efficiency, but lesser than natural diets (Quintana et al., 2008; Cerezo Valverde et al., 2008). On the other hand, high disaggregation rates represented the main problem of gelatine addition (Cerezo Valverde et al., 2008). Cerezo Valverde et al. (2008) performed a comparison between a diet based on a mixture of a fish-prawn paste, with alginate or gelatine, in *O. vulgaris* juveniles. In this study, the alginate agglutinated diet had greater stability in water and also resulted in better feed efficiency, feeding and growth rates, compared to the gelatine agglutinated diet. Contrariwise, an experiment performed by Garcia-Garrido et al. (2011) reported better growth rates by the gelatine aggregated diet (with a difference on 4.5±0.1 g Kg^{-1} bw day^{-1}) compared to alginate diet. A similar trial was

performed in *O. maya* by Rosas et al. (2008). The alginate agglutinated diet resulted in a nutrient absorption limitation, low digestibility and acceptability. On the other hand, the gelatinized diet obtained high digestibility, a decrease on enzyme activity requirements (for external digestion) and an enhancement on nutrient absorption performance. Garcia et al. (2009b), stated that better yields might be obtained by animal protein binders (such as gelatine) than those of vegetal origin (such as soy lecithin). These same authors also made a comparison between protein and carbohydrate binders from vegetal sources (soy lecithin and starch, respectively) and obtained better performances using the first.

In addition to raw materials and binders, the preparation of feeds also plays an active part on the final feeding product. In fact, raw materials are used in the preparation of artificial diets through the application of different processes.

Specific dry feeds (normally extruded) are recommended for intensive aquaculture. Dry diets present adequate organoleptic and ensure constant nutritional requirements supply, are more available, are easily long-term stored and conserved, require less manipulation, have better stability in water, reduce kg-production costs and reduce disease transmission risks. However, dry pellets (also when made with pure ingredients) have low acceptance and very poor growth in cephalopod species (Castro, Lee, 1994; Domingues et al., 2005; Domingues et al., 2007; Lee et al., 1991). In the same way, surimi diets (fish myofibrillar protein concentrates which have a heat treatment) resulted in null or practically non-existent food-intake (Lee et al., 1991). Gairin et al. (2011) compared an extruded (with 14% moisture) and a semi-moist (with 71% moisture) diet. Both shared the same raw materials (krill meal and soy meal) that were heated in preparation. The obtained food conversion rates (FCR) were not very different between diets; being 1.51±2.10 and 1.27±0.60, respectively. However, the reported specific growth rates (SFR) for the extruded diet was almost 3.7 times lower than semi-moist and 6.8 times lower when compared with the natural diets (Querol et al., 2012a; Querol et al., 2012b). When dry feeds are accepted by *O. vulgaris*, their feeding pattern is close to the use of natural diets. The daily feeding rates are lower and great ingestion is observed at the beginning, but an intake decrease is observed around two weeks later (Tomás et al., 2009).

A negative effect on diet or nutritional quality was identified when preparation requires heat application (Domingues et al., 2009). Heat causes protein denaturation, lipid oxidation and consequently amino acids, polar lipids and vitamins loss. Hence, better performances were

achieved by fresh, defrosted or fresh-dried products and alginate instead of gelatine agglutinated diets. In this sense, meal composed diets displayed poor acceptability (Morillo-Velarde et al., 2013). These diets produce rejection or low intake when accepted, resulting in non-existent or negative growth (Águila et al., 2007; Estefanell et al., 2009a; Garcia-Garrido et al., 2011; López et al., 2009; Rosas et al., 2007) which notes the inadequacy of its addition on cephalopod diets. The freeze drying process extracts water without heat treatment, maintaining molecular structure and nutritional properties. Freeze-dried ingredients were included recently for the first time in cephalopod diets. Morillo-Velarde et al. (2011b) reported higher preference for freeze-dried than meal ingredients by common octopus. Its inclusion improved nutritional quality, texture and palatability of *O. vulgaris* diets. It also promoted good weight gain (9.6±1.8 g/day), FCR (1.0±0.1), feed efficiency (97.8±13.5%) and digestibility (Morillo-Velarde et al., 2012b). Anyway, dry ingredients are an interesting option for their inclusion on feeds since storage, conservation and transport are easier than those needed by fresh products. In this way, ingredients processing represent a significant part of the expenses of diets production. A reduction on production costs might be achieved by using dehydrated freeze-dried ingredients.

Preference and ingestion of diets might be increased by including attractants in its formulation. Attractants have also been tested in diets of *O. vulgaris*. The inclusion of taurine produced no significant results (Garcia et al., 2009b). In a similar way, glutamate inclusion did not stimulate ingestion but slightly enhanced growth rates and feed efficiency; probably due to high levels of this amino acid in natural diets and an specific enzyme system for glutamate oxidation inherent in common octopus (Cerezo Valverde et al., 2012a). In a recent report, yolk powder was identified as an usefulness flavour enhancer (Morillo-Velarde et al., 2011b).

Current research performed in cephalopods nutrition is directed to artificial diets development in order to improve growth by taste enhancers and/or by supplying good balanced proteins (Querol et al., 2013).

1.7. Objectives

The objective of this thesis was to test the use of two semi-moist diets, having as common base liofilized raw materials, namely *Carcinus mediterranus* and *Todarodes sagittatus*, and as variable the process of heating (either freeze dried or meal) for *Boops boops*. In addition, the suitability of extending the period of starving (2 *vs.* 3 days), using the semi-moist based on freeze-dried *B. boops*, was tested as way to increase profitability.

2. Materials and methods

2.1. Rearing Systems

The experiments were performed at Estación de Acuicultura del Instituto Murciano de Investigación y Desarrollo Agroalimentario (IMIDA; 37.822919N, -0.758768W) facilities in San Pedro del Pinatar (Murcia, Spain).

The seawater used in the marine system comes from a water intake situated at San Pedro harbour. It is pumped by three pumps through a PVC pipe before it enters four decantation ponds of 500m^3 (Fig. 2.1.1). Prior to its storage in a reservoir tank of 20m^3 (Fig. 2.1.1), water passed by an outdoor protein skimmer (100m^3/h) and biological filter, to remove dissolved organic matter.

Figure 2.1.1. Aerial view of Estación de Acuicultura del Instituto Murciano de Investigación y Desarrollo Agroalimentario (adapted from Google Earth on 16/09/2013 under the Creative Commons Licence).

Each individual seawater system used in the present set of experiments was semi-open (Fig. 2.1.2). The water entered the system in an expansion tank ("e" in Fig. 2.1.2) with two chambers, for solids decantation. Then, water went through mechanical filtration (sand filter, Fig. 2.1.2), with decreasing granulometry towards the water exit tube. After, water was pumped to a protein skimmer ("sk" in Fig. 2.1.2) with bio-balls for biological filtration. Before returning to the tanks, seawater was disinfected through the use of ultra-violet filtration ("uv" in Fig. 2.1.2). Water temperature was maintained between 18-20˚C in both performed experiments, into the optimum range for this species (Aguado Giménez, García García, 2002), through the use of a heat pump (Air Energy, Mod. 400, Til, "hp" in Fig. 2.1.2). This pump model has a reverse cycle which gives the ability to heat or cool the water to maintain temperature within pre-established limits. The water exchange of the system was regulated in the expansion tank.

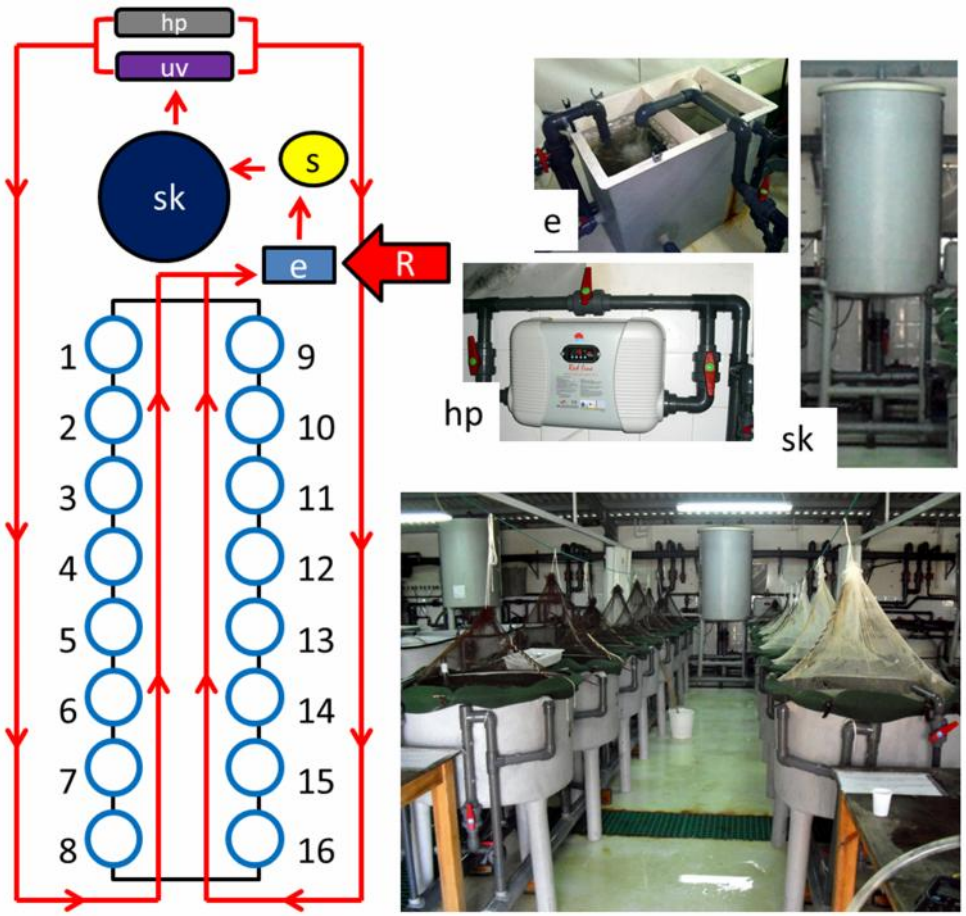


Figure 2.1.2. Semi-open seawater system with detailed flow-water and elements: R - water renewal; e - expansion tank; s - sand filter; sk - Skimmer; uv - ultra-violet filtration; hp - heat pump.

The experiments were carried in 24 replicates of 216L (83cm of diameter, 50cm of height and 40cm of filling height) circular tanks (Fig. 2.1.2). The tanks belonged to two independent seawater systems, according to the feed being tested, one with 16 and another with 8 tanks. Tanks had a plain bottom – for an exact determination of growth and food intake, to avoid cannibalistic behaviour and to facilitate faeces collection. In each tank, water entered by the top and exited by the bottom (Fig. 2.1.3). The upper edge of the tanks was filled with green scourer and an external net to prevent that octopus escaped (Fig. 2.1.3). In addition, to provide a good welfare environment during the trials, each tank was covered by an almost opaque textile (Fig. 2.1.3), which promoted a low light intensity environment, and a PVC tube (20mm diameter) was placed inside the tank to act as shelter (Fig. 2.1.3). As for the high oxygen saturation requirements, the water flow was controlled and adjusted accordingly, and aeration was supplied by an airlift (Fig. 2.1.3) inside the rearing tank. Dissolved oxygen was always maintained above 80% saturation to prevent limitations due to this parameter (Cerezo Valverde, García García, 2005). Tank culture parameters (water temperature and oxygen saturation) were measured, by an YSY 550A probe and registered daily at 9.00 a.m.

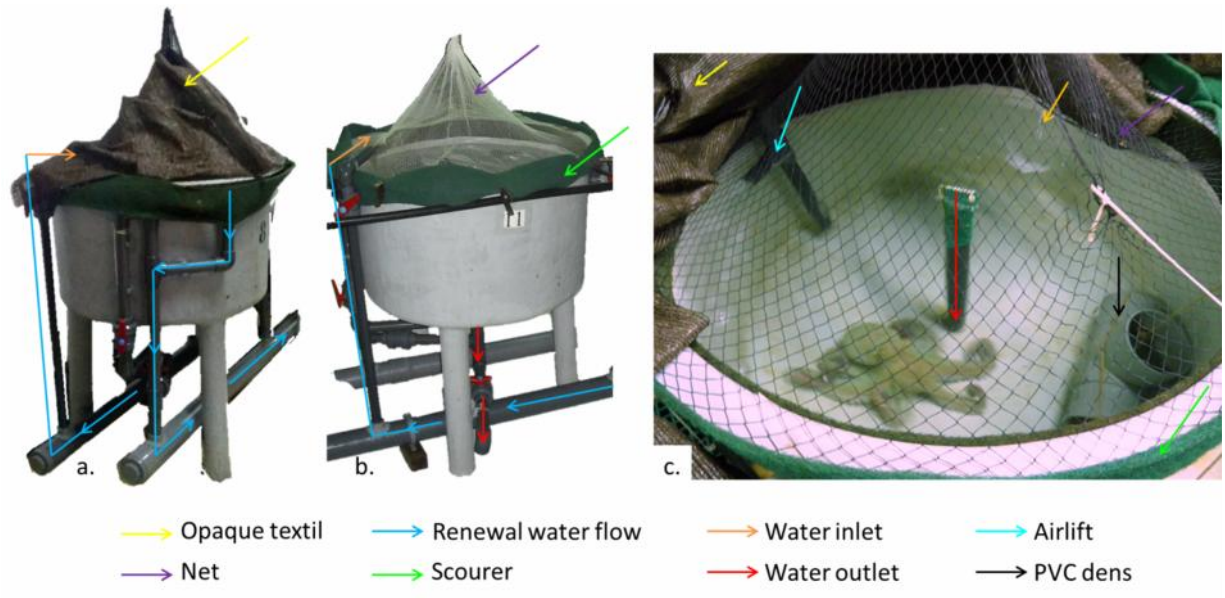


Figure 2.1.3 Circular experimental tank (216L): external (a. and b.) and internal (c.) views. Arrows refer to tank elements and water flow.

2.2. Experimental animals and maintenance

O. vulgaris individuals were caught in the Mediterranean Sea in late February of 2013, near San Pedro del Pinatar (Murcia, S.E. Spain), by commercial fishery vessels using bottom trawls.

While on-board, octopi were placed in 300L tanks with constant marine water supply to maintain oxygen up to 90% saturation. As a safeguard against water contaminants, pumping of water to the 300L tank was stopped before entering the harbour. Upon arrival in the harbour, animals were transferred into bag nets (10-12 octopuses, with ≈600g, in each 50x80cm nylon net with 8mm mesh; Fig. 2.2.1.a.) by lowering the tank water level. This procedure is known to be non-stressful to octopuses; and, in previous trials, longer survival was found with decreasing handling. These nets were then introduced into a 200L portable tank (Fig. 2.2.1.b.), allowing the animals to move out. The seawater of this tank (at 14-16˚C temperature) was oversaturated in oxygen and preceded from the rearing facilities.

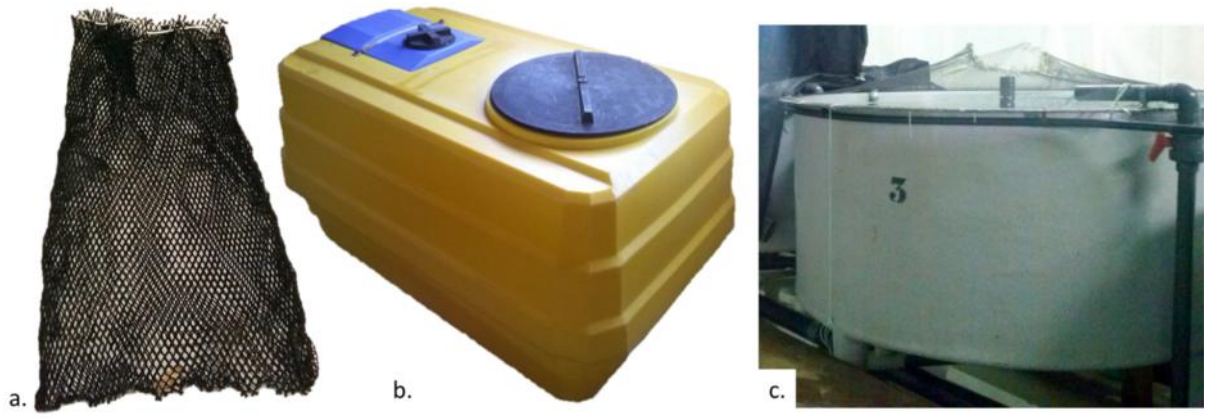


Figure 2.2.1 Transport and acclimatization. a. bag net(50x80cm), b. portable tank (200L), c. circular acclimatization tank (1970L).

The transport to the facilities took less than 2h. After, the octopuses were transferred to a 1970L (176 cm of diameter, 100 cm of height, and 81 cm of filling height) circular acclimation tank (Fig. 2.2.1.c.), which had PVC pipes as dens. Octopuses were daily fed on crab (*Carcinus mediterranus*). Temperature and oxygen saturation were controlled during acclimatization and the experiments, which lasted for 2 and 8 weeks, respectively. The

photoperiod used during both was natural (12L : 12D) and obtained through the use of fluorescent daylight lamps.

A week before the beginning of the experiments, 24 males were selected and transferred individually to the experimental tanks for adaptation (Fig. 2.1.2), continuing to be daily fed *ad libitum* on crab.

2.3. Preparation, conservation and water stability of the diets

Two experimental diets were prepared using bogue (*Boops boops*) from traditional fisheries, either freeze-dried or as meal. The comparative ingredients (10% of total raw materials) were: freeze-dried bogue (diet FDb) and bogue-meal (diet Mb) prepared under 60°C (Vegenat, S.A., Ctra. Badajoz-Montijo km 24.9, Pueblonuevo del Guadiana, Badajoz, Spain). The remaining ingredients are presented in Table 2.3.1.

Freeze-dried ingredients were prepared at IMIDA facilities. The preparation of raw ingredients for freeze-drying required the removal of bones and viscera from bogue and squid, and a whole crab trituration. After being cut into fillets, portions were distributed in plates and maintained at -80°C in a Thermo Scientific- REVCO VALUE PLUS before being used. The freeze-drying was done in a HETO Power Dry LL3000. Afterwards, all freeze-dried ingredients were triturated in a blender (Retsch Grindomix GM200) to obtain a fine powder (<200µm), which was vacuum packed (in a LeaderVac V500) and maintained at -4°C until use.

Feeds preparation was performed using a cooking blender (Professional LACOR My Cook 1.8; Taurus, S.L., Lleida, Cataluña, Spain; Fig. 2.3.1.B). Ingredients were accurately weighted and added (Fig. 2.3.1.A). An established order of addition was followed to facilitate ingredients dissolution. The addition of the next ingredient was done when a homogeneous mixture was obtained with the previous. Water was introduced first. When warmed (up to 40°C), starch and glucose were added at the same time. Then, fish oil and gelatine were slowly included to avoid lumps. At that time, the food processor's mixture speed was increased. When a homogenous mixture was obtained, the remaining ingredients (egg yolk powder; freeze-dried squid and crab and either freeze-dried or bogue-meal) were mixed and added inchmeal. Finally, the mixture was transferred to anti-adherent plates (Fig.

2.3.1.C) and stored in a fridge (for 24h at 4°C). After, feeds were vacuum-packed and maintained frozen in the freezer (-20°C) until use.

Table 2.3.1. Basal mixture (%), proximate composition (% dry weight) and statistical analysis of freeze-dried bogue (FDb) or bogue meal (Mb) formulated diets.

		Diet FDb	Diet Mb			
Basal Mixture (%)	**Water**	40	40			
	Gelatin[c]	22	22			
	Egg yolk[d]	10	10			
	Bogue[e] **(*B.boops*)**	10	10			
	Squid[e] **(*T.sagittatus*)**	5	5			
	Crab[f] ***(C.mediterraneus*)**	5	5			
	Fish oil	2	2			
	Glucose[g]	3	3			
	Starch[h]	3	3	**df**	**Statistic**	**P**
Proximate composition (%)	**Moisture**	43.48±0.13	43.59±1.00	4	0.164	0.878
	Crude protein	66.14±0.01	69.98±0.31	4	-4.249	0.013 [a]
	NFE[i]	5.93±0.51	5.58±0.07	3	-1.415	0.252
	Crude lipid	22.17±0.46	20.19±3.46	4	-1.766	0.215
	Ash	5.77±0.02	6.20±0.04	4	9.899	0.001 [a b]
	AIA[j]	0.0938±0.0247	0.0920±0.0118	4	-0.128	0.904
	Gross energy (kJ/100 g)	2400±9.5	2417±71.3	2.071	0.427	0.710
	P/E (g/MJ)	26.22±0.10	28.04±0.90	2.047	3.460	0.072

Data as mean±S.D.; [a] significant for P<0.05; [b] significant for P<0.01; [c] Granulated Gelatin, Bloom 220, supplied by Productos Sur, S.A. (Pol. Ind. Oeste, San Ginés, Murcia, Spain); [d] Egg yolk powder, supplied by Avícola San Isidro S.L. (Los Belones, Cartagena, Murcia, Spain); [e] Freeze-dried ingredients; [f] Whole animal freeze-dried; [g] Glucose anhidre, supplied by Guinama S.L.U. (Alboraya, Valencia, Spain); [h] Starch from potato soluble, supplied by Panreac Química S.L.U., (Castellar del Vallés, Barcelona, Spain); [i] NFE, Nitrogen-free extract, calculated by difference; [j] AIA, Acid Insoluble Ash; [k] P/E= protein/energy ratio; df-degrees of freedom.

Figure 2.3.1. Raw materials used in formulated diets (A): a. - bogue; a1. - bogue meal; a2. - freeze-dried bogue; b. - freeze-dried squid (*T.sagittatus*); c. - freeze-dried crab (*C. mediterranus*); d. - egg yolk powder; e. - commercial glucose; f. - commercial starch; g. - commercial gelatin; h. - water; i. - fish oil. Cooking blender Professional LACOR My Cook 1.8, Taurus (B). Formulated feed on anti-adherent plate and after being demolded (C).

The water stability of both feeds was determined and was expressed in disintegration percentage of dry weight after soaking in seawater during 24h. Three replicates (≈20 g each) of feed were introduced into closed pots filled with seawater, where they remained for 24 hours for stability determination. Then, replicates were removed from pots, were placed into moisture crucibles (previously dried and weighed), and dried in an oven (at 105˚C for 24 hours). Finally, the replicates were weighed, when cold, to calculate the dry matter. The disaggregation (WSI) was calculated using the mean values, according to the formula presented:

$WSI\ (\%) = \frac{(DWf - DWi)}{DWi} x\ 100$, where DW_i and DW_f are initial and final dry weights, respectively.

2.4. Experimental design

The experiments were performed for 56 days (from 28th February to 25th April, 2013). After acclimation, the experimental groups (n=8) were defined. Only males were selected, in order to avoid any influence of maturation and reproduction. The selection was made by visual recognition of the hectocotylus (a visible structural modification in the third arm; Fig. 1.6.1.3).

Feeds were supplied *ad libitum* (determined later as 5% of the body weight of each individual per day) in cubed shaped pieces (Fig. 2.4.1). In order to reduce stress, conditioning acceptability or intake; the daily food supply was performed with a time lag after collection of remaining food. The food remains collection was performed using a small net, daily in the morning (9.30 h approx.). At this time, oxygen saturation and temperature were also measured.

Figure 2.4.1. Cubed shaped feed pieces (a.) and feed supply (b.).

The collected food remains were dried in an oven (105˚C for 48 h) to obtain its dry weight, needed for the calculation of daily intake by each individual.

In each experiment, octopuses were only weighed (Fig. 2.4.2) three times to avoid handling and consequent stress: at the beginning (day 0), intermediate (day 28) and at the end (day 56). In the final sampling, the animals were weighed and anesthetized (by immersion in cold seawater) before being sacrificed to meet EU (Directive 2010/63/EU) and national welfare legislation. In this Directive, ethical principles of reduction, replacement and refinement are established to avoid/minimize any suffering, pain and anxiety (Sykes et al., 2012).

Figure 2.4.2. Sampling procedure. a. - transport of octopus individuals from the rearing tanks to the sampling area in a net; b. - weighing; c. - octopus transfer to the net before being placed back into the corresponding rearing tank.

2.4.1. Experiment 1

Two experimental groups of 8 replicates were defined by the tested diets: freeze dried bogue (FDb, n=8) and bogue-meal (Mb, n=8) feed. Octopuses were fed at 12h00, five days a week. They were starved on Wednesdays and Saturdays (Table 2.4.1.1).

Table 2.4.1.1. Feeding protocol 1.

Week / Day	1	2	3	4	5	6	7	8
Monday	1	8	15	22	29	36	43	50
Tuesday	2	9	16	23	30	37	44	51
Wednesday	3	10	17	24	31	38	45	52
Thursday	4	11	18	25	32	39	46	53
Friday	5	12	19	26	33	40	47	54
Saturday	6	13	20	27	34	41	48	55
Sunday	7	14	21	28	35	42	49	56

Colored cells indicate starvation.

The mean initial wet weights were of 661±62g for FDb and 696±58g for Mb, and presented no statistical difference ($p<0.05$). Mean temperature and oxygen saturation over the experimental period were 19.8±1.5˚C and 85.1±5.6%, and 19.3±1.0˚C and 83.4±4.8% for FDb and Mb, respectively.

2.4.2. Experiment 2

Experimental groups were defined by two different starvation protocols: three (3FDb, n=8) or two (2FDb, n=8) days per week without eating. The first group was not fed on Mondays, Wednesdays and Saturdays (Table 2.4.2.1), while the second was not fed on Wednesdays and Saturdays (Table 2.4.1.1). Octopuses were fed at 12h00 with the freeze-dried bogue diet (FDb).

Table 2.4.2.1. Feeding protocol 2.

Week / Day	1	2	3	4	5	6	7	8
Monday	1	8	15	22	29	36	43	50
Tuesday	2	9	16	23	30	37	44	51
Wednesday	3	10	17	24	31	38	45	52
Thursday	4	11	18	25	32	39	46	53
Friday	5	12	19	26	33	40	47	54
Saturday	6	13	20	27	34	41	48	55
Sunday	7	14	21	28	35	42	49	56

Colored cells indicate starvation.

The mean initial wet weights were 661±62g for 2FDb and 689±67g for 3FDb, and presented no statistical difference ($p<0.05$). Mean temperature and oxygen saturation over the experimental period were 19.8±1.5˚C and 85.1±5.6%, and 19.9±1.4˚C and 85.5±6.2% for 2FDb and 3FDb, respectively. In a similar way, both temperature and dissolved oxygen presented no differences ($P>0.05$).

2.5. Sample collection and preservation

After being sacrificed, animals were placed in numbered bags (Fig. 2.5.1.a.) and moved to the laboratory. All individuals were dissected and classified (according to final weights) in groups for tissues sampling. The digestive gland was weighed in each replicate (Fig. 2.5.1.b.), for the digestive gland index (DGI) calculation.

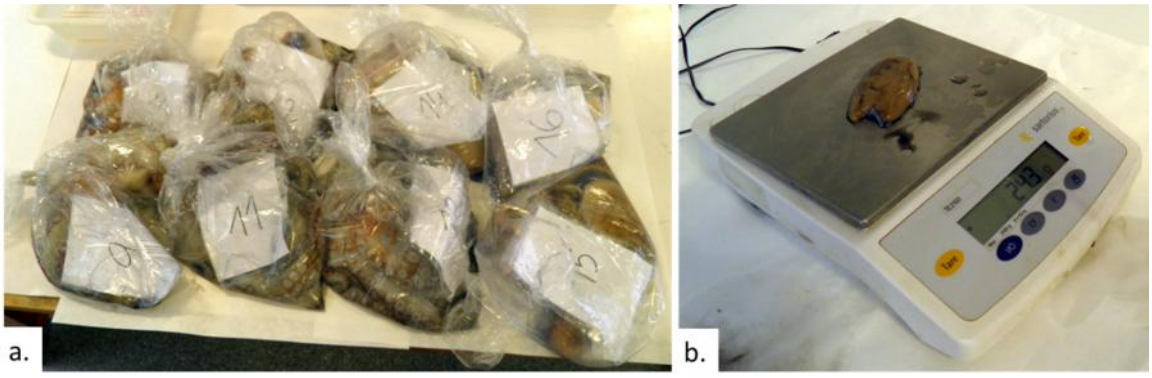


Figure 2.5.1. Numbered bags containing sacrificed octopuses (a.) and digestive gland weighting (b.)

Individuals were sampled (Table 2.5.1) following two protocols: a) in portions, when digestive gland ("DG" sample) and muscle tissue ("M" sample, three arms and part of the mantle) were introduced in separated numbered bags, or b) the whole animal, when digestive gland and carcass ("C" sample, animal excluding digestive gland) were introduced in the same sample bag (Fig. 2.5.2).

Table 2.5.1. Sampling protocol applied to replicates by treatment in both experiments.

Treatments	**Sampling protocol**	
	In portions (DG+M+C)	Whole animal
Experiment 1		
FDb	n= 5	n= 3
Mb	n= 4	n= 4
Experiment 2		
2FDb	n= 5	n= 3
3FDb	n= 5	n= 3

Mb, diet composed with bogue meal; FDb, diet composed with freeze-dried bogue.

All samples were frozen at -20°C to facilitate handling for trituration and mixture. After being homogenized, samples were placed in numbered plastic bags and frozen at -20°C before the analyses were performed.

Figure 2.5.2. Tissue sample preparation. a. - type of samples; b. - frozen sample; c., crushing machine; d. - sample cut as cubes into the crushing machine; e. - homogenous sample; f. - plastic bag with the sample.

During the experiments, faeces were collected daily in each replicate (after food remains collection) using a small net and removing water excess. These were placed in a plastic Petri dish and frozen at -80°C until the end of the experiment. At the end, both faeces and formulated diets samples were freeze-dried and triturated before being analysed (Fig. 2.5.3).

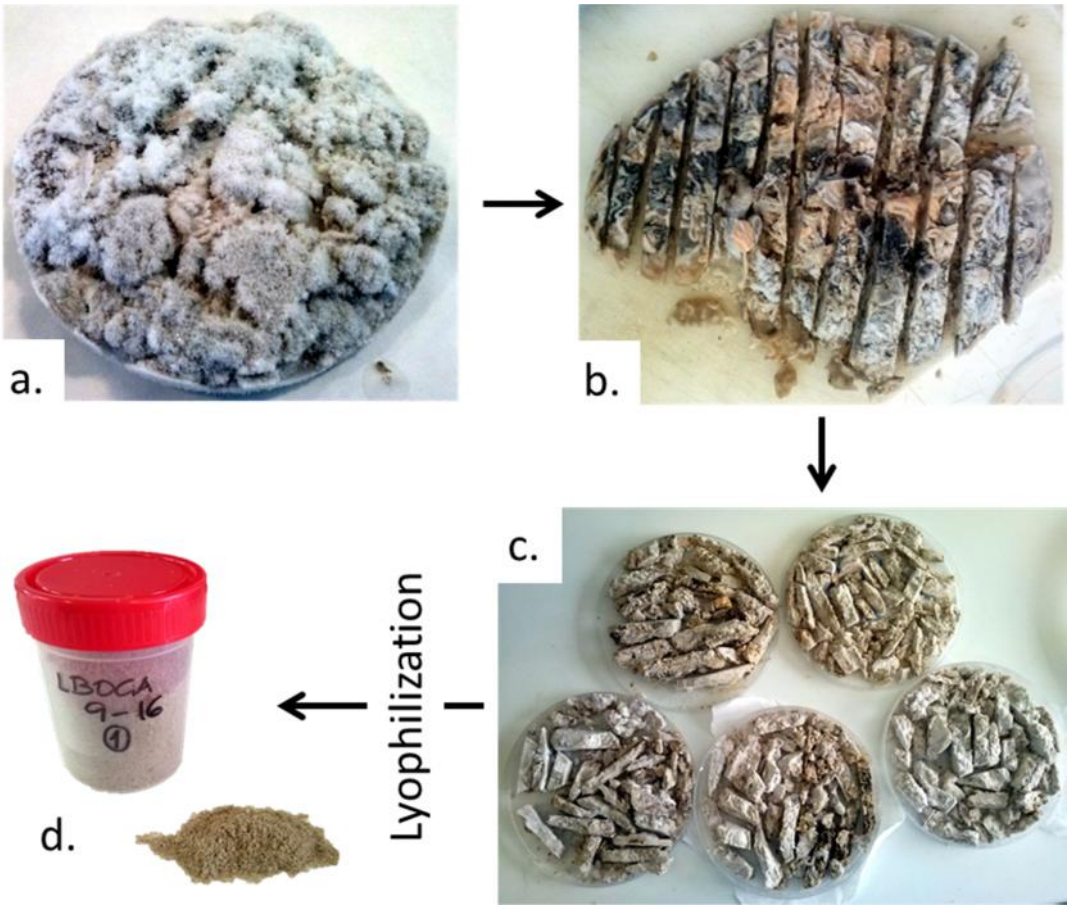


Figure 2.5.3. Faeces samples preparation. a. - Frozen faeces; b. - Cut frozen faeces; c. - freeze-drying plates prepared; d. - freeze-dried faeces sample.

2.6. Biochemical determinations

The macronutrient analyses were carried out in all samples: in duplicate on octopus samples (digestive gland, muscle and whole animal) and in triplicate on formulated diets and faeces samples.

Proximate composition was determined following Association of Official Analytical Chemists [AOAC; (Cunniff, AOAC, 1997)] methodologies. Moisture was obtained by drying the samples (at 105±1˚C for 24h) in a Heraeus Typ. UT12 oven, until constant weight, (Method 930.15; Fig. 2.6.1). Ash quantification was performed by incineration (at 450±2˚C for 16h) in a Heraeus Typ. M110 muffle oven, until constant weight (Method 942.05).

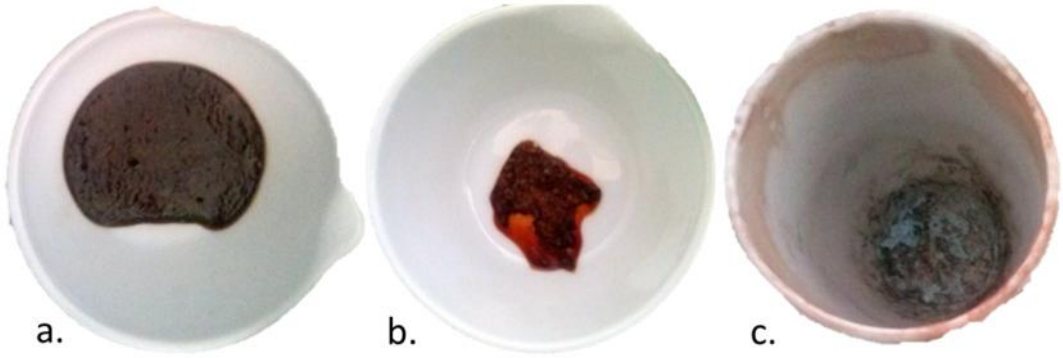


Figure Error! Use the Home tab to apply 0 to the text that you want to appear here.**.** Moisture samples for moisture determination [Method 930.15 (Cunniff, AOAC, 1997)]: a. - digestive gland sample; b. - muscle sample; c. - mineral sample.

The crude protein content was determined by the Kjeldhal method (commercial catalyst) applying a conversion factor of 6.25 for nitrogen transformation (Method 955.01; Fig. 2.6.2). This process includes a protein digestion to convert nitrogen from protein to ammonia (Fig. 2.6.2. a-d); a sample distillation to separate ammonia and solubilize it in an acid solution with known concentration (Fig. 2.6.2. e-g); and a valuation (quantity of neutralized acid by dissolved ammonia in the sample) to quantify nitrogen of the sample (Fig 2.6.2. h-i).

The total lipid content was determined using ethyl ether extraction in a SOXTEC AVANTI 2058 by Method 920.39 (Cunniff, AOAC, 1997). Prior to extraction, moisture was removed from the samples, crushing the samples with a salt (sodium sulphate anhydrous; Fig. 2.6.3. a-c). After lipids extraction samples were dried in an oven to evaporate ethyl ether remains.

The nitrogen-free extract (NFE), referred to carbohydrate content, was determined by subtracting the sum of the crude protein, total lipid, moisture and ash from the total weights.

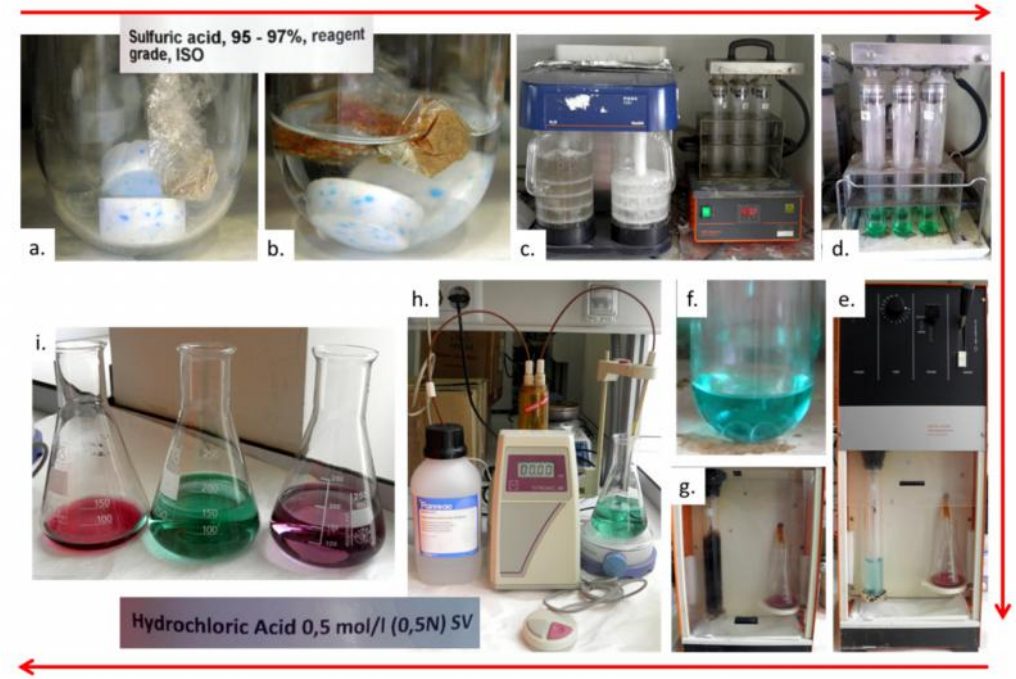


Figure Error! Use the Home tab to apply 0 to the text that you want to appear here.**2.6.2.** Crude protein determination by the Kjeldahl Method 954.01 (Cunniff, AOAC, 1997). Arrows indicate the sequence: a. - sample and commercial catalyst; b. - sample, commercial catalyst and sulfuric acid (95-97%); c. - sample digestion (≈420˚C) and gas condenser; d. - green color of the samples denote the end of the digestion; f. - cold sample mixed with 20mL of distilled water; e. - distiller, the tube contain the sample (left) and the Erlenmeyer a color solution (right); g. - distillation, the sample became black when sodium hydroxide (NaOH) is added, a volume of 150mL was established as the end of distillation; h. - determination; i. - color solution (left), distilled sample (middle) and established white for the determination (right).

Figure 2.6.3. Crude lipid extraction by Method 920.39 (Cunniff, AOAC, 1997). a. and b. - moisture extraction; c. - capsules for lipid extraction; d. - SOXTEC AVANTI 2058; e. - extracted crude lipid.

2.7. Determination of variables

The data obtained from macronutrient analysis were used for index calculation applying the following expressions.

2.7.1. Growth

- Average weight: $Wa\ (g) = \frac{(Wi+Wf)}{2}$, where W_i and W_f are the initial and final weight in g.
- Weight gain: $Wg\ (g) = Wf - Wi$
- Absolute growth rate: $AGR\ \left({}^{g}/_{day}\right) = \frac{(Wf-Wi)}{t}$, where t is the time in days.
- Specific growth rate: $SGR\ \left({}^{\%BW}/_{day}\right) = \frac{(LnWf-LnWi)}{t}\ x\ 100$
- Digestive gland index: $DGI\ (\%) = \frac{DGW}{Wf}\ x\ 100$, where DGW is the digestive gland weight.

The food intake data, referred to each experimental individual, was corrected to avoid overestimation. Considering feed disintegration in water, the dry weight of uneaten diets was multiplied by a correction factor (F), $F = \frac{DWi}{DWf}$.

2.7.2. Ingestion

- Absolute feeding rate: $AFR\ \left({}^{g}/_{day}\right) = \frac{IF}{t}$, where IF (wet weight in g) is the corrected ingested food value, after correction.

IF is calculated through the disaggregation rate suffered in water by the formulated diet; applying the following formula:

$IF =$

$(Dry\ feed\ supplied\ (g) - uneaten\ dry\ feed\ (g)\ x\ F) + Moisture\ feed\ supplied,$

where F values were 1.81 for the FDb diet and 1.75 for the Mb diet.

- Absolute protein/lipid feeding rate: $AyFR\ \left({}^{g}/_{day}\right) = \frac{Iy}{t}$, where *y* could be Lipid or Protein, and I_y is the ingested lipid/protein in g.
- Specific feeding rate: $SFR\ \left({}^{\%BW}/_{day}\right) = \frac{AFR}{Wa}\ x\ 100$

In addition, the variation of the daily intake was analysed in experiment 2 through the instantaneous feeding rate (IFR). This index was expressed as a percentage of the daily ingested food, by each individual, with respect to its body weight estimation (BW_{est}) from the obtained SGR for each one.

- $BWest = BWi + \left({}^{BWi * SGR}/_{100}\right)$, where BWi was the initial body weight (corresponding to the day before).
- $IFR = \frac{IF}{BWest*100}$

In the same way, the variation of the weekly intake was analysed in experiment 2 through the feeding rate per week (WFR). This index was expressed as a percentage of the weekly ingested food, by each individual, with respect to its body weight estimation (BW_{est}), from the obtained SGR for each one, at the 4th day of each week (BW_{est4}).

- $WFR = \frac{\sum_1^n IF}{BWest4}*100$

2.7.3. Feed efficiency

- Feed efficiency: $FE\ (\%) = \frac{(Wf-Wi)}{IF}\ x\ 100$
- Feed conversion ratio: $FCR = \frac{IF}{(Wf-Wi)}$
- Productive value: $y\ PP(\%) = 100x\ \frac{retained\ (y)}{IP}$, where y could be Lipid or Protein.
- Digestibility: The apparent digestibility coefficients were calculated for protein (ADC_{PROT}), lipids (ADC_L) and dry matter (ADCDM) applying the standard equation according to Maynard, Loosli (1969):

$ADC_N 100 - \left(100\ x\ \frac{\%Mdiet}{\%Mfaeces}\right) x \left(100\ x\ \frac{\%Nfaeces}{\%Ndiet}\right)$, where M is the inert marker and

N the nutrient (dry matter, protein, lipid, and NFE). Acid insoluble ash (AIA) was used as inert marker, applying the method described by Atkinson et al. (1984). The formulated diets and faeces samples were freeze-dried. All analysis was performed in triplicate.

2.8. Statistical Analysis

The obtained results were expressed as mean ± standard deviation (S.D.). All data was tested for both normal distribution and homogeneity of variances using the Shapiro-Wilk test and the Levene's test (Zar, 2010), respectively. Arcsine square root transformation was applied to all data expressed as percentage (Fowler et al., 1998) and to other not achieving normality and/or homoscedasticity. When significant difference was found at $P<0.05$ a more restrictive significance, $P<0.01$, was used to analyse data dissimilarities.

A mean comparison was performed, applying a T-test for independent samples (Zar, 2010), to compare: culture parameters (temperature and oxygen saturation); both diets (by their stability in water, their macronutrient composition and their associated energetics); the growth, ingestion and feed efficiency indices for each experimental group; the collected faeces macronutrient composition; digestibility; the proximate composition in octopus tissues fractions (digestive gland, muscle and whole animal) in both experiments; and the IFR comparison day by day in experiment 2.

When a normal distribution and/or homogeneity of the variances were not achieved after arcsine square root transformation, a non-parametric (Mann-Whitney U test) test was used (Zar, 2010).

A Repeated Measures General Linear Model (Zar, 2010) was applied to initial and final weight data of both experiments to analyse the effect of the different treatments (diets and starvation). The same test was performed to WFR data of experiment 2 to analyse its variation and possible interaction with the starvation treatments. The interaction between treatments and variables, diets-weight (experiment 1) and starvation-weight (experiment 2), were tested by a Multivariate test (Pillai's Trace). Reliance on Multivariate tests depends on sphericity; which was verified through the Mauchly's test. When significant differences were identified on sphericity the degrees of freedom were adjusted applying a correction, dependant on "epsilon" value (ε). When $\varepsilon>0.75$ a Huynh-Feldt correction was applied; in contrast, a Greenhouse-Geisser correction was used when $\varepsilon<0.75$.

The effect of the different diets (experiment 1) or starvation protocol (experiment 2) on weight gain, between samplings, was analysed by an Univariate General Linear Model (Zar, 2010).

3. Results

3.1. Water stability and macronutrient composition of the diets

Both formulated diets had a firm texture before being placed in the water. However, both displayed high disaggregation rates (Table 3.1.1). The Mb diet displayed similar stability in water (Table 3.1.1; P>0.05). Feed cubes became softer after been sunken in water for 24 hours and firmness was not maintained.

Table 3.1.1. Results of the water stability tests for diets based on bogue freeze-dried (FDb) or bogue meal (Mb), and statistical results.

Diet	Disaggregation (%) [a]	F [b]	df	Statistic	P
FDb (n=3)	44.71±1.48	1.81	4	-1.911	0.129
Mb (n=3)	42.69±1.09	1.75			

[a] Mean variation in dry weight of diet (% alter after 24 h immersion in water).[b] Correction factor for calculation of real intake. df – degrees of freedom.

Both diets were regarded as semi-moist diets and showed no significant differences in moisture, lipids, NFE and AIA (Table 2.3.1). However, the Mb had higher protein and mineral content (Table 2.3.1). The Energy and P/E ratio presented no differences among diets (P>0.05; Table 2.3.1).

3.2. Experiment 1

The culture parameters (oxygen saturation and temperature) displayed consistent values throughout the experiment (Fig. 3.2.1). The oxygen saturation was similar between experimental groups (P>0.05, Table 3.2.1). Contrariwise, significant differences on temperature were identified (P<0.05; Table 3.2.1). Nonetheless, these didn't exceed 1°C.

Table 3.2.1. Statistical results for temperature and oxygen saturation in experiment 1.

	Statistic	P
Temperature	-2.546	0.011 [a b]
Oxygen saturation	-1.736	0.083 [b]

[a] - significant for P<0.05; [b] –Nonparametric test for two independent samples (Mann-Whitney U); df-degrees of freedom.

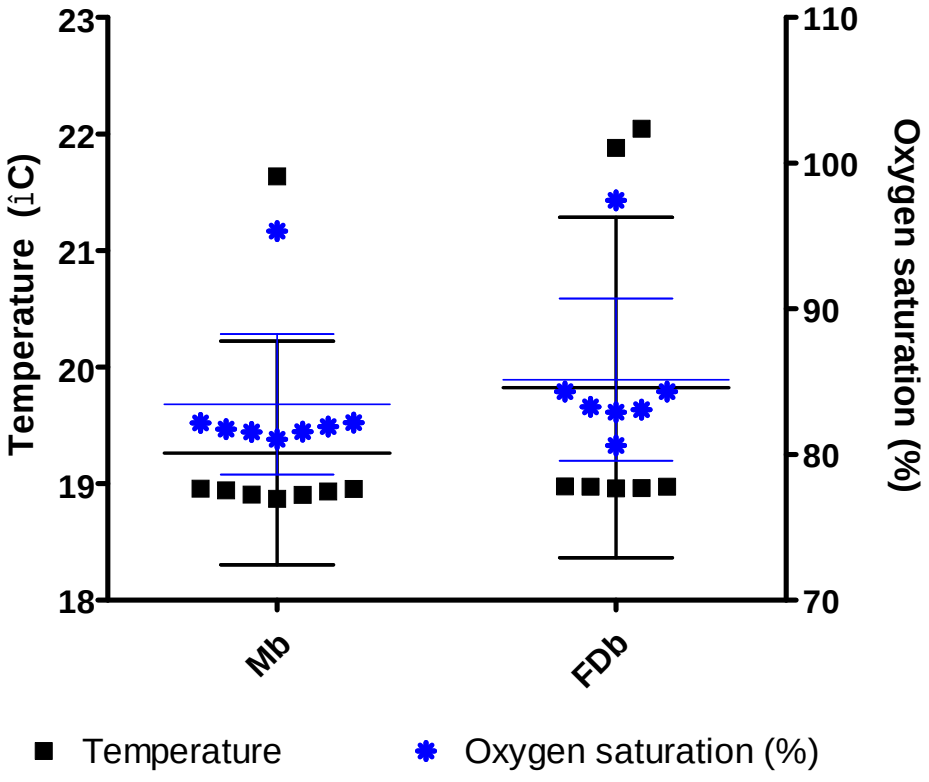


Figure 3.2.1. Mean temperature and oxygen saturation for replicates of Mb and FDb.

At the beginning of the trial, no significant differences were observed between the mean weights of octopi used for both treatments, (P>0.01, Table 3.2.2). Likewise, no-differences were identified in weight between diets throughout and at the end of the experiment (P>0.05, Table 3.2.2). All animals accepted the diets, grew, and produced faeces. No mortality was verified at the end of the experiment.

Table 3.2.2. Weight (W), weight gain (Wg), absolute growth rate (AGR), specific growth rate (SGR), absolute feeding rate (AFR), absolute protein feeding rate (APFR), absolute lipid feeding rate (ALFR), specific feeding rate (SFR), feed efficiency (FE), feed conversion ratio (FCR), protein productive value (PPV), lipid productive value (LPV) and digestive gland index (DGI) of each experimental group fed with formulated diets with freeze-dried bogue (FDb) or bogue meal (Mb) and statistical results.

Index		FDb	Mb	df	Statistic	P
Growth	**W(g)**					
	Day 0	661±62	696±58	13	1.142	0.274
	Day 28	872±102	926±102	13	1.029	0.322
	Day 56	1029±144	1123±83	13	1.574	0.140
	Wg (g)	368±112	426±50	3	1.838	0.165 [d]
	AGR (g/day)	6.57±2 .01	7.61±0.89	13	1.332	0.206
	SGR (%BW/day)	0.78±0.19	0.85±0.09	7.962	1.028	0.334 [c]
Ingestion	**AFR (g/day)**	13.41±2.07	19.47±2.47 [a]	13	0.880	0.000 [a]
			[b]	13	5.113	0.000 [b]
	APFR (g/day)	5.01±0.77	7.69±0.97 [a]	13	5.829	0.000 [a]
			[b]	13	5.113	0.000 [b]
	ALFR (g/day)	1.68±0.26	2.22±0.28 [a]	13	3.829	0.002 [a]
			[b]	13	3.829	0.002 [b]
	SFR (%BW/day)	1.59±0.14	2.14±0.22 [a]	-	-3.246	0.001 [acd]
Feed efficiency	**FE (%)**	48.31±9.70	39.22±2.92	6.977	-2.371	0.050 [c]
	FCR	2.15±0.47	2.56±0.19	7.622	2.150	0.065
	FCR_{dm}	1.22±0.27	1.45±0.11	7.616	2.124	0.068
	PPV (%)	16.80±4.85	16.64±0.92	5	0.008	0.994 [c]
	LPV (%)	10.12±4.44	13.45±7.07	5	0.713	0.508 [c]
DGI (%)		4.34±1.23	6.75±1.00 [a]	-	-2.838	0.005 [acd]

Data as mean±S.D. [a] - significant for P<0.05, [b] - significant for P<0.01. c- applied arcsine square root transformation to data statistical test, [d]- non-parametric test (Mann-Whitney test), df - the degrees of freedom.

The Mb fed experimental group showed a more homogenous Wg but similar growth rates (AGR and SGR) to FDb (P>0.01; Table 3.2.2). The Wg did not display differences regarding diets, time or their interaction (P>0.05, Table 3.2.3).

Table 3.2.3. Statistical results of Univariate General Linear Model applied to weight gain between diets.

Factor	ss	df	Statistic	P
Model	21466.607	3	1.838	0.165
Diet	6364.821	1	1.635	0.212
Time	14691.086	1	3.773	0.063
Diet*Time	800.952	1	0.206	0.654

ss- sum of squares; df- degrees of freedom

Contrariwise, the ingestion rates were significantly different between groups, being higher in Mb ($P<0.05$). In contrast, feed efficiency indices did not show statistical differences between treatments ($P>0.05$, Table 3.2.2). However, higher FE and PPV mean values were observed in the FDb group (Table 3.2.2). In contrast, the DGI was significantly higher in Mb group, ($P<0.01$, Table 3.2.2).

The FDb octopi produced a higher amount of faeces. The macronutrient content of faeces was significantly different between both experimental groups ($P<0.05$ and $P<0.01$; Table 3.2.4). Specifically, ash and AIA presented restrictive statistical significant differences ($P<0.01$, Table 3.2.4). However, a higher protein, mineral and AIA content was identified on Mb faeces. The FDb fed group faeces was more oleaginous and floated more than those of the Mb group. Accordingly, lipid content was higher on FDb (Table 3.2.4). In contrast, no-differences were identified in carbohydrate (NFE) content between both treatments (Table 3.2.4).

Digestibility was different in both treatments. All digestibility coefficients presented statistical differences ($P<0.01$; Table 3.2.4). The mean values obtained for all macronutrients digestibility coefficients (namely ADC_{DM}, ADC_{PROT}, ADC_{L} and ADC_{NFE}) were higher on Mb (94.93±0.72%, 98,13±0,27%, 96,49±0,41% and 65,90±6,45%, respectively) than those obtained for FDb (86,22±3,25 , 96,30±1,02 , 84,78±3,67 and 35,21±13,68; Table 3.2.4). Accordingly, a higher digestibility was observed in the Mb group.

Table 3.2.4. Statistical results and data of freeze-dried faeces proximate composition (% dry weight) and apparent digestibility coefficients obtained with formulated diets with freeze-dried bogue (FDb) and bogue meal (Mb)..

		FDb	Mb	df	Statistic	P
Proximate composition	**Crude protein**	17.69±2.42	25.03±0.04 [a]	2	5.110	0.036 [ac]
				2	5.110	0.036 [c]
	Crude lipid	24.46±1.03	13.60±1.13 [a]		-2.023	0.043 [acd]
	Ash	29.70±1.50	37.05±0.44 [ab]	4	8.079	0.001 [ac]
				4	8.079	0.001 [bc]
	NFE	28.15±3.33	24.32±1.58	4	-1.748	0.155 [c]
	AIA	0.7092±0.1811	1.7826±0.2731 [ab]	4	5.876	0.004 [ac]
				4	5.876	0.004 [bc]
Digestibility coefficients	**ADC_{DM}**	86.22±3.25	94.93±0.72 [ab]	4	5.163	0.007 [ac]
				4	5.163	0.007 [bc]
	ADC_{PROT}	96.30±1.02	98.13±0.27 [ab]	4	3.182	0.033 [ac]
				4	3.182	0.033 [bc]
	ADC_{L}	84.78±3.67	96.49±0.41 [ab]	4	6.760	0.002 [ac]
				4	6.760	0.002 [bc]
	ADC_{NFE}	35.21±13.68	65.90±6.45 [ab]	4	3.357	0.028 [ac]
				4	3.357	0.028 [bc]

[a] - significant for P<0.05; [b] - significant for P<0.01; [c]- applied arcsine square root transformation to data before statistical test; df- are the degrees of freedom;. NFE- Nitrogen-free extract, calculated by difference; AIA- Acid Insoluble Ash; ADC_{DM}- apparent digestibility coefficients of the dry matter; ADC_{PROT}- apparent digestibility coefficients of the protein; ADC_{L}- apparent digestibility coefficients of the lipids; ADC_{NFE}- apparent digestibility coefficient of the Nitrogen-free extract.

The observed macronutrient composition in octopus fractions (digestive gland, muscle and whole animal) was similar between treatments (P>0.05; Table 3.2.5). On the other hand, significant differences (P<0.05, Table 3.2.5) were observed on the ash/mineral content in muscle of different treatments, which was slightly higher in Mb.

Table 3.2.5. Macronutrient composition (% dry weight) of the different fractions of common octopus fed diets formulated with freeze-dried bogue (FDb) or bogue meal (Mb), and statistical results.

		FDb	Mb	df	Statistic	P
Digestive gland	**Moisture**	62.12±6.57	61.52±1.61	6	-0.197	0.850 [c]
	Crude protein	43.57±11.16	51.70±4.14	6	1.371	0.219 [c]
	NFE	8.39±2.83	5.60±2.42	6	-1.450	0.197 [c]
	Crude lipid	44.28±13.77	39.79±3.68	6	-0.568	0.591 [c]
	Lipids digestive gland (g)	8.09±4.87	11.21±2.99			
	Ash	3.77±1.41	2.91±0.35	6	-1.201	0.275 [c]
Muscle	**Moisture**	81.09±0.81	79.94±1.18	6	-1.648	0.151 [c]
	Crude protein	81.76±3.02	80.80±1.46	6	0.589	0.577 [c]
	NFE	4.24±3.14	7.39±1.87	5	1.313	0.246 [c]
	Crude lipid	2.44±0.18	1.81±0.72	3.241	-1.670	0.187 [c]
	Ash	11.56±0.73	10.01±0.67 [a]	6	-3.126	0.020 [ac]
				6	-3.126	0.020 [c]
Whole animal	**Moisture**	79.66±1.27	78.92±1.54	5	-0.686	0.523 [c]
	Crude protein	73.55±3.02	75.11±5.32	5	0.484	0.649 [c]
	NFE	9.82±4.12	7.53±3.30	5	-0.800	0.460 [c]
	Crude lipid	5.89±1.84	6.32±1.22	-	-0.707	0.480 [cd]
	Ash	10.74±1.23	9.29±1.34	5	-1.401	0.220 [c]

[a] - significant for P<0.05, [b] - significant for P<0.01, [c]- applied arcsine square root transformation to data before statistical test, [d]- non-parametric test (Mann-Whitney test), df are the degrees of freedom, P the significance, NFE = Nitrogen Free Extract.

3.3. Experiment 2

Temperature and oxygen saturation, were similar between treatments (P>0.05, Fig. 3.3.1 and Table 3.3.1).

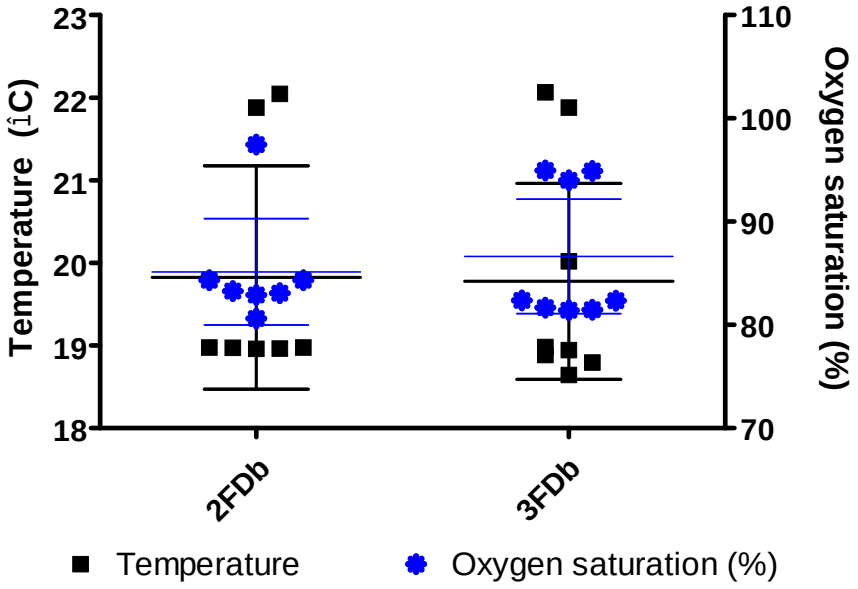


Figure 3.3.1. Mean temperature and oxygen saturation for replicates of 2FDb or 3FDb feeding protocols

Table 3.3.1. Statistical results for temperature and oxygen saturation in experiment 2.

	Statistic	P
Temperature	-0.694	0.487
Oxygen saturation	-0.579	0.563

In contrast, when temperature and oxygen saturation (Fig. 3.3.2) were analysed by week statistical differences between groups were identified on oxygen saturation (P<0.05, Table 3.3.2) in all weeks although temperature was similar (P>0.05, Table 3.3.2).

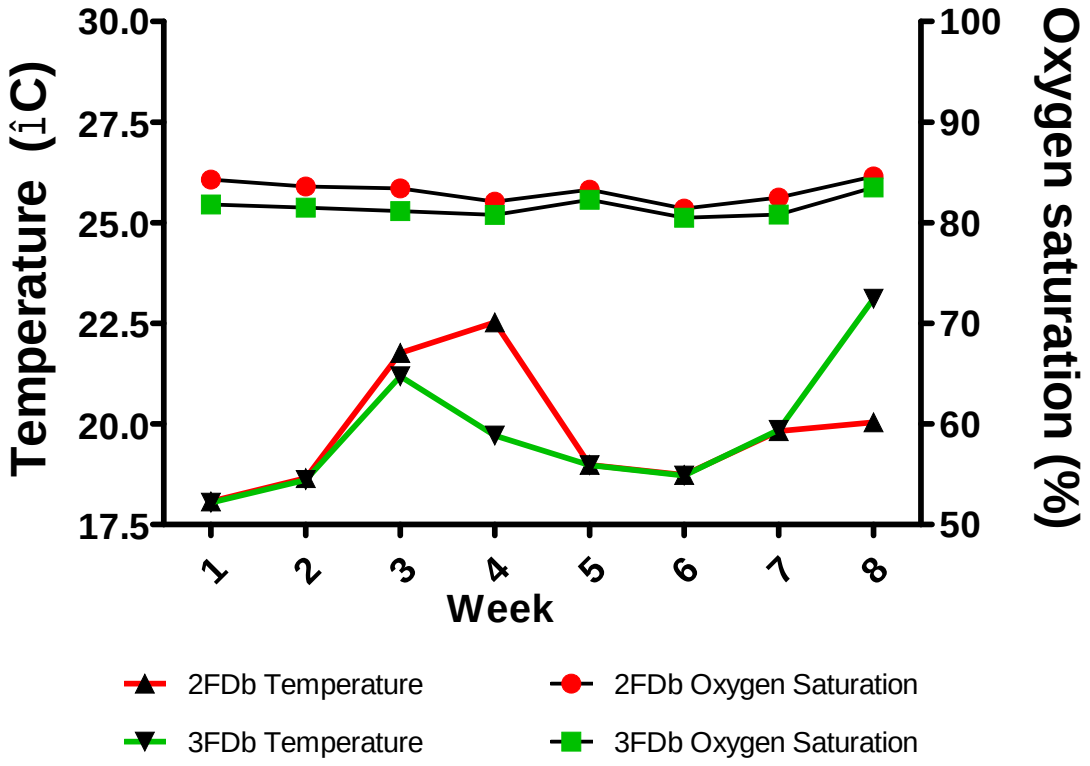


Figure 3.3.2. Mean temperature and oxygen saturation for each feeding protocol (2FDb or 3FDb) by experimental week.

Table 6. Statistical results of weekly temperature and oxygen saturation of both experimental groups, 2FDb and 3FDb

		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Temperature	**Statistic**	-0.661	-0.927	-1.551	-0.971	-0.299	-0.230	-0.446	-0.327
	P	0.508	0.354	0.121	0.332	0.765	0.818	0.656	0.744
Oxygen saturation	**Statistic**	-5.236	-4.835	-4.012	-2.261	-4.813	-3.062	-2.194	-4.175
	P	0.000 [abc]	0.000 [abc]	0.000 [abc]	0.024 [abc]	0.000 [abc]	0.002 [abc]	0.028 [abc]	0.000 [abc]

[a]- significant for P <0.05; [b]- applied arcsine square root transformation to data statistical test; [c]- Nonparametric test for two independent samples (Mann-Whitney U).

Table 3.3.3. shows results of experiment 2 regarding weight, weight gain, absolute growth rate, specific growth rate, absolute feeding rate, absolute protein feeding rate, absolute lipid feeding rate, specific feeding rate, feed efficiency, feed conversion ratio, feed conversion ratio in dry matter, protein productive value, lipid productive value and digestive gland index of each experimental group for diets formulated with freeze-dried bogue two(2 FDb) and three (3 FDb) days starvation.

Table 3.3.37. Weight (W), weight gain (Wg), absolute growth rate (AGR), specific growth rate (SGR), absolute feeding rate (AFR), absolute protein feeding rate (APFR), absolute lipid feeding rate (ALFR), specific feeding rate (SFR), feed efficiency (FE), feed conversion ratio (FCR), protein productive value (PPV), lipid productive value (LPV) and digestive gland index (DGI) of each feeding protocol, two (2FDb) and three (3FDb) day starvation, and statistical results.

Index		2FDb	3FDb	df	Statistic	P
Growth	**W(g)**					
	Day 0	661±62	689±67	12	-0.828	0.424
	Day 28	872±102	939±126	12	-1.090	0.297
	Day 56	1029±144	1127±123	12	-1.392	0.189 [c]
	Wg (g)	368±112	437±80	12	-1.068	0.307
	AGR (g/day)	6.57±2.01	7.81±1.42	12	-1.336	0.206
	SGR (%BW/day)	0.78±0.19	0.88±0.12	12	-1.197	0.255 [c]
Ingestion	**AFR (g/day)**	13.41±2.07	13.37±2.46	12	0.027	0.979
	APFR (g/day)	5.01±0.77	5.00±0.92	12	0.027	0.979
	ALFR (g/day)	1.68±0.26	1.68±0.31	12	0.027	0.979
	SFR (%BW/day)	1.59±0.14	1.47±0.16	-	-1.476	0.140 [cd]
Feed efficiency	**FE (%)**	48.31±9.70	58.65±6.47 [ab]	12	-2.346	0.037 [ac]
				12	-2.346	0.037 [bc]
	FCR	2.15±0.47	1.72±0.18	7.737	2.230	0.057
	FCR_{dm}	1.22±0.27	0.97±0.10	7.737	2.230	0.057
	PPV (%)	16.80±4.85	23.90±2.61 [a]	-	-1.993	0.046 [acd]
	LPV (%)	10.12±4.44	9.74±0.85	2.133	0.22	0.985 [c]
DGI (%)		4,34±1,23	5.55±1.09	-	-1.535	0.125 [cd]

Data as mean±S.D. [a] - significant for P<0.05, [b] - significant for P<0.01. c- applied arcsine square root transformation to data statistical test, [d]- non-parametric test (Mann-Whitney test), df - the degrees of freedom.

The formulated diet (FDb) was accepted by both groups (2FDb and 3FDb) and promoted growth, faeces production and 100% survival.

Mean weight (Table 3.3.3) of different groups were similar throughout the experiment. No significant differences were observed on initial, intermediate and final sampling weights between groups (P>0.05, Table 3.3.3). Nonetheless, differences between initial and final weight (P<0.05, Table 20) were observed.

In a same way, the weight gain between samplings was similar for both treatments (P>0.05, Table 3.3.3).

Table 8. Statistical results of Repeated Measures General Linear Model applied to weight data.

Factor	ss	df	Statistic	P
Weight	1142717.571	2	194.226	0.000 [a]
Starvation	43650.381	1	1.489	0.246
Weight*Starvation	8496.619	2	1.444	0.256

[a] - significant for P<0.05; ss- sum of squares; df- degrees of freedom.

Growth and ingestion were similar between experimental groups (P>0.05; Table 3.3.3). Nonetheless, significant differences were found for FE and PPV (P<0.05, Table 3.3.3) between feeding protocols. The higher mean values were presented by the three day starved group (3FDb).

The mean IFR values presented statistical differences between groups (P<0.05, Table 3.3.3). When IFR values were compared, on a daily basis (Fig. 3.3.3), significant differences were identified at various levels of significance (P<0.05 and P<0.01, Table 3.3.5). The 2FDb protocol exceeded 4% IFR three times; while 3FDb never exceed this value (Fig. 3.3.3). In both groups high IFR peaks were observed after the starvation day; but 2FDb group presented great difference on IFR at the following day than 3FDb.

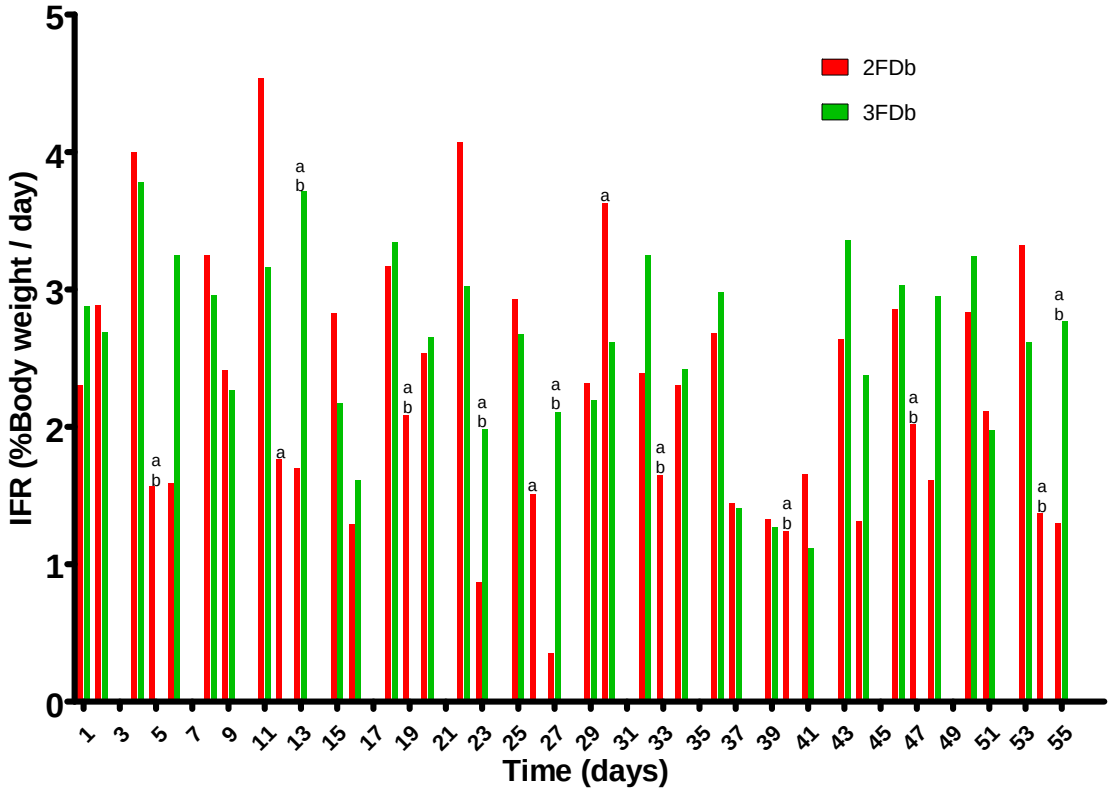


Figure 3.3.3. Instantaneous Feeding Rate (IFR) for two (2FDb) and three (3FDb) day of starvation. a - significant for P<0.05; b - significant for P<0.01.

Table 3.3.59. Statistical results of instantaneous feeding rate (IFR) analysis.

	df	Statistic	P
Day 5	13	7.509	0.000 [a b c]
Day 12	6	2.788	0.032 [a c]
Day 13	6.911	-2.989	0.021 [a c]
	13	-3.176	0.007 [b c]
Day 19	6	7.988	0.000 [a b c]
Day 23	13	-3.347	0.005 [a b c]
Day 26	6	3.341	0.016 [a c]
Day 27	13	-4.500	0.001 [a b c]
Day 30	8.574	2.913	0.018 [a c]
Day 33	6	16.048	0.000 [a b c]
Day 40	6	5.555	0.001 [a c]
	13	5.971	0.000 [b c]
Day 47	6	8.277	0.000 [a b c]
Day 54	6	6.976	0.000 [a b c]
Day 55	13	-3.106	0.008 [a b c]

[a] - significant for P<0.05; [b] - significant for P<0.0;. [c]- applied arcsine square root transformation to data; df- degrees of freedom.

The analysis of IFR on a daily basis applying a Repeated Measures General Linear Model displayed inconclusive results due to the higher number of repeated measures (days) referred to a low number of replicates. Therefore, ingestion was also analyzed weekly (WFR) Results are presented in Fig. 3.3.4.

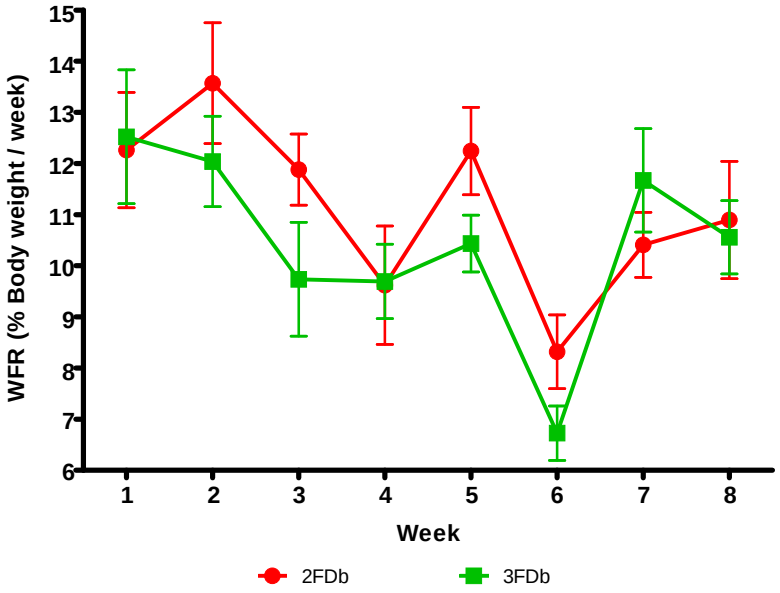


Figure 3.3.4. Feeding Rate per Week (WFR) for both starvation treatments, 2FDb and 3FDb.

The WFR displayed dissimilarities between different weeks ($P<0.05$, Table 3.3.6) but were similar for both starvation protocols ($P>0.05$, Table 3.3.6, which displayed a similar consumption trend.

Table 3.3.6. Statistical results of Repeated Measures General Linear Model applied on weekly feeding rates (WFR).

Factor	ss	df	Statistic	P
Weight	0.078	7	6.514	0.000 [abc]
Starvation	0.005	1	1.980	0.183 [c]
Weight*Starvation	0.10	7	0.868	0.535 [c]

[a] - significant for $P<0.05$; [b] - significant for $P<0.01$; [c]- applied arcsine square root transformation to data; ss- sum of squares: df- degrees of freedom.

Faeces production was higher in 2FDb (personal observation), which were also more oleaginous and floating; 3FDb produced thinner and less floating faeces. The proximate/macronutrient composition of faeces was similar between both feeding protocols (P>0.05; Table 3.3.7). Alike, the digestibility coefficients did not present statistical differences (P>0.01; Table 3.3.7).

Table 3.3.7. Statistical results and data of freeze-dried faeces proximate composition (% dry weight) and apparent digestibility coefficients obtained with formulated diets with freeze-dried bogue two (2 FDb) and three (3 FDb) days starvation.

		2FDb	3FDb	df	Statistic	P
Proximate composition	**Crude protein**	17.69±2.42	16.14±1.43	4	1.007	0.371 [c]
	Crude lipid	24.46±1.03	26.28±5.22	-	-0.664	0.507 [cd]
	NFE	28.15±3.33	29.25±5.03	4	-0.288	0.787 [c]
	Ash	29.70±1.50	28.34±1.98	4	0.883	0.427 [c]
	AIA	0.7092±0.1811	0.8654±0.1894	4	-1.010	0.369 [c]
Digestibility coefficients	**ADC_{DM}**	86.22±3.25	88.78±2.57	4	-1.059	0.349 [c]
	ADC_{PROT}	96.30±1.02	97.23±0.86	4	-1.183	0.302 [c]
	ADC_{L}	84.78±3.67	86.46±4.83	4	-0.499	0.644 [c]
	ADC_{NFE}	35.21±13.68	46.06±3.29	-	-1.528	0.127 [cd]

[a] - significant for P<0.05; [b] - significant for P<0.01; [c]- applied arcsine square root transformation to data before statistical test; df- are the degrees of freedom;. NFE- Nitrogen-free extract, calculated by difference; AIA- Acid Insoluble Ash; ADC_{DM}- apparent digestibility coefficients of the dry matter; ADC_{PROT}- apparent digestibility coefficients of the protein; ADC_{L}- apparent digestibility coefficients of the lipids; ADC_{NFE}- apparent digestibility coefficient of the Nitrogen-free extract.

The whole animal proximate composition was similar between groups (P<0.05, Table 3.3.8). However, digestive gland and muscle showed more variability on macronutrient mean values; while whole animal samples mean values were almost equal (Table 3.3.8).

Table 3.3.8. Proximate composition (% dry weight) of the different fractions of common octopus fed diets formulated with freeze-dried bogue with two (2 FDb) or three days (3 FDb) starvation and statistic results.

		2FDb	3FDb	df	Statistic	P
Digestive gland	**Moisture**	62.12±6.57	56.38±0.93	3.089	1.710	0.183
	Crude protein	43.57±11.16	39.40±3.69	7	0.794	0.453 [c]
	NFE	8.39±2.83	4.34±4.76	6	1.198	0.276 [c]
	Crude lipid	44.28±13.77	53.14±6.91	7	-1.267	0.246 [c]
	Ash	3.77±1.41	3.12±0.89	7	0.863	0.417 [c]
Muscle	**Moisture**	81.09±0.81	80.86±1.26	-	-0.865	0.387 [cd]
	Crude protein	81.76±3.02	76.72±3.55	7	2.259	0.058 [c]
	NFE	4.24±3.14	9.13±4.52	6	-1.189	0.279 [c]
	Crude lipid	2.44±0.18	2.72±1.80	7	-0.130	0.901 [c]
	Ash	11.56±0.73	11.43±1.36	-	-0.738	0.461 [cd]
Whole animal	**Moisture**	79.66±1.27	79.04±1.04	4	0.654	0.549 [c]
	Crude protein	73.55±3.02	74.24±3.17	4	-0.277	0.795 [c]
	NFE	9.82±4.12	10.71±3.74	4	-0.287	0.789 [c]
	Crude lipid	5.89±1.84	4.95±0.20	-	-0.655	0.513 [d]
	Ash	10.74±1.23	10.10±0.75	4	0.730	0.506 [c]

[a] - significant for P<0.05, [b] - significant for P<0.01, [c]- applied arcsine square root transformation to data before statistical test, [d]- non-parametric test (Mann-Whitney test), df are the degrees of freedom, P the significance, NFE = Nitrogen Free Extract.

4. Discussion

4.1. Experiment 1

This work tested two formulated diets (FDb and Mb). These diets had a similar raw material composition (Table 2.3.1), differing only in a comparative ingredient (bogue from discards), which was thermally processed differently (either freeze-dried or plain meal). It was interesting to verify that these thermal changes provoked differences on protein and ash contents of formulations. Hence, differences on diets proximate composition and performance were attributed to this comparative ingredient and the way in which they were prepared. Both diets displayed similar water stability ($P>0.05$, Table 3.1.1), which was characterized by high disaggregation rates that resulted on softening and firmness loss of the supplied feed cubes. This might be considered as a handicap for octopuses feed manipulation.

Bogue (*B. boops*) was used because of its previous results on octopus on-growing and of having an adequate amino acid balance (Cerezo Valverde et al., 2013) and lipid profile (Cerezo Valverde et al., 2012c). Additionally, this species is abundant as a by-product from fisheries and aquaculture from off-shore production cages (Estefanell et al., 2011). In this sense, the aquaculture by-products processing (as fish meal) should facilitate its use by the aquaculture industry. The European squid (*T. sagittatus*) also presents a good lipid profile (Cerezo Valverde et al., 2012c) and high protein content (Rosa et al., 2005). In fact, Morillo-Velarde et al. (2012b) obtained good performances adding freeze-dried squid as raw material in a tested diet. Likewise, crustacean species are recognized as natural preys. The best growth performances ever obtained used crustaceans as raw material. Aguado Giménez, García García (2002a) used a mixture of crab species such as *Macropipus depurator, Calappa granulate, Squilla mantis, Dorippe lanata, Partenope anguligrons* and *Homola barbata*; while Quintana et al. (2008) used shrimp (*Palaemonetes varians*). Hence, freeze-dried crab (*C. mediterranus*) was also included as raw material in both tested diets. Crustaceans are interesting from the nutritional point of view, but also act as an attractant. Egg yolk was also included to maximize the attraction of the prepared feeds. In fact, egg yolk was identified as an efficient attractant by Morillo-Velarde et al. (2011b). Glucose was added to enhance protein utilization for growth, but a PPV increase was not observed in the results. Gelatine and starch were used as binders and provided a suitable presentation for octopus.

According to Morillo-Velarde et al. (2013) meal based diets display low acceptability. Nonetheless, in this study both diets (FDb and Mb) were readily accepted by octopus from the first day and throughout the experiment and promoted 100% survival.

The macronutrient composition of the formulated diets was different than those of presented by other natural diets, such as crab (Cerezo Valverde et al., 2008) or bogue (Aguado Giménez, García García, 2002), Fig. 4.1.1. In general terms, crab and bogue presented less moist and mineral/ash content; and a practically inexistent carbohydrate (NFE) content. In contrast, formulated diets contained a double amount of protein and lipid, which incremented its gross energy content up to eight times than that presented by natural diets (determined in previous studies). Nevertheless, the P/E ratio was relatively similar between formulated and natural diets. On the other hand, the P/E ratios of both formulations (26.22±0.10g/MJ for FDb diet and 28.04±0.90g/MJ for Mb diet) were roughly only half the Lee's (Lee, 1994) suggested value required for optimum growth (>50g/MJ) of cephalopods.

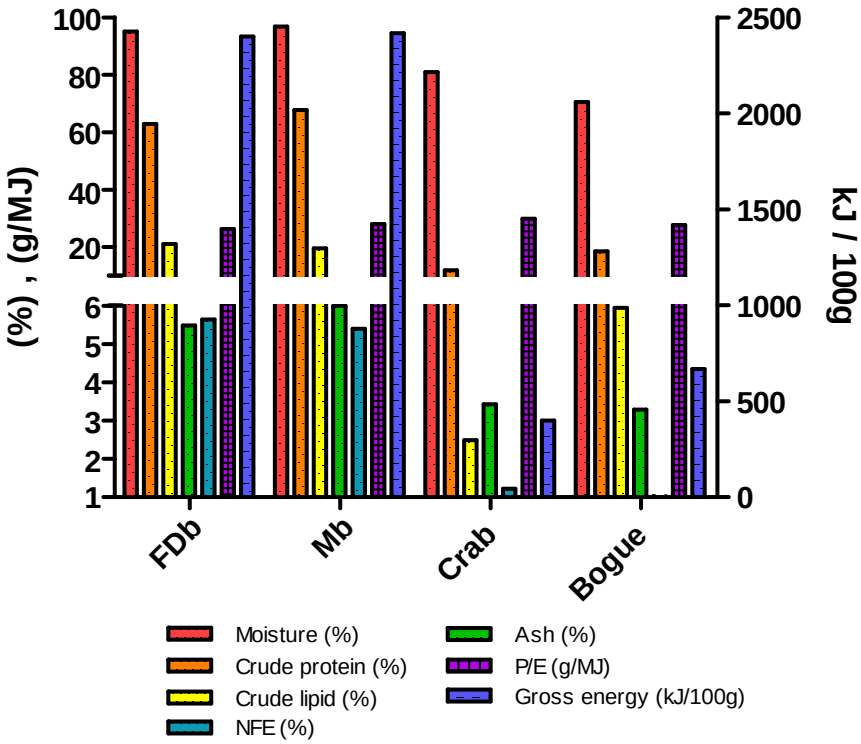


Figure 4.1.1. Proximate composition (% wet weight), protein-energy ratio (P/E, g/MJ) and gross energy (kJ/100g) of diets formulated with bogue, freeze-dried (FDb) or meal (Mb), and natural diets based on crab[a] (Cerezo Valverde et al., 2008) and bogue[b] (Aguado Giménez, García García, 2002).

Despite the ingestion rates were significantly different between groups ($P<0.05$ and $P<0.01$ in some cases, Table 3.2.2), with higher values displayed by Mb (Table 3.2.2), no differences were found between groups in growth and feed efficiency. Nonetheless, FE and FCR values were on the verge of showing significant differences, which might point to a better use of lyophilized bogue by *O. vulgaris*. However, this can only be speculated. .

Fig. 4.1.2 compares growth (AGR and SGR), ingestion (AFR and SFR) and feed efficiency (FE and FCR) indices obtained with the two tested diets in this work (FDb and Mb) with the values obtained for a European flying squid freeze-dried based diet (Morillo-Velarde et al., 2012b), with diets containing fish and krill meals (Querol et al., 2013; Querol et al., 2012a); but also for crab (Cerezo Valverde et al., 2008) and bogue (García García, Aguado Giménez, 2002). All of these studies were performed in similar culture systems and with similar rearing temperature (≈18.5°C). The profile of feeding based on bogue did not differ so much of that obtained when formulated diets containing freeze-dried ingredients were used (Fig. 4.1.2). Up till know, prepared diets which contained meal as raw material (Table 4.1.1 and Fig. 4.1.2) had performed worse than natural diets and feeds based on dry ingredients.

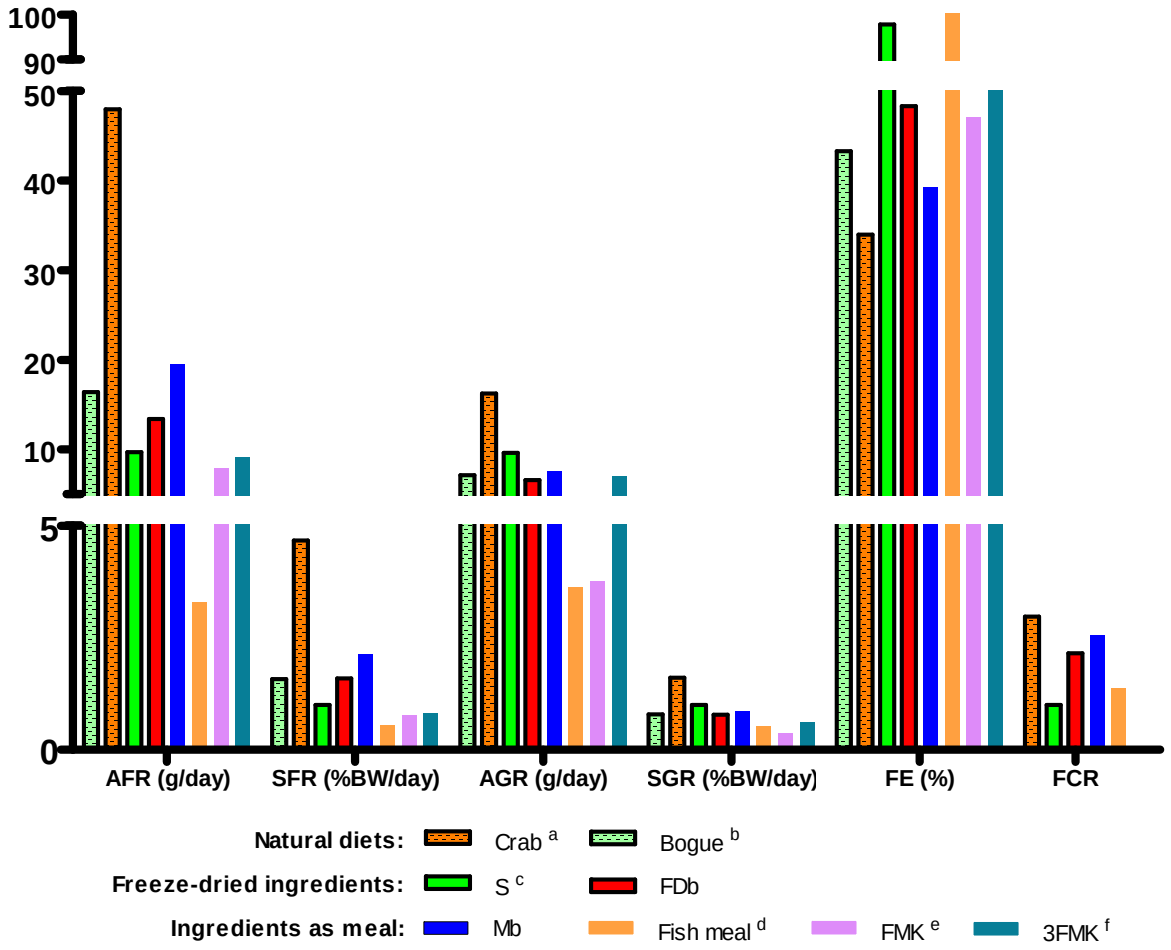


Figure 4.1.2. Comparison of AGR, SGR, AFR, SFR, FE and FCR obtained for diets in the present study (FDb = freeze-dried bogue; Mb= bogue meal), for natural diets [crab[a] (Cerezo Valverde et al., 2008)] and bogue[b] (García García, Aguado Giménez, 2002)] and for other formulated diets: S[c]= freeze-dried European flying squid, (Morillo-Velarde et al., 2012b), fish meal[d] (Querol et al., 2012b) and a mixture of fish and krill meals [ef] (Querol et al., 2013), where the 3FMK diet contained fish meal in a triple proportion than the FMK diet.

It is interesting that Mb and FDb displayed similar AFR, SFR and FCR to bogue of García García, Aguado Giménez (2002), roughly half of that obtained with crab (Cerezo Valverde et al., 2008) but higher than other meal (Estefanell et al., 2012; Estefanell et al., 2009a; López et al., 2009; Querol et al., 2013; Querol et al., 2012a; Querol et al., 2012b) or even lyophilized diets, such as squid (Morillo-Velarde et al., 2012b). On the other hand, Mb displayed higher AGR and SGR than any meal that used exclusively fish (Querol et al., 2012b) or even of that containing fish and krill on a 1:1 ratio (Querol et al., 2013), but was similar to a diet containing fish and krill meal but with a 3:1 ratio (Querol et al., 2013). It

was already expected that FDb would have promoted increased FE over Mb. Nonetheless, octopus fed FDb displayed lower FE when compared with the octopus that were fed fish meal by (Querol et al., 2012b).

In the present study, the highest ingestion (AFR) was obtained with diet Mb (19.47±2.47 g/day); although FDb intake was different but not so markedly lower (13.41±2.07g/day). In contrast, fish (Querol et al., 2012b) and fish-krill meal diets (Querol et al., 2013) displayed lower values of AFR. None ever reached the values of a crab-based diet [≈50 g/day; (Cerezo et al., 2008)].

In terms of weight gain, it was interesting to verify similar values for either formulations of the present study (368±112 g, for FDb, and 426±50 g, for Mb) with those of Morillo-Velarde et al. (2012b), which fed octopus juveniles with a formulation based on freeze-dried squid and obtained an increased weight of 401±78 g. On the other hand, these values were up to 8 times higher than those obtained by Querol et al., 2012b, which used fish meal as raw material and obtained only 50.9±15.9g of weight increment. No comparisons on weight gain were made with (Querol et al., 2013) because the initial weight of octopus was of ≈950g.

The higher FCR obtained by (Morillo-Velarde et al., 2012b) is surely related to use of squid in the formulation. It is quite interesting that a semi-moist formulation attained a higher FCR than an extruded diet, such as that evaluated by Querol et al., 2012b. The process of fabrication is in part responsible for retaining the nutritional properties of the diet while it is being exposed to sea water for hours and semi-moist diets are known to leach more than extruded ones (Tacon, 1987). Therefore, the squid formulation has to have the most balanced nutritional composition from all tested diets, compared in Fig. 40, to obtain a FCR of ≈1. On the other hand, the fish meal formulation attained lower FCR values than those of this study (either FDb or Mb) and AFR was almost 4x higher in these diets. This in accordance to a higher leaching of FDb and Mb, which is confirmed with disaggregation values of approximately 7% against only 4% after 4h, verified in the fish meal diet of Querol et al., 2012b.

Table 4.1.1 shows a comparison of AGR, SGR, AFR, SFR, FE and FCR of the present results with other not so successful trials that used meal in the formulation of prepared feeds for *O. vulgaris*. All of these diets promoted negative growth despite the increased ingestion.

Table 4.1.110. Comparison of AGR, SGR, AFR, SFR, FE and FCR obtained for diets in the present study (FDb = freeze-dried bogue; Mb= bogue meal) and for other formulated diets with: a mixture of bogue and crab meals [ab] (Estefanell et al., 2012; Estefanell et al., 2009a) and krill meal [c] (López et al., 2009)

Diet	AGR (g/day)	SGR (%BW/day)	AFR (g/day)	SFR (%BW/day)	FE (%)	FCR
FDb	6.57	0.78	13.41	1.59	48.31	2.15
Mb	7.61	0.85	19.47	2.14	39.22	2.56
BC meal [a]		-0.11			-5.00	
BC meal [b]	-1.90	-	-	3.10	-	-
Krill meal [c]	-1.06	-	200.79	22.72	-0.53	-190.21

The protein and lipid retention values obtained for the tested diets (Table 3.2.2) were lower than those obtained for natural diets. Crab [PPV= 49.01±6.74 % and LPV= 23.65±3.53 %; Cerezo Valverde et al. (2008)] promoted up to 3 to 5 times higher PPV, and 2 times LPV, than those obtained with both tested diets. In contrast, bogue [PPV= 36.48±2.87 % and LPV= 3.30±0.64 %; García García, Cerezo Valverde (2006)], promoted 2 times more PPV and 3 times less LPV than the ones of the present study.

As regards nutrient digestibility, diet Mb presented the better performance of both tested diets and similar values to crab tested by (Hernández, García García, 2004). The protein digestibility (ADC_{PROT}) of the diets tested in this study was similar to those of natural diets tested by (Hernández, García García, 2004). In all cases, ADC_{PROT} exceeded 95% (Fig. 4.1.3 and Table 3.2.4); which highlights the capability and efficiency of octopus to digest the protein present in the tested formulations of this study. The higher value of ADC_{PROT} obtained in Mb might be eventually related to the higher protein content of this diet (Table 2.3.1), since the differences of excreted protein of FDb to Mb were roughly similar in percentage (5%) to the differences verified in the diets composition.

In contrast, a variation was observed on lipid digestibility coefficients (ADC_L). The diet Mb displayed a higher ADC_L than the natural crab or bogue tested by (Hernández, García García, 2004), (Fig. 4.1.3) and FDb. Since no growth differences were verified between the tested diets and diets shared similar lipid content, this difference might be related to the eventual use of lipids for energy purposes because the total lipid of the digestive gland was similar between tested groups. This would mean that the protein and lipid quality of FDb

and Mb was different and those of the first would have higher quality or were more well balanced. This could be related to the preparation of bogue meal at a temperature below 60°C, which might have promoted lipid desaturation. This will be discussed below. According to Sánchez et al. (2009), a higher dietary lipid content diminishes digestion, as this species display poor capacity for mitochondrial lipid oxidation (O'Dor et al., 1984).

On the other hand, octopus fed FDb used up to two times more carbohydrates than those fed Mb. The use of dietary CH for energy purposes was widely studied in fish species, such as sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), as a possibility to spare protein (Enes et al., 2011a). However, carnivorous fish species display a limited capability of using CH for energy purposes (Enes et al., 2009). Nonetheless, the use of dietary CH by fish depends on various factors; such as species, botanical origin, physical state, dietary level and molecular complexity (Enes et al., 2011b). In this sense, several fish species (marine and freshwater) displayed a better efficiency with polysaccharides (e.g. starch and dextrin) than with mono- or disaccharides [e.g. glucose, sucrose or maltose (Enes et al., 2011b)]. *D. labrax* and *S. aurata*, enhanced starch digestibility with the processing of this raw material, from >70% with native starch to >90% when it was processed (Enes et al., 2011a). In this way, better performance was obtained by glucose than starch by *D. labrax*; while the opposite was observed by *S. aurata* (Enes et al., 2011a). However, lower starch digestibility was observed with high dietary starch in both species (Enes et al., 2011a). In this sense, an enhancement on CH digestibility was reported for *O. vulgaris* by Sánchez Morillo-Velarde (2013) when glucose were added to prepared feeds (97.56%, compared to 83.91% in those where CH were not added). In contrast, CH digestibility was practically null when only starch was included (Sánchez Morillo-Velarde, 2013). The previous evidenced a more efficiency in the use of monosaccharide than of polysaccharides by *O. vulgaris*. However, in some cases, polysaccharides (such as starch) are insoluble in cold water; but an enhancement on solubility is observed with increasing temperature, which enhances polysaccharide gelatinization. According to O'Dor et al. (1984), after a meal containing CH a rapid CH catabolism is observed. These authors also referred to little accumulation of CH in the muscle, as muscular glycogen, and poor removal from muscle in starvation situations. Accordingly, Morillo-Velarde et al. (2011a) reported a quick dietary CH catalysis when explosive activities, such as apprehension and escaping from predators, occur. Additionally, these authors also referred a ≈10% CH contribution on the daily energetic waste of *O. vulgaris* in short-term starvation situations. The CH tissues removal

for energy proceeded from muscle and digestive gland, 8.6% and 1.3%, respectively (Morillo-Velarde et al., 2011a). Hence, CH inclusion in feeds needs to be considered to sustain its contribution to fuelling metabolism in this species under starvation situations (Sykes, Pers. Comm).

The dry matter digestibility coefficient (ADC_{DM}) was analogous in trend and in values with the described ADC_L. In contrast, carbohydrate content in diets was similar (around 6% of total composition) but the nitrogen-free extract (ADC_{NFE}) shown very poor digestibility values, from 35 to 66% in tested diets (Fig. 4.1.3).

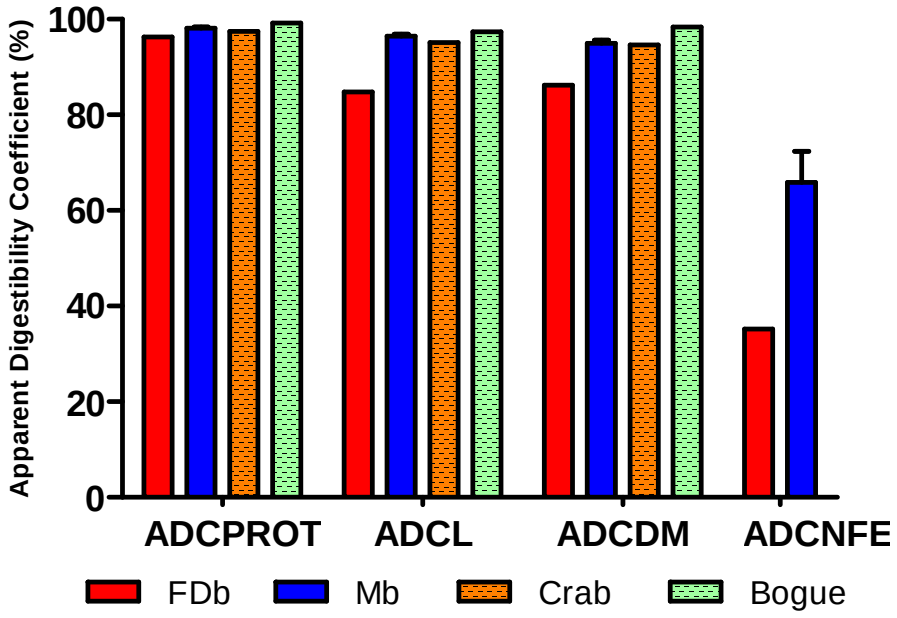


Figure 4.1.3. Comparison of apparent digestibility coefficients of protein (ADCPROT), lipid (ADCL), dry matter (ADCDM) and nitrogen-free-extract (ADCNFE) obtained for the experimental diets formulated with freeze-dried bogue (FDb) or bogue meal (Mb) and with natural diets (crab and bogue) in similar conditions (Hernández, García García, 2004)

In terms of nutritional composition of *O. vulgaris* tissues, non-significant differences were found in between both tested diets (Fig. 3.2.5). As previously reported by Kreuzer et al. (1984), lipids were mostly found in the digestive gland and proteins were mainly stored in muscle tissue. These results were similar to those reported for the different fractions of common octopus fed with the freeze-dried squid diet tested by (Morillo-Velarde et al., 2012b).

The digestive gland index (DGI) is used to analyse the condition of the animals and is applicable to octopus species. In this sense, (Cerezo Valverde et al., 2008) reported a DGI value of ≈5% using crab, which was similar to that verified with FDb in the present study. The higher value of DGI verified in Mb fed octopus might be eventually related to an increase of the digestive gland area to increase its capability of lipid digestion. Nonetheless, those can only be speculated since no histological cuts of the digestive gland were performed in this study.

Until now, the use of freeze-dried instead of meals as ingredients in octopus feeds promoted better performances. However, in the present study results are not conclusive. Domingues et al. (2009) reported better performance when lower temperature was applied to ingredients, prior to their inclusion in formulated diets, for *S. officinalis*. In this study, the best results were obtained through freeze-dried (-40˚C), compared to dried (60˚C) or boiled (100˚C) shrimp. Differences in growth and survival were related to the temperature processing of shrimp. Heat application might origin disturbances on weak interactions (as primarily hydrogen bonds) on proteins. These might cause an alteration on protein's native conformation (three-dimensional: tertiary and quaternary structures), also called protein denaturation. This process implies the loss of biological activity by proteins (reducing bounding probability with other molecules), which is traduced in a nutritional value and quality reduction. According to Lehninger (1981), the native form of proteins remains stable between a temperature range. Additionally, temperature and stability are directly proportional. However, when this range is exceeded at ≈57˚C, proteins collapse (Fig. 4.1.4). Considering this, the meal used in this study was prepared under 60˚C and the negative effect on diet protein quality, caused by denaturation and loss of proteins and amino acids, was not observed. Accordingly, Ariyawansa (2000) reported higher functional properties retention by low temperature fish meals. Fish meal is considered a raw material that provides essential amino acids, phospholipids and fatty acids, such as DHA and EPA, but also fat-soluble vitamins and steroid hormones (Miles, Chapman). In addition, fish lipids represent an excellent source of polyunsaturated fatty acids (PUFA), being predominant those of group omega-3 fatty acids (linoleic, DHA and EPA) than omega-6 fatty acids. Despite the high nutritional value of lipids, lipid oxidation/degradation especially affects those feeds which are dehydrated, exposed to high temperatures or cooked at after being stored (Addis, 1986). The molecular structure of PUFA (with a great number of unsaturated carbon-carbon bonds in the long fatty acid chains) promotes its oxidation. The lipid

oxidation process includes three steps: auto-oxidation (molecular oxygen reacts directly with lipid and hydroperoxide is obtained); and secondary reactions (molecular oxygen reacts with hydroperoxide and diperoxide is formed; other consecutive oxidation provides ketoglycerides). However, diperoxide dehydration provides hydroperoxide. The division of hydroperoxides produces hydroxi and carbonyl groups. Then, those react with other compounds and secondary products are formed. These new formed products are associated with rancidity highlighting an acute quality loss. Lipid degradation might be fast-tracked by exposure to light and heat (Turner et al., 2006). In fact, the use of bogue meal promoted similar results to the use of lyophilized bogue.

Hence, it seems that temperature control on raw materials preparation is an important issue for octopus nutrition and development of diets.

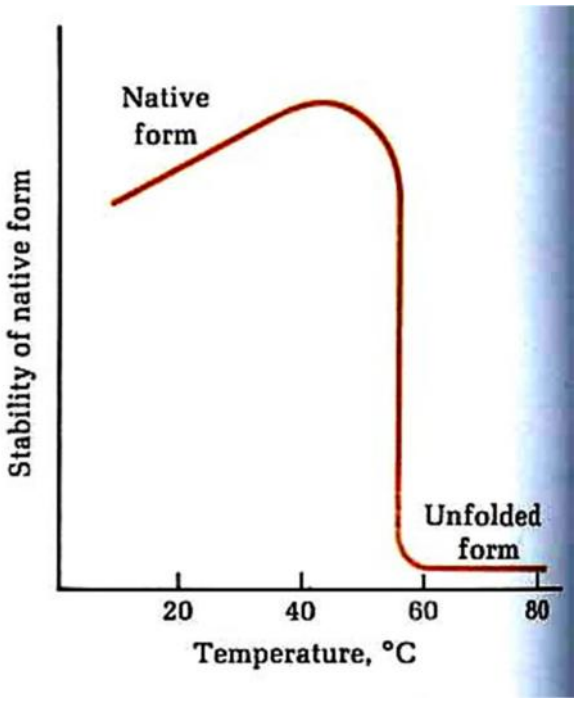


Figure Error! Use the Home tab to apply 0 to the text that you want to appear here.**4.1.4.** Thermal melting point of a protein [adapted from (Lehninger, 1981)]

This study revealed no loss on protein diet quality when dehydration of raw materials was performed below 60˚C; but also a similar yield between meal and freeze-dried bogue based diets.

4.2.Experiment 2

This experiment tested effects of two (2FDb) or three starvation days (3FDb) per week on a weekly feeding schedule. The feeding of both treatments was performed with the FDb diet (Table 2.3.1). The application of this feeding scheme was a success, since no significant differences on growth and ingestion were observed between treatments (Table 3.3.3). In addition, a 100% survival was registered in both groups. However, a previous starvation study performed on the species by García García, Cerezo Valverde (2004), and using crab as food, resulted in better SGR when starvation days increased from no starvation up to two days of starvation (Fig. 4.2.1).

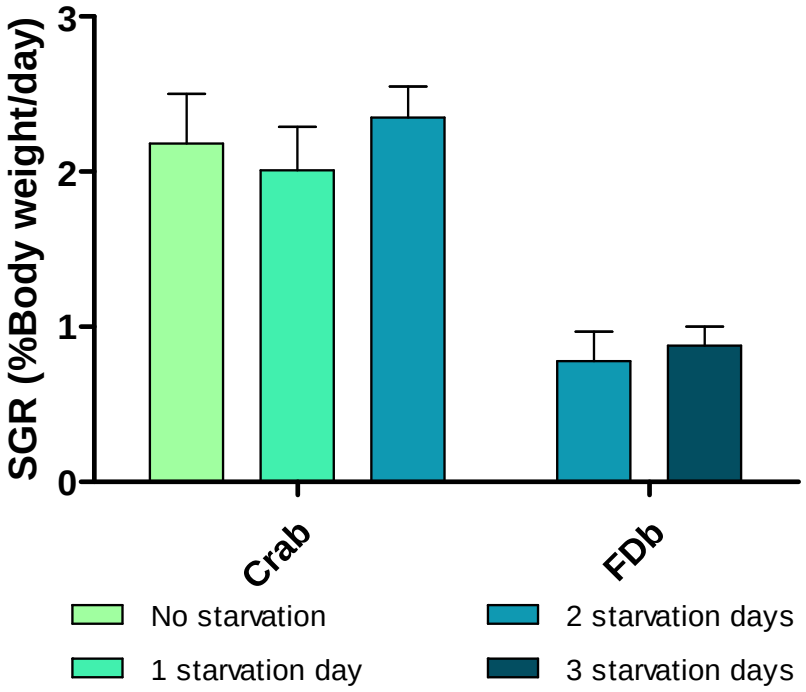


Figure 4.2.1. Specific growth rate (SGR, % body weight.day^{-1}) obtained for different feeding protocols (differing by starvation days per week) feeding with crab (García García, Cerezo Valverde, 2004) and with the tested diet (FDb).

In terms of ingestion (Fig. 4.2.2), while previous results with crab (García García, Cerezo Valverde, 2004) showed a decline in SFR with increasing starvation days, values of SFR were still 5 times higher after 2 days of starvation than those observed in the present study with FDb. In addition, no differences were found between 2 or 3 days of starvation while using the FDb diet. This means that FDb provided enough nutrients to sustain short term

starvation through the use of reserves. Also, probably, *O. vulgaris* individuals are used to undergo this type of starvation in nature and have adapted physiologically to this short-term starvation condition. In this sense, Nixon (1987) reported the existence of some regulation of food intake, since this species reduces its interest on a new attack against other preys when a previous meal was ingested in short-term.

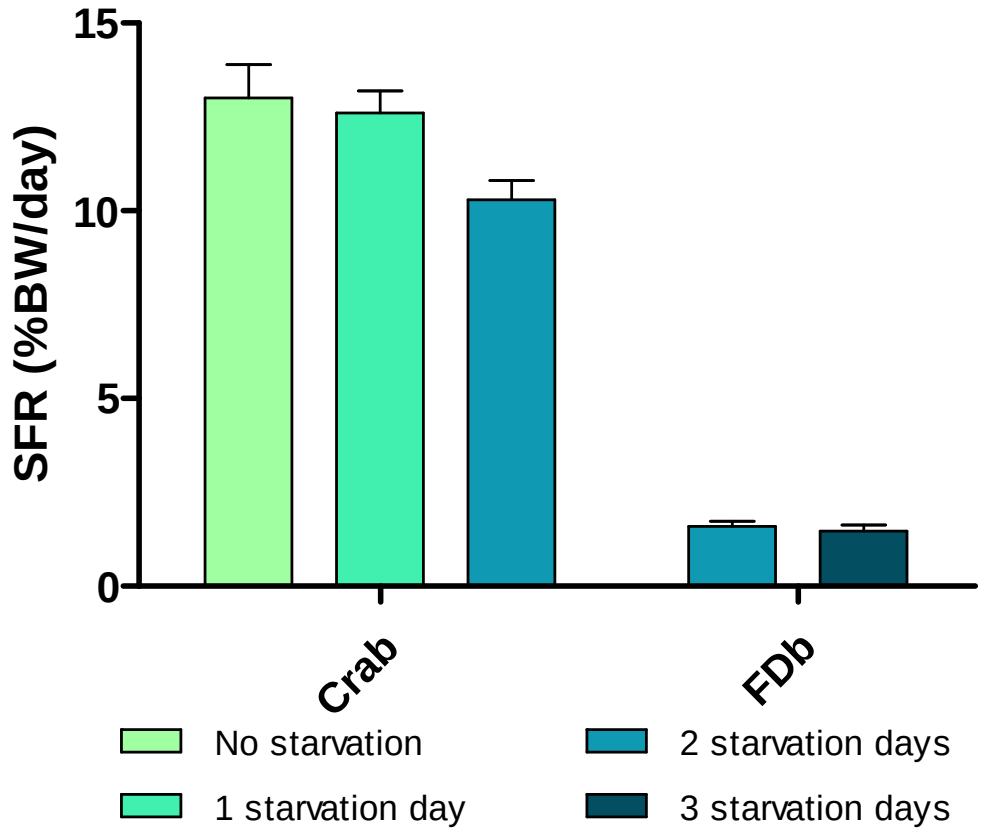


Figure Error! Use the Home tab to apply 0 to the text that you want to appear here.**4.2.2.** Specific feeding rate (SFR, % body weight·day^{-1}) obtained for different feeding protocols (differing by starvation days per week) feeding with crab (García García, Cerezo Valverde, 2004) and with the tested diet (FDb).

After these starvation days, both groups displayed a higher food-intake (Fig. 3.3.3). Therefore, it seems that this species compensates starvation with an immediately increase of food intake afterwards. This compensation-intake was the reason behind the significant differences on IFR between groups at given days. This conclusion is supported by the fact that, when performing a weekly food-intake analysis (Fig. 3.3.2), both groups displayed a similar trend of food intake ($P>0.05$, Table 3.3.4).

FE was better with the 3FDb protocol. In contrast, no statistical differences were identified on FCR but statistical significance was on the verge of being identified. It was interesting to verify that the use of FDb promoted a better use of food by octopus. In fact, octopus fed FDb display a lower FCR than that observed by (García García, Cerezo Valverde, 2004) on octopi fed crab (Fig. 4.2.3).

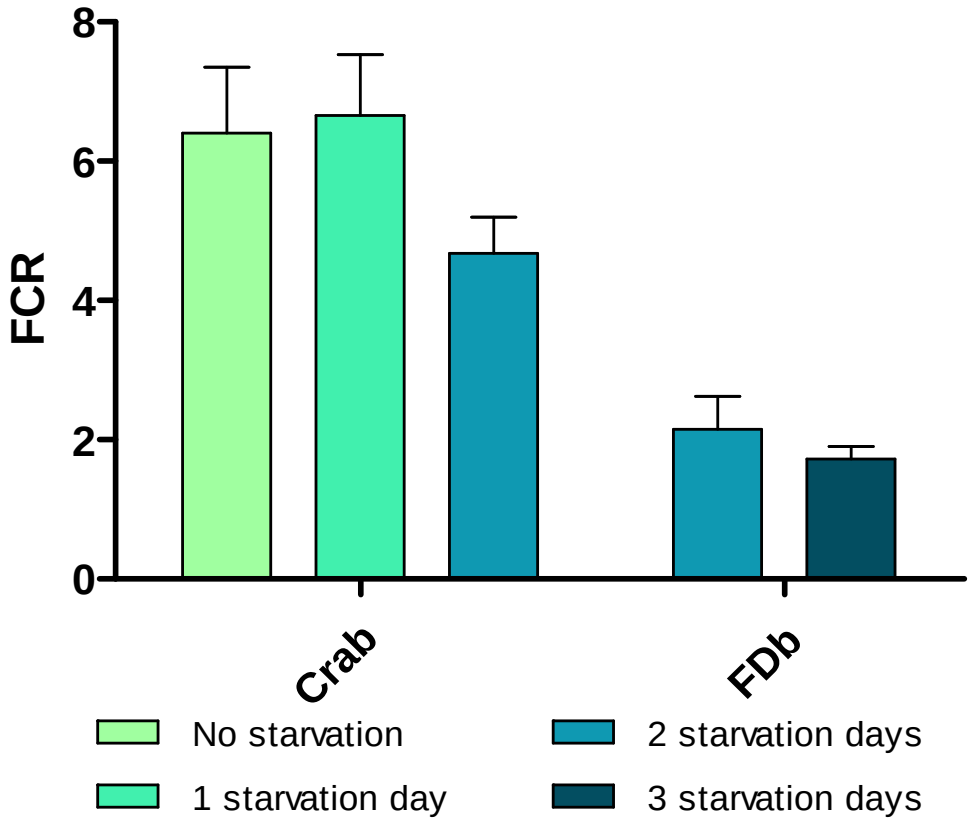


Figure 4.2.3. Food conversion rate (FCR) obtained for different feeding protocols (differing by starvation days per week) feeding with crab (García García, Cerezo Valverde, 2004) and with the tested diet (FDb).

In conclusion, it seems that growth, survival and proximate composition was not influenced by the applied starvation/feeding protocols. It also seems that *O. vulgaris* has the ability to compensate starvation through an increase in food intake on the subsequent day. The application of feeding protocols that include starvation days might be an interesting option for industrial application since FE and FCR were enhanced. This will promote a reduction in operational costs, such in manpower and food.

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