UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal Seguridad Alimentaria

Anexo I

Dª MARÍA SORAYA DÉNIZ SUÁREZ, SECRETARIA DEL INSTITUTO UNIVERSITARIO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Departamento en su sesión de fecha 21 de diciembre de 2011 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral europea titulada: **"Evaluación de ingredientes alternativos a la harina de pescado en dietas de engorde para bocinegro (pagrus pagrus)",** presentada por la doctoranda Dª Josefa García Romero, dirigida por la Dra. Dª. Lidia Robaina Robaina y Dr. D. Rafael Ginés Ruiz.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a veintidós de diciembre de dos mil once.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal Seguridad Alimentaria

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Programa de Doctorado: Acuicultura

Título de la Tesis

"Evaluation of alternative ingredients as fish meal replacers in on-growing diets for red porgy (*Pagrus pagrus*)"

Tesis Doctoral presentada por **Josefa García Romero**

Dirigida por la **Doctora Lidia Esther Robaina Robaina** y el **Doctor Rafael Ginés Ruiz**

La Directora **El Director** **La Doctorando**

Las Palmas de Gran Canaria, a 16 de Diciembre de 2011

LIDIA ESTHER ROBAINA ROBAINA, PROFESORA EN EL ÁREA DE CONOCIMIENTO DE ZOOLOGÍA DEL DEPARTAMENTO DE BIOLOGÍA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

INFORMA:

Que **Doña Josefa García Romero**, Licenciada en Ciencias de Mar, ha realizado bajo mi dirección y asesoramiento el presente trabajo titulado: **"Evaluation of alternative ingredients as fish meal replacers in on-growing diets for red porgy (*Pagrus pagrus*)". (Evaluación de ingredientes alternativos como sustitutos de la harina de pescado en dietas de engorde para el bocinegro (*Pagrus pagrus*)"),** el cual considero reúne las condiciones y calidad científica para optar al Grado de Doctor.

FDO.: LIDIA ESTHER ROBAINA ROBAINA

RAFAEL GINÉS RUIZ, PROFESOR TITULAR DE UNIVERSIDAD EN EL ÁREA DE CONOCIMIENTO DE PRODUCCIÓN ANIMAL DE LA FACULTAD DE VETERINARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

INFORMA:

Que **Doña Josefa García Romero**, Licenciada en Ciencias de Mar, ha realizado bajo mi dirección y asesoramiento el presente trabajo titulado: **"Evaluation of alternative ingredients as fish meal replacers in on-growing diets for red porgy (*Pagrus pagrus*)". (Evaluación de ingredientes alternativos como sustitutos de la harina de pescado en dietas de engorde para el bocinegro (*Pagrus pagrus*)"),** el cual considero reúne las condiciones y calidad científica para optar al Grado de Doctor.

FDO.: RAFAEL GINÉS RUIZ

Evaluation of alternative ingredients as fish meal replacers in on-growing diets for red porgy (*Pagrus pagrus*)

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Thesis for the degree of *Doctor of Phylosophy*
University of Las Palmas de Gran Canaria, 2011

Directors:
Dra. Lidia Esther Robaina Robaina & Dr. Rafael Ginés Ruiz

A mi familia,

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Abbreviations

AA Amino acid

ADC Apparent digestibility coefficient

AE Ammonia-N excretion

AI Atherogenicity index

ARA Arachydonic acid

BHT Butilated hydroxitoluene

CD Control diet

DHA Docosahexaenoic acid

EAA Essential amino acid

EFA Essential fatty acid

EPA Eicosapentaenoic acid

FAMES Fatty acid methyl esters

FM Fish meal

HUFA Highly unsaturated fatty acids

MC Marine crab

MCM Marine crab meal

MDA Malonaldehyde

MUFA Monounsaturated fatty acids

EAA Essential amino acids

N-NH_4^+ Ammonia nitrogen

P Phosphorus

PUFA Polyunsaturated fatty acid

RC River crab

RCM River crab meal

SFA Saturated fatty acids

TBARS Thiobarbituric acid reactive substances

TI Thrombogenecity index

TLC Thin layer chromatography

Acknowledges

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hecho y terminado nunca……por tu confianza y sacrificio de entender que era lo que tenía que hacer…..por decirme todos los días y a pesar de las dificultades por las que hemos pasado de que estabas orgulloso de mí…que momento mas ideal para expresarte mi amor profundo y que quede grabado, decirte que no olvidaré nunca tu ayuda, apoyo y tu amor.

1. GENERAL INTRODUCTION

1.1 AQUACULTURE PRODUCTION

The accessibility of people to sufficient, safe and nutritious food to maintain a healthy and active life is expressed by the term of "Food Safety". Improving food security constitutes a necessary precursor for the global intention towards reduction of hunger and poverty (Seligman *et al.*, 2010). Fish has long been recognized as a relative cheap source of high-quality protein, essential vitamins and minerals, playing an important role in the food safety and the nutrition of the developing countries (FAO, 2003).

Fish supply has been depending of fishing wild captures for many years. However, global capture fisheries has almost stopped growing since the mid-1980s, especially marine fisheries resources which have been exploited to their maximum or beyond the level of sustainability, whereas the worldwide demand for fish is still increasing (FAO, 2010). Aquaculture is perceived as the faster growing food-producing sector with a great potential to meet the increasing worldwide demand of sea food. Thus, in order to alleviate the pressure on fish stocks the aquaculture appears as the most feasible complement to fisheries. According to FAO (2010), world aquaculture output has increased substantially over the last 50 years from a global food fish production of 3.9% in early 1950s to 47% in 2008 (excluding aquatic plants), being estimated this production bring off more than 50% of global food fish consumption by 2012.

In contrast to world capture fisheries production, which is almost stopped, the aquaculture sector maintained a worldwide annual growth rate of 8.3 % between 1970 and 2008, with a per capita supply from aquaculture increasing by ten times from 0.7 kg to 7.8 kg in the same period (Fig. 1.1). World aquaculture production from seawater represents a 36% of the global aquaculture production and produces many high value finfish, crustaceans and molluscs. Marine fish represents a 3.4% (1.8 million of tonnes) of the total aquaculture production in 2008 (FAO, 2010).

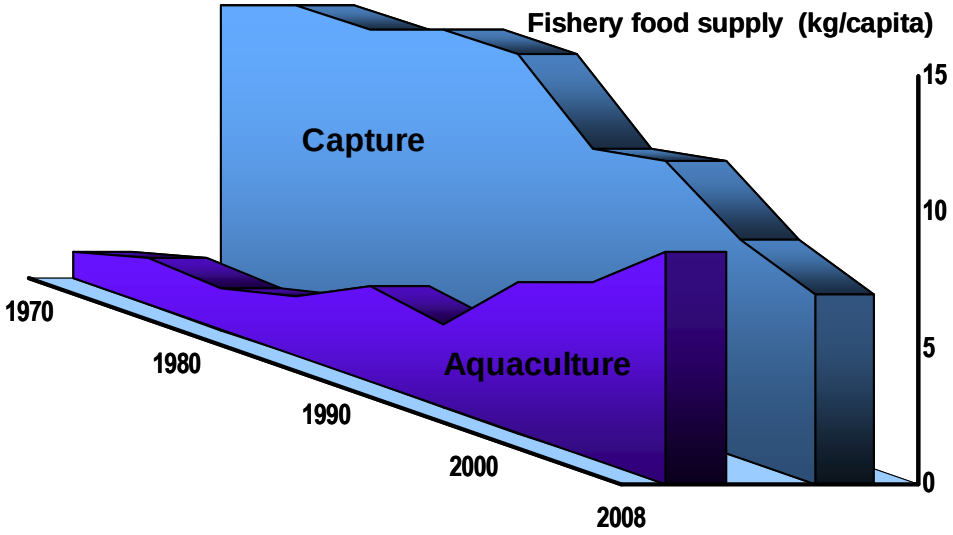

Figure 1.1 Relative contribution of aquaculture and capture fisheries to food fish consumption from 1970 to 2008 (adapted from FAO, 2010).

1.2 PROTEIN IN DIETS FOR FISH SPECIES

In the context of animal feeding, protein mainly refers to crude protein content that is commonly determined as nitrogen content x 6.25. This definition is based on the assumption that the nitrogen content of most protein found in animal is about 16% (NCR, 1993). Amino acids are the building block of protein. Thus, protein are composed of up to 20 α-amino acids linked into chains by peptide bonds and cross-linked between chains by sulfhydryl bonds, hydrogen bonds and Van der Waals forces.

Fish consume protein to obtain free amino acid, certain of them are essential components as fish can not synthesize them *de novo* and must be supplied in the diet (Laird and Needham, 1988). Amino acids, after being absorbed by the gastrointestinal tract, are transported to different tissues and organs to be used for fish growth (protein and/or synthesis of several other components or even to produce energy through catabolic processes) (NRC, 1993). Therefore, fish have a dietary protein requirement which means the minimum amount of protein needed to meet requirements of amino acids for achieving

optimum growth and metabolic necessary process. Quantitative dietary protein requirements has been established in many fish species mainly by the dose-response method, in which graded amounts o high-quality protein were proved in a reference diet (NCR, 1993). Carnivorous species requirements vary between 35-55% of the diet, being appreciably higher than those in diets of terrestrial warm-blooded animals (Merzt, 1972; Smith, 1989). One of the main factors affecting nitrogen excretion is the quantity and quality of protein in the diet (Lied and Braaten, 1984; Liu *et al.*, 2009). Thus, protein is one of the key nutrients in the diets of aquatic species, not only as a nutrient but also as a factor involved in water quality.

1.2.1 Importance of protein in compounds feed for farming finfish

Formulated feeds are obviously the first step to develop intensified aquaculture systems. Feeds is still the most expensive operating cost in most fish farming (Deutsch *et al.*, 2007), often ranging from 30% to 60% of the total variable expenses, where proteins are the most expensive components of formulated feed (Tacon and Metian, 2008; FAO, 2010).

The production of aquafeed compounds for aquaculture in 2006 was around 22.73 million tonnes (Tacon and Metian, 2008). With the increase of the aquaculture growth rate, is estimated that this production reached 29.3 million tonnes in 2009 (Fig. 1.2). Chinese carp constitutes the mayor group in compound feed production with 11.40 millions tonnes or 47% of the total production. On a global basis, it is estimated that 3.72 million tonnes of fish meal, or the equivalent of 16.6 million tonnes of pelagic forage, was used in the production of 22.73 million tonnes of compound fed in 2006 (Tacon and Metian, 2008; Hardy, 2010). It is therefore important to consider these protein needs in the context of sustainability of aquaculture development.

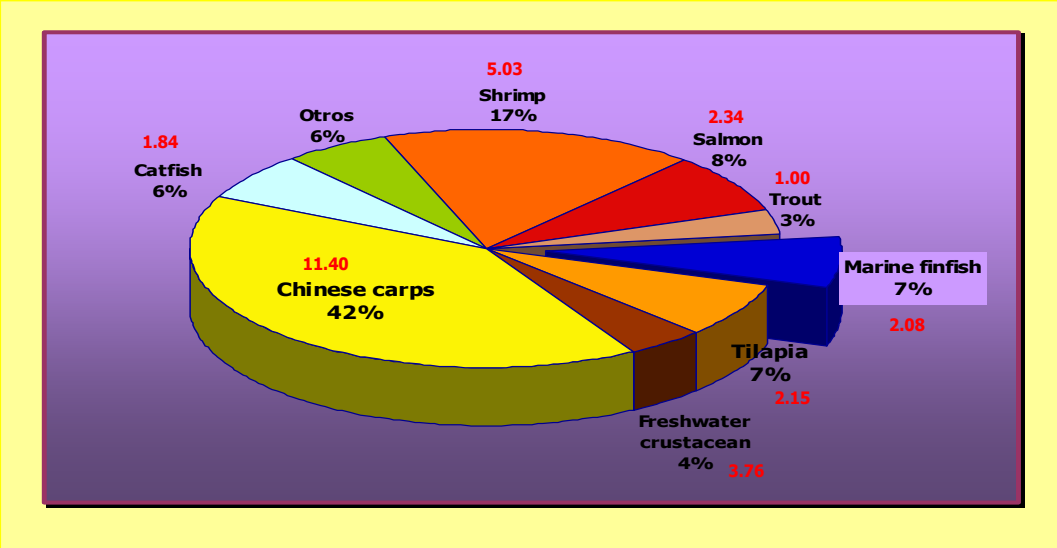

Figure 1.2 Total estimated compound feed production (millions tonnes) for the main farmed aquaculture species in 2009 (adapted from FAO 2004-2009).

1.2.2 Fish meal as the main protein source in fish diets

Due to its elevated nutritional quality, including high protein content, good balance of amino and fatty acids, low concentrations of anti-nutrients and being highly digestible, fishmeal (FM) has long been used as the main protein source for compound feed in farms, not only from aquaculture but also from terrestrial origin. Standard fishmeal typically has a crude protein ranges from 64% to 67%, although special high quality product contents between 68% and 72%, with up to 12% fat. In addition, freshness of the used raw material is reflected by a low biogenic amine concentration (e.g. maximum 500 ppm histamine), which is an important parameter to define the final quality of meals.

Traditionally, demand sectors for fish meal was to feeding poultry, pigs and ruminants. However, the recent expansion development of aquaculture has been an important factor for increasing global demand for this product. For aquaculture, the demand of fish meal since 1980 has increased by almost 50% in 2008, and according to New (2002) it may estimate to increase by 70% in 2012. Contrary, in other sectors of animal feed the use of fish meal has considerably

declined (IFFO, 2009) (Fig. 1.3). The biggest aquaculture consumers group of fishmeal in 2006 was marine shrimp followed by marine fish and other farming fish species such as salmon, Chinese carp, trout, eel, catfish and tilapia (Hardy, 2010).

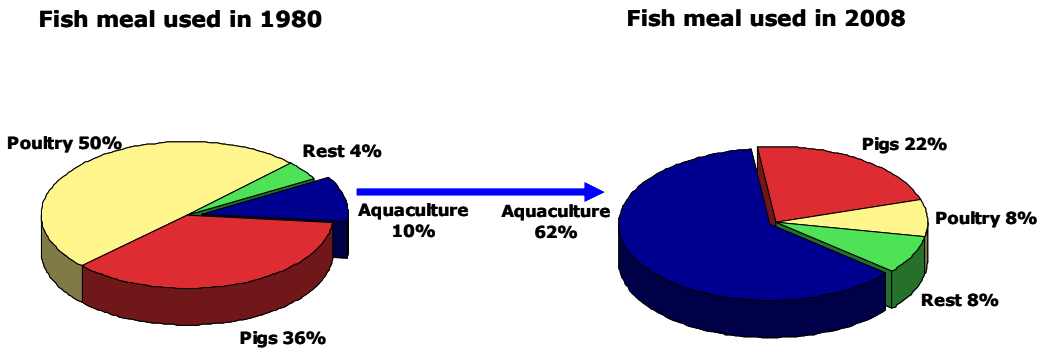

Figure 1.3 Fish meal used in different farmed animals sectors from 1980 to 2008.

1.2.3 Fish meal current world production and situation

Since 1950 increased demand of fish meal, mainly for using in animal feed, has made this a global high value commodity. However, anchovy (*Engraulis ringens*) stocks, main species used to obtain fish meal are very unstable as their populations decline notably when the climate phenomenon "*el Niño*" occurs. Moreover, there is a strong administrative scheme controlling closed seasons for fishing sustainable and preserving the health of stocks. Because of this the production of fishmeal in recent years has suffered a gradual decline in global production passing from 2.3 million tons in 2000 to 1.34 million tonnes in 2009, which represents a reduction of 58% in their production (FAO-Fish Globe, 2010). These lower yields are due mainly as a result of lower Peruvian catches, which were 70% less than those achieved in 2000 (FAO-Fish Globe, 2010).

The main country producing and exporting fishmeal, mostly coming from anchovy species is Peru followed by Chile, while the main consumer market is China, followed by Japan, Taiwan and the European Union (FAO-Fish Globe,

2010). However, Chinese stocks of fish meal have been one of the lowest levels providing a substantial price increase. Respect to Chile, it increased its production to 2009 due mainly to the demand of high quality meal from China. Nonetheless, the earthquake occurred in February of 2010 seriously damaged the Chilean fish meal factories causing a cut of 200,000 tons in the production and export. On the other hand, the possible reduction of Peru's fishing quota for the following years along with the impact of the climatic phenomenon "*el Niño*" would lead to reduced even more the availability of fishmeal in the market (FAO-Fish Globe 2010).

Based on this scenario, the predictions for the production of fishmeal could not be more pessimistic and uncertainty. Moreover, if the demand was already strong this will cause an even more rise in fish meal prices making it as a finite commodity. The reliance on fish meal makes the aquaculture industry less sustainable and because of this, predictions for fish meal consumption in marine fish feed is estimated to be reduced in a 31.3% from 2006 to 2020 (Gatlin *et al.*, 2007; Tacon *et al.*, 2006), being needed replacing it with other protein sources that are readily available and at lower prices.

1.3 ALTERNATIVE PROTEIN INGREDIENTS TO FISH MEAL IN FISH DIETS

1.3.1 Alternative protein sources from terrestrial origin

Recently, a large number of studies have been focused on the replacement of fish meal with alternative protein ingredients, especially those from plant origin and terrestrial animal by-products, in compound aquaculture feeds to alleviate problems of supply and high price of fish meal.

In this sense, the effect of partial or total replacement of fish meal by plant protein sources, which are generally less expensive and more ready available, has been accomplished in many bred fish species with different success degree in growth performance, feed utilization and fish quality (Moyano *et al.*, 1992; Gomes *et al.*, 1995; Robaina *et al.*, 1997, 1999; Hardy, 1996; Kaushik *et al.*, 2004; Francesco *et al.*, 2007; Shafaeipour *et al.*, 2008). Soybean is so far the plant source

widely used instead of fish meal (Robaina *et al.*, 1997, 1999; Mambrini *et al.*, 1999; Refstie *et a*l., 2000; Wang *et al.*, 2006; Venou *et al.*, 2006; Overland *et al.*, 2009); other sources as those from peas (Hardy *et al.*, 1996; Gouveia *et al.*, 2000; Borlongan *et al.*, 2003; Sánchez-lozano *et al.*, 2009; Davies *et al.*, 2010), different lupine species (Robaina *et al.*, 1995; De la Higuera *et al.*, 1988; Glencross *et al.*, 2003; Serrano *et al.*, 2011) and other plant meals (Watanabe *et al*, 1997; Davies *et al.*, 1997; Mente *et al.*, 2003; Palmegiano *et al.*, 2006; Sánchez-lozano *et al.*, 2007; Sánchez-Lozano *et al.*, 2009).

However, it is well recognized that utilization of high dietary levels of several plant protein sources in carnivorous species could induce problems linked to the deficiency in the essential amino acids, presence of many specific antinutritional factor, palatability, poor digestibility and absence of n-3 highly unsaturated fatty acids (HUFA) (Francis *et al.*, 2001). In addition, specific gastrointestinal damage has also being associated with high dietary levels of plant sources (Caballero *et al.*, 2003; Penn *et al.*, 2011). On the other hand, omnivorous species have shown greater tolerance with higher dietary inclusion level (Kaushik *et al.*, 1993; Robinson and Li, 1994; El-Saidy and Gaber, 2002). Although improvements in the raw materials and feed processing technologies have enabled promising results in overcoming plant protein sources, problems such as presence of anti-nutrients can not be avoided (Drew *et al.*, 2007). Some author such as Tacon and Metian (2008) argued that while trying to make cost-effective diets by using cheaper ingredients, the use of plant protein sources even may increase the costs since it may be necessary to treat anti nutritional factors, and to add certain amino-acids in order to improve the nutritional profile of the diets.

Regarding to by-product meals from animal protein, these appear to have the greatest potential as cost-effective replacements for fish meal especially in carnivorous fish species (Bureau *et al.*, 2000). However, the use of this type of protein sources there may be to produce variable growth results and reduction in

digestibility, due to high levels of lipid, ash, and indigestible fibre content (Robinson and Li, 1996; Kureshy *et al.*, 2000). In addition, there exist concerns that extensive replacement of fish meal in aquaculture diets could adversely affect the marketability of the fish because of consumer perception of altered taste, or reduced health benefit due to lowered omega-3 fatty acid content (Pigott 1989; Williams *et al.*, 2003).

1.3.2 Products and by-products from marine sources as novel ingredients in aquaculture feed

Extending the range of raw materials from which suitable fish meal alternatives ingredients could be obtained, derived resources from marine origin have been received much more attention during the last few years. The presence of long chain omega-3 fatty acids and amino acid protein profile, which resembles more closely fish natural diet, would result from feeding marine origin meals in better fish response and final quality advantages rather than those being derived from either vegetable or terrestrial animal protein origin.

These resources include by-catch (the "no-target specie" and undersized individuals of target species which are unintentionally taken), discards (by-catch and deformed or damaged target individual species), and processing waste and by-products (edible or inedible rest raw material). These resources contain an extraordinary nutritional potential, based on its high added value compounds content (minerals, lipids, amino acids, polysaccharides, vitamins, pigments…etc) from marine origin, which have unique features and can be still used in many application, yet most end up as residues.

Fisheries worldwide annual discards are estimated to represent 25% of the total catch (Rustad, 2003; Ferraro *et al.*, 2010), while the amount of fish by-products vary depending on species size, season and fishing ground, but it reach up to 50% of seafood industrially prepared (Falch *et al.*, 2006; Rustad *et al.*, 2011). In the European Union, discards and by-products represent a total of 5.2 million

tons per year (AWARENET, 2004; Mahro and Timm, 2007; Ferrearo *et al.*, 2010). Fish filleting, salting and smoking generate the major amount of solid wastes and by-products (50–75% of the fish), with a total of 3.17 million tons per year (AWARENET, 2004; Ferrearo *et al.*, 2010). The currently increasing of fish product for human consumption together with the changing consumer trend towards ready to use product, will be raising even more the discharged waste by-products in the future.

Utilizing these resources arises from a more integrated and sustainable fisheries practices and a responsible proper management of the wastes disposal, which constitute at present a serious environmental problem (Arvanitoyannis and Kassaveti, 2008). The year 2015 is by far the late year under European regulation for the start up obligation to recovering by-products from the industry, fisheries and aquaculture sectors included (Rustad *et al.*, 2011).

The use of marine by-product is not new, thus are wide research to explore different useful applications such as material for fertilizer (Thingoc and Kyun, 2011), animal feed (Naylor *et al.*, 2008), human food (Falch *et al.*, 2006; Rustad *et al.*, 2011) and for extracting diversity of bioactive compounds (Kim *et al.*, 2006; Ferraro *et al.*, 2010). In aquaculture industry, the inclusion of fishery discards (including by-catch and over-catch) and fish processing waste, even from aquaculture, could reduce dependence on expensive imported fish meals and present an opportunity to recycle these valuable resources.

The widely protein material produced from this potential raw material include fish meal and fish protein concentrate from either as silage (fish or waste fish treated with different acids as lactic acid derived from fermentable carbohydrate substrate) or hydrolysate (enzimatically treated). The experimental use of fish silage as an alternative protein ingredient has been tested in some aquaculture species such as Atlantic salmon *Salmo salar* (Jackson *et al.*, 1984; Lie *et al.*, 1988; Parrish *et al.*, 1991; Heras *et al.*, 1994; Espe *et al.*, 1999; Hevrry *et al.*, 2005), rainbow trout *Oncorhynchus mykiss* (Rungruangsak and Utne, 1981;

Hardy *et al.*, 1983; Stone *et al.*, 1989), tilapia *Oreochromis niloticus* (Fagbenro, 1994; Fagbenro and Jauncey 1994) and abalone *Halotis fulgens* (Viana *et al.*, 1996). The results generally indicate that fish silage is a suitable protein resource for aquaculture with some studies showed highly digestible and effective replacement for up to 75% of fish meal in diet (Vidotti *et al.*, 2002; Goddard *et al.*, 2003).

Fish meal is the most valuable non-edible commodity produced from marine by-products (Ferraro *et al*, 2010), with a global production ranges between 5.5 and 7.5 million tons/year (Hardy and Tacon, 2002). Its composition reflects the material from which it is made, typically on a weight basis composition with 56-66% of proteins, 6.3-8.0% of lipids and 19-23% of ash content (Rathbone *et al.*, 2001). Evidently, that quality composition is lower than traditional fish meal (Table 1.1).

Table 1.1 Nutritional profiles of meals from fish by-catch and processing wastes (adapted from Goddad *et al.*, 2008).

	COM	MBM	SPM	CAM	TCW
Proximate composition (g kg^{-1})					
Crude protein	672	662	629	565	643
Lipid	114	63	68	72	79
Ash	130	161	224	244	173
Moisture	71	82	59	84	70
Essential amino acids (g 100 g^{-1}) protein					
Arginine	5.49	6.04	5.52	6.38	5.62
Histidine	1.86	2.36	2.37	2.52	2.71
Isoleucine	3.12	3.73	3.32	3.75	3.49
Leucine	6.75	7.70	6.94	8.10	7.01
Lysine	6.66	8.06	7.15	8.44	6.84
Methionine	3.55	4.10	3.75	3.49	3.64
Phenylalanine	6.20	7.39	6.70	7.50	6.77
Threonine	4.17	4.73	4.21	4.86	4.39
Tryptophan	1.04	1.29	1.15	1.29	1.17
Valine	3.96	4.32	3.92	4.37	4.05
Mineral composition (% dry matter)					
Calcium	1.29	3.61	2.56	2.74	2.94
Phosphorous	0.88	1.85	1.32	1.48	1.54

COM, commercial anchovy meal; MBM, mixed benthic meal; SPM, small pelagic meal; CAM, catfish meal; TCW, tuna cannery waste meal.

Fish meal manufactured from fisheries by-catch and processing by-products have been evaluated in diets for gilthead seabream *Sparus aurata* (Kotzamanis *et al.*, 2001), coho salmon *Oncorhynchus kisutch* (Rathbone *et al.*, 2001), rainbow trout *Oncoryhnchus mykiss* (Hardy *et al.*, 2005), red drum *Scianops ocellatus* (Li *et al.*, 2004; Whiteman and Gatlin III, 2005) and Nile tilapia *Oreochromis niloticus* (Goddard *et al.*, 2008; Saidi *et al.*, 2010). The results show that by-catch and by-product meals are suitable as total or partial replacements of commercial fish meal in diets for fish aquaculture species.

Although using fishery discards and processing wastes as alternative protein sources is feasible, these have some constraints related with the final quality of product and fluctuations in abundance of species. The raw materials are extremely variable in composition and, in addition, regular supplies of sufficient quantity must be available to support the profitable operations of fish meal plants. Moreover, the high ash content which reflects the higher original bone and cartilage fraction in the raw material may exceed regulations introduced in some countries to prevent excess phosphorus discharge (Goddard *et* al., 2008).

1.3.2.1 Crab products and by-products

Among all discards and by-products generated by the seafood industry, these coming from crustacean species represents a highly valuable source of nutrients especially protein, minerals, caroteonid pigments and chitin (Lee, 1990; Negro *et al.*, 2000; Cremades *et a*l., 2003; Perez-Galvez *et al.*, 2008). Crabs represent 20% of all crustaceans caught and worldwide farmed (FAO, 2009). Generally, crab by-catch and no target species are discarded while the head, shell and tail portions of edible crabs are removed during processing and accounting for approximately 50% of the volume of raw materials (Islam *et al.*, 2004). Tons and tons of them derived from the meat crab industry currently constitute a large amount of underutilized waste.

Crab residues have been usually processed to obtain materials of added value such as chitin, enzymes, and pigments, particularly astaxanthin (Stewart

and Noyes-Hull, 2010). In addition, they may serve as excellent source of raw material for feed ingredients preparation (Shahidi and Synowiecki, 1991). In this line, composition of meal from crabs widely change regarding to raw material specie, origin (whole animal or offal) and processing methods. Generally, residues have a high amount of protein (25-50%), carotenoids (14-37%) and mineral salts (15-35%), (Lee *et al.*, 1982; Sachindra *et al.*, 2005).

In finfish species, crab meal has been successfully used as a replacement for fish meal protein and as a carotenoids source to promote coloration of skin and flesh. Synthetic astaxanthin and canthaxanthin are the most commonly carotenoids used for pigmentation of aquaculture species (Torrisen, 1990; Metusalach *et al.*, 1996; Higuera-Ciapara *et al.*, 2006). However, the dietary synthetic carotenoids is not favoured mainly due to their high cost and also because of human concern regarded to these complex chemical compounds; thus, during last years natural sources of caroteonid have being studied as an alternative to synthetic ones. Carotenoids content from different crab sources have found to enhance pigmentation of cultured fish such as brook trout *Salvelinus fontalis* (Satio and Regier, 1971), salmonids (Spinelli and Mahnken, 1978; Coral-Hinostroza *et al.*, 1997; 1998; Lyons *et al.*, 2001) and southern flounder *Paralichthys lethostigma* (Gonzalez *et al.*, 2006).

On the other hand, partial replacement of fish meal by crab meal from *Pleuroncodes planipes* and *Cancer pagurus* in diets for white shrimp *Litopenaeus vannamei* and Atlantic cod *Gadus morhua*, respectively, has yielded successful growth results in both species (Goytortua-Bores *et al.*, 2006; Toppe *et al.*, 2006; Villarreal *et al.*, 2006). In the above studies it was found that dietary crab meal greatly induce feed consumption, showing the potential of crab products as highly attractant and palatable. This fact was reflected by an increase of feed intake which positively affected animal growth performance.

Furthermore, better feed efficiency ratio was observed in shrimp fed on diets containing crab, suggesting the presence of growth-stimulating compounds

in crab products which may improve dietary protein quality or utilization. Although high dietary inclusion of crab meal is not desirable due to its high ash and chitin content, which are known to reduce dietary nutrients digestibility, some studies have presented good data for crab meal digestibility. Thus, good crab meal digestibility values have been found in haddock *Melanogrammus aeglefinus* (82%) (Tibbetts *et al.*, 2004) and higher in cod (89%), Atlantic halibut *Hippoglossus hippoglossus* (88%) (Tibbetts *et al.*, 2006) and cobia *Rachycentron canadum* (89%) (Fines and Holt, 2010).

According to different authors, fish show specific abilities to properly digest these types of feed ingredients without any negative effect on nutrient digestibility. Different ability is given by biological differences related to digestive acid secretion and adequate enzymatic system (Danulat and Kausch, 1984; Wood *et a*l., 1992; Gutowska *et al.*, 2004; Toppe *et al.*, 2006). New processing technologies can improve the nutritional value of industrial by-products, if is applied to crab waste, thus providing new aquaculture ingredients with interesting crude protein and amino acid profile content.

Regarding to crab by-catch species, related to capture fisheries, include particularly small crab or mixed crab/fish which are caught and commonly discarded. Little information is available regarding to crab by-catch species and quantity. Pingguo (2005) reported that a total of 767,000 kg of porcupine crab *Neolithodes grimaldii* was caught and discarded by gillnet vessels fishing, during turbot fishery in Canadian coast. However, no regular supplies or sufficient quantity to support the profitable operations of processed plants limits the utilization of these resources as protein sources. Local utilization of crab wastes could generate small industries and could be an efficient way to use by-catch species in aquaculture, particularly in small farms where fish diets can be produced in an artisanal manner with low cost and minimal equipment (Match *et al.*, 2010).

1.3.2.1.1 Chitin long-change polysaccharide as potential bio-components from crustacean meals

Marine crustacean shell wastes have long been the primary source of the polysaccharide known as chitin (Healy *et al.*, 2003). Chemically, the structure of chitin consist in units of N-acetyl-2-amino-2-deoxy-D glucopyranose ($C_8H_{15}O_6N$) bound by b (1→4) glycosidic linkages (Díaz-Rojas *et al.*, 2006). Chitin is obtained from crustacean shell waste such as shrimp, prawn, krill, crab and lobster, with shrimp carcass contenting the higher amount of chitin (30-40%) followed by crab (15-30%) (Qin and Aboh, 1997). Commercially, it is mostly used as a raw material to produce chitosan oligosaccharides and glucosamine in a worldwide amount of 37,000 tons/year (Ferraro *et al.*, 2010).

Chitosan is obtained mainly from chitin isolated where the industrial extraction process used can be tentatively divided into three major step: 1) shells grinding, 2) decalcified process which consist in an elimination of inorganic matter (metals, salts and calcium carbonate) through treating shell with dilute hydrochloric acid in acidic medium, and 3) de-proteinization with the extraction of protein matter in alkaline medium or by proteases derived from bacteria (Diaz-Rojas *et al.*, 2006).

In aquaculture, chitin and chitosan has been proved in some cultured species as immunostimulant showing its protective effect against bacterial diseases (Anderson and Siwicki, 1994; Esteban *et al.*, 2000; Ortuno *et al.*, 2000; Esteban *et al.*, 2001; Cuesta *et al.*, 2003; Wang and Chen, 2005; Gopalakannan and Venkatesan, 2006).

Regarding to fish growth parameters, the dietary effects of chitin and chitosan have been yielded different results. For instance, Shiau and Yu (1999) observed depressed growth in tilapia (*Oreochromis niloticus* × *O. auratus*) after feeding chitin and chitosan at the 2%, 5% and 10% inclusion level, suggesting an interference of chitosan and chitin in the absorption of the nutrients. Later on, in common carp *Cyprinus carpio*, lower dietary chitosan content (1%) has proved to

enhance growth performance (Gopalakannan and Venkatesan, 2006), explained by the crucial role of chitosan to enhance the digestion and absorption of nutrients at low dietary inclusion levels. However, in the same study, fish fed on chitin (1%) showed depressed growth after 30 days of feeding due to the fish developing intolerance to chitin. Contrary, diet containing higher dosage (up to 10%) of chitin or chitosan did not affect fish growth when feeding to red sea bream *Pagrus major*, Japanese eel *Anguila japonica* and yellow tail *Seriola quiqueradiata* (Kono *et al.*, 1987).

A recent research was done to study the presence of chitinolytic enzymes system and the apparent digestibility of chitinous waste meal in cobia fish (Fines and Holt, 2010). Results showed that fish stomach chitinase and chitobiase activities were high, even when antibiotic were supplemented suggesting that chytinolytic bacteria are not significant. In addition, the apparent digestibility of chitin was high either for crab meal (67%) or shrimp meal (78%), and the crude protein, lipid and gross energy digestion for crab and shrimp meals were not significantly different respect to fish meal. With a caloric content for the chitin of 17.1 kJ g^{-1} (Gutoska *et al.*, 2004), it could constitute a great percentage of the total energy intake in species which natural feeding are based on crustacean and therefore chitin intake are considerable. In this respect, chitinous waste material can be utilized to replace carbohydrates and protein from fish meal if we consider the available energy from the chitin (Fines and Holt, 2010). Moreover, same authors pointed out the indeed to realise similar feeding experiments in other species with similar feeding behaviour to known more about the potential use of these kind of ingredients in aquaculture diets.

1.3.2.1.2 Dietary crab meal and fish flesh quality

Fish quality involves different concepts depending on fish farmer, the processing industry or consumer understanding. Thus, organoleptic properties, nutritional value and flesh self life, are sets of characteristic that contribute to fish quality as perceived by the consumer. All mentioned characteristic are

strongly related with fish chemical composition, which depends on many quality aspects, among them diet composition is highly important (Lie *et al.*, 2001; Grigorakis, 2007). It seems that all acquired quality attributes of fish fillet are well correlated with fat content and lipid class in tissue (Waagbo *et al.*, 1993; Guillou *et al.*, 1995; Morris *et al.*, 1995). Lipid metabolism and accumulation in fish as response to dietary protein sources has been showed (Lupatsch *et al.*, 2002; Francesco *et al.*, 2007), affecting to flesh quality parameters such as sensory characteristic (Williams *et al.*, 2003 b; Karlsen *et al.*, 2006; Suontama *et al.*, 2007b) and the fillet texture of the farmed fish (Hernández *et al.*, 2007).

Few studies exist about the effect of dietary crustacean meal on final flesh quality with most of them being realized to examine crustacean krill meal. Fernández (2006) reported a significant increase of ω-3/ω-6 ratio in muscle of blackspot *Pagellus bogaraveo* fed on a 30% dietary inclusion level of crustacean meal (10% krill meal and 20% shell shrimp meal) related to the high dietary ω-3/ω-6 relation in the crustacean meals. Suontama *et al.* (2007a) showed that a substitution of 20% the fish meal protein by protein from krill *Thysanoessa inermes* did not modified fillet fatty acid profile in Atlantic salmon and halibut *Hippoglossua hipoglossus*, while in the same work higher substitution of the fish meal, either for krill meal (60%) or amphipodo *Themso libellula* meal (40%), produced significant changes in fillet fatty acid content. Furthermore, substituting fish meal by krill meal did not appear to influence muscle sensory evaluation or texture to any major extent in Atlantic salmon (Karlsen *et al.*, 2006; Suoantama *et al.*, 2007b).

Regarding to crab meal and flesh quality, Gonzalez *et al.* (2006) studied the effect of crab meal-supplemented diets (5%) on southern flounder *Paralichthys lethostigma* muscle quality. Results indicated that flounder fed on crab meal significantly increased ARA (20:4n-6), adrenic (22:4n-6) and DHA (22:6n-3) muscle fatty acids content with an overall higher n-3/n-6 ratio, improving the

nutritional value of fish fillet. This clearly suggests that crab meal finishing diets could be utilized to enhance final product quality.

1.3.2.2 Other potential ingredients from marine sources: by-products processing from echinoderm industry

Some research has been done in order to explore the possible uses of other marine products and by-products such as those from echinoderm species. From green sea urchin only gonads and for cucumber only muscle bands and body wall are desirable, while the remaining tissue together with the unwanted edible material, that not meet commercial requirement of quality and are discarded. In case of by-product from Atlantic cucumber *Cucumaria frondosa* and green sea urchin *Strongylocentrotus droebachiensis* estimated discard amount may reach up to 50% and 80% respectively (Mamelona *et al.*, 2010). Nutritional composition reveals that these undesirable raw materials are rich in many valuable nutrients as proteins (4.5-14.5% wet weight) and lipids (1.9-4.6%) together with a 35% of essential amino acids of the total ones and also rich in polyunsaturated fatty acids (44% of total fatty acid), with high EPA (17%) but low DHA (0.2%). In terms of vitamins, they are mainly rich in alpha-tocopherol or vitamin E (Zhong *et al.*, 2007; Mamelona *et al.*, 2010a; b).

1.4 THE RED PORGY (*Pagrus Pagrus*; Linnaeus, 1758)

1.4.1 External appearance, geographical distribution, habitat and reproduction

Red porgy (Fig. 1.4) is a sparid marine fish with a wide geographical distribution extends along the Western Atlantic coasts, from North Carolina to Argentina, and Eastern, from British Island to South of Senegal including the archipelagos of Madeira, Canaries and Cape Verde. It is also present in the Mediterranean and the Adriatic seas (Manooch and Hassler, 1976; Bauchot *et al.*, 1981; Pajuelo *et al.*, 1996).

Figure 1.4 Taxonomic classification of red porgy.

Red porgy is a bottom fish that generally lives in warm temperatures and subtropical habitats (Afonso *et al.*, 2008). Juveniles specimens are often found on sandy bottoms, at 20-50 m depth, while higher fish size are frequently found on rocky bottoms in a depth around 50 m. According to Manooch (1976) and Alekseev (1983), red porgy is a hermaphrodite protogynous reproductive specie, where individual are females at smaller sizes and males at larger sizes. The change of sex occurs in fish sizes between 32.5 to 42.5 cm, and gonads maturation occurs at 3-4 years of age (Manoocher and Alekseev, 1990); although this species undergoes a first sexual maturity of females when reach about 250-300 grams whole weight during their second and third year of life. The spawning season appears between January and April, with water temperatures between 16 °C and 21 °C.

1.4.2 Natural diet of the red porgy

Similar feeding habit have been observed for individuals of both the Eastern Mediterranean (Labropoulou *et al.*, 1999) and the western Atlantic (Manooch, 1977), as well as in Tunisian coast (Chakroun-Marzouk and Kartas, 1987). Detailed studies about feeding habits of juvenile red porgy such as

Castriota *et al.* (2006) reveals that from the 78 mostly epibenthic identified species consumed, crustacean are the dominant group followed by molluscs and fish, with echinoderms sea urchin also consumed in a lower proportion. Furthermore, despite the large number of taxa found, few species accounted for most of the diets, suggesting specialized feeding. The individual feeding specialization was based on hermit crabs, brachyuran crabs and prosobranch gastropods (Castriota *et al.*, 2006).

The knowledge of red porgy natural diet and feeding habits helps to understand the nutritional mechanisms involved in their feeding biology, which are necessary for commercial cultured red porgy diet improvement.

1.4.3 Red porgy culture

The diversification of aquaculture through the production of new species is one of the most important initiatives towards stabilization of the productive sector, currently characterized by high production of a small number of species. In selecting a new candidate species for aquaculture, some requirements or aspects of the species are considered important such as good growth rate, high market value and a large demand for human consumption. In addition, biological and technical aspects of the culture conditions must also be overcame by the new species (Suquet *et al.*, 2002).

In this sense, red porgy has a high meat quality prized, making it a species of interest to both commercial fisheries and recreational anglers. Fillets are characterised by delicate flavour, firm flesh with high protein and low fat content. Red porgy has an important market demand on the Mediterranean and Atlantic coast (Kokokiris *et al.*, 2006). Unfortunately, owing to prolonged exploitation, some red porgy populations have become over fished (Haimovici, 1998: Vaughan et al., 2002; Afonso *et al.*, 2008), and consequently wild catches of this species are very limited or insufficient to meet the current market demand.

Preliminary studies carried out with this species under culture conditions have shown good adaptability to growing conditions, spontaneous spawning, high growth rates and no serious problems of disease or mortality (Kentouri *et al*, 1994; Kokokiris, 1998; Cejas *et al.*, 1999). These facts make red porgy one of the priority marine species for aquaculture diversification of the Spanish Atlantic coast or Mediterranean Sea since the nineties of the last century (Kentouri *et al.*, 1995; Hernandez-Cruz *et al.*, 1999).

1.4.3.1 Dietary protein and carotenoids pigments requirements.

One of the main constraints in the culture of the red porgy is the loss of its characteristic red-pink skin colour under cultured conditions (Kentouri *et al.*, 1995; Klios *et al.*, 1997; Basurco and Abellán, 1999). The preservation of the natural colour and the similarity in external appearance towards the wild specimens has a great importance from a commercial point of view, associated with the acceptance or rejection of the product by consumers (Shahidi *et al.*, 1998; TECAM, 1999). Fish are unable to bio synthesize carotenoids *de novo* (Goodwin, 1984), hence, carotenoids must be provided in the red porgy diet. Thus, most nutritional studies realized on this species have principally focussed on different carotenoid sources and inclusion levels about the effect on skin coloration. From the different carotenoids tested, only astaxanthin, in its esterified form, has given desirable results (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; Tejera *et al.,* 2007). In wild specimens, the astaxanthin carotenoid is provided by small crustaceans that constitute the natural feed of this species (Labropoulou *et al.*, 1999; Castriota *et al.*, 2005). Therefore several studies have used crustacean meals as a source of astaxanthin in order to overcome the red porgy skin pigmentation problem, given interesting results (Cejas *et al.*, 2003; Kalinowski *et al.*, 2005; 2007).

Another key constriction in cultured red porgy is their high protein dietary requirement, around 50% dry weight, in artificial diets (Schuchart *et al.*, 2008), where fish meal has currently been used as protein source. Some works has been realized to study the partial replacement of fish meal protein by protein from hydrolyzed krill meal in red porgy diet (Schchardt, 2005). In general, good feed utilization, growth performance as well as skin colour enhanced results were obtained in a diet with a 20% inclusion of krill meal and low dietary lipid level (15%). In the same study, a reduction of the dietary protein level by increasing the dietary fish oil, clearly resulted in a reduction on fish growth when fat levels where above to 15%. Red porgy is considered a lean fish (Ruedas *et al.*, 1997).

In this line, both dietary protein quantity and quality are considered important in these animals compared to other types of less expensive energy ingredients such as fats.

Consequently, for red porgy being competitive in the market remains that not only return their natural skin pigmentation is necessary, but also replacing the expensive fish meal in their diet is important as this would reduce feed cost. In this sense, development of new supplies of protein and natural pigments, especially from marine sources, in on-growing red porgy diets would assist in their economical rearing to reach a marketable size.

The present study is focused on identifies potential fish meal alternative ingredients and natural pigment, especially from marine origin, for red porgy diets. The results from this work could give valuable answers to reduce the inclusion level of the expensive fish meal in red porgy diets. Moreover would greatly improve basic nutritional and metabolic knowledge of the red porgy and their effects by dietary changes. All studied aspects will contribute to producing diets for farmed red porgy that more closely resembles their natural diet, thus promoting fish growth and colour towards the wild specimens.

2. OBJECTIVES

The main objective of this thesis was to evaluate potential feed ingredients which promote the reduction of fish meal in on-growing red porgy diets. Overall studies were propouse to elucidate the fish dietary response on growth, nutrient utilization and the final fish flesh quality, as well as skin coloration towards the wild specimens. Moreover, some studies were carried out to gain knowledge of the red porgy metabolic responses.

To achieve this purpose the following objectives were addressed in the present thesis:

1. To evaluate the effect of the partial replacement of fishmeal with two different crab meal origin, river or marine, on feed utilization, final growth performance and skin colouration in red porgy until commercial size.

2. To determine the nutrient digestibility of the river and marine crab meal, as well as to determine the effect of dietary crab meals inclusion on ammonia nitrogen excretion and calcium and phosphorous mineral deposition.

3. To study the effect of both dietary river and marine crab meal inclusion on final flesh composition and quality and muscle lipid oxidation during refrigerate storage.

4. To determine the overall effect of two other dietary marine ingredients, spider crab meal and sea urchin meal, on feed utilization, growth performance, skin colour, ammonia excretion, final flesh composition and quality and muscle lipid oxidation during refrigerate storage.

3. GENERAL MATERIAL AND METHODS

The all materials and methods used in the four studies of the present thesis are described in general with further detail provided in each study section. All experiments were carried out at the Marine Culture facilities of the Grupo de Investigación en Acuicultura (ULPGC & ICCM) (Canary Islands, Spain) and were included in the Spanish Ministry of Science and Innovation funded project (Harinas de algas y de cangrejo y subproductos de las mismas como ingredientes alternativos a la harina de pescado en dietas para bocinegro, *Pagrus pagrus*: efectos digestivos y metabólicos y repercusiones en la calidad del filete) (**AGL2006-12888/ACU**).

Figure 3.1 The Canarian Institute of Marine Sciences (ICCM)

3.1. ANIMALS

All juveniles red porgy used in the experiments were produced at the research facilities of the "Instituto Canario de Ciencias Marinas" localized in Gran Canaria, Spain (Figure 3.1). At the beginning of each experiment, weight and total length were taken from all fish, previously anesthetised with 2-

phenoxyethanol (0.1 mL L^{-1}), and then randomly distributed into the circular fibreglass tanks.

3.2 CULTURE CONDITIONS

3.2.1 Facilities

The feeding trials described in the present work were performed at the Marine Culture facilities of the Canary Institute of Marine Sciences (ICCM), belonging to the Canary Island Government (Agencia Canaria de Investigación, Innovación y Sociedad de la Información del Gobierno de Canarias).

3.2.2 Experimental tanks

Tanks system were composed by a group of cylinder conical tanks with a total volume of 500 litres and disposed with a central drainage channel. Each tank had dimensions of 1.5 m of diameter and 1.0 m of depth in the central part and was continuously supplied with natural sea water under experimental conditions. All tanks were individually covered to prevent the fish jumping out the tanks.

3.2.3 Experimental conditions

Experiences were realised with tanks held indoors and natural light condition with a natural photoperiod (latitude 28º, 10' North). Fish were fed until apparent satiation 2 time a day and six day per week. Daily mortality was recorded as well as the supplied and waste feed in order to determine the daily feed intake. Water dissolved oxygen and temperature were weekly measurement with the oximeter YSI 95 Dissolved Oxygen, 95/10 FT CE model.

3.3 EXPERIMENTAL FEED INGREDIENTS

3.3.1 River crab, *Procambarus clarkii*, meal

Figure 3.2 Taxonomic classification of the river crab *Procambarus clarkii*.

The crab *Procambarus clarkii* (Fig. 3.2) is a freshwater crayfish specie native to North-eastern of Mexico and the South-eastern of United States. However, it can be found in a wide range of places due to the deliberate introduction of this species in many countries. In Spain, this crayfish was introduced in 1974 for commercial purposes. The first introduction occurred in the Guadalquivir River at the South of Spain (Hadsburgo-Lorena, 1979; Algarin, 1980, Ocete and López, 1983). Nowaday, Spain is among the top three countries that most contribute to the world market of this river crab (Laurent, 1990).

The red river crab meal, of the present work was provided by a company located in the southern of Spain (Seafood Sevilla S.L.). This company processes this river crab for human consumption. The meal was generated from the crab by-products and processing waste mainly consisting of shells, legs, viscera, deformities animal and out market size discards, which all still has a considerable nutritional value. The raw material was processed at high temperature by the company towards to obtain the river crab meal used in the present work (Fig. 3.5).

3.3.2 Marine crab, *Chaceon affinis* and *Paramola cuvieri*, meals

Figure 3.3 Taxonomic classification of the marine crab *Chaceon affinis*.

The red crab *Chaceon affinis* (Fig. 3.3) is the largest epibenthic brachyuran which inhabit oceanic water throughout the eastern Atlantic Ocean, Iceland to Senegal and around all the Macaronesian Islands (López Abellán *et al.*, 2002). In the Canary Islands, this species was first collected during a scientific survey in July 1985 (Lozano *et al.*, 1992). It is has been caught at depths ranging from 550 to 1200 m by scientific surveys and by fishermen (Pinho *et al.*, 2001; López *et al.*, 2002). In spite of the stock having an economic potential and the presence of some local fisheries of this specie in north-western Spain (Galicia Bank), there is no still commercial market development of this species for human consumption.

The red crab *Paramola cuvieri* (Fig. 3.4) it is widely distributed throughout the eastern Atlantic, from southern Iceland (63 º N) and Norway to South Africa (36 º S), including the Azores, Madeira, Canaries and Cape Verde, also in some areas of the Mediterranean. In the Canaries, it is occasionally captured between 120 and 860 m depth and until now, there is not a local market for this species and constitutes a discard by-catch in the local fisheries (Sanchez *et al.*, 2004). It is caught with bottom traps as a secondary species in fisheries, especially shrimp (*Plesionika edwardsii*) between 250 and 350 m and with the red crab (*Chaceon affinis*) between 600 and 900 m.

Figure 3.4 Taxonomic classification of the marine crab *Paramola cuvieri*.

3.3.2.1 Preparation of the two marine crab meals.

The two marine crabs, *C. affinis*, and *P. cuvieri*, were caught close to the Canaries' Coast in Spain by local fishermen. Both meals were prepared locally at our laboratory facilities from the whole crabs individuals using a process adapted from Sudaryono *et al.*, (1996). First, animals were individually cleaned and autoclaved at high pressure for 15 min, then oven-dried for 12 hours at 55ºC and finally ground in a hammer mill through a 0.5 mm mesh and stored in vacuum plastic bags at 4ºC. The subsequent batch meals were finally mixed until obtained an only homogenous batch (Fig. 3.5).

Figure 3.5 River and marine crabs meals used in the experimental diets.

3.3.3 Sea urchin, *Diadema antillarum*, meal

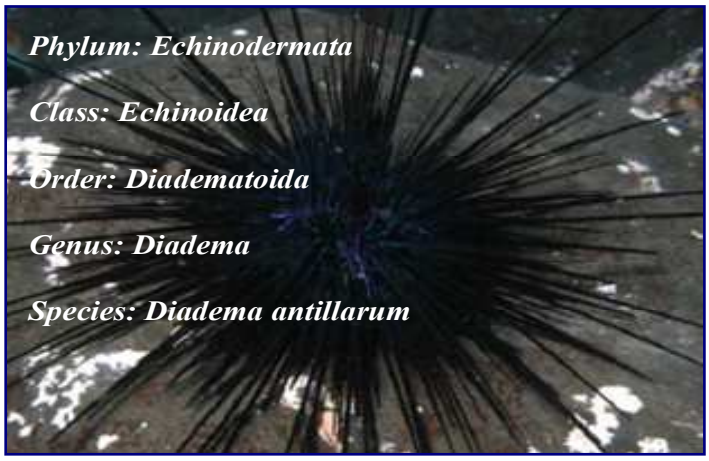

Figure 3.6 Taxonomic classification of the long-spined sea urchin *Diadema antillarum*.

The long-spined black sea urchin *Diadema antillarum* (Philippi) (Fig. 3.6), is an amphi-Atlantic warm species that plays a key-role in benthic communities as the main herbivorous across the temperate warm-waters of the central-east Atlantic (26-36 º N) (Alves *et al.*, 2003; Tuya *et al.*, 2004). In this sense, the long-spined black sea urchin is directly involved in the transformation of large rocky-reefs covered by erect fleshy algae to overgrazed, deforested, stable substrates dominated by encrusting organisms. These so-called "urchin-grazed barrens" are a global phenomenon; generally support less biodiversity and productivity that nearby vegetated area (Pinnegar *et al.*, 2000).

Individuals of the sea urchin were caught from the seafloor Canaries' coast and meal was prepared locally from whole animals, previously removing part of the surrounding whole body spines, and using the same adapted process to obtain the marine crab meals (Sudaryono *et al.*, 1996). After drying, the material was also grounded in a hammer mill through a 0.5 mm mesh and stored in vacuum plastic bags at 4ºC. The different batch meals were mixed until obtained an only homogenous batch.

3.3.3 Other feed ingredients

Fish meal and fish oil used in all experiments were from Peruvian origin and provided by the company Proaqua Nutrición S.A, España. The used pre-gelatinized starch "Merigel 100" and the vitamin C "Stay C" were donated by Spanish companies Especialidades Puma, S.A. (Amylum Group) and DMS-Nutrición S.A, respectively.

3.4 FEED PROCESSING

3.4.1 Formulation

Proximate composition and total carotenoids content of all used feed ingredients were analysed prior to diet formulation. In all experiences, diets were formulated to be isocaloric and isoproteic with 12% lipid and 50% protein, in dry weight basis, to meet the specie requirements as previously reported by Schuchardt *et al.* (2008). A diet based on high quality fish meal (FM) was used as a control diet. The treatments were formulated by replacing the desirable % of FM protein in the control diet with protein from the alternative tested meals. Fish oil was the main lipid source in all experimental diets. Dietary starch content varied between diets and was used as a filler to keep the same protein and lipid levels in all experimental formulation.

3.4.2 Diets elaboration

A mix of minerals and hydro-soluble vitamin (Table 3.1) were prepared and stored at 4ºC until diets elaboration. In both mixtures α-cellulose was used as a carrier component. The other mix was the fat-soluble vitamins (Table 3.1), which was prepared at the time of diets elaboration, using ethoxyquin as antioxidant.

To prepare the diets, all dry ingredients were added and mixed sequentially, from the lower to the higher amount. Fat-soluble vitamins were dissolved in the fish oil and added to the whole dry ingredients mixture, being gently mixed to obtain a homogenous mass. Vitamin C was introduced at the end

of the process to minimize losses due to possible oxidation. The last component added was the water, about 10%, in which the choline chloride vitamin was dissolved. Finally, the resultant mixture diet was pelleted with a CMP (Mod CL3, USA) pellet machine, throughout 5mm die diameter to obtain an adequate feed pellet according to the fish size. The obtained pellets were disposed into different trays and dried in an aerated oven at a temperature of about 38ºC for 12 hours for then being disposed in closed plastic bags and stored at 4ºC.

Table 3.1 Vitamins and minerals premixes used in all experimental diets.

Vitamins (g/ kg diet)		Minerals (g/ kg diet)	
Hydro-soluble			
Cianocobalamine (B_{12})	0.5	$(H_2PO_4)Ca$	1.605
Biotin (B_7)	0.001	$CaCO_3$	4.0
Folic acid (B_9)	0.01	$FeSO_4.7H_2O$	1.5
Pyridoxine (B_6)	0.04	$MgSO_4.7H_2O$	1.605
Riboflavine (B_2)	0.05	K_2HPO_4	2.8
Thiamine (B_1)	0.04	$Na_2PO_4H_2O$	1
Choline	2.7	$Al(SO_4)_3.6H_2O$	0.02
Pantotenic acid	0.12	$ZnSO_4.7H_2O$	0.24
Niacin (B_3)	0.2	$CuSO_4.5H_2O$	0.12
Ascorbic acid (C)	0.7	KI	0.02
Mio-inositol	2	$CoSO_4.7H_2O$	0.08
Lipid-soluble		$MnSO_4H_2O$	0.08
∝-tocopherol (E)	0.25		
Retinol acetate (A)	0.025		
Medianone (K_3)	0.02		
Cholecalciferol (D_3)	0.005		

3.5 BIOLOGICAL AND FEED UTILIZATION PARAMETERS

The following equations were used to study the effect of the experimental diets on growth performance and feed utilization.

3.5.1 Relative growth

Represent the relation between the biomass (g) increase and initial weight.

Growth (%) = [(final weight (g) – initial weight (g))/ initial weight (g)] x 100

3.5.2 Condition factor (K)

Represent the relation between the total animal weight and length.

$K = weight\ (g)/ (length\ (cm))^3$

3.5.3 Specific growth rate (SGR)

Is the percentage of daily growth increase.

SGR= [(Ln final weight -Ln initial weight)/nº experiment days)] x 100

3.5.4 Feed conversion ratio (FCR)

Represent the relation between feed intake and weight gain.

FCR= feed intake (g)/weight gain (g)

3.5.5 Hepatosomatic index (HSI)

Represent the relation between the liver weight and total animal weight.

HSI= [liver weight (g)/ fish weight (g)] x 100

3.5.6 Visceral index (VSI)

Represent the relation between total viscera weight and fish weight.

VSI= *[viscera weight (g)/ fish weight (g)] x 100*

3.5.7 Protein utilization ratio (PER)

This index relates the fish weight with total protein intake, and constitutes a measurement of dietary protein utilization.

PER= weight gain (g)/ protein intake (g)

3.6 INSTRUMENTAL COLOUR DETERMINATION

For skin colour measurements, a portable colorimeter (Hunter Lab MiniScan™ XE, USA) was used in all experiments. Measurements were taken directly from the left front lateral skin zone (Kalinowski *et al.*, 2005). The colour parameters obtained were *L**, *a** and *b** from the Hunter system (Fig. 3.7).

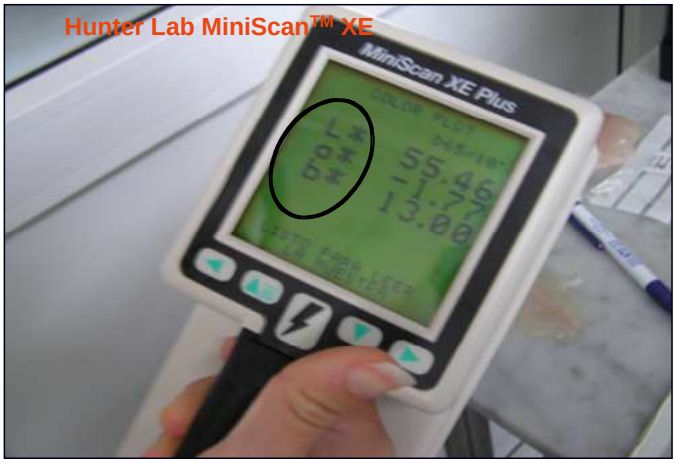

Figure 3.7 Colour measurements taken with the portable colorimeter.

*L**, is the lightness and ranges from 0 for black to 100 for white. The parameter *a** represents chromaticity ranging from green (negative values) to red (positive values), while *b** represents chromaticity ranging from blue (negative values) to yellow (positive values), according with the recommendations of the international Commission on Illumination, CIE (1976). From *a** and *b** values, Hue (H_{ab}) and chroma (C_{ab}) values were calculates by the following equations:

$H_{ab} = arctan\ (a^*/b^*)$, (Hunt, 1977).

$C_{ab} = ((a^*)^2 + (b^*)^2)^{1/2}$, (Hunt, 1977).

Hue is an angular variable and represents the observable colour. Values of 0°, 90°, 180° and 270° indicate a red, yellow, green and blue hue respectively. Chroma parameter expresses the saturation or intensity of the observable colour.

3.7 ANALYSIS OF PROXIMATE AND FATTY ACIDS COMPOSITION.

All samples were properly collected and stored at -80ºC until analysis.

3.7.1 Moisture

Moisture was determined by drying the samples at 105ºC until a constant weight was reached (AOAC, 1995).

3.7.2 Ash

For determining ash content, sample was dried in an oven at a temperature of 405ºC until a constant weight was attained (AOAC, 1995).

3.7.3 Crude Protein

Protein sample content was determined following the kjeldhal method (AOAC, 1995). Sample was previously digested with concentrated sulphuric acid at a temperature of 420ºC.

3.7.4 Crude Lipids

Total lipid was extracted from the samples by the method of Folch *et al.* (1957), using a mixture of chloroform:methanol (2:1, v/v) containing 0.01% of BHT. After lipid extraction, solvent was dried under nitrogen atmosphere and then weighted.

3.7.5 Fatty acid profile

Fatty acid profile from lipid was obtained as described by Christie (1982). Thus, the extracted total lipids were trans-esterified by adding to the samples a solution of Toluene with BHT and another with methanol and sulphuric acid at 1%. Later, samples were incubated for 16 hours at 50ºC. Afterwards, pure distilled water and hexane: dyetil ether (1:1) with BHT at 0.01% was added to the sample. The obtained fatty acid methyl esters (FAMES) were evaporated to dryness under nitrogen atmosphere, weighed and diluted with hexane at 20mg/ml concentration. For the identification and quantification of FAMES, a gas

chromatograph (Mod. Shimadzu GC-14A; Analytical instrument division, Kyoto, Japan) was used under conditions described by Izquierdo *et al.* (1992). Each metilesters was identified and compared with an external standard EPA 28, Nippai, Ltd. Tokyo, Japan.

From fatty acid results, the indexes of atherogenicity and thrombogenecity were calculated with the following equations, as proposed by Ulbricht and Southgate (1991):

Index of atherogenicity (AI)

$AI= [(12:00) + (4 \times 14:00) + (16:00)] \times [(PUFA\ n\text{-}6\ and\ n\text{-}3) + MUFA]^{-1}$

Index of thrombogenicity (TI)

$TI= [(14:00) + (16:00) + (18:00)] \times [(0.5 \times MUFA) + (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3/n\text{-}6)]^{-1}$

3.8 CAROTENOID ANALYSIS

3.8.1 Carotenoid extraction

Carotenoids from meals and diets were extracted according to Barua *et al.* (1993). First, sample is homogenized with ethyl acetate:methanol (1:1), followed by ethyl acetate and finally hexane. For skin sample, due to their high water content, the method utilized to carotenoids extraction was Schiedt and Liaaen-Jensen (1995). In the phase extraction of this method, only acetone was used at rate of 5ml per gram of sample. Acetone is used repeatedly until no colour of initial sample was observed. Then, 5 ml of hexane and 2 ml of water is added to acetone extract and the mix was shaken cautiously until observed two separated phases. The hexane upper layer, which contains the dissolved carotenoids, was selected.

In both methods of carotenoids extraction, final hexane extract is filtrated and evaporated to dryness under nitrogen atmosphere. Dry carotenoids were dissolved in a known volume of hexane to give an absorbance range from 0.2 to

0.8. Total carotenoids concentration was calculated spectrophotometrically (Genesys UV10, Thermo Fisher Scientific Inc. Waltham, USA) at maximum absorbance (λ_{max}) using a $E_{1\%,1\ cm}$ = 2500, or at λ=470 nm using $E_{1\%,1\ cm}$= 2100 if carotenoids was expressed as astaxanthin equivalent, by following equation:

$$\mu g\ g^{-1}\ sample = 10000*V*A/W*E_{1\%,1\ cm}$$

where *V (mL)* = volume of the extract, *W (g)* = weight of sample, *A*= absorbance and $E_{1\%,1\ cm}$= extinction coefficient, is the absorbance of an 1% solution read in a 1cm cuvette.

3.8.2 Carotenoids separation and relative quantification (%) by thin layer chromatography (TLC)

For the separation and relative quantification (%) of red and yellow pigments contented in red porgy skin, TLC methodology was used. Thus, final n-hexane solutions, containing total skin carotenoids, were analyzed by TLC using pre-coated plates of silica gel (G60 Merck glass plates, 20 x20 cm). Hexane/diethylether 20:1 was used as a developing system. Separated skin pigments were tentatively identified on the basis of relative motility on a TLC plate (Fig. 3.8).

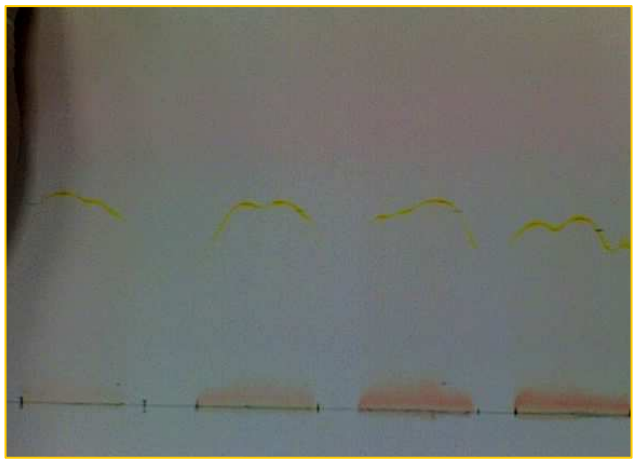

Figure 3.8 Red and yellow pigments of red porgy skin, separated by TLC

For quantification, each separated component was scraped off the silica plate and washed several times with acetone until no colour was observed. Thereafter, the acetone volume was filtered, evaporated to dryness and dissolved in a volume of hexane for further quantification by UV/Vis spectroscopy, reading at λ maxima and using an extinction coefficient ($E_{1\%,1\ cm}$) of 2500 for unknown compounds (Britton *et al.*, 1995).

3.9 AMMONIA EXCRETION ASSESSMENT

3.9.1 Water sampling for ammonia-N determination

Measurements were carried out in three 24-h ammonia-N excretion cycles at 8 days interval. In each cycle, feeding was performed at apparent satiation between 08:00 and 08:30 am. Inlet and outlet water samples (50 mL) were taken after that from each treatment tanks every 2 h between 08:30 am and 08:30 pm to analyse the levels of ammonia-N excretion from fish fed the different diets. Samples of water were kept into dark recipients at 4ºC and immediately analysed after collected them during the two hours interval. Samples from one tank without fish and other one with unfed fish were identically treated for measurements of blank values.

3.9.2 Analytical method

Water ammonia-N concentrations were determined by the colorimetric indophenol blue method of Koroleff *et al.* (1983). In the method, the alkaline phenol and the chloride react with ammonia to form indophenol blue, which is proportional to the ammonia concentration (Fig. 3.9).

3.9.2.1 Solutions

Previously to realize the method, the following solutions and reagents were needed:

Standard solution: 2.360 g of dry ammonium sulphate was weighed (preheated to 100ºC for 24 hours) and dissolved in 500 ml of water.

Stock solution: 500 ml of water is added to 500 ml of previous solution.

Phenol reagent: 17.5 g of crystalline phenol and 0.2 g of sodium nitroprusside were weighted and dissolved in 500 ml of water.

Sodium citrate reagent: 170 g of tri-sodium citrate and 10 g of sodium carbonate were dissolved in 400 ml of water. The solution was heated to boil for 20 minutes. Then, 2 g of dichlorinecyanuric acid and 500 ml of water were added to the solution.

3.9.2.2 Standards

Firstly, a calibration curve from standards solutions was performed. From the stock solution standards were prepared with the following volumes: 0.5, 1, 1.5, 2 and 3 ml. Then sea water was added to each standard until given a total volume of 100 ml. Thus, final ammonia concentrations of standards were 50, 100, 150, 200 and 300 ppm respectively (Fig. 3.9).

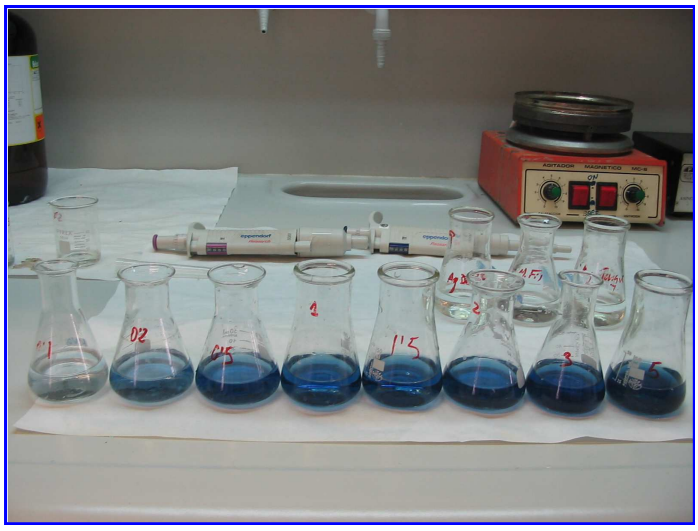

Figure 3.9 The colorimetric indophenol blue method.

3.9.2.3 Procedure

0.25 ml of phenol reagent was added to 5 ml of the collected sample. The mix was shaken in a vortex and added another 0.25 ml of a second sodium citrate

reagent. The new mix was again shaken and heated to 100ºC for 30 minutes and finally read at the wavelength of 625 nm.

3.10 DIGESTIBILITY DETERMINATION

3.10.1 Experimental tanks and faecal samples collection

To determine the digestibility of the experimental ingredients, modified Guelph system type (CYAQ-2) tanks, proposed by Cho *et al.* (1975; 1982), were used. The tanks were cylindrical, conical-bottomed (125 L), each fitted with a faeces collection column (Robaina *et al.*, 1995). Faecal samples were colleted from the settle column each morning, before feeding, in 50 ml centrifuge tubes. All samples were centrifuged at 10,000 rpm for 20 min and the supernatants discarded. The solid phase, which was pooled by tank, was stored at -20ºC for their later analysis.

3.10.2 Chromium (III) oxide (Cr_2O_3), determination

For digestibility determination, all diets were supplemented with 0.5% Cr_2O_3 as an external inert digestion marker (Austreng, 1978). The percentage of Cr_2O_3 content of the diets and faecal samples were determined as previously described (Furukawa and Tsukahara, 1966). First, the diets and faecal samples were exposed to a nitric acid digestion, followed by concentrated perchloric acid solution and then absorbance was measured in spectrophotometer at the wavelength of 350 nm.

3.10.3 Apparent digestibility coefficients determination (ADCs)

Total diet digestibility for the diets was calculated using the formula:

$$\textit{Total diet digestibility (\%)} = 100\,[1 - (Cr_2O_3 \textit{ in diet} / Cr_2O_3 \textit{ in faeces})]$$

The ADCs (%) of the nutrients in the diets were expressed as the fractional net absorption of nutrients according to Maynard and Loosli (1979):

$$ADC\ (\%) = 100\ [1 - (Cr_2O_3\ in\ diet / Cr_2O_3\ in\ faeces) \times (Nutrient\ level\ in\ faeces / Nutrient\ level\ in\ diet)]$$

Using these data, the ADCs of nutrients in the tested meals were calculated according to Forster (1999) and Hardy *et al.* (2002):

$$ADC_{ing}\ (\%) = [(Nutr_{td} \times AD_{td}) - (0.7 \times Nutr_{bd} \times AD_{bd})] / (0.3 \times Nutr_{td})$$

where ADC_{ing} (%) is the digestibility of a given nutrient in the meal, $Nutr_{td}$ is the nutrient concentration of the test diet, AD_{td} is the apparent digestibility of the nutrients in the test diet, $Nutr_{bd}$ is the nutrient concentration in the C diet, AD_{bd} is the apparent digestibility of the nutrients in the reference diet, and $Nutr_{ing}$ is the nutrient concentration of the meal in the test diet.

3.11 FLESH TEXTURE PROFILE ANALYSIS

For determination of the flesh texture profile, measured instrumentally, the sacrificed fishes were kept with ice in polyethylene boxes packing at 4ºC for 24h. The texture analysis was conducted in a Stable Micro System texture analyser (TA.XT2, Surrey, England) equipped with a 5 kg load cell. After removal of the skin, shear test were performed in triplicate using three 2x2x1.2 cm^3 blocks of muscle excised from each fillet above the lateral line. All tests were carried out at refrigeration temperature, keeping the blocks cooled with ice. Fish fillets were compressed up to breaking, following the methodology described by Ginés *et al.* (2004). Compression test was defined as the force exerted to deform a block fillet using a flat-ended (Type P/100) aluminium compression plate and a test speed 0.8 mm/s at 80% penetration. From the force vs. time, seven mechanical texture parameters were calculated: hardness (N), fracturability (N), adhesiveness (J), springiness (m), cohesiveness (N) and chewiness (J).

Table 3.2 Parameters description of the texture profile analysis.

Parameters	Description	(units)
Hardness	Mechanical required force to compress the sample to certain level	Force (N)= $(kg \times m \times s^{-2})$
Fracturability	Required force to produce the rupture of the sample piece	Force (N)= $(kg \times m \times s^{-2})$
Cohesiveness	Maximum extent of the sample before breaking	ratio without unit
Springiness	The full or partial recovery of the sample, after stopping the compression force, to its original shape	Distance (m)
Adhesiveness	Required work to separate the sample from the compression cylinder	Work (J)= $(kg \times m^2 \times s^{-2})$
Gumminess	Is the product of hardness and cohesiveness.	Force (N)= $(kg \times m \times s^{-2})$
Chewiness	The required energy to break up the sample	Work (J)= $(kg \times m^2 \times s^{-2})$

3.12 SENSORY ANALYSIS

The sensory profile analysis was determined on the cooked fillets of red porgy fed with the different experimental diets. A preliminary training session was aimed before to select the profile attributes, to establish the sample evaluation procedures and to define the score sheet. The dorsal half part of each left fillet was divided into three portions of approximately 3x3 cm and cooked in aluminium boxes, previously identified with codes, for 10 min in a steam oven at 150ºC. Immediately after cooking, fillets were offered to a panel of selected trained judges (ISO, 1985; 1993). Judges were randomly offered closed food boxes labelled with codes containing the fillets. Attributes of odour (marine, oily and atypical), appearance/aspect (whiteness, shininess and cohesiveness), texture (juiciness, firmness and adhesiveness), flavour (marine, oily and atypical) and residual taste (permanence, marine and earthy) were tested for samples of fish fed the experimental diets, and classified by the judge in a continuous scale from 0 to 100 for each parameter. The sensory analysis was carried out in two sessions,

and to reduce the panellists' effect an analysis of repeated measurements was done.

3.13 FISH FILLET LIPID OXIDATION

The lipid oxidation of fish muscle was determined by measuring the 2-thiobarbituric acid reactive substances (TBARS), according to the methodology of Shahidi and Hong (1991). Firstly, fish muscle sample was mixed with a volume (2 × grams of sample) of 10% trichloroacetic acid (w/v) in a test tube and homogenized (Ultraturrax T25, Jane and Kukel GmbH, Geermany) for 1 minute. The process was carried out at refrigeration temperature, keeping the tubes cooled with ice. Then the samples were centrifuged at 4,000 rpm and 4ºC for 30 minutes. After that, 2 ml of the final extract, previously filtrated, were added to 2 ml of 0.02 M aqueous TBARS in a test tube. The test tubes were heated at 100ºC for 1 hour; after this time, samples were refrigerated under a continuous flow of water and read at 532 nm in a spectrophotometer (Genesys UV10, Thermo Fisher Scientific Inc. Waltham, USA). The results were obtained using a standard curve with 1,1,3,3 tetrametoxipropano (TMP) and expressed as tribarbitúrico acid reactive substances (TBARS) in mg of malonaldehyde per kilogram of sample.

3.14 STATISTICAL ANALYSIS

All scalar data type was tested for normality of distribution and homogeneity of variance (Sokal and Rohlf, 1995). To compare treatments, parametric and homoscedastic data were analysed by one-way *ANOVA* followed by Tukey's test for multiple comparisons. For non-normal data and/or data not showing homogeneity of variance, the Kruskal-Wallis multiple range test was employed. When two treatments were established, data were compared statistically by means of T-Student test (Sokal and Rohlf, 1995). In case of considering two factors and their interaction, data were submitted to a two-way *ANOVA* by using linear model procedure. All statistical analyses were performed

using the SPSS v. 13.1 statistical package. Significance was set at $P<0.05$.

The angular colour parameter, hue, was analysed for statistical differences following circular statistical procedures. The estimation of the mean value and standard deviation was performed with descriptive statistics for circular distributions. The Rayleigh test was applied to check the uniformity of circular distribution (Zar, 1999). As sampled populations was not unimodal, differences in the hue variables between and experimental groups were tested using the non parametric Watson's U^2 test and accepted at $P<0.05$ (Zar, 1999). All tests were performed with Oriana, version 3, statistical software (Kovach Computing Services, Pentraeth, Wales, UK).

STUDIES

STUDY I: Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): effect on growth, fish composition and skin colour (Aquaculture Research 41 (2010), 1759-1769)

STUDY II: Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): digestibility, ammonia excretion, phosphorous and calcium retention

STUDY III: Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): effect in fillet fatty acid profile and flesh quality parameters

STUDY IV: Meals of spider marine crab (*Paramola cuvieri*) and sea urchin (*Diadema antillarum*) in diets for red porgy (*Pagrus pagrus*): effect on growth performance, ammonia excretion, skin colour and flesh quality

STUDY I

Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): effect on growth, fish composition and skin colour

(Aquaculture Research 41 (2010), 1759-1769)

Abstract

River crab (RC) meal (*Procambarus clarkii*) and marine crab (MC) meal (*Chaceon affinis*) were tested as a partial replacement for fish meal in diets for red porgy (*Pagrus pagrus*), and their effect on growth performance, fish proximate composition and skin coloration were evaluated. Red porgy were fed during 165 days with five diets. High-quality fish meal diet was used as a control diet (CD). Protein of fish meal in the control was replaced by increasing the dietary levels of protein derived from RC and MC by up to 10% and 20% of each of them (RC10, RC20, MC10 and MC20). Fish fed on MC20 showed the highest values in feed intake, weight gain and growth (%). No differences were found in FCR and protein efficiency ratio (PER) among treatments. Inclusion of both crab meals in diets significantly decreased the lipid content in whole fish compared to control animals. Feeding both crab meals resulted in colour improvement compared to that of the control fish, with better hue values for the RC meal group than those for the MC meal group. The crab meals tested in the present study are suitable as a partial replacement for fish meal in diets for the red porgy, with the MC meal improving growth and both crab meals improving skin colour, with further improvements in skin colour produced in fish-fed diets containing the RC meal.

Keywords: carotenoids, crab meal, fish composition, *Pagrus pagrus*, skin colour

4.1 Introduction

Red porgy, *Pagrus pagrus*, is a marine fish species with an important market demand and greatly appreciated by consumers on the Mediterranean and Atlantic coasts (Kokokiris *et al.*, 2006); therefore, it is considered, in this region, to be a priority species for aquaculture diversification. Preliminary studies carried out with this species under culture conditions have shown good adaptability together with efficient growth rate (Divanach *et al.*, 1999). However, one of the main constraints in the culture of this marine species is the loss of its characteristic red-pink skin colour, which is a huge problem from a commercial point of view, associated with the acceptance or rejection of the product by consumers (TECAM, 1999; Cejas *et al.*, 2003). One of the key factors responsible for the discoloration of cultured red porgy skin is the fact that fish are unable to biosynthesize carotenoids *de novo* (Goodwin, 1984); hence, under aquaculture conditions, carotenoids must be provided in the diet. Thus, most recent nutritional studies done on this species have principally focussed on different carotenoid sources and levels and their effect on skin coloration. In fact, the inclusion of dietary astaxanthin, mainly in its esterified form, has been shown to be an effective carotenoid for farmed red porgy skin to acquire the characteristic red-pink coloration (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Pavlidis *et al.*, 2006; Tejera *et al.*, 2007).

Another important limitation in farmed red porgy is the high protein requirement (around 50%) for this species. To date, only one study has focused on determining the protein requirement for this species at the on-growing stage, utilizing a high-quality fish meal tested as a sole protein source (Schuchardt *et al.*, 2008). Due to the global fish meal production situation (Tacon and Metian, 2008) there is a great need for studying alternative protein sources for this species, ideally by-products from different industries.

By-products from the crab industry have an attractive potential not only as a protein source, but also as a carotenoid source (Shahidi and Synowiecki, 1991;

Cremades *et al.*, 2003). The partial replacement of fishmeal by crab meal from *Pleuroncodes planipes* and *Cancer pagurus* in white shrimp (*Litopenaeus vannamei*) and Atlantic cod (*Gadus morhua*) diets, respectively, has yielded successful growth results in both species (Goytortúa-Bores *et al.*, 2006; Toppe *et al.*, 2006; Villarreal *et al.*, 2006). Also, the inclusion of crab meal from *P. planipes* has been shown to be an effective source of astaxanthin for rainbow trout (Coral-Hinostroza *et al.*, 1997; 1998).

Regarding freshwater crabs, in Spain the crayfish *Procambarus clarkii* is an introduced crustacean from North America but nowadays Spain is one of the main producers in the global crayfish market. Factories that process this animal for human consumption produce large quantities of waste products that are utilized as supplementary animal feed ingredients, constituting a good source of protein, astaxanthin and chitin (Lee, 1990; Negro and Garrido-Fernandez, 2000; Cremades *et al.*, 2003; Pérez-Gálvez *et al.*, 2008).

Together with crustacean species, there are others with great potential such as the marine crab *Chaceon affiniss.* This species is distributed throughout the Eastern Atlantic Ocean, from Iceland to Senegal and around all the Macaronesian Islands. It is a deep-sea crab caught at depths ranging from 550 to 1200 m by scientific surveys and by fishermen (Pinho *et al.*, 2001; López-Abellán *et al.*, 2002). In spite of the stock having economic potential and the presence of some local fisheries, there is no commercial market development of this species for human consumption. Therefore it is an interesting source of protein and carotenoids for fish diets.

The aim of the present study is to partially replace dietary fishmeal protein with proteins from crab meal of *P. clarkii* and *C. affiniss* species and to evaluate their effect on red porgy growth performance, skin coloration and whole proximate composition.

4.2. Materials and methods

4.2.1 Crab meals

Two different crab meals were used for fish meal protein replacement in the diets. The first was a MC meal from the benthonic deep-sea crab, *C. affinis,* commonly caught close to the Canaries' coast in Spain. The meal was prepared locally from whole crabs using a process adapted from Sudaryono (1996). First, individuals were autoclaved at high pressure for 15 min, then oven-dried for 12 hours at 55ºC and finally ground in a hammer mill through a 0.5 mm mesh and stored in vacuum plastic bags at 4ºC. The second used in the present trial, river crab meal (RC), was provided by a company located in the South of Spain (Seafood Sevilla S.L.) and obtained by processing the red river crab *P. clarkii*. The proximate composition and total carotenoid content of both crab meals are shown in Table 1.4

Table 4.1 Proximate composition (% dry wt) and total carotenoid content (mg kg^{-1}) of fish (FM), river (RC) and marine (MC) crab meals used in experimental diets

	FM	RC	MC
Crude	67.29	42.24	45.85
Crude	11.61	6.05	5.85
Ash	15.34	30.69	28.48
Moisture	13.96	8.87	8.17
Carotenoid	3.37	15.74	5.90

4.2.2 Fish and culture conditions

Red porgy with initial mean weight of 233 ± 36 g [(mean ± standard deviation (SD)] were distributed in circular fibreglass tanks, of 500 L, at an initial density of 5.59 kg m^{-3}. Tanks were provided with natural seawater with 17-19 ºC temperature and 7-10 mg L^{-1} dissolved oxygen range. Fish were carefully fed until apparent satiation 2 times per days (9:00 and 15:00), 6 days per week, with daily feed intake recorded.

4.2.3 Experimental diets

Five isocaloric (12%) and isoproteic (50%) diets were formulated (Table 4.2) and tested in triplicate groups of fish for 165 days. A control diet consisted of high quality fish meal and fish oil (CD) and four diets were formulated where 10% and 20% of fish meal protein in CD was replaced by either marine crab meal protein (diets MC10 and MC20) or river crab meal protein (diets RC10 and RC20). Diets were prepared by mixing ingredients in a horizontal ribbon mixer and pelleting them (CPM, CL3 Pellet mill model. USA) through a 5-mm- diameter die.

Table 4.2 Ingredients (g kg^{-1}) and proximate analyses of the experimental diets

	CD	RC10	RC20	MC10	MC20
Ingredients					
FM [a]	672	608	543	608	543
RC [b]	-	105	215	-	-
MC [c]	-	-	-	98	213
Fish oil	70	70	70	71	70
Gelatinized starch [d]	213	172	127	178	128
Vitamin premix	20	20	20	20	20
Mineral premix	20	20	20	20	20
CMC	0.5	0.5	0.5	0.5	0.5
Proximate composition (% dry wt)					
Crude protein	46.68	46.75	46.64	46.30	47.08
Crude lipids	11.59	10.68	11.61	11.42	11.53
Ash	11.21	14.61	16.74	14.73	17.32
Carbohydrate [e]	30.52	27.96	25.01	27.55	24.07
Moisture	8.33	6.61	7.8	6.20	5.83
Gross Energy (MJ kg^{-1}) [f]	20.73	20.10	19.93	20.21	19.84
Total carotenoid (mg kg^{-}	2.86	3.36	5.56	3.02	3.58

[a] Fish meal.
[b] River crab *Procambarus clarkii* meal.
[c] Marine crab *Chaceon affinis* meal.
[d] Merigel 100 Amylum Group.
[e] Carbohydrate = 100 – protein – lipid – ash.
[f] Gross Energy = (23.6 MJ kg^{-1} x % protein + 39.8 MJ kg^{-1} x % lipid + 17.2 MJ kg^{-1} x % carbohydrate)/100.

4.2.4 Sampling and colour evaluation

Skin colour parameters, whole body weight and total length were taken in all fish previously anesthetised with 2-phenoxyethanol (0.1 mL L^{-1}) at the beginning of the experiment and at 30, 60, 120, 165 days feeding. For proximate analysis of whole fish, nine individuals per treatment were sacrificed. Nine fish per treatment were also sampled for skin carotenoids concentration at the end of the experiment at the same zone where skin colour was measured. All samples were stored at -80ºC until analysis.

4.2.5 Colour measurements

The skin colour parameters evaluated were lightness (L^*), redness (a^*) and yellowness (b^*) in accordance with the recommendations of the International Commission on Illumination (1976). Colour variables were measured from the left front lateral zone as suggested by Kalinowski *et al.* (2005), using a portable colorimeter (Hunter MiniScan™ XE plus). Hue which is the colour [$H_{ab}=arctan(b^*/a^*)$] and chroma, which is the intensity of the colour [$C_{ab}= (a^{*2}+b^{*2})^{1/2}$], were calculated from a^* and b^* (Hunt, 1997).

4.2.6 Biochemical analysis

Moisture, crude protein and ash content were determined according to AOAC (1995). For total lipids extraction, the methodology of Folch *et al.* (1957) was used. Carotenoids from meals and diets were extracted according to Barua (1993) and for skin samples, due to their high water content, according to Schiedt and Liaaen-Jensen (1995). Total carotenoid concentration was calculated spectrophotometrically at λ=470nm using $E_{1\%,1cm}$= 2100 and expressed as astaxanthin equivalent by following equation:

$$\mu g\ g^{-1}\ sample = 10000*V*A/W*E_{1\%,1\ cm}$$

where V (mL) is the volume of the extract, W (g) the weight of sample, A the absorbance and $E_{1\%,1\ cm}$ the extinction coefficient. Final n-hexane solutions,

containing total skin carotenoids, were analyzed by TLC using pre-coated plates of silica gel (G60 Merck glass plates, 20 x 20 cm). Hexane/diethylether 20:1 was used as a developing system. Separated skin pigments were tentatively identified on the basis of relative motility on a TLC plate. For quantification, each separated component was scraped off the silica plate and washed several times with acetone until no colour was observed. Thereafter, the acetone volume was filtered with a Pasteur pipette filled with cotton, evaporated to dryness and dissolved in a volume of hexane for further quantification by UV/Vis spectroscopy, reading at λ maxima and using an extinction coefficient ($E1_{\%,1cm}$) of 2500 for unknown compounds (Britton *et al.*, 1995). Unpublished colour variables of wild red porgy skin from our research group were used as referential values.

4.2.7 Statistical analysis

All scalar data types were tested for normality of distribution and homogeneity of variance (Sokal and Rohlf, 1995). In order to determine significant differences between treatments, parametric and homoscedastic data were analyzed with one-way ANOVA using Tukey's test for multiple comparisons. For data not displaying normality and/or homogeneity of variance, a non-parametric analysis and multiple range test (Kruskal-Wallis) were used. All statistical analyses were tested at 0.05 significance level using the SPSS (13.1) statistical package.

The angular colour parameter, hue, was analysed for statistical differences following circular statistical procedures. The estimation of the mean value and standard deviation was performed with descriptive statistics for circular distributions. The Rayleigh test was applied to check the uniformity of circular distribution (Zar, 1999). As sampled populations was not unimodal, differences in the hue variables between and experimental groups were tested using the non parametric Watson's U^2 test and accepted at $P<0.05$ (Zar, 1999). All tests were performed with Oriana, version 3, statistical software (Kovach Computing Services, Pentraeth, Wales, UK).

4.3 Results

4.3.1 Growth and feed utilization

All diets were well accepted from the start of the trial, with higher feed appetence observed in those fish fed with MC meal diets, which resulted in higher feed intake, although only the higher inclusion level, MC20, caused significantly greater feed intake than the other treatments (Table 4.3). At the end of the experiment, final fish weight, absolute weight gain and percent growth increase showed a pattern similar to that of the feed intake, with higher values for the MC-based diets, and significantly greater in the high inclusion level. Results for the RC-based diets did not differ from the CD. SGR values were similar to the control treatment in fish fed RC10, RC20, MC10 and MC20 diet, with only the SGR of fish fed the MC20 diet significantly higher than the RC10 diet. No difference was found for FCR, protein efficiency ratio (PER), condition factor (K) or hepatosomatic or visceral indices among diets (Table 4.3).

4.3.2 Proximate composition of fish

Proximate composition of whole fish showed similar protein and lipid content among fish fed with diets containing both crab meals, of marine or river origin, at either of the two tested levels. Compared to the control fish, all the crab meal diets produced significantly lower whole body lipid content; however, the only difference for protein was for the MC20-fed fish with significantly lower values compared to the control group. Body moisture content was the same between river crab meal groups and the control group; although significantly higher water content was observed for the marine crab meal groups (Table 4.4).

Table 4.3 Growth performance and feed utilization parameters at the end of the trial

	CD	RC10	RC20	MC10	MC20
Initial weight (g)	239.49±39.77	234.39±31.75	228.74±35.94	228.65±35.73	233.68±36.70
Final weight (g)	433.00±56.19 [b]	420.27±59.25 [b]	412.53±52.14 [b]	430.99±58.59 [b]	481.91±59.34 [a]
Weight gain (g) [a]	193.60±2.84 [b]	185.59±24.41 [b]	184.03±4.09 [b]	211.93±26.22 [b]	247.73±10.29 [a]
Growth (%) [b]	80.84±0.32 [b]	79.21±8.93 [b]	80.38±1.70 [b]	88.61±8.74 [b]	106.24±1.43 [a]
FI (g fish $^{-1}$) [c]	342.09±20.50 [b]	331.58±22.32 [b]	320.96±25.43 [b]	383.02±29.61 [ab]	422.24±59.61[a]
SGR [d]	0.38±0.00 [ab]	0.37±0.03 [b]	0.38±0.00 [ab]	0.42±0.04 [ab]	0.46±0.01 [a]
FCR [e]	1.77±0.13	1.80±0.12	1.75±0.18	1.82±0.16	1.71±0.26
PER [f]	1.22±0.09	1.19±0.08	1.23±0.13	1.19±0.11	1.26±0.18
K [g]	3.20±0.22	3.07±0.06	3.17±0.07	3.09±0.02	3.11±0.09
HSI [h]	1.62±0.08	1.70±0.06	1.60±0.05	1.51±0.03	1.58±0.07
VSI [i]	8.19±0.38	8.64±0.31	8.18±0.08	8.29±0.44	8.35±1.24

Different letters in same row denote significant differences among groups.

[a] **Weight gain** = (final weight- initial weight)
[b] **Growth (%)=** ((final weight-initial weight)/initial weight)x100
[c] **Feed intake (g)**= per fish for 165 day experiment
[d] **Specific growth rate=** 100x(ln final weight-ln initial weight)/n° days
[e] **Feed conversion ratio=** feed intake (g)/weight gain (g)
[f] **Protein efficiency ratio=** weight gain (g)/protein intake (g) (dry matter).
[g] **Condition factor (%)=** 100x(final weight /(final length)^3)
[h] **Hepatosomatic index (%)=** 100x(liver weight/final weight)
[i] **Visceral index (%)=** 100x((final weight-final eviscerated fish weight)/final weight)

Table 4.4 Whole fish proximate composition (% wet wt)

	CD	RC10	RC20	MC10	MC20
Protein	18.73±0.77 ^{a}	18.13±0.38 ^{ab}	18.49±0.51 ^{ab}	18.50±0.96 ^{ab}	17.72±0.06 ^{b}
Lipid	15.03±0.9 ^{a}	12.94±0.50 ^{b}	13.04±0.41 ^{b}	12.50±1.51 ^{b}	12.75±0.72 ^{b}
Moisture	63.66±0.11^{b}	63.50±0.12 ^{b}	64.58±2.00 ^{b}	67.17±1.68 ^{a}	66.86±1.46 ^{a}

Means with no letter or common letter denote no significant differences.

4.3.3 Skin colour parameters

From day 60 until the end of the trial, all treatment groups, except MC10, attained significantly higher skin redness (a^*) values, in comparison to red porgy fed on CD (Table 4.5). Skin yellowness (b^*), from day 120 until the end of the trial, was the highest in fish fed on RC20 in comparison with red porgy fed on CD. Regarding hue values, RC10, RC20, MC10 and MC20 fish were significantly lower than the values of control animals (Fig. 4.1). At the end of the experiment, cultured red porgy skin colour was affected positively by the inclusion of both marine and river crab meal-based diets, showing redder hue skin colouration. Concerning chroma, only red porgy fed RC20 presented significantly higher values than CD fish (Fig. 4.2). Finally, no significant differences were found in lightness (L^*) among fish from all dietary treatment groups (Fig. 4.3).

Table 4.5 Skin redness (a^*) and yellowness (b^*) at 0, 30, 60, 120, 165 days of experiment and total carotenoid content (μg g^{-1}) of red porgy skin at the end of the experiment

Day	Colour Parameters	CD	RC10	RC20	MC10	MC20
30	a^*	3.19±1.32 ^{b}	3.96±1.14 ^{ab}	4.00±1.51 ^{a}	2.72±1.14 ^{c}	3.47±1.25 ^{b}
	b^*	10.53±1.98 ^{b}	10.94±1.76 ^{ab}	10.64±1.88 ^{b}	9.08±2.14 ^{b}	11.22±1.72 ^{a}
60	a^*	3.10±0.97 ^{c}	4.02±1.16 ^{ab}	4.25±1.42 ^{a}	3.27±1.25 ^{bc}	3.97±1.11 ^{ab}
	b^*	7.19±2.06 ^{ab}	10.72±2.43 ^{a}	11.33±2.23 ^{a}	9.01±2.13 ^{b}	10.08±2.36 ^{ab}
120	a^*	2.87±1.13 ^{c}	4.48±1.30 ^{b}	5.82±1.06 ^{a}	3.25±1.08 ^{c}	4.81±1.50 ^{ab}
	b^*	7.19±2.06 ^{b}	8.24±2.06 ^{ab}	9.82±2.39 ^{a}	6.59±2.35 ^{b}	7.16±1.96 ^{b}
165	a^*	4.21±1.01 ^{c}	6.27±0.99 ^{ab}	7.07±0.87 ^{a}	4.70±0.77 ^{bc}	5.70±1.47 ^{ab}
	b^*	8.58±1.42 ^{b}	8.76±1.36 ^{bc}	11.24±2.04 ^{a}	7.08±1.10 ^{c}	8.70±1.18 ^{b}
	Total Carotenoids	2.86±0.25 ^{c}	8.00±0.69 ^{b}	10.88±1.04 ^{a}	3.95±0.83 ^{c}	5.81±0.71 ^{b}

Means with common letter denote no significant differences. Initial skin redness *(a*)* and yellowness *(b*)* (mean ± SD, n=50 fish) = 1.53 ± 0.89 and 9.07 ± 1.90 respectively

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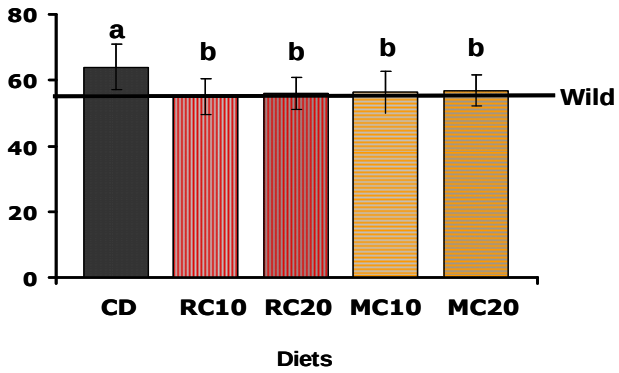

Figure 4.1 Skin Hue values (º) of red porgy at the end of the experiment. Means with common letters denote no significant differences.

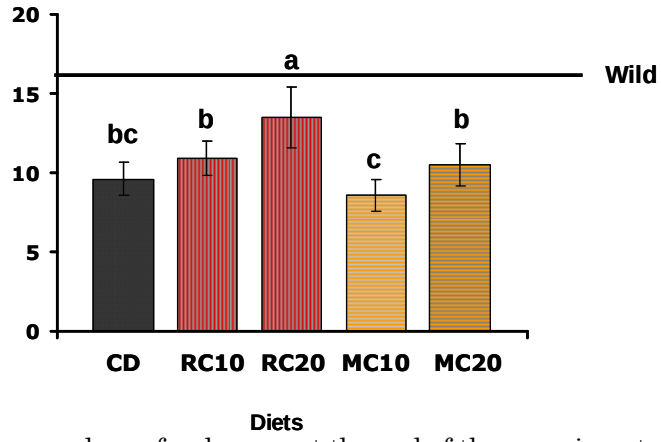

Figure 4.2 Skin chroma values of red porgy at the end of the experiment. Means with common letters denote no significant differences.

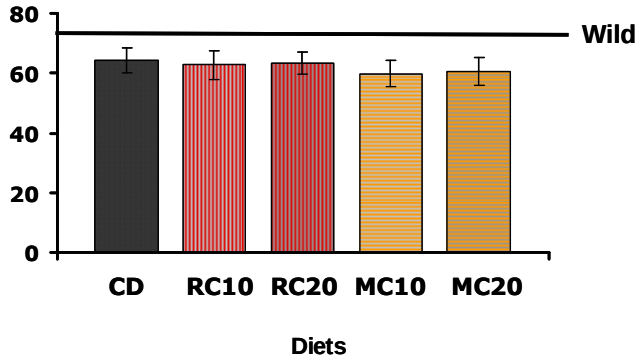

Figure 4.3 Lightness values skin fish of red porgy fed at the end of the experiment. Means with no letters denote no significant differences

4.3.4 Skin carotenoid concentration and TLC analysis

Skin carotenoid concentration was enhanced by the addition of both crab meals. The highest skin carotenoid concentration was observed in fish fed with RC20, followed by RC10 and MC20 diets, which did not differ from one another, only from fish fed on CD and MC10 diets (Table 4.5).

Skin total carotenoid separation carried out by thin-layer chromatography revealed two fractions: a red and a yellow fraction (Table 4.6). Including crab meal in the diets resulted in lower yellow pigment proportions while increasing the proportion compared to the control fish. Red porgy fed on RC10 and RC20 presented a higher percentage of red, hence a lower percentage of the yellow fraction, in comparison to the rest of the treatment groups. Comparing results obtained from cultured individuals against wild red porgy, fish fed with RC meals had values closest to wild red porgy.

Table 4.6 Red and yellow pigments fractions, separated on TLC silica gel plates, as a percentage of the total pigments (red+yellow) from fish skin carotenoid extracts

	Red pigment (%)	Yellow pigment (%)
CD	31.65±6.69 ^{c}	68.35±6.97 ^{a}
RC10	48.45±3.66 ^{ab}	51.55±4.35 ^{bc}
RC20	54.20±3.66 ^{a}	45.80±3.66 ^{c}
MC10	40.53±0.26 ^{cb}	59.47±0.26 ^{ab}
MC20	39.33±1.38 ^{cb}	60.67±1.38 ^{ab}

Column means with common letter denote no significant differences.

4.4 Discussion

4.4.1 Growth performance

From the beginning of the experiment all RC and MC meal-based diets were well accepted and utilized by cultured red porgy. This was clearly reflected in growth performance results that were similar to control animals. Nevertheless, fish from MC20 treatment group, presented higher growth performance compared to the rest of the experimental groups. Previous work in smaller-size red porgy reported no improvement in growth parameters by feeding fish with a combination of commercial pellets (Trouw Spain, 16% lipid) and defrosted shrimp, *Pleisonika sp.* (88% and 12%, respectively), with FCR values around 2 (Cejas *et al.*, 2003). In earlier work carried out on the same species, Kalinowski *et al.* (2007) reported a similar SGR (0.42–0.48%) but higher FCR (2.1–2.5) rates when 16% of fish meal was substituted with shrimp shell meal over a longer feeding period (180 days) and smaller initial fish size. Moreover, growth enhancement and better protein utilization measured by PER were also observed. In the present study, the PER values in all experimental groups were statistically similar suggesting that the replacement of fish meal protein by either crab meal did not significantly affect dietary protein utilization. Since the PER value from fish fed on MC diets did not differ from the rest of treatments, the better growth found in MC-fed fish cannot be explained by a better protein utilization from MC meal.

The reason for a higher growth in MC-fed fish could be related to the higher feed intake also presented for this group. The increase of feed intake is often in line with a better appetence of the diet but also with a reduction in dietary energy, as a consequence of poorer digestibility in diets containing high ash levels. Therefore the fish compensates reduced dietary energy by eating more. Meals prepared from crustaceans and their by-products are usually high in ash content (about 20% or higher), which adversely affects fish feeds digestibility (NRC, 1993; Robaina *et al.*, 1997).

Few studies have presented data on crab meal digestibility in fish, showing species-specific abilities; thus lower crab meal digestibility values have been found in haddock *Melanogrammus aeglefinus* (82%) (Tibbetts *et al.*, 2004), and higher in Atlantic cod *Gadus morhua* (89%) and halibut *Hippoglossus hippoglossus* (88%) (Tibbetts *et al.*, 2006). In the latter study, although comparable protein digestibility coefficients were found for the fish meal (92.8%) and crab meal (89.4%) was found in diets for cod, a medium range energy digestibility coefficient for crab meal (82.4%) in comparison to the fish meal (86.4%) was obtained, which could affect fish feed intake. Toppe *et al.* (2006) also reported a negative correlation between the dry matter digestibility and the dietary ash in an experiment with cod fed dietary inclusion levels of crab meal from to 54 to 176 g kg^{-1}. Moreover, a linear increase in feed intake was found with increasing dietary ash content, resulting in an improvement in growth performance. In the present study, together with MC diets, RC diets also presented high ash content and red porgy fed on these diets did not show higher feed intake; so, from this point of view, the higher feed intake observed for MC meal diets cannot be easily explained by a lower digestibility as a result of high ash content in these diets.

It is also important to mention that crustacean shells contain 50–80% chitin, a complex amino polysaccharide [(poly-ß-(1→4)-N-acetyl-glucosamine)], which is normally poorly digested. The natural diet of red porgy includes a high proportion of chitin-rich crustaceans (Labropouluo, 1999; Castriota *et al.*, 2005), thus suggesting, as in Atlantic cod, a high activity of the digestive enzyme chitinase in this species (Danulat and Kausch 1984; Danulat, 1986), supporting the idea that crustacean products are identified as good candidates to replace fish meal in diets for this species (Toppe *et al.*, 2006).

Apart from this, the increase of feed intake due to the greater appetence for marine crab meal should be considered. Prey attraction in fish is mostly a consequence of visual, olfactory and gustatory stimulus that detects soluble

compounds present in the surrounding environment. These soluble compounds are often low molecular weight substances containing nitrogen (Jones, 1992; Carr *et al.*, 1996; Yacoob and Browman, 2007). Crustacean meals are used as dietary attractants and stimulants in many aquatic species, due to the presence of certain amino acids that stimulate the reception and palatability (smell and taste) of animal and feeding behaviour (Shimizu *et al.*, 1990; Smith *et al.*, 2005; Goytortúa-Bores *et al.*, 2006). Therefore, in the present study the higher feed intake observed in CM fed fish is probably related more to the attractant and stimulant power of marine crab meal. Also, CM diets had a lighter colour compared to the other diets and could therefore have affected attractability and feed intake as has been suggested for cod (Toppe *et al.* 2006).

4.4.2 Fish proximate composition

The addition of both crab meals to the diets significantly reduced the total lipid content in whole fish. This lipid reduction could be due in part to a higher proportion of chitin and chitosan in these diets, which is known to bind lipid in the digestive tract and lower its absorption in humans and rats (Mun *et al.*, 2006; Zhang *et al.*, 2008). Although this species should be able to highly digest this complex polysaccharide as mentioned previously, some of them may act in this manner. On the other hand, accumulation of lipids in fish put on the crab meal diet could also be related to a dietary imbalance between saturated and unsaturated fatty acids, as a consequence of the high level of saturated fatty acids normally found in crab meals. In the present work, the proximate composition of the experimental diets was similar, but changes in relative proportions of fatty acids by crab meal inclusion could affect lipid utilization and deposition. The fatty acid analysis of diets and tissues were not determined in the present experiment, so more research is needed to better understand the nutritional value of fatty acids in the crab meals used for the red porgy. Differences in the minerals content of the crab meal diets compared to the control fish meal diet should also be considered regarding growth parameters and body lipid content. Calcium and

phosphorus content are normally higher in crab meal than fish meal. At the levels of crab meal tested in the present experiment and according to data from Toppe *et al.* (2006), the final dietary phosphorus content should be higher than that of the control diet. Phosphorus deficiency in fish diets normally results in body fat accumulation, as a result of cellular hypoxia and inhibition of oxidative phosphorylation (Sakamoto and Yone 1980; Sugiura *et al.*, 2004). Non-polar lipid accumulation in muscle and liver, mainly oleic and palmitic acid, has been found in some species when fed a P-deficient diets (Takeuchi and Nakazoe, 1981). On the contrary, the lower body lipid content in the present experiment could be related to higher dietary phosphorus content in the crab based diets compared to the fish meal diet. Red porgy is a lean fish species, so more research is also needed to elucidate the effect of these changes on fatty acid body lipid content, fish behaviour and fish quality. Moreover, mineral excretion parameters should be considered to finally define the appropriate level of inclusion of these types of ingredients in diets; other aspects such as feed levels and retention of unwanted substances like fluorine, heavy metals and organic pollutants should be considered.

4.3 Skin coloration

From the different carotenoids tested to return the characteristic skin coloration to cultured red porgy, only dietary astaxanthin has given optimistic results (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; Tejera *et al.*, 2007), with esterified astaxanthin being better utilized than unesterified astaxanthin by red porgy for skin pigmentation purposes (Tejera *et al.*, 2007). In several crustacean species, astaxanthin is quantitatively the most prevalent carotenoid, and a major part is often in an esterified form (Matsuno and Hirao, 1989). Therefore several studies have used crustacean meals as a source of astaxanthin in order to overcome the red porgy skin pigmentation problem, given interesting results (Cejas *et al.*, 2003; Kalinowski *et al.*, 2005; 2007). In the

present trial, a positive effect of carotenoids from both crab meals was clearly observed in red porgy skin also. This visual analysis was corroborated with results from colorimetric variables (*a**, *b**, hue and chroma). The only skin colour variable not affected positively by crab meals was lightness. Variations of this parameter have often been attributed to different environmental factors such as background colour, light and intensity spectrum and density (Van der Salm *et al.*, 2006).

From the two crab meals used, the RC meal produced better skin colour results, as indicated by both visual and colorimetric evaluation, especially at the highest level of inclusion (RC20). These results were expected since river crab meal and river crab meal based diets presented higher total carotenoid content than marine crab meal and its diets. It is important to mention that results obtained in our laboratory showed a total carotenoid concentration of 15 μg g^{-1} and 5 μg g^{-1} for RC meal and MC meal respectively, with a lower concentration of RC meal than reported by other authors due mainly to different batches of raw products and also to processing differences.

Together with visual and colorimetric evaluation, skin total carotenoid concentration was also determined and once again red porgy fed on the river crab meal based diets presented the highest concentrations in this tissue.

Using the TLC chromatographic technique, two fractions were obtained, red and yellow, tentatively identified as astaxanthin and tunaxanthin respectively. Wild red porgy presents approximately 64% and 36% of red and yellow fractions respectively; therefore the RC20 treatment group presented the closest profile to wild individuals (54 and 46% for the red and yellow fractions respectively). In the study carried out by Tejera *et al.* (2007), astaxanthin and its esters (ranging from 65 to 84% of total carotenoid) were the major carotenoid present in the skin of the red porgy and tunaxanthin diesters were the second most abundant carotenoid comprising 16 to 35% of the total carotenoid in the astaxanthin-supplemented treatments. In the present study, the low percentage

of red fraction found in the skin of fish fed on RC10, MC20 and MC10, could be attributed to the lower total carotenoid content present in the respective diets (3 to 3.58 μg g^{-1}) in comparison to the levels tested by Tejera *et al.* (2007) (ranging from 25 to 50 μg g^{-1}).

4.5 Conclusions

In conclusion, the present work clearly shows the good potential of crab meals as a dietary ingredient for red porgy. The partial replacement of fish meal protein by up to 20% from MC meal positively influenced the red porgy growth performance. On the other hand, MC meal at the highest inclusion level tested achieved a more intense red colour than control fish, but RC meal revealed as more efficient pigment source for this species at the levels used in this study. More research is needed to correctly define the appropriate levels of inclusion of these ingredients.

STUDY II

Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): digestibility, ammonia excretion, phosphorous and calcium retention

Abstract

Two trials were conducted to evaluate the nutrient digestibility of river crab (RC) (*Procambarus clarkii*) and marine crab (MC) (*Chaceon affinis*) meals in diets for red porgy (*Pagrus pagrus*) and the effect on ammonia-N excretion and P and Ca retention. In trial I, the apparent digestibility coefficient (ADC) of nutrients and energy contents in RC and MC meals was determined. Results showed that, the ADC values for protein, lipids, ash, dry matter and gross energy were significantly higher for MC than RC meal. Both crab meals were efficiently digested despite their high chitin and ash contents. In trial II, red porgy were fed over 6 months with five diets: a control diet (CD) based on high quality fish meal (FM), and four diets where FM protein was replaced at rate of 10% or 20% by protein derived from either RC or MC meals (RC10, RC20, MC10 and MC20). Replacement of the FM protein by RC or MC proteins had no effect both on protein utilization and ammonia-N excretion rates, which ranged from 105 to 119 mg $N\text{-}NH_4^+$ kg^{-1} day^{-1} for all diets. A linear increase was observed in the dietary Ca/P ratio by the increasing of the dietary RC and MC meal inclusion, showing in a reduction in the whole fish body content of P, Ca and ash with interesting similar whole body Ca/P ratio (1.59-1.63) for all treatments. Accordingly, Ca, P and ash retention rates were significantly reduced by increasing the dietary proportion of both crab meals. Moreover, a negative relationship between fish growth and final whole body Ca, P and ash content was obtained. However, no significant differences were observed in total P excretion value (kg P t^{-1}).

Keywords: alternative ingredient; crab meal; digestibility; ammonia-N excretion; calcium retention; phosphorus retention; red porgy.

5.1 Introduction

The red porgy, *Pagrus pagrus*, is a sparid bottom fish that lives in warm and subtropical waters on both sides of the Atlantic and in the Mediterranean Sea. Its meat quality is highly prized, making it a species of interest to both commercial fisheries and recreational anglers. Unfortunately, owing to prolonged exploitation, some red porgy populations have become overfished (Haimovici, 1998: Vaughan *et al.*, 2002; Afonso *et al.*, 2006). However, the positive results obtained in studies on red porgy development under aquaculture conditions (Divanach *et al.*, 1993; Kentouri *et al.*, 1994, 1995; Hernández-Cruz *et al.*, 1999; Cejas *et al.*, 1999), together with the capacity of red porgy to acquire its characteristic red-pink skin coloration when fed by diets containing carotenoid (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; Pavlidis *et al.*, 2006; Tejera *et al.*, 2007), make this species a potential candidate for aquaculture in Mediterranean and Atlantic coastal regions.

An important limitation to farmed red porgy is their high dietary protein requirement (around 50%). This, coupled with the low global production of fish meal (FM) (Tacon and Metian, 2008), highlights the need to search for alternative protein sources. In this sense, crabs and crab waste products are a source of protein and pigments (Cremades et al., 2003; Toppe *et al.*, 2006), with potential use of these products to feed farmed red porgy. A recent study with this species showed that diets including crab meal are highly attractant and palatable promoting better fish growth. In addition, the required skin colour was achieved, and growth rates and feed performance were even better than fish fed a control FM-based diet (Garcia-Romero *et al.*, Study I).

Certainly, crab meals contain chitin and large amount of ash that can adversely affect the digestibility of nutrients (NCR, 1993; Robaina *et a*l., 1997; Shi-Yen *et al.*, 1999; Krogdahl *et a*l., 2005). However, some studies on crab meal digestibility in fish have shown species-specific differences, with digestibility values of 82% in haddock (*Melanogrammus aeglefinus*) (Tibbetts *et al.*, 2004), 89%

in cod (*Gadus morhua*) and 88% in Atlantic halibut (*Hippoglossus hippoglossus*) (Tibbetts *et al.*, 2006). The ability of a species to consume these types of feed with no adverse effect on nutrient digestibility depends on the adequate secretion of gastric acid and appropriate enzyme activity (Wood *et al.*, 1992; Gutowska *et al.*, 2004). High chitinase production has been found in fish that feed naturally on crustaceans. The high intake of chitin with such natural diets suggests that chitinolytic enzymes play an important role in digestion (Gutowska *et al.*, 2004; Fines and Holt, 2010).

For cultured species, data about the effects on total ammonia-N ($N\text{-}NH_4^+$) excretion patterns would be very helpful to validate feed nutrient utilization. The main factors affecting fish total ammonia-N excretion are those that influence the catabolism and deposition (or retention) of protein by fish (Lied and Braaten 1984; Liu *et al.*, 2009), especially the quantity and quality of protein in the diet.

Another crab meal characteristic is the high amount of Ca content, found as mineral way making up the exoskeleton. In fish, bones take up a large proportion of Ca and P through the formation of hydroxyapatite. Both Ca and P have numerous essential biological functions (Lall and Lewis-McCrea, 2007). The dietary Ca requirements of fish depend on the water chemistry, species differences, dietary P levels and the available sources of P. Dietary Ca can, however, inhibit the absorption of other dietary minerals such as P, Mg and Zn (Nakamura, 1982; Hardy and Shearer, 1985; Gatlin and Philips, 1989).

The present work was undertaken to determine nitrogen utilization from two different crab meal origin included in diets for red porgy (*Pagrus pagrus*), by measuring postprandial ammonia nitrogen ($N\text{-}NH_4^+$) excretion patterns. Since crab meals are normally rich in Ca, a further aim was to evaluate the effects of the dietary Ca they provide on whole fish Ca and P contents as well as their retention rates. In a parallel experiment, the apparent digestibility coefficients (ADCs) for nutrients in the two crab meals were determined.

5.2 Materials and Methods

5.2.1 Crab meals tested in trial I and II

The river crab (*Procambarus clarkii*) meal was provided by a company (Seafood Sevilla S.L.), while the marine crab (*Chaceon affinis*) meal was processed at the laboratory according to Sudaryono *et al.* (1996). See extended procedure in Study I. Table 5.1 shows the chemical composition of the two crab meals.

Table 5.1 Chemical composition (% dry wt) of fish (FM), river crab (RC) and marine crab (MC) meals tested in red porgy

	FM	RC	MC
Crude protein	67.29	42.24	45.85
Crude lipids	11.61	6.05	5.85
Ash	15.34	30.69	28.48
Moisture	13.96	8.87	8.17
Carbohydrate [a]	5.76	21.02	19.82
Calcium	2.95	11.78	12.07
Phosphorus	2.22	1.11	1.15
Chitin	-	11.01	13.11

[a] Carbohydrate = 100 – (% protein + % lipid + % ash)

5.2.2 Trial I. Digestibility

5.2.2.1 Diets

The ADCs of the RC and MC meals were determined using the 70/30 method (Cho and Slinger; 1979; Cho *et al.*, 1982). A FM-based diet previously tested for this species (Shuchardt *et al.*, 2008) was prepared and used as a control diet (CD). Two test diets (RCD and MCD) were produced by mixing 70% of the CD diet and 30% of each one of crab meals. All diets were supplemented with 0.5% of chromium (III) oxide, Cr_2O_3, to act as an external inert digestion marker (Austreng, 1978). Table 5.2 shows the chemical composition of the final diets.

Table 5.2 Proximate composition (% dry wt) of diets containing fish meal (CD), river crab meal (RCD) and marine crab meal (MCD) used in the trial I.

	CD	RCD	MCD
Crude protein	50.77	46.20	48.20
Crude lipids	15.98	14.28	12.80
Ash	11.40	17.29	16.75
Moisture	8.11	9.08	8.37
Carbohydrate	21.85	22.23	22.25
Gross Energy [a] (MJ kg-	22.06	20.37	20.26
Calcium [b]	2.06	6.46	6.84
Phosphorus [c]	1.55	1.41	1.43
Chitin [d]	-	3.3	3.9

[a] Gross energy = (23.6 MJ kg^{-1} x % protein + 39.8 MJ kg^{-1} x % lipid + 17.2 MJ kg^{-1} x % carbohydrate) (Maynard et al., 1981)

[b, c, d] Theoretical values calculated from the chitin content of the RC and MC meals.

5.2.2.2 Fish and experimental conditions

Red porgy (mean weight 70±6 g) were randomly distributed into groups of 15 fish per treatment (in triplicate) and placed in cylindrical, conical-bottomed tanks (125 L), each one fitted with a faeces collection column as described by Robaina *et al.* (1995). Each tank was provided with natural seawater at a constant flow rate (2.0 L/min); the water temperature was 19.0-19.2ºC and the dissolved oxygen concentration of the tank around 7 mg L^{-1}. Fish were hand-fed the assigned diets until apparent satiation twice per day (8:00 and 15:00), 6 days per week.

5.2.2.3 Faecal sample collection and apparent digestibility coefficients

Faecal samples were colleted from the collection column each morning, before feeding, in 50 mL centrifuge tubes, over an eight week period. All samples were centrifuged at 10,000 rpm for 20 min and the supernatants discarded. The solid pellets were pooled by tank and stored at -20ºC for later analysis.

Total diet digestibility (%) was calculated using the formula:

$$ADC\ (\%) = 100\ [1 - (Cr_2O_3\ in\ diet\ /\ Cr_2O_3\ in\ faeces)]$$

The ADCs (%) of the nutrients were expressed as the fractional net absorption of nutrients according to Maynard and Loosli (1979):

$$ADC\ (\%) = 100\ [1 - (Cr_2O_3\ in\ diet / Cr_2O_3\ in\ faeces) \times (Nutrient\ level\ in\ faeces / Nutrient\ level\ in\ diet)]$$

From these data, the ADCs of nutrients in the crab meals were calculated according to Forster (1999) and Hardy *et al.* (2002):

$$ADC_{ing}\ (\%) = [(Nutr_{td} \times AD_{td}) - (0.7 \times Nutr_{bd} \times AD_{bd})\ ] / (0.3 \times Nutr_{td})$$

where ADC_{ing} (%) is the digestibility of a given nutrient in the meal, $Nutr_{td}$ is the nutrient concentration of the test diet, AD_{td} is the apparent digestibility of the nutrients in the test diet, $Nutr_{bd}$ is the nutrient concentration in the C diet, AD_{bd} is the apparent digestibility of the nutrients in the C diet, and $Nutr_{ing}$ is the nutrient concentration of the meal in the test diet.

5.2.3 Trial II. Nitrogen utilization and Ca and P retention

5.2.3.1 Diets

Five isocaloric (12%) and isoproteic (50%) diets based on fish meal (FM), river crab (RC) (*Procambarus clarkii*) and marine crab (MC) *(Chaceon affinis)* meals were formulated (Table 5.3). The control diet (CD) consisted of high quality FM and fish oil. The other four diets were formulated by replacng 10% or 20% of the FM protein in the CD with MC meal protein (diets MC10 and MC20) or RC meal protein (diets RC10 and RC20). All diets were prepared by mixing the ingredients in a horizontal ribbon mixer and pelleting (California Pellet Mill, CL3 model, USA) throughout a 5 mm diameter matrix. Table 5.3 shows the chemical composition of the experimental diets.

Table 5.3 Ingredients (g kg^{-1}) and proximate composition of the experimental diets used to feed red porgy in the trial II.

	CD	RC10	RC20	MC10	MC20
Ingredients					
FM [a]	672	608	543	608	543
RC [b]	-	105	215	-	-
MC [c]	-	-	-	98	213
Fish oil	70	70	70	71	70
Gelatinised starch [d]	213	172	127	178	129
Vitamin premix	20	20	20	20	20
Mineral premix	20	20	20	20	20
CMC	5	5	5	5	5
Proximate composition (% dry wt)					
Crude protein	46.68	46.75	46.64	46.30	47.08
Crude lipids	11.59	10.68	11.61	11.42	11.53
Ash	11.21	14.61	16.74	14.73	17.32
Carbohydrate [e]	30.52	27.96	25.01	27.55	24.07
Moisture	8.33	6.61	7.8	6.20	5.83
Gross Energy (MJ kg^{-1}) [f]	20.73	20.10	19.93	20.21	19.84
Calcium	2.66	3.55	5.00	3.56	5.59
Phosphorous	1.74	1.66	1.58	1.61	1.56
Ca/P	1.52	2.13	3.16	2.28	3.47
Chitin [h]	-	1.16	2.37	1.28	2.79

[a] Fish meal (*Peruvian origin*)
[b] River crab (*Procambarus clarkii*) meal
[c] Marine crab (*Chaceon affinis*) meal
[d] Merigel 100 Amylum Group
[e] Carbohydrate = 100 –(% protein + % lipid + % ash)
[f] Gross energy = (23.6 MJ kg^{-1} x % protein + 39.8 MJ kg^{-1} x % lipid + 17.2 MJ kg^{-1} x % carbohydrate) (Maynard et al., 1981)
[h] Calculated from the analysed content in the RC and MC meals.

5.2.3.2 Fish and experimental conditions

Fifteen groups of 10-12 red porgy with a body weight of 233±36 g (mean ± SD), were distributed among 15 circular circular fibreglass tanks of 500 L. Experimental treatments were performed in triplicate. Fish were fed the diets by hand to apparent satiation twice per day (8:00 and 15:00), six days per week for 165 days. Tanks were provided with a constant flow rate of natural seawater at a temperature range of 18-19.2ºC. The dissolved oxygen content of the tank was 8.7-9.4 mg L^{-1}.

5.2.3.3 Water sampling for ammonia-N determination

Ammonia-N determinations were made over 24 h cycles on days 130, 139 and 148. In each cycle, feeding was performed to apparent satiation at 08:00 am. Inlet and outlet water samples (50 mL) were then taken from each tank every 2 h between 08:30 am and 08:30 pm to analyse the ammonia-N excretion of fish fed the different diets. Samples of water were kept in dark recipients and stored at 4ºC until analysis. Samples from one tank without fish and from one with unfed fish were taken to determine the blank-corrected value.

5.2.3.4 Ca and P content

Whole fish samples (3 fish per tank; 9 fish per treatment) were taken at the initial (day 0) and end of the trial (day 165), and pooled accordingly to determine whole body Ca and P and ash content. The same analyses were performed on wild red porgy of the same size captured in local fisheries.

5.2.4 Chemical analysis

Moisture, crude protein and ash contents were determined as previously described (AOAC, 1995). Lipids were extracted with chloroform-methanol (2:1, v/v), as described by Folch *et al.* (1957). The chitin content was determined by hydrolysing with 5% HCl at 50ºC for 60 min, and then with 4% NaOH at 80ºC for 90 min. After that, the nitrogen content in the undissolved fraction was determined by the Kjeldahl method and the chitin content calculated as *N**14.51. The Ca and P contents were analysed by atomic absorption spectrometry (RD2257/1194) and by a spectrometric UV-Vis method (RD2257/1994) respectively. The amino acid (AA) profile of samples was determined by HPLC method following the directive commission of 98/64/EC 1998.

The Cr_2O_3 content of the diets and faecal samples was estimated by acid digestion (Furukawa and Tsukahara, 1966). Water ammonia-N concentrations were determined by colorimetric indophenol blue method (Koroleff *et al.*, 1983).

5.2.5 Statistical analysis

All data were tested for normality of distribution and homogeneity of variance (Sokal and Rohlf, 1995). To compare treatments, parametric and homoscedastic data were analysed by one-way ANOVA followed by Tukey's test for multiple comparisons. For non-normal data and/or data not showing homogeneity of variance, the Kruskal-Wallis multiple range test was used. When two treatments were established, data were compared statistically by means of T-Student test (Sokal and Rohlf, 1995). All statistical analyses were performed using the SPSS v. 13.1 statistical package. Significance was set at $P<0.05$.

5.3. Results

5.3.1 Trial I.

5.3.1.1 Diets biochemical content

Table 5.1 shows the proximate composition of diets used in the digestibility trial. The ash, calcium and chitin levels of diets ranged from 11 to 17%, 2 to 7% and 0 to 4% respectively, reflecting the contents of their respective ingredients, i.e., with values increasing after addition of RC or MC meal.

5.3.1.2 Total diet digestibility and apparent digestibility coefficients of river and marine crab meals

Table 5.4 shows total digestibility of diets containing fish meal (CD), river crab meal (RCD) and marine crab meal (MCD). The digestibility of the CD diet was 88%, similar to that of the MC diets (86%) and higher than that of the RC diet (74%). Table 5.5 shows the ADCs of crude protein, crude lipids, ash, dry matter and gross energy in the RC and MC meals. The MC meal had significantly higher ADCs values than RC meal for all tested nutrients (crude protein, crude lipids, ash, dry matter and gross energy).

Table 5.4 Total digestibility of diets containing fish meal (CD), river crab meal (RCD) and marine crab meal (MCD) used in the trial I.

	CD	RCD	MCD
Diet ADC (%)	88.09±0.44[a]	74.04±0.37[b]	86.71±0.78[a]

Column, means ± SD, with different letter denote a significant difference.

Table 5.5 Apparent digestibility coefficients (ADCs) of crude protein, crude lipids, ash, dry matter and gross energy in the river crab (RC) and marine crab (MC) meals.

ADCs (%)	RC	MC
Protein	70.69 ± 0.23[b]	95.21 ± 0.88[a]
Lipid	75.74 ± 0.88[b]	99.74 ± 0.78[a]
Ash	13.00 ± 3.67[b]	27.00 ± 3.54[a]
Dry matter	69.04 ± 1.37[b]	82.75 ± 1.37[a]
Gross Energy (MJ	74.14 ± 0.86[b]	89.73 ± 2.71[a]

Column, means ± SD, with different letter denote a significant difference.

5.3.2 Trial II.

5.3.2.1 Biochemical content of the diets

The proximate diet composition (Table 5.3) reflected the compositions of the included ingredients. Thus, higher ash values were observed with increasing proportions of any crab meal. Ca levels increased with the dietary ash content, with comparable values for the diets containing RC or MC meal. A slight reduction in the P content of all diets with crab meal respect to the CD was also observed. Thus, the Ca/P ratio increased in RC and MC diets. Table 5.6 shows the crude amino acids profile for the FM, RC meal and MC meal, together with experimental diets. The FM had more essential (EAA) and non-essential amino acids (NEAA) than the RC or MC meals (with similar levels). However, the amino acid profiles of all the crab meal-based diets closely resembled that of the CD. In addition, all diets showed a similar EAA and NEAA profile with a similar ratio of EAA/NEAA, ranging from 0.91 to 0.95.

Table 5.6 Crude essential (EAA) and non-essential (NEAA) amino acid profile of fish meal (FM), river crab (RC) and marine crab (MC) meals, and the experimental diets (g AA x 100g protein^{-1}) used to fed red porgy in trial II.

	Meals			Diets				
	FM	RC	MC	CD	RC10	RC20	MC10	MC20
EAA								
Arginine	5.88	4.43	4.99	2.66	2.60	2.55	2.63	2.59
Histidine	3.46	1.25	1.24	1.57	1.47	1.38	1.47	1.34
Isoleucine	3.79	3.03	2.53	1.71	1.68	1.66	1.66	1.66
Leucine	7.49	5.45	4.69	3.39	3.30	3.23	3.27	3.20
Lysine	9.18	3.93	3.51	4.15	3.93	3.71	3.91	3.65
Methionine	5.05	1.47	1.64	2.29	2.13	1.98	2.14	2.03
Phenylalanine	4.03	3.41	2.77	1.82	1.80	1.78	1.77	1.76
Threonine	4.47	3.05	2.92	2.02	1.96	1.91	1.96	1.93
Tryptophan	1.17	0.76	0.70	0.53	0.52	0.50	0.51	0.48
Valine	4.58	3.39	3.29	2.07	2.02	1.98	2.02	1.99
Sum of EAA	49.12	30.16	28.29	22.21	21.41	20.68	21.34	20.63
NEAA								
Aspartic acid	9.64	7.67	6.19	4.36	4.29	4.22	4.22	4.10
Glutamic acid	14.61	11.25	9.71	6.61	6.48	6.36	6.41	6.20
Alanine	6.78	5.02	3.95	3.06	2.99	2.93	2.95	2.95
Cystine	2.23	2.49	2.31	1.01	1.02	1.04	1.02	1.11
Glycine	6.29	4.45	4.14	2.84	2.77	2.70	2.76	2.70
Proline	4.00	3.05	3.47	1.81	1.77	1.74	1.79	1.77
Serine	4.29	3.08	3.10	1.94	1.89	1.85	1.9	1.84
Tyrosine	3.05	2.70	2.64	1.38	1.37	1.36	1.37	1.39
Hydroxyproline	0.74	0.19	0.14	0.34	0.31	0.29	0.31	0.29
Sum of NEAA	51.63	39.89	35.64	23.35	22.89	22.49	22.73	22.35
EAA/NEAA	0.95	0.77	0.79	0.95	0.93	0.91	0.93	0.92

5.3.2.2 Postprandial ammonia-N excretion patterns and nitrogen retention

The postprandial ammonia-N excretion rates (mg kg^{1} body weight) showed similar patterns of change in all experimental diet groups (Fig. 5.1). An increasing ammonia-N excretion was detected after feeding, reaching a maximum value at 6 h in all treatments. The inclusion of crab meals at two probed levels had no effect on total ammonia-N excretion (mg kg^{1} body weight) even when the total excretion rate was expressed as units of N intake percentage. Thus, the total ammonia-N excretion rates (mg kg^{-1} d^{-1}) obtained were 105, 113, 117, 119 and 112 for the CD, RC10, RC20, MC10 and MC20 diets respectively, while the total ammonia-N excretion per unit of N intake was 14, 16, 15, 13 and 11%. An increase of N retention percentage linked to higher proportions of RC and MC meal was observed (Table 5.7).

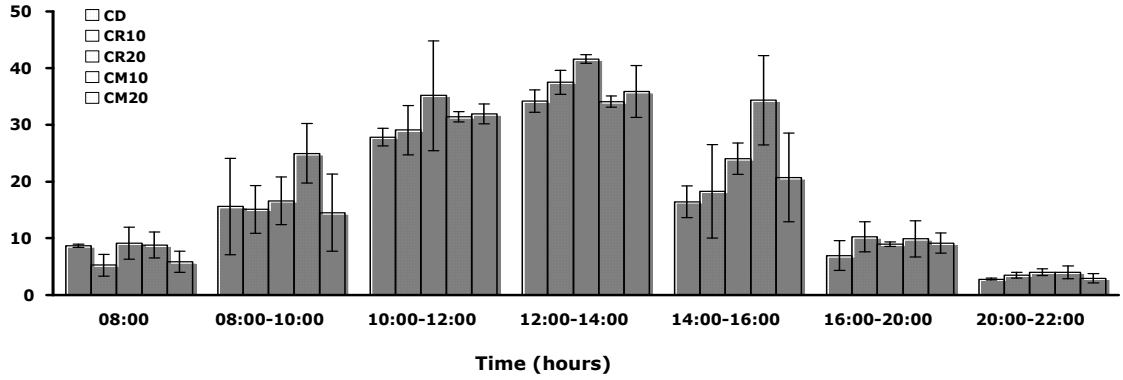

Figure 1.5 Daily ammonia nitrogen excretion pattern in red porgy after feeding the experimental diets (Means ± SD). Means without letters denote no significant difference between diets at any interval.

Table 5.7 Nitrogen intake (N) and ammonia-N excretion ($N\text{-}NH_4^+$) of red porgy fed the different diets in trial II.

	CD	RC10	RC20	MC10	MC20
N [a] (mg kg^{-1} d^{-1})	780 ± 110	721 ± 180	750 ± 131	979 ± 152	1014 ± 159
$N\text{-}NH_4^+$ (mg kg^{-1} d^{-1})	105 ± 12	113 ± 11	117 ± 15	119 ± 14	112 ± 20
$N\text{-}NH_4^+$/N (%)	14%	16%	15%	13%	11%
Nitrogen retention [b]	21.92±0.19	21.57±1.05	23.22±0.17	22.79±0.86	23.09±0.72

[a] Total nitrogen intake in the ammonia-N excretion tests of trial I.
[b] Nitrogen retention rate (%)={(Final nutrient content − initial nutrient content) / nutrient intake}x100.
Values are means ± standard deviations (n=3) for each treatment. Means with no letters denote a lack of a significant difference.

5.3.2.2 Whole body Ca and P content

Compared to fish fed control diet, whole body Ca and P contents were reduced in all fish fed RC and MC diets, fish fed CM20 diet showed the lowest values (Table 5.8). The Ca and P and ash contents of the CD fish were the closest to those of wild red porgy.

Ca retention was high in CD fish but it fell significantly with increasing dietary proportions of crab meals, with the lowest value for the MC20 fish (Table 5.6). All crab meal-based diets also resulted in lower P retention (Table 5.6), with a reduction of around 10% in RC10, RC20 and MC10 compared to CD fish, while the MC20 fish showed a reduction of 24%. The ash content and ash retention values for fish fed any crab meal diets also fell significantly. However, the whole body Ca/P ratio resulted very similar among treatments (1.59-1.63), being those values lower than that showed for wild red porgy (1.85) (Table 5.7).

Table 5.8 Whole body calcium (Ca), phosphorus (P) and ash composition (g kg^{-1}), retention rates (%) and phosphorus excretion (PE) of red porgy fed the experimental diets.

	Wild	CD	RC10	RC20	MC10	MC20
Ash	37	34.63	31.15	27.94	29.68	26.05
Ca	3.99	3.72	3.02	2.76	2.81	2.56
P	2.15	2.27	1.89	1.71	1.75	1.61
Ca/P	1.85	1.63	1.60	1.62	1.60	1.59
Ca R [a]	-	55.19±0.96 [a]	33.54±2.16 [b]	22.10±0.73 [c]	30.52±2.70 [b]	15.54±0.77 [d]
P R [a]	-	48.87±0.87 [a]	43.85±2.70 [b]	44.11±1.47 [b]	43.94±1.63 [b]	36.63±1.78 [c]
Ash R [a]	-	44.97±0.66 [a]	29.57±1.92 [b]	24.89±0.80 [c]	30.23±0.83 [b]	21.43±1.01 [c]
PE [b]	-	12.34±1.88	13.12±2.73	12.92±2.48	13.89±2.61	10.71±3.30

Different letters in the same row denote significant differences among groups.

[a] R: retention (%)={(Final nutrient content − initial nutrient content) / nutrient intake} x100.

[b] PE: P excretion (kg t^{-1})=[{FCR x nutrient in diet (g) – nutrient retained in fish (g)} /production (t)] x1000

5.4 Discussion

5.4.1 Digestibility

The ADC of protein (ADCP) was high in MC meal (95%) and medium-high in RC meal (71%). These results, especially those for the MC meal, are similar to those reported by other authors. For example, Tibbetts *et al.* (2006), who worked

with cod (*Gadus morhua*), reported ADCP values of 96%, 89% and 67% for krill meal (obtined from whole animals), crab by-product meal and shrimp meal. Tibbetts *et al.* (2004) also reported an ADCP value of 82% in haddock (*Melanogrammus aeglefinus*) fed crab meal, while Peach (2005) give a value of 88%, also fed with crab meal, in halibut (*Hippoglossus hipoglossus*).

The lower ADCP value associated with the RC meal might be a consequence of its higher ash content (307 g/kg; 173 g/kg as part of the entire diet) compared to MC meal (284 g/kg; 167 g/kg as part of the entire diet). Ash has a well known negative effect on the assimilation of nutrients (NRC, 1993; Robaina *et al.*, 1997). However, in other species, dietary ash contents higher than those of this study (184 g/kg), also from crab meal, appeared to have no effect on the ADCP (Toppe *et al.*, 2006).

Origin and processing of meals can also affect the bioavailability of nutrients. Although MC meal was produced from whole crabs, and the RC meal from crab by-products, their amino acid profiles were very similar. Thus, differences in processing would appear to be responsible for differences in digestibility. Many authors indicate that the high-temperature heating of protein ingredients during processing could negatively affect the nutritional value of the final meal, leading to differences in ADCP associated with changes in the chemical or conformational composition of the proteins (Opstevedt *et al.*, 1984; Anderson *et al.*, 1993; Oduguwa *et al.*, 1998; Ljokjel *et al.*, 2004; Sorensen *et al.*, 2005). The present MC meal was made from fresh, whole crabs at low temperature, while the RC meal was produced by a high temperature process.

The ADC of lipids (ADCL) was higher for MC meal (99%) than RC meal (75%). Crustaceans generally have high contents of unsaturated fatty acids, triglycerides and phospholipids, the later known to be highly digestible by fish (Colombo-Hixon *et al.*, 2010). Since no differences were seen in the lipid profiles of the diets in trial I (data not shown), the differences in ADCL would once again appear to be related to the processing.

The ADC of ash (ADCA) differed strongly between CD, RC meal and MC meal of diets used in the trial I (38%, 13% and 27% respectively). In a similar study to the present work, Toppe *et al.* (2006) observed a significant reduction in the ADCA of crab meal compared to a control where ash content of the meal was increased, however, the ADCL and ADCP values were not affected.

The high calcium content of RC and MC meals is largely responsible for their high ash content. That feature may prevent the assimilation of minerals (Nakamura, 1982; Hardy and Shearer, 1985; Galtin and Philips, 1989), explaining why both RC and MC meals had a lower ADCA than CD in trial I. However, the calcium content of both these meals was practically the same; thus, this cannot explain the differences in ADCA (13% and 27% for RC and MC meal respectively). Sorensen *et al.* (2005), indicate that the bioavailability of minerals on feed is even more sensitive to thermal processing than other nutrients since they may undergo physico-chemical changes. Such changes might have occurred during the processing of RC meal.

The ADC of energy (ADCE) was high in MC meal (90%), in agreement with the results reported in cobia (*Rachycentron canadum*) (90%) (Fines *et al.*, 2010), and somewhat lower for the RC meal (74%) with respect to values reported for crab meal in haddock (83%) and cod (82%) (Tibbets *et al.*, 2004; 2006),

5.4.2 Postprandial ammonia-N excretion patterns

The partial substitution of FM by crab meal (at rate of 10 or 20%) had no effect on protein utilization as determined by ammonia-N excretion. This was confirmed by the N retention results (Table 5.7) and other indices such as protein efficiency ratio (PER) (García-Romero *et al.*, 2010; Study I). This good protein utilization is partly explained by the adequate amino acid profile of the diet, which was not altered by the inclusion of the crab meals (Table 5.6). These results agree with those reported by García (2002), who indicated that red porgy fed a diet of FM with 20% substituted for shrimp meal led to no significant changes in

ammonia-N excretion. However, in the same work, substitution at the rate of 40% did lead to an increase in ammonia-N excretion perhaps indicating an imbalance in the diet's amino acid profile and/or the interaction of some components when such ingredients are included at higher rates.

Sub-optimal levels of dietary energy from non-protein (lipid and carbohydrate) sources are known to increase the catabolism of proteins leading to the excretion of ammonia-N (Cho and Kaushik, 1990). In the present work, although carbohydrate content of the diet fell from 213 g kg^{-1} in the CD to 128 g kg^{-1} and 127 g kg^{-1} in the RC20 and MC20 diets respectively, no effect on ammonia-N excretion was seen. This might be due to the level of dietary lipids was enough to meet energy demands, or an efficient use of carbohydrates from the crab meals. In agreement with Shuchardt *et al.* (2008), red porgy shows greater capacity to use carbohydrates than lipids. In cobia, strong chitinase activity has been detected in the stomach, allowing them to use ingested chitin as an energy source (Fines and Holt, 2010).

5.4.3 Calcium and phosphorus retention

Ca and P concentrations in whole fish fell with the inclusion of RC and MC in the diet. Most studies report that the differences between P content and its retention in different tissues are associated to dietary P levels and bioavailability of the P source (Buyukates *et al.*, 2000; Sathoh *et al.*, 2003). Dietary Ca, however, can interfere with P assimilation (Nakamura, 1982; Davies *et al.*, 1993; Peñaflorida, 1999; Cheng *et al.*, 2006). Lee *et al.* (2010) reported lower available P concentrations in rainbow trout (*Oncorhynchus mykiss*) fed diets based on seafood processing waste-product meals as they have high calcium concentrations. In the present work, the high Ca concentrations of the crab meals could have inhibited the assimilation of P, leading to a reduction in the whole body P content. In return, this lower quantity of available or assimilatable P may have affected the deposition of Ca, thus reducing the whole body content; several authors have

shown that the amount of available P in the diet limits the deposition of Ca (Magbenka *et al.*, 2005; Lee *et al.*, 2009). The last group of authors also showed that, with respect to the transport of dietary Ca in the blood, supplementation with P is more important than supplementation with Ca since fish have the capacity to absorb calcium from the environment.

It has been reported that minerals such as Zn influence bone mineralisation by acting as cofactors to enzymes involved in the process (Gómez *et al.*, 1999; Ye *et al.*, 2006). When calcium is present in the diet at over 2% it can interfere with the assimilation of Zn and Mg (Hardy and Shearer, 1985; Hossain and Furuichi, 2000; Apines *et al.*, 2003; Ye *et al.*, 2006), a fact reflected in the reduction of whole body ash contents. In this sense, the smaller amounts of ash in fish fed the RC and MC meal diets may not only be due to a reduction in whole body Ca and P, but also to a reduction in other minerals as result to the inhibition of assimilation by dietary Ca.

In the present work, it would be interesting to metion that although with same dietary level of Ca and P for the RC10 and RC20 respect to the MC10 and MC20, the poorer ash digestibility for the RC, half of that for MC, should indicate a lower Ca and P bioavailability for the RC diets compared to the MC ones. This fact was not however related with the observed Ca and P content in the whole fish, which was some higher for the RC with respect to the MC diets. On the other hand, a negative relationship (R^2=-0.44) between the whole body ash content and the SGR (Study I) of the fish for the different treatments was observed. This suggest that, in addition to the anterior described interaction between Ca and P, an import effect of the fish weight increasing rates by feeding the different diets on the final fish ash ca and P content seems to occurr. Contrary, in spite of the similar growth rates showed for CD, RC10 and RC20 feeding fish (Study I), clear differences in body ash, Ca and P exist among them; in this sense the lower contents in RC fish could be due to the lower ash and dry matter digestibility for this meal.

Although Ca/P ratios appreciably differed between diets, the same ratio (about 1.6) was seen in all fish, irrespective of diet. Thus, red porgy appear to be able to adjust the levels of Ca in their body to maintain a constant Ca/P ratio as occur in other species (Ogino *et al.*, 1979; Watanabe *et al.*, 1980; Lee *et al.*, 2009). However, the values showed in the present work were higher than those reported in other species (Nordrum *et al.*, 1997; Lee *et al.*, 2009).

Although the P retention declined with an increasing of crab meal proportion in the diet, no significant differences were seen in the estimate of excreted P (loading phosphorus). Nevertheless, the values obtained are slightly higher than those estimated for rainbow trout (Satoh *et al.*, 2003), as well as those found in red sea bream (*Pagrus major*) (Sarker *et al.*, 2007). Fish meal is the usual source of phosphorus, but it is reported to be poorly assimilatable; therefore, it is excreted (Watanabe *et al.*, 1980). The present results suggest that the amounts and type of available P required by red porgy be further investigated.

5.5 Conclusion

The present results show that the tested crab meals are suitable for inclusion in the diet of farmed red porgy. The partial substitution of FM protein by up to 20% with protein from RC or MC meal did no affect dietary protein utilization. The RC meal was readily digestible but the MC meal even more so, being related this difference to the meals processing. The Ca content of the crab meals appeared to reduce whole body Ca and P contents. However, compared to the control, no significant differences were seen in terms of estimated total P excretion (kg P t^{-1}) by feeding with crab meals.

STUDY III

Marine and freshwater crab meals in diets for red porgy (*Pagrus pagrus*): effect in fillet fatty acid profile and flesh quality parameters.

Abstract

The present study was conducted to evaluate the effect of using river crab (*Procamburus clarkii*) meal (RC) and marine crab (*Chaceon affinis*) meal (MC) on the red porgy (*Pagrus pagrus*) fillet fatty acid profile and quality. Red porgy were fed during 165 days with five diets: a control diet based on high-quality fish meal (CD); four diets replacing 10% and 20% fish meal protein by protein from both RC or MC meals (RC10, RC20 & MC10, MC20). Fillet fat content resulted significantly higher in MC20 fish than the rest of diets. Feeding with both RC and MC meals slightly increased muscle contents of polyunsaturated (PUFA), 20:4n-6 (ARA), 20:5n-3 (EPA), 22:6n-3 (DHA) and n-3/n-6 ratio as well as decrease of n-9 fatty acids, although difference are only significant for the ARA content. After inclusion of both crab meals, values of atherogenicity index (AI) resulted similar among treatments while thrombogenecity index (TI) were reduced, but not significantly, reflecting the higher n-3 fatty acid content in fillets from crab diets. Sensory analysis showed that red porgy fillets of all treatments were very well appreciated with some differences detected in only MC20 fillets, which had significantly higher scores in the attributes of oily odour and flavour, cohesiveness aspect and firmness texture compared to the rest of diets. RC meal dietary inclusion did not promote any changes in sensory evaluation panel. Instrumental muscle texture did not revealed differences between treatments. Results from TBARS of raw fillets showed that the inclusion of both crab meals clearly delay lipid oxidation compared to a FM based diet, during refrigerated storage at 4ºC. Thus, for 4 and 7 days of storage, fillets of RC or MC diets, attained significantly lower TBARS values than those fed on CD.

Keywords: alternative ingredients; crab meal; flesh quality; lipid oxidation; red porgy.

6.1 Introduction

Red porgy *Pagrus pagrus* is a highly appreciated fish in the commercial fisheries of the Mediterranean region and the Atlantic coast, from Spain to Senegal, characterised by delicate flavour and firm flesh with low fat content. However, wild catches of this species are very limited and insufficient to meet the market demand. These facts, together with good response under culture condition (Divanach *et al.*, 1993; Kentouri *et al.*, 1994, 1995; Hernández-Cruz *et al.*, 1999; Cejas *et al.*, 1999; Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; Pavlidis *et al.*, 2006; Tejera *et al.*, 2007), make red porgy one of the priority marine species for aquaculture diversification in the Mediterranean region. An important limitation in farmed red porgy is the high dietary protein requirement, around 50% (Schuchardt *et al.*, 2008). Due to the situation on global fish meal production (FAO 2010), success in introducing this new specie in aquaculture, among other things, depend on to find potential alternative and suitable protein sources to reduce the dependence from the fish meal and improve the feeding cost.

By-products from the crab industry have an attractive potential as protein source (Shahidi and Synowiecki, 1991; Cremades *et al.*, 2003). Factories that process crabs for human consumption produce large quantities of waste products that are used as supplementary animal feed ingredients, constituting a good source of protein, astaxanthin and chitin (Lee, 1990; Negro and Garrido-Fernandez, 2000; Cremades *et al.*, 2003; Toppe *et al.*, 2006; Pérez-Gálvez *et al.*, 2008). Accordingly, crab by-product meals from *Pleuroncodes planipes* and *Cancer pagurus* have been identified as good candidates to replace fish meal in diet for white shrimp (*Litopenaeus vannamei*) and Atlantic cod (*Gadus morhua*), respectively (Villarreal *et al.*, 2004; Goytortúa-Bores *et al.*, 2006; Toppe *et al.*, 2006;).

However, special attention on the inclusion of a new ingredient should be given since dietary modification could affect to the quality of the fish and thus altering its value for human consumption or effective market appreciation. Fish

quality involves different concepts depending on fish farmer, the processing industry or consumer understanding. Thus, organoleptic properties, nutritional value and self life, are sets of characteristic that shape as it is perceived fish quality by the consumer. All mentioned characteristic strongly depend on chemical composition of the fish, in turn depending on many quality aspects, among them, diet composition are important (Lie *et al.*, 2001; Grigorakis, 2007). It seems that all acquired quality attributes of fish fillet are well correlated with fat content and lipid class in tissue (Waagbo *et al.*, 1993; Guillou *et al.*, 1995; Morris *et al.*, 1995). Although a clear relationship normally exist between dietary and fish muscle fatty acids profile, lipid metabolism and accumulation in fish related to dietary protein source has been showed (Lupatsch *et al.*, 2002; Francesco *et al.*, 2007) and may significantly affect fish flesh quality such as sensory characteristic (Bjerkeng *et al.*, 1997; Williams *et al.*, 2003 b; Karlsen et *al.*, 2006; Suontama *et al.*, 2007), and texture of the farmed fish (Hernández *et al.*, 2007).

A major concern to extend the self life of fillet fish exist since fish fillet is rich in polyunsaturated fatty acids and hence is high sensitive to lipid oxidation compared with other animal fillets. In this sense, the oxidation susceptibility of fish muscle in response to the dietary protein source has been previously studied (Alvarez *et al.*, 1999; López-Bote *et al.*, 2001).

Thereby, changes in the origin of dietary protein could result in changes of the nutritive fish fillet quality. Thus, if crab meals have been used in red porgy diets, with success results as alternative ingredient from growth to digestibility, it is also essential investigate if they may affect the final quality of the product. According to this, the aim of the present study is to evaluate the impact of crab meal dietary inclusion on the sensory properties, texture and fatty acid composition as well as self life, in term of lipid oxidation, in red porgy muscle.

6.2 Material and Methods

6.2.1 Experimental diets

Five isocaloric (12%) and isoproteic (50%) diets were formulated (Table 6.1) and tested in triplicate groups of fish. A control diet based on high quality fish meal and fish oil (CD) and other four diets where 10% and 20% of the fish meal protein in CD was replaced by marine crab (MC) (*Chaceon affinis*) meal protein (diets MC10 and MC20) or river crab (RC) (*Procambarus clarkii*) meal protein (diets RC10 and RC20). Diets were prepared by mixing ingredients in a horizontal ribbon mixer and pelleting (California Pellet Mill, CL3 model, USA) throughout a 5 mm diameter matrix. The proximate composition and total carotenoid content of the used crab meals and experimental diets are shown in Table 6.1.

Table 6.1 Ingredients (g kg^{-1}) and proximate analysis of the experimental diets

Ingredients	CD	RC10	RC20	MC10	MC20
Fish meal [a]	672	608	543	608	543
River Crab meal [b]	-	105	215	-	-
Marine Crab meal [c]	-	-	-	98	213
Fish oil	70	70	70	71	70
Gelatinized starch [d]	213	172	127	178	128
Vitamin premix	20	20	20	20	20
Mineral premix	20	20	20	20	20
CMC	5	5	5	5	5
Proximate composition (% dry wt)					
Crude protein	46.68	46.75	46.64	46.30	47.08
Crude lipids	11.59	10.68	11.61	11.42	11.53
Ash	11.21	14.61	16.74	14.73	17.32
Carbohydrate [e]	30.52	27.96	25.01	27.55	24.07
Total carotenoids* (mg	2.86	3.36	5.56	3.02	3.58

[a] Fish meal (*Peruvian origin*); proximate composition (% dry wt.): protein (67.29); lipid (11.65); ash (15.34).

[b] River crab, *Procambarus clarkii*, meal; proximate composition (% dry wt.): protein (42.29); lipid (6.05); ash (30.7) and carotenoids* (15.74).

[c] Marine crab, *Chaceon affinis*, meal; proximate composition (% dry wt.): protein (45.85); lipid (5.85); ash (28.48) and carotenoids* (5.90).

[b, c] The river crab meal was provided by a company (Seafood Sevilla S.L.), while the marine crab meal was processed at the laboratory (Sudaryono *et al.*, 1996). See extended procedure in Study I.

[d] Merigel 100, Amylum Group

[e] Carbohydrate = 100 –(% protein + % lipid + % ash)

* Total carotenoids concentration expressed as astaxanthin equivalent calculated at λ=470nm and using an extinction coefficient value of 2100 ($E_{1\%,1\,cm}$).

6.2.2 Fish and experimental conditions

Red porgy of initial weight 233 ± 36 g (mean ± SD) were randomly distributed in 15 circular fibreglass tanks of 500 l each, at an initial density of 5.59 kg m^{-3}. Tanks were provided with natural seawater with 17-19 ºC temperature and 7-10 mg L^{-1} dissolved oxygen range. Triplicate groups of fish were hand fed until apparent satiation with the experimental diets, 2 times per day (9:00 am and 15:00 pm), and 6 days per week, until commercial fish size was reached after 165 days.

6.2.3 Proximate biochemical content and fatty acid profile analysis.

Proximate analysis and fatty acid profile was performed on crab meals (three sub-samples per meal), diets and fish muscle samples (nine per diet). Moisture, crude protein and ash content were determined according to AOAC (1995). Lipids from experimental samples were extracted with chloroform-methanol (2:1, v/v) mixture, as described by Folch *et al.* (1957). The fatty acid methyl esters were obtained by transesterification with 1% sulphuric acid methanol (Christie, 1982), purified by absorption chromatography on NH_2 Sep-pak cartriges (waters, S.A., Milford, MA, USA) and separated and quantified by gas-liquid chromatography as described by Izquierdo *et al.* (1992) Fatty acids were then identified by comparison with external standards. Data on crab meals, diets and muscle samples fatty acid composition were expressed as percent of the total identified fatty acids (Table 6.2 and Table 6.4) and as percent of wet weight of fillet (Table 6.5). From fatty acid results, the indexes of atherogenicity and thrombogenecity were calculated with the following equations, as proposed by Ulbricht and Southgate (1991):

Index of atherogenicity (AI)

$$AI = [(12{:}00) + (4 \times 14{:}00) + (16{:}00)] \times [(PUFA\ n\text{-}6\ and\ n\text{-}3) + MUFA]^{-1}$$

Index of thrombogenicity (TI)

$$TI = [(14{:}00) + (16{:}00) + (18{:}00)] \times [(0.5 \times MUFA) + (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3/n\text{-}6)]^{-1}$$

Table 6.2 Fatty acid profile (g/100 g fatty acids) of the meals and diets.

Fatty acids	FM	RC	MC	CD	RC10	RC20	MC10	MC20
14:00	0.05	0.64	2.24	7.25	7.83	7.51	7.86	7.66
15:00	0.23	0.53	0.49	0.67	0.76	0.81	0.70	0.74
16:00	4.24	9.33	13.18	21.55	21.67	21.83	22.05	21.71
17:00	1.68	1.35	0.94	1.59	1.59	1.54	1.67	1.62
18:00	10.25	3.13	5.41	4.38	4.16	4.41	4.34	4.36
20:00	0.42	n.d.	0.50	0.24	0.24	0.27	0.24	0.25
∑ SFA	16.87	14.98	22.76	35.68	36.25	36.37	36.86	36.34
14:1n-7	10.21	0.35	0.29	0.02	0.08	0.08	0.06	0.07
14:1n-5	0.06	0.62	0.02	0.28	0.28	0.33	0.23	0.23
15:1n-5	0.90	n.d.	0.02	0.09	0.12	0.14	0.11	0.10
16:1n-5	0.62	1.08	0.56	0.13	0.25	0.27	0.24	0.22
16:1n-7	8.68	7.27	4.84	9.03	9.41	9.52	9.39	9.03
18:1n-5	0.12	0.75	0.43	0.11	0.10	0.12	0.12	0.10
18:1n-7	4.37	6.38	4.27	3.26	3.28	3.42	3.39	3.21
18:1n-9	10.88	22.17	18.31	9.79	9.62	10.54	9.87	9.38
20:1n-5	n.d.	n.d.	0.93	0.22	0.23	0.23	0.27	0.24
20:1n-7	0.43	1.77	1.68	0.24	0.29	0.31	0.32	0.31
20:1n-9	0.95	4.98	1.68	0.81	0.98	1.03	1.08	1.04
22:1n-9	0.44	0.75	0.52	0.10	0.18	0.19	0.18	0.20
22:1n-11	0.57	0.77	0.56	0.73	0.71	0.73	0.77	0.82
∑MUFA	38.23	46.89	34.11	24.81	25.53	26.91	26.03	24.95
16:2n-6	1.55	0.30	0.90	0.39	0.46	0.48	0.47	0.44
16:2n-4	1.53	0.68	0.65	0.32	0.37	0.18	0.36	0.34
16:3n-4	0.06	0.09	n.d	0.64	0.66	0.71	n.d.	0.64
16:3n-3	0.41	0.88	0.61	1.63	1.68	1.66	1.68	1.65
16:3n-1	1.70	0.11	0.85	0.25	0.21	0.24	0.24	0.21
16:4n-3	n.d.	1.27	0.36	1.28	1.37	1.24	1.26	1.35
16:4n-1	n.d.	n.d.	0.38	n.d.	n.d.	n.d.	0.04	n.d.
18:2n-9	0.12	0.19	0.16	0.07	0.08	0.08	0.09	0.09
18:2n-6	1.17	1.03	1.30	3.84	3.93	4.78	4.07	3.95
18:2n-4	0.54	0.32	0.18	0.38	0.41	0.41	0.41	0.40
18:3n-6	0.42	0.27	0.22	0.31	0.33	0.35	0.33	0.32
18:3n-4	0.22	0.17	0.52	0.25	0.33	0.34	0.35	0.32
18:3n-3	0.48	0.91	0.34	0.99	1.06	1.20	1.02	1.00
18:4n-3	0.21	0.13	0.34	1.69	1.70	1.61	1.60	1.66
18:4n-1	n.d.	n.d.	0.05	0.22	0.21	0.20	0.21	0.21
20:2n-9	0.07	1.04	0.15	0.09	0.09	0.09	0.10	0.11
20:2n-6	0.20	n.d.	1.03	0.19	0.17	0.21	0.18	0.15
20:3n-6	0.10	0.15	0.08	0.10	0.12	0.20	0.12	0.12
20:3n-3	n.d.	n.d.	0.17	n.d.	0.07	0.08	0.08	0.07
20:4n-6	1.90	9.19	8.16	1.10	1.54	1.63	1.48	1.83
20:4n-3	0.54	6.75	0.25	0.78	0.77	0.75	0.78	0.79
20:5n-3	14.02	0.78	9.62	11.45	10.76	9.83	10.70	11.11
22:4n-6	0.44	1.08	0.12	0.49	0.49	0.44	0.45	0.48
22:5n-6	n.d.	1.00	0.95	0.32	0.30	0.28	0.32	0.32
22:5n-3	2.19	1.82	2.01	1.87	1.73	1.59	1.70	1.78
22:6n-3	15.18	9.05	13.47	10.31	9.64	8.48	9.05	9.86
∑PUFA	43.05	37.21	42.87	38.46	38.48	37.06	37.09	39.20
∑n-3	33.04	21.59	27.18	30.01	28.78	26.43	27.86	29.26
∑n-6	5.78	13.02	12.51	7.12	6.93	7.91	7.31	6.97
∑n-9	12.47	29.14	20.81	10.86	10.55	10.96	10.63	11.94
∑n-3 HUFA	31.94	18.40	25.52	24.41	22.97	20.71	22.30	23.60
n-3/n-6	5.72	1.66	2.17	4.22	4.15	3.34	3.81	4.20
ARA/EPA	0.14	0.96	0.85	0.09	0.14	0.17	0.13	0.16
EPA/DHA	0.92	1.06	0.71	1.11	1.12	1.16	1.18	1.13

6.2.4 Sensory attributes

Nine fish from each treatment were chosen at random, gutted, filleted and kept at 4ºC for 24h until sensory tests were carried out. Fish had been previously starved for 24h before being slaughtered in a small tank with ice and seawater. Each left fillet was divided in three portions of 3x3 cm and cooked in aluminium boxes in a steam oven at 150ºC for 10 min. A preliminary training session was aimed before to select the profile attributes, to establish the sample evaluation procedures and to define the score sheet. Immediately after cooking, fillets were offered to a panel of 12 selected trained judges (ISO, 1985, 1993). Sensory tests were conducted in isolated and air conditioned rooms with standardized light (ISO, 1988).

Judges were randomly offered closed food boxes labelled with codes containing the fillets. Attributes of odour (marine, oily and atypical), appearance/aspect (whiteness, shininess and cohesiveness), texture (juiciness, firmness and adhesiveness), flavour (marine, oily and atypical) and residual taste (persistence, marine and earthy) were tested for samples of fish fed the experimental diets and classified by the judge in a continuous scale from 0 to 100 for each parameter.

6.2.5 Texture profile analysis

Nine fish from each treatment were used for texture analysis and after slaughtering they were kept with ice in polyethylene boxes packing at 4ºC for 24h. The texture analysis was conducted in a Stable Micro System texture analyser (TA.XT2, Surrey, England) equipped with a 5 kg load cell. After removing the skin, shear test were performed in triplicate using three 2x2x1.2 cm^3 blocks of muscle from each fillet above the lateral line. All tests were carried out at refrigeration temperature, keeping the blocks cooled with ice. Fish fillets were compressed up to breaking, following the methodology described by Ginés *et al.* (2004). Compression test was defined as the force exerted to deform a block fillet

using a flat-ended (Type P/100) aluminium compression plate with a test speed 0.8 mm/s and a strain to 80%. During the test, seven mechanical texture parameters were calculated: hardness (N), fracturability (N), adhesiveness (J), springiness (m), cohesiveness, chewiness (J) and gumminess (N).

6.2.6 Determination of lipid oxidation in fish fillet

To determine the oxidative stability of fish fillet during refrigerated storage, nine fish from each treatment were collected, filleted and kept at 4ºC until analysis. Oxidative changes in samples were monitored at 0, 4 and 7 days of storage. The index of lipid oxidation was determined as thiobarbituric acid reactive substances (TBARS) (Shaidi and Hong, 1991). Fish sample (3 g) was mixed with 6 ml of 10% trichloroacetic acid (w/v) and homogenized for 60 s and centrifuged at 4,000 rpm at 4ºC for 30 minutes. After filtration, 2 ml of the filtrate were added to 2 ml of 0.02 M TBA solution and heated at 100ºC for 1 hour. Then the absorbance was measured at 532 nm by UV/Vis spectrophotometer (Genesys UV10, Thermo Fisher Scientific Inc. Waltham, USA). TBARS value was expressed as mg of malonaldehyde (MDA) per kg of fillet.

6.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was conducted for the all data. The homogeneity of variance was tested using Levene's test and the normal distribution of data was checked (Sokal and Rohlf, 1995). When the differences were significant at the $P < 0.05$ level, Tukey's range test was utilized to compare the mean values among the treatments due to the main effects. For data not displaying normality and/or homogeneity of variance, a non-parametric analysis and multiple range test (Kruskal-Wallis) were used. In case of the thiobarbituric acid-reactive substances data, a general linear model with two fixed factors, time and dietary level inclusion, was used. All statistical analyses were tested at 0.05 significance level using the *SPSS* (13.1) statistical package.

6.3 Results

6.3.1 Fatty acid composition of the lipid fraction in crabs meals and experimental diets

Some differences were observed on fatty acid profile (g/100 g fatty acids) in two different crab meals (Table 6.2). Thus, higher proportion of saturated fatty acids (SFA) (22.76%) and lower of monounsaturated fatty acids (MUFA) (34.11%) were obtained for the CM respect to the RC (14.98% and 46.89%, for SFA and MUFA respectively). Similarly, MC contained higher concentrations of total PUFA (polyunsaturated fatty acids), n-3, n-3 HUFA (highly unsaturated fatty acids) and DHA (docosahexaenoic acid; 22:6n-3) (42.87%, 27.18%, 25.52% and 13.47%, respectively), compared to those for RC meal (37.21%, 21.59%, 18.40% and 9.05%, respectively). On the other hand, RC meal showed the higher concentrations of total n-9 fatty acid (29.14%) and linolenic acid (0.91%) s, respect to those for MC meal (20.81% and 0.34%, respectively). ARA (arachidonic acid; 20:4n-6) fatty acid content was similar for both crab meals but 4.5 fold higher than the observed in the fish meal. According to total PUFA content, MC meal profile resulted closer to that of FM.

Meanwhile, fatty acid analyses of experimental diets showed similar profile without significant differences among diets (Table 6.2). The main dietary fatty acids were 16:00, 18:1n-9, EPA (eicosapentaenoic acid; 20:5n-3) and DHA. Compared to the control diet, a general increase in the ARA fatty acid content with increase the dietary inclusion of both crab meals were detected, being 48% and 66% higher for RC20 and MC20, respectively.

6.3.2 Proximate fillet composition

The proximate composition of fish muscle at the end of the trial is shown in Table 6.3. Results were very similar for protein, ash and moisture content among fish fed with diets containing both crab meals at the two tested levels, and the fish fed with the control diet. Significantly higher lipid content (4.73%) was obtained

for the MC20 feeding fish compared with the other treatments (between 3.67% and 3.80%).

Table 6.3 Muscle proximate composition (%wet wt) of red porgy fed the experimental diets.

	CD	RC10	RC20	MC10	MC20
Protein	23.00±0.13	22.88±0.18	22.91±0.11	23.05±0.13	22.91±0.22
Lipid	3.67±0.39 [b]	3.73±0.55 [b]	3.77±0.24 [b]	3.80±0.65 [b]	4.73±0.30 [a]
Ash	1.52±0.03	1.54±0.05	1.59±0.06	1.59±0.05	1.58±0.06
Moisture	73.58±1.16	73.89±1.16	73.33±0.23	73.44±1.02	72.26±1.92

Different letters in same row denote significant differences among diets (P<0.05).

6.3.3 Fillet fatty acid composition

The fatty acid composition (g/100 g fatty acids) of fish fillet at the end of the trial is shown in Table 6.4. Very similar SFA and MUFA content were obtained in all treatments. Replacing FM with both RC and MC meals slightly increased PUFA, n-3, n-3 HUFA, ARA, EPA and DHA as well as decrease n-9, especially oleic acid (18:1n-9). Only ARA content was found significantly different with the lowest value for CD feeding fish (0.86%). Consequently, dietary inclusion of crab meals promote higher fillet overall n-3/n-6 ratio although the differences were not significant yet (P<0.05). AI index resulted similar between different treatments while TI index showed a trend to reduce with inclusion crab meals (Table 6.4).

Fatty acid content was also calculed as percentage of wet tissue (Table 6.5). The results were determined by the different fillet fat content, in fact, the increment of total lipid in fish fed MC20 diet promote differences in the correspondent fatty acid content. Thus, significantly higher content of MUFA, PUFA, n-3, n-3-HUFA, ARA and DHA, (25%, 40%, 47%, 50%, 100% and 40% higher, respectively) respect to fish fed CD diet, were obtained when fish was feeding with CM20 diet.

Table 6.4 Muscle fatty acid profile (g/100 g fatty acids) of red porgy fed the experimental diets

Fatty acids	CD	RC10	RC20	CM10	CM20
14:00	4.63	4.84	4.80	4.86	4.78
15:00	0.88	0.76	0.90	0.97	0.78
16:00	22.40	21.93	22.14	22.57	21.59
17:00	0.87	0.96	0.98	0.94	0.98
18:00	6.97	6.55	6.90	6.95	6.40
20:00	0.23	0.23	0.25	0.22	0.22
∑SFA	35.98	35.27	35.97	36.51	34.74
14:1n-7	0.07	0.08	0.08	0.08	0.08
14:1n-5	0.15	0.17	0.19	0.17	0.15
15:1n-5	0.08	0.08	0.10	0.09	0.09
16:1n-5	0.21	0.23	0.25	0.23	0.24
16:1n-7	7.52	7.77	7.76	7.72	7.75
18:1n-5	0.11	0.11	0.11	0.11	0.11
18:1n-7	3.31	3.33	3.37	3.36	3.34
18:1n-9	18.32	16.91	16.39	16.66	16.74
20:1n-5	n.d	0.19	0.20	0.19	0.19
20:1n-7	0.75	0.65	0.49	0.80	0.71
20:1n-9	0.70	0.60	1.00	0.75	0.70
22:1n-9	n.d	0.24	0.24	0.23	0.23
22:1n-11	0.67	0.66	0.62	0.62	0.60
∑ MUFA	31.89	31.02	30.80	31.01	30.93
16:2n-6	0.24	0.22	0.12	0.26	0.27
16:2n-4	0.86	0.92	0.92	0.93	0.91
16:3n-4	n.d	0.03	0.04	0.01	0.04
16:3n-3	0.13	0.13	0.12	0.14	0.14
16:3n-1	0.20	0.19	0.23	0.22	0.21
16:4n-3	0.61	0.66	0.66	0.63	0.65
16:4n-1	0.06	0.06	0.08	0.07	0.06
18:2n-9	0.24	0.21	0.15	0.21	0.25
18:2n-6	6.97	6.24	5.70	5.48	6.07
18:2n-4	0.29	0.32	0.31	0.31	0.31
18:3n-6	0.27	0.28	0.28	0.27	0.29
18:3n-4	0.27	0.29	0.30	0.31	0.31
18:3n-3	0.93	0.87	0.84	0.79	0.87
18:4n-3	0.72	0.79	0.77	0.74	0.81
18:4n-1	0.15	0.16	0.15	0.15	0.18
20:2n-9	0.21	0.19	0.17	0.20	0.23
20:2n-6	0.20	0.20	0.21	0.20	0.21
20:3n-6	0.19	0.19	0.20	0.20	0.21
20:3n-3	0.06	0.07	0.07	0.06	0.07
20:4n-6	0.86[b]	1.11[b]	1.32[a]	1.27[a]	1.32[a]
20:4n-3	0.59	0.64	0.62	0.63	0.68
20:5n-3	5.51	6.16	6.16	5.96	6.20
22:4n-6	0.28	0.31	0.30	0.29	0.32
22:5n-6	0.30	0.35	0.37	0.36	0.36
22:5n-3	1.98	1.91	1.92	1.86	2.07
22:6n-3	10.14	11.04	11.12	10.79	11.27
∑PUFA	31.76	33.54	33.13	32.34	34.31
∑n-3	20.68	23.56	22.29	21.35	22.76
∑n-6	8.45	8.72	8.20	7.84	8.76
∑n-9	19.03	16.59	16.95	17.31	17.44
∑n-3 HUFA	18.28	21.11	19.90	19.08	20.29
n-3/n-6	2.44	2.71	2.72	2.72	2.62
ARA/EPA	0.15	0.17	0.21	0.21	0.21
EPA/DHA	0.54	0.52	0.56	0.56	0.56
AI[a]	0.67	0.65	0.67	0.68	0.65
TI[a]	0.40	0.35	0.37	0.38	0.36

[a]AI, atherogenic index; TI, thrombogenecity index.

[*] Letter by rows denotes significant difference between treatments ($P<0.05$, *ANOVA*)

Table 6.5 Muscle fatty acid profile (g / 100 g wet fillet) of red porgy fed the experimental diets.

Fatty acids	CD	RC10	RC20	MC10	MC20
∑ SFA	1.31	1.39	1.24	1.15	1.65
∑ MUFA	1.17 [b]	1.12 [b]	1.06 [b]	1.07 [b]	1.47 [a]
∑ PUFA	1.15 [b]	1.32 [b]	1.14 [b]	1.02 [b]	1.61 [a]
∑ n-3	0.73 [b]	0.87 [b]	0.77 [b]	0.70 [b]	1.07 [a]
∑ n-6	0.34	0.35	0.28	0.26	0.41
∑ n-9	0.70	0.65	0.58	0.56	0.82
∑ n-3 HUFA	0.64 [b]	0.78 [b]	0.69 [b]	0.60 [b]	0.95 [a]
ARA	0.03 [b]	0.04 [b]	0.04 [b]	0.04 [b]	0.06 [a]
DHA	0.38 [b]	0.43 [b]	0.38 [b]	0.35 [b]	0.53 [b]
EPA	0.20	0.24	0.21	0.20	0.29
Linolenic	0.03	0.03	0.03	0.02	0.04
Linoleic	0.25	0.24	0.20	0.18	0.28
Oleic	0.67	0.65	0.56	0.54	0.79

Letter by rows denotes significant difference between treatments.

6.3.4 Sensory analysis of fillets

The results of sensory evaluation of cooked red porgy fillets are presented in Table 6.6. Fillets were very well appreciated by the judges, independently of the experimental diets. All differences found in values provided by the sensory analyses were for fish fed on MC meal supplemented diets, specially at the highest level (MC20), whereas the dietary inclusion of RC meal, both RC10 and RC20 level, did no showed appreciable changes respect to CD group. Thus, fish fed on MC meal seemed to produce an oilier perception with higher oily odour and flavour scores, being significantly higher in MC20 fillets than fillets of fish fed CD or the rest of treatments. Also, fillets from fish fed on MC meal based diets appeared to have more cohesiveness aspect, with only significant difference for MC20 diet. Regarding to texture attributes, flesh firmness by feeding on MC meal was significantly higher than those of fish fed CD, RC10 and RC20 diet, and once again different for the MC20 diet. No significant differences were observed in the rest of the assayed attributes.

Table 6.6 Sensory analysis of cooked fillets from red porgy fed the experimental diets.

Attributes		Experimental Diets					
		CD	RC10	RC20	MC10	MC20	*ANOVA*
Odour	Marine	46	46	41	47	46	$p = 0.97$
	Oily	5 ^{b}	5 ^{b}	4 ^{b}	6 ^{ab}	8 ^{a}	$p = 0.03$
	Atypical	3	3	3	3	3	$p = 0.87$
Aspect	Whiteness	53	55	54	53	49	$p = 0.86$
	Shine	45	50	38	44	42	$p = 0.54$
	Cohesivenes	40 ^{b}	41 ^{b}	39 ^{b}	44 ^{b}	54 ^{a}	$p = 0.03$
Texture	Juiciness	39	43	41	42	42	$p = 0.76$
	Firmness	30 ^{b}	39 ^{b}	34 ^{b}	45 ^{ab}	51 ^{a}	$p = 0.00$
	Adhesivenes	39	35	32	39	35	$p = 0.76$
Flavour	Marine	44	45	40	45	40	$p = 0.74$
	Oily	4 ^{b}	4 ^{b}	4 ^{b}	5 ^{ab}	8 ^{a}	$p = 0.04$
	Atypical	3	2	4	4	4	$p = 0.53$
Residual flavour	Persistance	36	41	42	38	35	$p = 0.85$
	Marine	33	43	35	41	38	$p = 0.19$
	Earthy	3	3	3	3	3	$p = 0.98$
Acceptance		73	74	73	75	73	$p = 0.78$

Means whit different letter denote significant differences ($P<0.05$)

6.3.5 Texture parameters

Data on texture parameters assayed on raw fillets of red porgy fed the different diets are showed in Table 6.7. Dietary inclusion of both crab meals at the two probed levels, 10% and 20%, had no significant effect on any of the seven texture parameters analysed

Table 6.7 Texture parameters of raw fillets from red porgy fed the experimental diets

	CD	RC10	RC20	MC10	MC20
Fracture (N)	1.87±0.61	1.90±0.48	1.60±0.38	1.82±0.44	1.79±0.54
Hardness (N)	2.80±0.76	3.07±0.67	2.95±0.75	3.09±0.64	3.00±0.56
Springiness	0.43±0.08	0.42±0.07	0.44±0.08	0.42±0.05	0.47±0.09
Cohesiveness	3.74±0.92	4.29±0.42	4.04±0.40	4.33±0.56	4.07±0.91
Gumminess	11.43±2.98	14.60±2.69	12.54±2.78	12.98±2.65	12.42±2.58
Chewiness (N)	5.01±0.94	6.43±0.93	4.29±0.88	5.72±0.90	5.48±0.82
Adhesiveness	-0.03±0.01	-0.03±0.01	-0.03±0.09	-0.03±0.01	-0.03±0.01

6.3.6 Lipid oxidation of red porgy fillets

The effect of dietary treatment on muscle lipid oxidation, measured as changes in TBARS values at 0, 4 and 7 days of storage after slaughtering is illustrated in Figure 6.1. A significant dietary effect on lipid oxidation was observed ($P<0.00$) in addition to a significant interaction effect of time and dietary treatments ($P<0.00$). No differences in TBARS values between diets were observed at the moment of slaughter (0 days). However, at days 4 and 7 of storage, fish giving diets containing both RC meal and MC meal, at the two inclusion levels, attained significantly lower TBARS values in comparison with red porgy fed with fish meal based diet (CD). A trend to reduce TBARS values by increasing the dietary crab meals was observed. The lowest TBARS value, at days 4 and 7, was observed in fish fed with RC20, followed by MC20, RC10 and MC10 diets, which did not differ from one to another (Fig. 6.1).

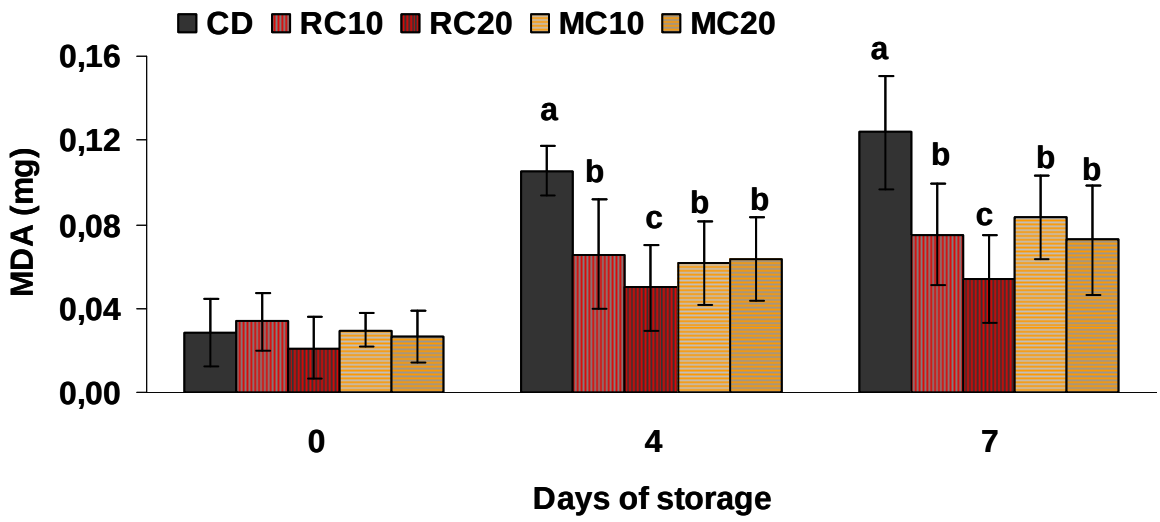

Figure 6.1 Evolution of fillet lipid oxidation during refrigerated storage of fillets from red porgy fed the experimental diets.

6.4 Discussion

Data of fillet chemical composition were in the range (lipids slightly higher) of results reported by Robaina *et al.* (2005) and Schuchard *et al.* (2008) for farmed red porgy of 400 g body weight. According to the nutritional classification cited by Nunes *et al.* (2003), based on the lipid and protein content of 27 consumed fish species, results obtained in the present study indicate that the red porgy even farmed is considered a lean fish specie (lipid < 5%; protein >20% wet weight), showing an excellent protein source with low fat content.

Different results are reported in the literature regarding the effect of crustacean meal on muscle composition in reared red porgy and other species. In a previous work, Schuchard (2005), reported an increase of 13% in muscle lipid content in red porgy fed diet with 20% of krill meal. Opposite results were showed by Chebbaki (2001), with a decrease in muscle fat in red porgy fed krill meal. However, no effect on total lipid was observed on Atlantic salmon (*Salmo salar*) and Atlantic halibut (*Hippoglossua hipoglossus.*) fed diets containing different crustacean meal levels (Olsen *et al.*, 2006; Suontama *et al.*, 2007). In the present study, the significant higher fat muscle content of fish fed with MC20 diet could be more related to their higher feed intake and weight (see García-Romero *et al.* Study I), since lipid tissue content in fish tend to increase with these two factors (Martínez *et al.*, 1992; Shearer, 1994; Ruedas *et al.*, 1997).

As in most fish, the fillet fatty acid composition reflected that of diet (Madsen *et al.*, 2000; Bell *et al.*, 2002; Glencross *et al.*, 2003). Nevertheless, in all treatments, some fillet fatty acids are presented in different proportion relative to diets. For instance, 18:1n-9 fillet content was higher (40-50%) regardless of dietary contents, which was also reported in gilthead sea bream (Arantzamendi, 1999). The level of the essential fatty acid EPA was reduced (40-50%) in muscle compared to diet, whereas DHA concentration was maintained near or slightly above diet value. This fact denote the preference of red porgy to utilize EPA for energy purposes while a selective deposition of DHA, as previously observed both

in fatter muscle species (Froyland *et al.*, 2000; Izquierdo *et al.*, 2003) and lean fish (Kennedy *et al.*, 2007; Valente *et al.*, 2011).

Regarding to differences between treatments, total SFA and MUFA muscle content appears to be unaffected by the dietary inclusion of the two different crab meals. The main fatty acid detected in SFA and MUFA were 16:00 (palmitic acid) and 18:1n-9 (oleic acid) respectively, common in other farmed marine fish (Krajnovic-Ozteric *et al.*, 1994; Glencross *et al.*, 2003; Izquierdo *et al.*, 2005; Grigorakis *et al.*, 2011). On the contrary, feeding red porgy with both RC and MC meal diets, promoted an increase of PUFA, n-3, n-3HUFA, ARA, EPA and DHA along with a lower n-9 fatty acid (mainly 18:1n-9) when compared to CD feeding fish. Yet, only the ARA content resulted statistically higher ($P<0.005$). Presumably, the level of ARA in muscle showed a linear increase with inclusion level of crab meals in agreement with the ARA meal value (Tables 6.2 and 6.4). Higher ARA content has been found in wild fish respect to farmed one, included red porgy, due to the fed lipids from marine organisms (Ohshima *et al.*, 1983; Ruedas *et al.*, 1997; Izquierdo *et al.*, 2005).

Furthermore, the increment of total lipid in CM20 fillets induce to higher level of some fatty acids, included essential fatty acids, even further improving the nutritional value of red porgy flesh for human consumption. Regarding to lipid quality, good values of atherogenicity (AI) and thrombogenicity (TI) indices (Ulbricht and Southgate, 1991; Sidhu, 2003; Ruxton *et al.*, 2004; Seierstad *et al.*, 2005) were obtained in all fillets (Table 6.4). In addition, the increase of n-3 fatty acids, and hence the n-3/n-6 ratio, found in fish fed by crab meals diets caused an improvement of TI index. Obtained values in present work for AI and TI are slightly higher and comparable to those reported for wild and farmed red porgy respectively (Rueda *et al.*, 1997; Miniadis-Meimaroglou *et al.*, 2007).

Regarding to results of sensory evaluation, some differences were showed in CM10 and CM20 fillets. However, no effect on the final fish acceptance was determined with high scores for all treatments (Table 6.6). The differences were

found in oily odour and flavour, being CM20 fillets statistically higher. These oily changes were no related with other characteristics as marine o atypical flavour intensity, and would be more related to the higher fat content in these fishes. In other species such as farmed southern flounder (*Paralichthys lethostigma*), cooked flesh acquired a less fish flavour intensity when fed a 5% crab meal supplemented diet (González *et al.*, 2006).

The results for other attributes are however quite not consistent with the above explanations mentioned for MC20 oily differences. Thus, although oily fillets are normally associated to lower fillet firmness (Lie *et al.*, 2001), in present case higher values for the firmness and cohesiveness were obtained for the MC20 feeding. In other lean farmed species such as cod (*Gadus morhua*), Karsel *et al.* (2006), showed that firmness texture of cooked flesh increased by increasing krill meal inclusion level. On the other hand, the instrumental texture measurements, performed in raw red porgy fillet, were unaffected by the inclusion of the two different crab meals in diet. This fact is in accordance with those reported by González *et al.* (2006) in southern flounder fed on crab meal and Karlsen *et al.* (2006) in cod fed on graded levels of krill (0-100%). Data of texture were similar to results obtained in the same species in a previous work (Robaina *et al.*, 2005), while texture parameters of cohesiveness, gumminess and chewiness were higher compared to gilthead sea bream (*Sparus auratus*) (Castro, 2010), both studies performed under the same fish sized and instrumental conditions.

The data reported about malonaldehyde (MDA) content in red porgy fillets ranged from 0.024-0.034 mg kg^{-1}, at slaughtering day, to 0.054-0.124 mg kg^{-1} at seventh day of refrigerated (4ºC) storage. Those values were lower than those observed in other marine commonly farmed fish species. Thus, both in European sea bass (*Dicentrachus labrax*) of 250 g (Poli *et al.*, 2001) and in gilthead sea bream of similar size at the present study (Castro, 2010), muscle MDA content increased from 0.04 to 0.115 mg kg^{-1} and from 0.160 to 0.260 mg kg^{-1} respectively, at seventh day of refrigerated (4ºC) storage.

In the present study, the partial substitution of FM by crab meal clearly decreased the lipid oxidation rate of fillets as indicated by TBARS values. The decrease in fish muscle lipid oxidation in response to dietary protein source has been observed when fish meal was replaced by vegetable protein sources (Lopez-Bote *et al.*, 2001; Menoyo *et al.*, 2004), being associated with the lower muscle content of PUFAs which are highly prone to oxidation by reactive oxygen species. Not only no relation was found between muscle PUFAs level and TBARS values, but also CM20 fillets had low oxidation values despite having higher fat muscle content and hence higher long chain fatty acids per weight unit. This indicates that the delay in lipid oxidation found from day 4 is a result of the antioxidant activity of some components present in both crab meals, protecting fatty acids against oxidation.

Exogenous dietary micronutrients acting as antioxidant protectors may be involved in the oxidative status of post mortem fish muscle (Undeland *et al.*, 1999; Monahan *et al.*, 2000; Hamre *et al.*, 2004), especially feeding during long time. Crab meals are rich in carotenoid pigments, mainly astaxanthin (Cremades *et al.*, 2003; Sachindra *et al.*, 2006; Vilasoa *et al.*, 2008), and dietary astaxanthin has been showed to act as potent antioxidant (Miki *et al.*, 1991; Britton, 1995; Bell *et al.*, 2000; Guerin *et al.*, 2003; Wang *et al.*, 2006), being even more than other carotenoid pigments (Kukarchuk *et al.*, 1997; Bell *et al.*, 2002). Most of works done in this sense have been realized in fatter muscle fish. Thus, in salmon *Salmo salar* Christiasen *et al.* (1995) showed a significant enhancement of the oxidative status in muscle and liver tissues of animals fed on dietary synthetic astaxanthin. However, it has been suggested that in lean fish the liposoluble antioxidant nutrients, such as vitamin E, may be mainly stocked in liver, the main reservoir of fat in lean fish (Hemre *et al.*, 2004).

Although the concentration of pigment in muscle was not determined, a relation between dietary astaxanthin level and TBARS values on days 4 and 7 was found. Thereby, fish fed with the highest astaxanthin level diet (CR20)

yielded significantly lowest values of lipid oxidation in their fillets (Figure 6.1). It would be also important to note in the present study that crab meals are rich in chitin, the precursor of chitosan, another antioxidant nutrient. Chitosan and other water-soluble derivates of chitin are bioactive components with a wide range of interesting proprieties useful in biology and medicine (Young, *et al.*, 2006; Ya *et al.*, 2007; Yen *et al.*, 2008). Among their studied benefits proprieties the antioxidant activity is of particular interest, as this component has the ability to inhibit lipid peroxidation by scavenging free radicals (Xue *et al.*, 1998; Xie *et al.*, 2001; Anraku *et al.*, 2009). For instance, dietary chitosan administration in rats showed to prevent oxidative damage in tissues (Santhosh *et al.*, 2006).

However, the manner in which these components could have the ability to prevent lipid oxidation in fish is unknown so far. More works are needed in order to identify antioxidant components in these ingredients and the potential benefits of incorporating them in terms of oxidative changes on the flesh quality, especially in lean fish species.

6.5 Conclusion

Dietary inclusion of both crab meals up to 20% favoured the deposition in muscle of n-3PUFA in red porgy fillets with an increase of ARA fatty acid content and n-3/n-6 ratio, together with a reduction of TI index in all treatments, promoting a positive effect on nutritional value for human consumption. Although some minor differences in sensory attributes for CM20 fillets were found, all fillets were well accepted. The inclusion of two crab meals in the diets, both at 10% and 20%, delay lipid oxidation rate in muscle during the refrigerated storage of fish fillets.

STUDY IV

Meals of spider marine crab (*Paramola cuvieri*) and sea urchin (*Diadema antillarum*) in diets for red porgy (*Pagrus pagrus*): effect on growth performance, ammonia excretion, skin colour and flesh quality

Abstract

The present study was conducted to evaluate the effect of using marine crab (*Paramola cuvierii*) meal (SMC) and sea urchin (*Diadema antillarum*) meal (SU) on growth performance, ammonia-N excretion, skin coloration and final flesh quality and lipid oxidation in on-growing diets for red porgy (*Pagrus pagrus*). Fish were fed during 180 days with five diets: a control diet based on high-quality fish meal (CD); two diets replacing 10% and 20% fish meal protein by protein from SMC meal (SMC10, SMC20), and other two diets with 8% and 16% inclusion of SU meal (SU8, SU16). The inclusion of both SMC and SU meal increased the fish appetite response reflected in a significantly higher feed intake, which turned in higher weight gain and SGR respect to the CD. Results were further encouraged for fish fed on SU diets, with better feed conversion (FCR) and protein utilization (PER) than those fed on CD and SMC diets. Ammonia-N excretion rates decreased in fish fed on SMC20, SU8 and SU16 diets respect to the CD, although only SU16 was significantly lower. Red porgy skin coloration improved by SMC meal inclusion, showing the highest redness values, while SU meal promoted yellowness coloration in fish skin. Higher muscle fatty acid content of PUFA, total n-3, n-3 HUFA and ARA, was concomitant with increased level of SMC in diets. The same trend was also found in fillet fish fed on SU meal diets although less accused, except for ARA content with highest values in SU16 followed by SU8 fillets. Results of TBARS in raw fillets showed that the dietary inclusion of SMC meal delay lipid oxidation compared to FM and SU diets during refrigerated storage at 4ºC.

Keywords: alternative ingredients; carotenoids; crab meal; sea urchin; fish composition; lipid oxidation; skin colour, *Pagrus pagrus*.

7.1 Introduction

Due to the compromising situation in the production of fish meal and oil, and increased risk expected in the future (FAO, 2010), the search for potential alternative ingredients sources has become a well known recognized priority for sustainable development of aquaculture industry. Among ingredients, these coming from seafood industry like by-product waste or discard from fisheries, either from fish and no-fish species, means a massive waste of food and a potential income based on their nutritional value and also on the necessary action response in waste minimize and recycling. Furthermore, the evidence of the fatty acids lipids and amino acid protein profiles which resembles more closely fish natural diet, feeding marine origin meals and oils would result advantages in fish health and quality rather than those derived from both vegetable and terrestrial animals origin. However, many of these marine resources are more complex than common fish meal and require an overall evaluation in order to determine their nutritional value and appropriate dietary inclusion rate.

Red porgy (*Pagrus pagrus*) is a sparid marine fish of great commercial importance (Kokokiris *et al.*, 2006). The excellent quality meat and the reduction in traditional fisheries have made it a priority candidate for Mediterranean aquaculture diversification (Kentouri *et al.*, 1995; Hernandez-Cruz *et al.*, 1999). It is well known that cultured red porgy fed diets without astaxanthin supplementation, mainly as esterified forms, exhibit a dark-grey skin colour that lead to the rejection by the consumer (Kalinowski *et al.*, 2007). Thus, most of the nutritional research have been focused to promote an adequate redness on red porgy fish skin, showing the benefit of different crustacean sources for that purpose (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; 2007; Tejera *et al.*, 2007).

Another key constriction in cultured red porgy is their high protein dietary requirement, around 50%, of diets (Schuchart *et al.*, 2008), in which fish meal is used as the main protein source. Therefore, research of new supplies of protein

and pigment sources of natural origin in on-growing red porgy diets would assist in their economical rearing.

Within marine ingredients sources, crustacean meals has been shown promising results in several aquaculture species as alternative protein source to fish meal and an enhancer for colour pigmentation (Goytortúa-Bores *et al.*, 2006; Toppe *et al.*, 2006; Villarreal *et al.*, 2006). In wild specimens, the astaxanthin carotenoid is provided by the small crustacean that constitutes the natural feed of this species (Labropoulou *et al.*, 1999; Castriota *et al.*, 2006). These kind of feed ingredients are highly palatable and provide different nutrients such as protein, lipids and carotenoids but also has high level of minerals, mainly calcium remained in ash, and chitin content. Although the high ash and chitin content could limit the value of the crab meals, biological differences in species associated to acid secretion and an adequate enzymes digestive system, could result in a specific ability to digest this type of ingredients (Word and Serfaty-Lacrosniere, 1992; Vielma *et al.*, 1998; Dunalat and Kaush, 1984; Toppe *et al*, 2006; Fines and Holt, 2010).

Together with the marine king crab *C. affinis* the spider crab *Paramola cuvieri* is another deep sea crab which is also an unexploited deep-water resources. Until now, there is not market for this specie and constitute a discard by-catch in the local fisheries (Sanchez *et al.*, 2003). This marine crab exhibits a well reddish colour suggesting its potentiality as valuable candidate ingredient for red porgy diet. Another valuable ingredient from marine origin is the long-spined black sea-urchin *Diadema antillarum*. At present, this specie represents a problem in most local ecosystem balance (i.e. Canary Islands), as they are directly involved in the transformation of large rocky-reefs covered by erect fleshy algae to poorly productive substrates devoid of seaweeds and dominated by these encrusting organisms (Alves *et al.*, 2003; Tuya *et al.*, 2004). Some papers have reported the nutritional potential of sea urchin by-products (De la Cruz-García *et al.*, 2000; Zhong *et al.*, 2007a; Mamelona *et al.*, 2010a).

Aside from promote fish growth enhancement, a new ingredient is able as suitable ingredient if their inclusion not negatively affect the final product quality as well as environment pollution. Likewise, the present study is focused on evaluate two natural ingredients from marine origin crab meal of *P. cuvierri* and sea urchin meal of *D. antillarum* as suitable ingredients for red porgy diet through an overall evaluation about the effect on fish growth performance, skin coloration, ammonia-N excretion, flesh quality and lipid flesh oxidation during refrigerated storage.

7.2 Materials and methods

7.2.1 Marine crab and sea urchin meals elaboration

Marine crab meal (SMC) was prepared from whole individuals of *Paramola cuvieri*, which were captured as by-catch in local crab fisheries in the Canaries' Coast, Spain. While sea urchin meal (SU), was obtained by processing *D. antillarum* specimens also caught in sea bottom of Canaries' coast. All animals were provided by fishermen's, being meals obtained at the laboratory using an adapted process from Sudaryono *et al.* (1996). First, individuals were autoclaved at high pressure for 15 min, then cold down quickly before oven-dried at 55ºC for 12 hours and finally ground in a hammer mill through a 0.5 mm mesh and stored in vacuum plastic bags at 4ºC. Results for proximate composition, total carotenoid content and fatty acid profile of meals are shown in Tables 7.1 and 7.3.

7.2.2 Experimental diets

Five isocaloric (13%) and isoproteic (48%) diets were formulated (Table 7.2) and tested in triplicate groups of red porgy. The control diet (CD) consisted of Peruvian high quality fish meal and fish oil. Two diets were formulated by replacing 10% and 20% of fish meal protein in CD with SMC meal protein (diets SMC10 and SMC20). Another two diets were formulated by inclusion 8% and 16% of the SU meal (diets SU8 and SU16). All diets were prepared by mixing the ingredients in a horizontal ribbon mixer before pelleting in a California Pellet Mill

(CL3 Pellet mill, USA) through out a 5 mm matrix diameter. The chemical composition of diets and fatty acid profile are reported in Tables 7.2 and 7.3.

Table 7.1 Proximate composition (% dry wt) and total carotenoid content (mg kg^{-1}) of the fish meal (FM), spider marine crab meal (SMC) and sea urchin meal (SU) used in the experimental diets

	FM	SMC [a]	SU [b]
Crude	68.12	37.59	15.83
Crude	10.11	5.11	5.87
Ash	15.34	31.98	48.13
Moisture	6.81	5.77	3.17
Carotenoid	3.28	11.96	6.50

[a] Spider marine crab *Paramola cuvieri* meal
[b] Sea Urchin *Diadema atillarum* meal

Table 7.2 Ingredients (g kg^{-1}) and proximate analysis of the experimental diets

	CD	SMC10	SMC20	SU8	SU16
Ingredients					
FM [a]	700	642	571	695	676
SMC [b]	-	124	248	-	-
SU [c]	-	-	-	80	160
Fish oil	86	86	86	82	80
Gelatinized starch [d]	169	103	50	98	39
Vitamin premix	20	20	20	20	20
Mineral premix	20	20	20	20	20
CMC	5	5	5	5	5
Proximate composition (% dry wt)					
Crude protein	47.23	46.75	46.69	47.08	46.73
Crude lipids	13.39	13.68	13.20	13.42	13.53
Ash	11.27	14.71	15.83	15.01	18.56
Carbohydrate [e]	28.11	24.86	24.28	24.49	21.18
Gross Energy (MJ kg^{-1}) [f]	21.31	20.75	20.44	20.66	20.05
Total carotenoid (mg kg^{-1})	2.62	3.58	5.04	2.79	3.08

[a] Fish meal (Peruvian origin)
[b] Spider marine crab, *Paramola cuvieri*, meal
[c] Sea Urchin, *Diadema Atillarum*, meal
[d] Merigel 100 Amylum Group
[e] Carbohydrate = 100 – (protein + lipid + ash)
[f] Gross Energy = (23.6 MJ kg^{-1} x % protein + 39.8 MJ kg^{-1} x % lipid + 17.2 MJ kg^{-1} x % carbohydrate)/100

Table 7.3 Fatty acid (g/100 g fatty acids) profile of the meals and experimental diets

Fatty acids	FM	SMC	SU	CD	SMC10	SMC20	SU8	SU16
14:00	0.05	3.15	16.83	7.25	6.78	7.28	7.13	7.87
15:00	0.23	0.73	1.24	0.67	0.45	0.56	0.46	0.57
16:00	4.24	17.46	21.64	21.55	18.32	19.82	18.73	20.64
17:00	1.68	1.05	0.44	1.59	1.57	1.64	1.52	1.53
18:00	10.25	5.86	3.93	4.38	3.62	4.15	3.68	4.36
20:00	0.42	0.54	5.32	0.24	0.20	0.26	0.20	0.26
∑SFA	16.87	28.79	49.41	35.68	30.94	33.71	31.73	35.23
14:1n-7	10.21	0.06	0.22	0.02	0.05	0.04	0.03	0.05
14:1n-5	0.06	0.19	0.06	0.28	0.16	0.16	0.13	0.16
15:1n-5	0.90	0.02	0.04	0.09	n.d	0.40	n.d	n.d
16:1n-5	0.62	0.42	0.20	0.13	0.37	n.d	0.38	0.37
16:1n-7	8.68	6.35	4.86	9.03	8.76	9.07	8.73	8.76
18:1n-5	0.12	0.38	0.40	0.11	0.13	0.14	0.13	0.13
18:1n-7	4.37	4.59	4.65	3.26	3.25	3.47	3.27	3.25
18:1n-9	10.88	23.30	4.77	9.79	9.62	9.70	9.40	9.62
20:1n-5	n.d.	0.75	0.50	0.22	0.26	0.43	0.35	0.26
20:1n-7	0.43	1.85	5.37	0.24	0.68	0.89	0.75	0.68
20:1n-9	0.95	1.85	5.76	0.81	0.68	0.89	0.75	0.68
22:1n-9	0.44	2.39	1.10	0.10	0.21	0.29	0.23	0.21
22:1n-11	0.57	1.30	4.69	0.73	0.67	0.87	0.64	0.67
∑MUFA	38.23	43.44	32.61	24.81	24.84	26.33	24.79	24.84
16:2n-6	1.55	0.49	0.12	0.39	0.44	0.45	0.39	0.40
16:2n-4	1.53	0.95	0.57	0.32	0.37	0.37	0.36	0.37
16:3n-4	0.06	0.16	0.13	0.64	0.69	0.68	0.69	0.61
16:3n-3	0.41	0.48	1.27	1.63	1.68	1.62	1.67	1.65
16:3n-1	1.70	1.11	0.90	0.25	0.25	0.27	0.26	0.25
16:4n-3	n.d.	0.10	0.20	1.28	1.39	1.37	1.40	1.39
16:4n-1	n.d.	0.20	0.04	n.d.	0.02	0.02	0.02	0.02
18:2n-9	0.12	0.22	1.75	0.07	0.09	0.08	0.08	0.07
18:2n-6	1.17	1.86	0.50	3.84	3.86	3.89	3.85	3.86
18:2n-4	0.54	0.19	0.30	0.38	0.21	0.21	0.21	0.21
18:3n-6	0.42	0.09	0.63	0.31	0.30	0.33	0.29	0.30
18:3n-4	0.22	0.24	0.28	0.25	0.33	0.29	0.26	0.28
18:3n-3	0.48	0.37	1.51	0.99	1.06	1.05	1.05	1.06
18:4n-3	0.21	0.34	1.44	1.69	1.70	1.70	1.65	1.70
18:4n-1	n.d.	0.05	0.21	0.22	0.24	0.20	0.23	0.24
20:2n-9	0.07	0.14	0.57	0.09	0.12	0.14	0.13	0.12
20:2n-6	0.20	1.08	1.34	0.19	0.16	0.22	0.20	0.16
20:3n-6	0.10	0.13	0.06	0.10	0.12	0.11	0.12	0.12
20:3n-3	n.d.	0.20	0.64	n.d.	0.09	0.10	0.09	0.12
20:4n-6	1.90	7.18	8.07	1.10	1.10	1.33	1.14	1.61
20:4n-3	0.54	0.32	0.42	0.78	0.87	0.83	0.84	0.87
20:5n-3	14.02	1.04	0.80	12.45	12.04	12.20	12.87	12.04
22:4n-6	0.44	0.18	0.20	0.59	0.66	0.55	0.65	0.66
22:5n-6	n.d.	n.d	n.d	0.32	0.33	0.34	0.30	0.33
22:5n-3	2.19	1.04	1.10	2.87	2.71	2.21	2.54	2.71
22:6n-3	15.18	12.71	0.77	10.31	10.06	10.48	10.95	10.06
∑PUFA	43.05	36.41	23.82	41.06	40.89	41.04	42.24	41.21
∑n-3	33.04	23.59	7.38	32.00	31.60	31.56	33.06	31.60
∑n-6	5.78	9.44	10.92	6.84	6.97	7.22	6.94	7.44
∑n-9	12.47	29.74	13.96	10.86	10.72	11.10	10.59	10.70
∑n-3 HUFA	31.94	22.31	3.73	26.41	25.77	25.82	27.29	25.80
AA/EPA	0.14	6.90	7.34	0.09	0.09	0.11	0.09	0.13
EPA/DHA	0.92	0.08	1.43	1.21	1.20	1.16	1.18	1.20
n-3/n-6	5.72	2.49	0.68	4.68	4.53	4.37	4.76	4.25

7.2.3 Fish and culture conditions

Red porgy with initial mean weight of 205 ± 39 g (mean ± SD) were distributed in 15 fibreglass circular tanks of 500 l, at an initial density of 6.3 kg m^{-3}. Tanks were provided with natural seawater with 17-18 ºC and 7-9.7 mg L^{-1} of temperature and dissolved oxygen respectively. Fish were carefully hand feeding to apparent satiation twice per day (8:00 and 15:00), six days per week for 180 days. Uneaten feed was daily recovered for feed intake correction.

7.2.4 Sampling and colour evaluation

Skin colour parameters, body weight and total length were taken in all fish previously anesthetised with 2-phenoxyethanol (0.1 mL L^{-1}) at the beginning of the experiment and at 45, 90, 135, 180 feeding days. For proximate analysis of muscle and liver, nine individuals per treatment were slaughtered. Nine fish per treatment were also sampled for skin carotenoids concentration at the end of the triall. All samples were stored at -80ºC until analysis.

2.5. Samples recollection and ammonia nitrogen concentration

Ammonia-N determinations were carried out over 24 h on days 145, 154 and 163. Feeding was performed to apparent satiation at 08:00 am. Inlet and outlet water samples (50 mL) were then taken from each tank every 2 h between 08:30 am and 08:30 pm to analyse the ammonia-N excretion of the fish fed the different diets. Samples of water were kept in dark recipients and stored at 4ºC until analysis. A tank without fish and another with unfed fish were also sampled to determine the blank-corrected value.

7.2.6 Colour measurements

Skin colour parameters were lightness (L^*), redness (a^*) and yellowness (b^*), in accordance with the recommendations of the International Commission on Illumination, CIE (1976). Colour variables were measured from the left front lateral zone (Kalinowski *et al.*, 2005) using a portable colorimeter (Hunter

MiniScan™ XE plus). Hue [$H_{ab}=arctan(b^*/a^*)$] and Chroma [$C_{ab}=(a^{*2}+b^{*2})^{1/2}$], were calculated according to Hunt (1997).

7.2.7 Carotenoids analysis

Carotenoids of meals and diets were extracted according to Barua *et al.* (1993), while for skin, due to the high water content, according to Schiedt and Liaaen-Jensen (1995). Total carotenoid concentration was calculated spectrophotometrically at λ=470nm using $E_{1\%,1\ cm}$= 2100, when carotenoids were expressed as astaxanthin equivalent, by the following equation:

$$\mu g\ g^{-1}\ sample = 10000*V*A/W*E_{1\%,1\ cm}$$

where V (mL) is the volume of the extract, W (g) the weight of sample, A the absorbance and $E_{1\%,1\ cm}$ the extinction coefficient.

For quantification of red and yellow pigments, final n-hexane solutions, containing total skin carotenoids, were analyzed by TLC using pre-coated plates of silica gel (G60 Merck glass plates, 20 x 20 cm). Hexane/diethylether 20:1 was used as a developing system. Separated skin pigments were tentatively identified on the basis of relative motility on a TLC plate. For quantification, each separated component was scraped off the silica plate and washed several times with acetone until no colour was observed. Thereafter, the acetone volume was filtered evaporated to dryness and dissolved in hexane for further quantification by UV/Vis spectroscopy, reading at λ maxima and using an extinction coefficient (E1%,1cm) of 2500 for unknown compounds (Britton *et al.*, 1995). Unpublished colour variables of wild red porgy skin from our research group were used as referential values.

7.2.8 Proximate analysis and fatty acid profile

Proximal analysis and fatty acid profile was performed for meals and diets in triplicate. For muscle samples three fish from each experimental tank were

used. Moisture, crude protein and ash content were determined according to AOAC (1995). Lipids were extracted with chloroform-methanol (2:1, v/v), as described by Folch *et al.* (1957). The fatty acid methyl esters were obtained by transesterification with 1% sulphuric acid in methanol (Christie, 1982), purified by absorption chromatography on NH_2 Sep-pak cartriges (Waters, S.A., Milford, MA, USA) and separated and quantified by gas-liquid chromatography as described by Izquierdo *et al.* (1992). Fatty acids were identified by comparison to external standards. Data were expressed as percentage of total identified fatty acids.

The index of atherogenicity and thrombogenecity related to effects of different fatty acids on human's health were calculated in concordance to Ulbricht and Southgate (1991):

Index of atherogenicity (AI)

$$AI = [(12{:}00) + (4 \times 14{:}00) + (16{:}00)] \times [(PUFA\ n\text{-}6\ and\ n\text{-}3) + MUFA]^{-1}$$

Index of thrombogenicity (TI)

$$TI = [(14{:}00) + (16{:}00) + (18{:}00)] \times [(0.5 \times MUFA) + (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3/n\text{-}6)]^{-1}$$

7.2.9 Texture analysis

After a starving period of 24 h, fish were slaughtered in a small tank with ice and seawater. Nine fish from each treatment were randomly sampled and kept with ice in polyethylene boxes at 4ºC for a day. The texture analysis was conducted in a Stable Micro System texture analyser (TA.XT2, Surrey, England) equipped with a 5 kg load cell. All tests were carried out at refrigeration temperatures, keeping the fillets on ice. After removal of the skin, three blocks (2×2×1.2 cm) were obtained from each fillet above the lateral line. Fish fillets were compressed up to breaking. Compression test was defined as the force exerted to deform a block fillet using a flat-ended (Type P/100) aluminium compression plate with a speed to 0.8 mm/s and strain to 80% penetration. The

total force, F_1 (N), needed to compress the fillet was recorded.

7.2.10 Measurement of Thiobarbituric acid-reactive substances (TBARS)

Nine fish from each treatment were collected for the 2-thiobarbituric acid-reactive substances (TBARS) analysis (Shaidi and Hong, 1991). At days 0, 4 and 7 of refrigerated storage, a fillet sample of 3 g was mixed with 6ml of 10% trichloroacetic acid (w/v) and homogenized for 60 s. After filtration, 2 ml of the filtrate were mixed with 2 ml of a TBARS solution (0.02 M) and heated at 100ºC for 1 hour. Then, the absorbance was measured at 532 nm by UV/Vis spectrophotometer (Thermo Scientific, Evolution 300 model, Chicago, USA). TBARS values were expressed as mg of malonaldehyde (MDA) per kg of fillet.

7.2.11 Statistical analysis

Scalar data was tested for normality of distribution and homogeneity of variance (Sokal and Rohlf, 1995). To compare treatments, parametric and homoscedastic data were used by one-way ANOVA followed by Tukey's test for multiple comparisons. For non-normal data and/or data showing homogeneity of variance, the Kruskal-Wallis multiple range test was used. In case of the thiobarbituric acid-reactive substances data, a general linear model with two fixed factors, time and dietary level inclusion, was used. All statistical analyses were tested at 0.05 significance level using the *SPSS* (13.1) statistical package.

The angular colour parameter, Hue, was analysed by circular statistical test. The estimation of the mean and standard deviation was performed with descriptive statistics for circular distributions. The Rayleigh test was applied to check the uniformity of circular distribution (Zar 1999). As sampled populations was not unimodal, differences in the Hue values were tested using the non parametric Watson's U^2 test and accepted at $P<0.05$ (Zar 1999). All tests were performed with Oriana, version 3, statistical software (Kovach Computing Services, Pentraeth, Wales, UK).

7.3 Results

7.3.1 Growth and feed utilization

All diets were well accepted from the start of the trial, with higher feed appetence observed in fish fed both SMC and SU meals diets. This fact resulted in higher feed intake and a positive effect on growth performance, although only the highest inclusion level of the two meals (SMC20 and SU16 diets) caused significantly greater feed consumption compared to CD diet (Table 7.4). Better growth response was observed for the inclusion of SU than for SMC meal. Thus, final fish weight, absolute weight gain and specific growth rate (SGR) values were significantly higher both to group with the highest inclusion of spider marine crab meal (SMC20), and groups with sea urchin meal (SU8 and SU16) compared to fish fed CD and SMC10, which did not differed among them.

Results for feed conversion ratio (FCR) and the efficiency of dietary protein utilization (PER) were positively affected by increasing dietary levels of SU meal, with significant lower FCR values for both inclusion levels (SU8 and SU16) and significant higher PER values for the highest inclusion level (SU16). Comparing between the two meal groups (SMC and SU), SU16 showed significantly better FCR and PER results than those for SMC10 and SMC20, while SU8 has similar values respect to SMC20 and better compared to SMC10 (Table 7.4).

Fish body appearance measured by condition factor, K, was better affected by feeding SU diets, showing significantly lower values respect to CD and SMC fish. Results for HSI had similar trend, with lowering indexes by increasing dietary levels of both meals. No effect was found for visceral index (VSI) between diets (Table 7.4).

Table 7.4 Growth performance and feed utilization parameters of red porgy fed the experimental diets (mean ± SD)

	CD	SMC10	SMC20	SU8	SU16
Initial weight (g)	201.61±22.11	204.60±25.09	201.10±22.89	202.92±24.09	200.68±21.55
Final weight (g)	421.91±43.57 ^{b}	433.42±44.03 ^{b}	463.78±45.32 ^{a}	476.54±46.24 ^{a}	479.24±52.18 ^{a}
Weight gain (g) [a]	221.02±35.47 ^{b}	229.44±45.51 ^{b}	253.95±34.87 ^{a}	273.92±41.88 ^{a}	279.27±43.14 ^{a}
FI (g fish $^{-1}$) [b]	379.1±1.59 ^{b}	407.00±23.35 ^{ab}	432.51±1.26 ^{a}	414.96±26.26 ^{ab}	444.57±26.61 ^{a}
SGR [c]	0.37±0.05 ^{b}	0.38±0.07 ^{b}	0.42±0.04 ^{a}	0.44±0.06 ^{a}	0.45±0.05 ^{a}
FCR [d]	1.73±0.05 ^{a}	1.84±0.07 ^{a}	1.70±0.09 ^{ab}	1.53±0.03 ^{bc}	1.48±0.05 ^{c}
PER [e]	1.22±0.04 ^{bc}	1.10±0.04 ^{c}	1.25±0.07 ^{bc}	1.34±0.09 ^{ab}	1.44±0.05 ^{a}
K [f]	3.10±0.28 ^{a}	3.02±0.37 ^{a}	2.88±0.30 ^{ab}	2.80±0.39 ^{b}	2.83±0.29 ^{b}
HSI [g]	1.44±0.29 ^{a}	1.31±0.20 ^{a}	1.11±0.30 ^{b}	1.09±0.25 ^{cb}	1.01±0.23 ^{c}
VSI [h]	5.99±1.42	5.75±1.09	4.98±0.68	5.93±0.85	5.53±0.92

Significant differences between treatments are indicated wth different letter.

[a] **Weight gain** = (final weight- initial weight)

[b] **Feed intake (g)** per fish for 165 day experiment

[c] **Specific growth rate=** 100x(ln final weight-ln initial weight)/n° days

[d] **Feed conversion ratio=** feed intake (g)/weight gain (g)

[e] **Protein efficiency ratio=** weight gain (g)/protein intake (g) (dry matter).

[f] **Condition factor (%)=** 100x(final weight /(final length)^{3})

[g] **Hepatosomatic index (%)=** 100x(liver weight/final weight)

[h] **Visceral index (%)=** 100x((final weight-final eviscerated fish weight)/final weight)

7.3.2 Postprandial ammonia nitrogen excretion

The postprandial ammonia-N excretion concentration (mg kg^{1} body weight) showed similar patterns in all feeding groups, with increasing ammonia-N excretion after feeding and reaching maximum values at 4 h in all treatments. Another peak, with less amplitude, was observed at 10 hours after feeding (Fig 7.1).

The inclusion of SMC up to 20% level did not significantly affected the total daily ammonia-N excretion (mg $N\text{-}NH_4^+$ kg^{-1} day^{-1}) respect to the CD, although a lower value in SMC20 fish was observed (Table 7.5). On the other hand, fish fed on SU meal diets showed lower ammonia-N excretion, only significant for the highest inclusion level (SU16). Per unit of nitrogen intake, total ammonia-N excretion reached 34%, 42%, 32%, 31% and 22% for fish fed on CD, SMC10, SMC20, SU8 and SU16 diets, respectively (Table 7.5).

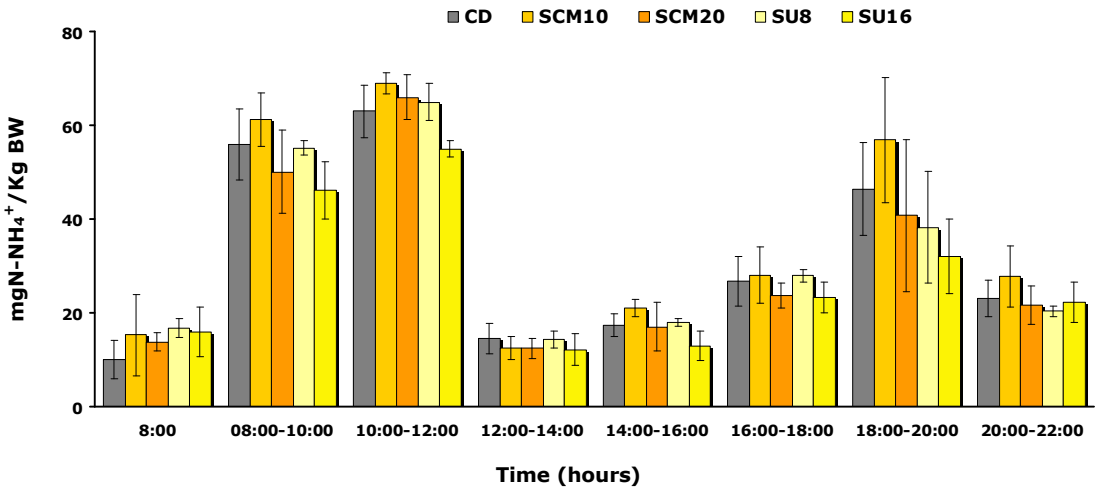

Figure 7.1 Daily ammonia nitrogen excretion patterns in red porgy after feeding the experimental diets. Means ± SD, (n=3) for each treatment. Means without letters denote no significant differences.

Table 7.5 Total daily ammonia-N excretion ($N\text{-}NH_4^+$) and per unit of nitrogen intake (%) of red porgy fed the experimental diets.

	CD	SMC10	SMC20	SU8	SU16
N [a] (mg Kg^{-1} d^{-1})	801 ± 107	682 ± 120	761 ± 130	814 ± 114	978 ± 132
$N\text{-}NH_4^+$ (mg Kg^{-1} d^{-1})	277 ± 32^a	292 ± 41^a	245 ± 25^a	255 ± 34^a	219 ± 20^b
$N\text{-}NH_4^+$/ N intake	34%	42%	32%	31%	22%

[a] Total nitrogen fish intake during the ammonia excretion trial.
Values are means ± standard deviations (n=3) for each treatment. Significant differences between treatments are indicated with different letter.

7.3.4 Skin colour parameters

From day 45 until the end of the trial, fish fed SMC meal- diets (SMC10 and SMC20), increased significantly skin redness (a^*) values compared to fish fed on CD and SU meal diets, which did not differ among them. Only after 180 days of feeding time, fish fed SMC20 diet differ from SCM10 once showing higher a^* value (Table 7.6). Meanwhile, skin yellowness (b^*) was higher in fish fed on SU meal diets, although only at 45 and 90 feeding days caused significantly greater values than the rest of treatments (Table 7.6).

Regarding Hue values (Fig 7.2), skin colour of farmed red porgy was positively affected by the inclusion of SMC meal diets as Hue decrease along the whole experiment and promotes more reddish skin coloration. Fish from SMC20 diet achieved the lowest Hue value, although not differing from SMC10 group until 180 days.

Concerning chroma, at 45 days fish fed on SMC20 diet and the two dietary level of SU meal (SU8 and SU16) diets presented similar chroma, with SU16 fish significantly higher than CD and SMC10 fish which did not differ between them (Fig 7.3). After 135 days, chroma was significantly higher in fish fed on SMC meal diets respect to the rest of treatments. At the end of the experiment SMC20 showed the highest value and CD fish the lowest. Finally, no significant differences were found in lightness (L^*) among fish in all dietary groups (Fig 7.4).

Table 7.6 Skin redness (a^*) and yellowness (b^*) values along the trial and total carotenoid content (μg g^{-1}) of red porgy skin at the end of the experiment

Day	Colour Parameters	CD	SMC10	SMC20	SU8	SU16
45	a^*	2.08±0.97 ^{b}	3.43±1.06 ^{a}	3.68±1.10 ^{a}	2.49±1.42 ^{b}	2.57±1.08 ^{b}
	b^*	8.41±2.50 ^{b}	7.89±2.60 ^{b}	8.98±1.95 ^{ab}	10.23±2.48 ^{a}	10.15±2.65 ^{a}
90	a^*	2.75±1.09 ^{b}	4.13±1.14 ^{a}	4.26±1.06 ^{a}	3.31±1.22 ^{b}	2.97±1.03 ^{b}
	b^*	7.30±2.28 ^{ab}	7.43±1.75 ^{ab}	7.15±2.08 ^{b}	8.27±2.02 ^{a}	7.68±2.39 ^{ab}
135	a^*	2.39±1.51 ^{b}	4.71±1.35 ^{a}	5.28±1.65 ^{a}	2.83±1.16 ^{b}	2.90±1.41 ^{b}
	b^*	7.10±2.17	7.72±2.81	7.66±2.93	7.79±2.97	6.76±3.04
180	a^*	3.27±1.03 ^{c}	5.39±1.22 ^{b}	6.79±0.70 ^{a}	3.79±1.24 ^{c}	3.71±1.06 ^{c}
	b^*	7.20±2.01	7.10±1.97	7.39±2.60	7.55±2.60	7.40±2.15
	Total Carotenoid	2.42±1.20 ^{d}	5.16±2.74 ^{b}	8.93±1.47 ^{a}	3.61±1.42 ^{c}	3.26±1.23 ^{c}

Significant differences between treatments are indicated wth different letter.
Initial skin redness (a^*) and yellowness (b^*) (mean ± SD, n=50 fish) = 2.35 ± 0.89 and 9.43 ± 0.97 respectively.

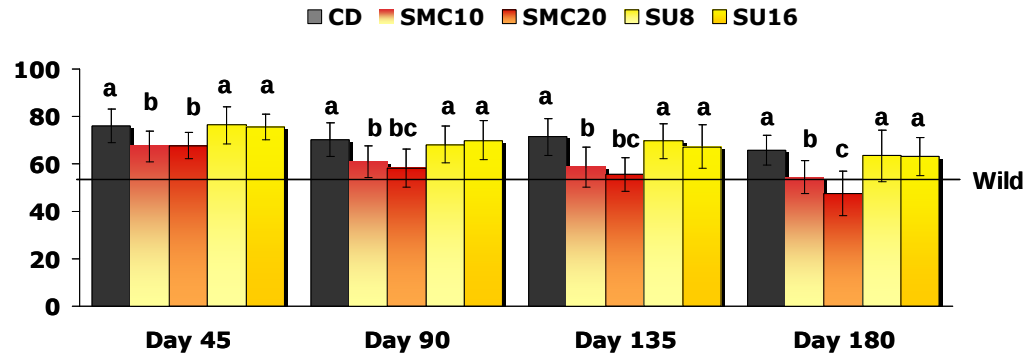

Figure 7.2 Skin Hue values (°) of red porgy along the experiment. Data of wild red porgy skin from Kalinowski *et al.* (2007) were used as referential values. Significant differences between treatments are indicated wth different letter.

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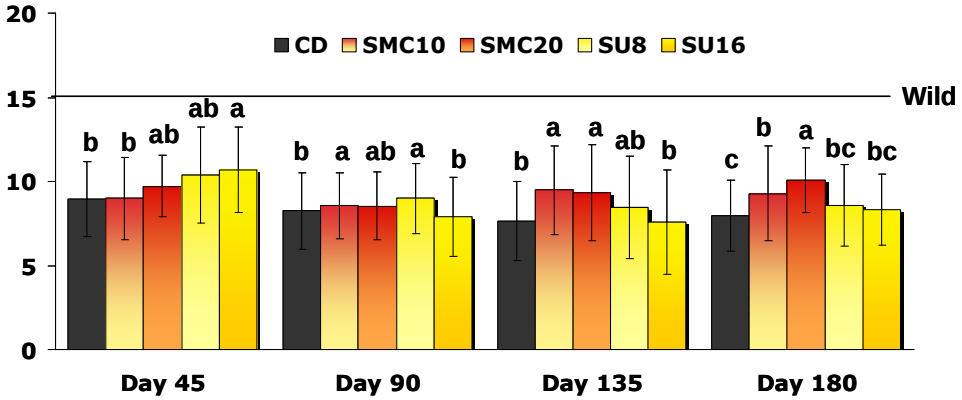

Figure 7.3 Skin chroma values of red porgy along the experiment. Data of wild red porgy skin from Kalinowski *et al.* (2007) were used as referential values. Significant differences between treatments are indicated wth different letter.

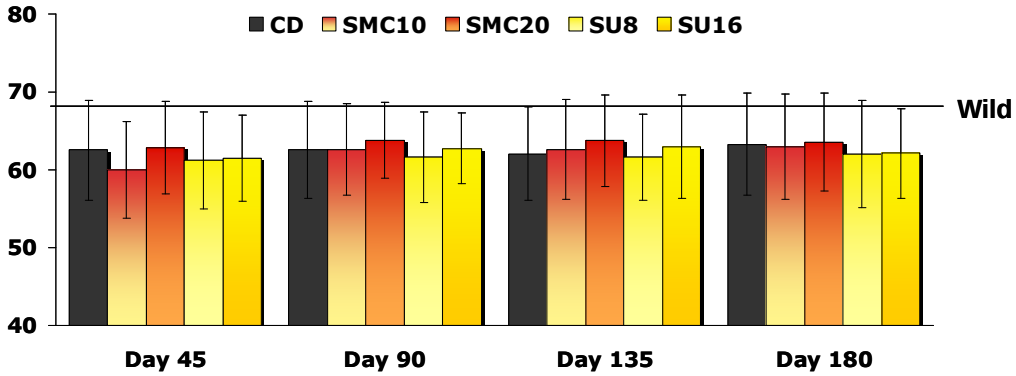

Figure 7.4 Lightness values skin fish of red porgy along the experiment. Data of wild red porgy skin from Kalinowski *et al.* (2007) were used as referential values. Significant differences between treatments are indicated wth different letter.

7.3.5 Skin carotenoid concentration and TLC analysis

Four significantly homogeneous groups were found regarding skin carotenoid concentration. Evidently, the concentration of the carotenoid pigments on skin was enhanced by the addition of SMC meal with the highest values observed in fish skin fed with SMC20 diet followed by SMC10 one. The third group corresponded to fish fed on SU8 and SU16 diets and the last group with the lowest concentration value for fish fed on CD (Table 7.6).

Skin total carotenoid separation carried out by thin layer chromatography revealed two fractions, red and yellow, fraction in all treatment groups (Table 7.7). The observed trend for the pigment fraction in fish skin reflected the content of the used meals. Thereby, including SCM meal resulted in significantly higher red pigment proportion in skin (60% and 39% for SCM20 and SCM10 respectively) respect to the CD (28%) and SU meal diets (9% and 12% for SU16 and SU8 respectively). Conversely, yellow pigment fraction was significantly found in major proportion in the skin fish fed on SU16 (91%) and SU8 (88%) diets. Skin colour results of SMC20 fish were closest to that from wild red porgy orgin (Table 7.7).

Table 7.7 Percentage distribution of red and yellow pigments fraction in skin of red porgy fed the experimental diets (means ± SD).

	Red pigment fraction %	Yellow pigment fraction %
CD	27.66±4.69 ^{c}	72.34±4.97 ^{b}
SMC10	38.65±2.96 ^{b}	61.34±2.96 ^{c}
SMC20	59.96±3.77 ^{a}	40.03±3.77 ^{d}
SU8	11.86±1.12 ^{d}	88.12±1.12 ^{a}
SU16	8.83±0.12 ^{d}	91.17±0.12 ^{a}

Different letter denote significant differences. Data of red and yellow fractions for the wild red porgy were 54 and 46% respectively (Study I).

7.3.6 Muscle proximate composition and fatty acid profile

Althouhgt SMC and SU meals fatty acid profile was different to that observed in FM, dietary fatty acid profile resembled in all experimental diets (Table 7.3). No significant differences were found in muscle proximate composition between dietary treatments. Only a tendency towards increase muscle lipid content by increasing the levels of SMC and SU meals in the diets was observed (Table 7.8).

Fatty acid composition (% of total identified fatty acids) of red porgy fillets is presented in Table 7.9. Although without any significantly difference, fillet from red porgy fed SMC meal showed a slightly higher level of polyunsaturated PUFA,(7 and 11% for SMC10 and SMC20 respectively), total n-3 (10 and 16% for SMC10 and SMC20 respectively) and n-3 HUFA (10 and 17% for SMC10 and SMC20 respectively), along with a lower level in saturated SFA (5 and 7% for SMC10 and SMC20 respectively) and total n-6 (6%) fatty acid content, compared to those found in CD fillets. The same trend respect to the CD fish was also observed for fish fed on SU meal diets, even if less marked (Table 7.9). The only statistical difference in the fillet fatty acid content was found in the 20:4n-6 (ARA) fatty acid, with a greater content observed in fish fed on SU16 than the rest of treatments (Table 7.9). The AI and TI indexes did not show any statistical differences between the experimental diets, decreasing with SMC inclusion and unaffected by the SU meal inclusion.

Table 7.8 Muscle proximate composition (% wet wt) of red porgy fed the different diets (means ± SD).

	CD	SMC10	SMC20	SU8	SU16
Protein	23.49±0.66	23.96±0.58	23.98±0.90	23.69±0.69	23.50±0.53
Lipid	1.46±0.23	1.52±0.24	1.66±0.39	1.76±0.36	1.83±0.70
Moisture	74.10±0.71	73.70±0.77	73.21±1.05	73.85±0.93	73.81±0.81

Table 7.9: Muscle fatty acid profile (g/100 g fatty acids) of red porgy fed the experimental diets.

ffa	CD	SMC10	SMC20	SU8	SU16
14:00	2.47	2.46	2.51	2.66	2.67
15:00	0.24	0.24	0.25	0.25	0.27
16:00	19.48	18.45	17.72	18.93	19.55
17:00	0.56	0.60	0.64	0.63	0.57
18:00	6.12	5.55	5.26	5.95	6.08
20:00	0.20	0.20	0.20	0.19	0.20
∑SFA	29.06	27.49	26.58	28.62	29.35
14:1n-7	0.05	0.05	0.06	0.06	0.06
14:1n-5	0.02	0.02	0.02	0.02	0.02
16:1n-5	0.16	0.18	0.18	0.20	0.20
16:1n-7	4.43	4.52	4.52	4.85	4.45
18:1n-5	0.13	0.13	0.12	0.14	0.15
18:1n-7	3.01	3.01	2.93	3.05	3.05
18:1n-9	13.48	12.85	12.38	13.28	12.42
20:1n-5	0.21	0.21	0.20	0.24	0.28
20:1n-7	0.78	0.78	0.76	0.77	0.83
20:1n-9	0.78	0.78	0.76	0.77	0.83
22:1n-9	0.35	0.35	0.32	0.32	0.32
22:1n-11	0.71	0.73	0.68	0.64	0.65
∑MUFA	24.11	23.63	22.92	24.31	23.26
16:2n-6	0.14	0.13	0.14	0.13	0.13
16:2n-4	0.55	0.58	0.56	0.61	0.57
16:3n-3	0.06	0.06	0.06	0.06	0.06
16:3n-1	0.45	0.38	0.44	0.37	0.42
16:4n-3	0.71	0.57	0.67	0.64	0.64
16:4n-1	0.23	0.20	0.23	0.19	0.22
18:2n-9	0.09	0.13	0.12	0.09	0.08
18:2n-6	0.08	0.07	0.08	0.09	0.08
18:2n-4	8.45	7.76	6.80	7.67	7.60
18:3n-6	0.10	0.10	0.26	0.12	0.11
18:3n-4	0.17	0.18	0.18	0.18	0.16
18:3n-3	0.87	0.95	0.71	0.93	0.88
18:3n-1	0.23	0.95	0.91	n.d	n.d
18:4n-3	0.38	0.44	0.45	0.50	0.43
18:4n-1	0.09	0.10	0.09	0.11	0.09
20:2n-9	0.08	0.07	0.08	0.09	0.07
20:2n-6	0.29	0.29	0.24	0.29	0.33
20:3n-6	0.16	0.16	0.22	0.18	0.18
20:3n-3	0.08	0.09	0.07	0.09	0.09
20:4n-6	1.28^{b}	1.32^{b}	1.38^{b}	1.42^{ab}	1.64^{a}
20:4n-3	0.46	0.48	0.48	0.52	0.47
20:5n-3	5.55	6.18	6.39	6.63	6.16
22:4n-6	0.18	0.14	0.18	0.18	0.20
22:5n-6	0.61	0.58	0.71	0.58	0.62
22:5n-3	2.48	2.66	2.70	2.66	2.57
22:6n-3	21.35	23.16	25.52	21.90	22.76
∑PUFA	45.11	47.74	49.65	46.27	46.56
∑n-3	31.93	34.59	36.26	33.95	34.05
∑n-6	11.21	10.49	10.51	10.59	10.80
∑n-9	12.68	14.18	13.66	14.54	13.73
∑n-3 HUFA	29.91	32.58	35.16	31.82	32.05
AA/EPA	0.23	0.21	0.22	0.21	0.27
EPA/DHA	0.26	0.27	0.25	0.30	0.27
n-3/n-6	2.84	3.29	3.45	3.19	3.15
AI [a]	0.44	0.41	0.40	0.44	0.44
TI [a]	0.19	0.17	0.16	0.19	0.19

[a] AI, atherogenic index; TI, index of thrombogenecity

[*] Different letter by rows denotes significant difference between treatments

7.3.7 Flesh texture

No significant differences were obtained for texture between CD and the rest of the fish diets, although a reduction trend was observed by increasing SU inclusion. It was only different the value of SU16 fish, lower than that for SMC10 fish (Table 7.10).

Table 7.10 Force, F_1 (N), required to compress the raw fillet of red porgy fed the experimental diets.

	CD	SMC10	SMC20	SU8	SU16
F_1	1.83 ± 0.70^{ab}	1.94 ± 0.75^{a}	1.76 ± 0.63^{ab}	1.65 ± 0.73^{ab}	1.40 ± 0.62^{b}

Means with different letter denote significant difference

7.3.8 Muscle lipid oxidation

The effect of dietary treatment on lipid oxidation measured as changes in TBARS values at 0, 4 and 7 days of storage after slaughtering is illustrated in Figure 7.5. A significant dietary influence ($p<0.00$) with an effect interaction with time storage ($p<0.00$) was observed. Thus, no differences in TBARS values were observed at 0 days while at days 4 and 7 of storage, fish fed SMC diets at the two inclusion level attained significantly lower TBARS values in comparison with CD and SU diets (Fig. 7.5).

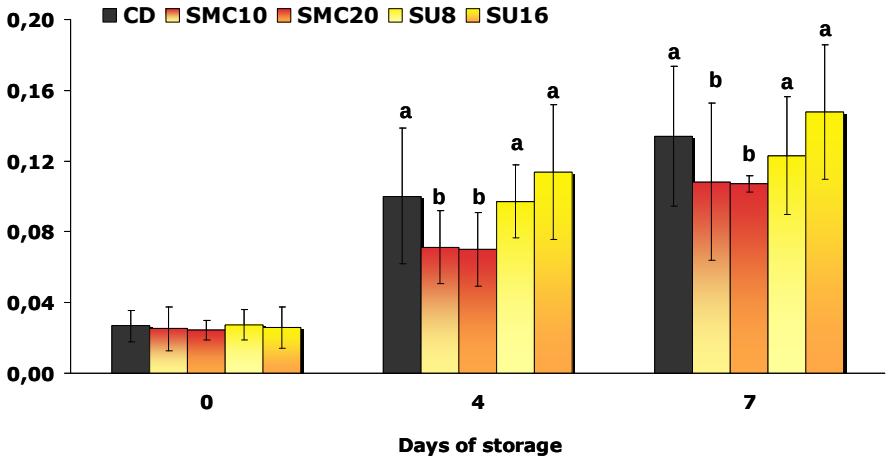

Figure 7.5 Evolution of fillet lipid oxidation during refrigerated storage of fillets from red porgy fed the experimental diets. Significant differences between treatments are indicated with different letter.

7.4 Discussion

7.4.1 Growth performance

In present study, the inclusion of both SMC and SU meals in diets positively affected on the final growth performance. As observed in a previous experiment (García-Romero *et al.*, 2010; study I) conducted also with red porgy fed on different crab meals origin, river (*Procambarus clarkii*) or marine (*Chaceon affinis*), dietary inclusion of marine origin ingredients highly induced the fish appetite response, clearly reflected in a significantly higher feed intake respect to the control diet together with a positive impact on fish growth. Results obtained are further encouraged for fish fed on SU meal, which attained significantly better feed efficiency (FCR) and protein utilization (PER) than SMC meal diets.

Crustacean meals have been used as alternative protein sources in several species showing successful results on diets appetence and growth performance (Smith *et al.*, 2005; Goytotúa-Bores *et al.*, 2006; Tibbetts *et al.*, 2006; Kalinowski *et al.*, 2007). Feed intake is regulated by the amount of digestible energy in diets but also by the palatability and attractant diet effect associated with the presence of certain stimulant soluble nutrients (Jonnes 1992; Yacoob and Browman, 2007). Digestible energy of SMC and SU meal based diets may be expected to be lower than that of CD, due to some nutrient limiting factors such as the greater ash and chitin content. Nevertheless, since a good digestibility (Study II) has been found in diet containing high amount of ash (from marine crab *Chaceon affinis* meal), the higher feed consumption observed may be also explained by attractant and good palatability properties conferred by certain components presented in both marine meals. It is important to mention that this fact was supported by the observed fish feeding behaviour of SU and SMC diets, showing a faster initial pellet intake response together with more feed competitiveness and longer sustained appetence. The same feeding behaviour was recently described by Tibbetts *et al.* (2010), where feeding with krill meal on cod (*Gadus morhua*) and halibut (*Hippoglossus hipoglossus*) significantly increased feed intake, even at

higher levels of digestible energy and protein, with increment on growth, FCR and PER rates enhancement krill, as a crustacean, has the role of meal attractant attributed to the soluble protein fraction content, preserved in meals with the adequate processing. In agreement with these authors, feed attractant properties should be highly potential as could affect in double growth in fish species. Both SU and SMC meals were gently processed at low drying temperature, which may preserve certain nutritional attractants components in meals and better general quality.

Feeding with SMC diets resulted in a tendency toward to decrease Ammonia-N excretion (AE) respect to those fed the control diet, while diets with SU meal significantly reduced AE by increasing the inclusion level. It is well known that AE is influenced by the fish size (Zhang *et al.*, 2004; Liu *et al.*, 2009), however, since SU8 and SU16 fish displayed similar K value condition, the lower AE value in SU16 diet may likely due to a better dietary protein utilization, or even to a "protein-sparing effect" of any non-protein component of SU meal. Furthermore, the lower AE along with the increase of PER value suggests that growth of animals fed SU diets was not only due to the higher feed intake but also better protein utilization of diets containing SU meal. More research should be performed to elucidate possible growth stimulating compounds in these ingredients.

The lesser amplitude second peak excretion at about 12 hours after morning feeding may be related with the rule of feeding the fish twice a day, while only once during ammonia experience trials. The presence of two peaks of excretion is widely reported to have two meals (Echavarria and Col, 1993; Engine and Carter, 2001), indicating that the second peak could be a result of metabolism as reflecting residual to these fish have been accustomed to two meals.

7.4.2 Skin colour performance

Several crustacean species are rich in astaxanthin carotenoid, which is often presented in esterified form (Matsuno and Hirao, 1989; Coral-Hinostroza and Bjerkeng, 2002). In the present trial, the carotenoids content from the spider marine crab meal, specially at the highest level of inclusion (SCM20), was an effective dietary ingredient for enhancing skin colour in red porgy based on *a**, *b**, Hue, Chroma values and skin carotenoids concentration. This observation is in line with the previous work (García-Romero *et al.*, 2010; study I) where the dietary inclusion crab meals positively affected skin colour, showing crab meals as a good source of esterified astaxanthin, which has proved to be more efficiently utilized than the unesterified form to improve skin colour in red porgy (Kalinowski *et al.*, 2005; Tejera *et al.*, 2007). From the two marine crabs species used in the different finding (*C. affinis*. in study I and *P. cuvieri* in present study IV), superior pigmentation efficacy from the *P. cuvieri* diets over the *C. affinis* crab diets (MC) were obtained since total carotenoids concentration in SMC meal (11.96 mg kg^{-1}) was higher respect to the MC meal (5.90 mg kg^{-1}).

Wild red porgy presents a red-pink skin coloration a blend of red (astaxanthin) and yellow (tunaxanthin) pigments (Tejera *et al.*, 2007). Thus, the adequate relative proportion between red and yellow pigments in the skin of farmed red porgy promotes the adequate overall colour appearance. In present study fish fed on SMC20 diet had similar pigments relative proportion (60%/ 40%, red/yellow) to wild origin (64%/36%, red/yellow). On the other hand, red porgy fed SU diets did not increase redness parameter colour (*a**) or deposit high amount of red pigment as we expected, since SU meal contain mainly yellow pigments and red sparids have shown limited ability to convert them (B-carotene, zeaxanthin and lutein) into astaxanthin (Nakazoe *et al.*, 1984; Chatzifotis *et al.*, 2005; Chatzifotis *et al.*, 2009). Although, SU meal was tested mainly under growth performance purposes, it was observed an increase of yellowness (*b**) supported with a significant major proportion of yellow pigment fraction (88%-91%) in skin

fish fed on SU diets. The echinenone (4-keto-β-carotene) is a yellow pigment well-known as major carotenoid found in gonads of sea urchins (Griffiths, 1966; Tsushima *et al.*, 1993b; Havardsson and imsland, 1999; Matsuno and Tsushima, 2001). This induces that the yellow pigment from SU was well utilized by the red porgy and deposited in the skin as tunaxanthin. Moreover, it has been proved that red porgy has the ability to oxidize dietary asthaxanthin into tunathanxin, so other precursors of tunaxanthin may be considered (Kalinoswski *et al.*, 2005). Results showed SU meal pigments content led to deposition of yellow carotenoid in fish skin contributing to a yellowish coloration and it could offer an alternative as yellow pigment source for enhance tunaxanthin content.

Skin lightness (L*) was not significantly affected by diets. As occurred in other species, a variation in this parameter seems mainly related with different environmental factors (Pavlidis *et al.*, 2006).

7.4.3 Composition and fish flesh quality

As shown by numerous species and previous works with red porgy, the fillet fatty acid composition reflects that from the diet (Madsen *et al.*, 2000; Bell *et al.*, 2002; Glencross *et al.*, 2003). In present study, there was an increase of PUFA, total n-3, n-3 HUFA, DHA (22:6n-6), EPA (20:5n-3) and ARA (20:4n-6) fatty acid content, as well as a decrease in total n-6 and saturated fatty acid concomitant with the inclusion of marine crab meal in diets. Similar results were found where feeding southern flounder (*Paralichthys lethostigma*) with crab meal supplemented diets (5%) also improved the muscle nutritional quality with higher n-3/n-6 ratio respect to fish fed on a commercial diet (Gonzalez *et al.*, 2006). The same trend also occurred by feeding with SU diets although lower accused, with only 10% increase in n-3/n-6 ratio respect to the control group in comparison with SMC10 and SCM20 (15% and 20% respectively). ARA content in diets increased in dose-dependent manner as the amount of crab meal or sea urchin meal increased (up to 13.3 g kg^{-1} in SMC20 and 16.1 g kg^{-1} in SU16). Effect of higher feed intake

with a feed from marine origin could have an effect on red porgy nutritional fillet enhancement.

The TI and AI index was also favourable for SMC fillets as result of the healthier combination on fillet fatty acid profile respect to the control diet. Regarding to SU fillet, those indexes were unaffected respect to the CD fish mainly due to the similar SFA content in fillets.

Regarding fillet texture, the reduction of the required force to compress fillets of fish fed on SCM20, SU8 and SU16 respect to the control diet could be related to the slightly increase of fillet fat content found in these diets (table 7.4). Fish tend to increased lipid muscle concentration with growth so differences in muscle hardness between dietary groups may be partially explained by the significantly higher size, and hence higher muscle lipid content, presented in fish fed on SCM20, SU8 and SU16 respect to the control group, as reported the same effect in other cultured fish species (Izquierdo *et al.*, 2005; Suontama *et al.*, 2007).

According to fillet shelf-life (lipid oxidation) results, the inclusion of crab meal in diet delayed the lipid oxidation in muscle stored at 4ºC respect to fish fed on CD diet. The rate of lipid oxidation in post mortem flesh has been proved to vary with factors such as multi type and level of pro- and antioxidant presented in fillet (Undeland *et al.*, 1999; Bell *et al.*, 2000; Hamre *et al.*, 2004). Exogenous antioxidants are provided by the diet and retained in fish tissues especially during long-time supplementation period. Among them, carotenoids have been showed to be potential antioxidants (Bell *et al.*, 2002). In present study SMC10 diets and SU16 diets have similar carotenoids concentration (3.58 mg kg^{-1} and 3.08 mg kg^{-1} respectively) but fillet of fish fed on SU16 diet did not show lower oxidation. This suggest that against-oxidation capacity of crab meal may be associated not only with the amount of carotenoids but also probably with the whole action of various constitutes with antioxidant activity. Moreover, astaxanthin has longer showed to present stronger antioxidant functions than other carotenoids (Miki *et al.*, 1991; Bell *et al.*, 2002; Guerin *et al.*, 2003; Wang *et*

al., 2006). Crab meal was demonstrated to be richer in astaxanthin respect to FM and SU meals. However, this is speculation since antioxidants were not measured in that particular study. From the promote results more experiments are required to evaluate and identify the antioxidant capacity of the different contain pigments, active lipid or protein constituents of the crab meals toward the adequate analytical methodology.

7.5 Conclusion

Results confirm the possibility to use SMC and SU meals as potential feed ingredients in on-growing diets for red porgy, improving feed appetite and growth performance. Furthermore, the inclusion of SMC meal has a positive effect on fish skin colour and especially on the extension of self life, delaying lipid oxidation during storage time. Meanwhile, SU meal promotes yellow skin coloration in red porgy.

8. CONCLUSIONS

8. CONCLUSIONS

1. All tested ingredients from marine origin, meals of *Paramola cuvieri* crab, *Chaceon affinis* crab and *Diadema antillarum* sea urchin, presented a great attractant potential, higher respect to the fish meal. Thus, a better fish feeding response and feed consumption were observed.

2. The partial replacement of fish meal by both tested marine crabs, *Chaceon affinis* and *Paramola cuvieri,* positively influenced on the red porgy growth performance. On the contrary, the same inclusion levels of the river crab *Procambarus clarkii* meal did not improved fish growth.

3. The inclusion of sea urchin *Diadema antilarum* meal up to 16% positively influenced red porgy growth performance and significantly enhanced feed conversion and protein efficient rates.

4. The inclusion of tested crab meals, *Procambarus clarkii*, *Chaceon affinis and Paramola cuvieri,* increased the PUFA content, n-3/n-6 ratio and significantly increased the ARA content of red porgy muscle, positively affecting its nutritional value for human consumption. While, the dietary inclusión of sea urchin *Diadema antilarum* meal, both at the 8% and 16% inclusion level produced the highest ARA content in muscle.

5. Marine crab *Chaceon affinis* meal at 20% of dietary inclusion improved the red skin colour compared to that of the control fish. While the river crab *Procambarus clarkii* meal and the crab *Paramola cuvieri* meal revealed to be more efficient as pigment source for this species at the two levels used in this study.

6. Dietary inclusion of sea urchin *Diadema antillarum* meal significantly increased yellow pigments content in red porgy skin.

7. Both river crab *Procambarus clarkii* and marine crab *Chaceon affinis* meals were efficiently digested by the red porgy. The MC meal had significantly higher ADCs values than RC meal for all tested nutrients.

8. Dietary Ca/P ratio did not affect the whole body Ca/P ratio, which remain at around 1.6 for all treatments.

9. Dietary river crab meal inclusion did not influenced on the tested sensory attributes of red porgy fillets, while the inclusion of marine crab meal at 20% significantly produced higher oily odour and flavour and higher firmness and cohesiveness.

10. The dietary inclusion of all crab meals used significantly decreased lipid oxidation rate of fish fillet during its refrigerated (4ºC) storage.

9. RESUMEN EN ESPAÑOL

9.1 INTRODUCCIÓN GENERAL

9.1.1 PRODUCCIÓN DE LA ACUICULTURA

La accesibilidad de las personas hacia alimentos suficientes, inocuos y nutritivos para mantener una vida sana y activa se conoce como "seguridad alimentaria". Mejorar la seguridad alimentaria constituye un paso necesario para la iniciativa global de reducir el hambre y la pobreza en el mundo (Seligman *et al.*, 2010). El pescado constituye no sólo una fuente barata de proteínas de alta calidad sino también de vitaminas, minerales y ácidos grasos esenciales, jugando un papel importante en la mejora de la nutrición y seguridad alimentaria (FAO, 2003).

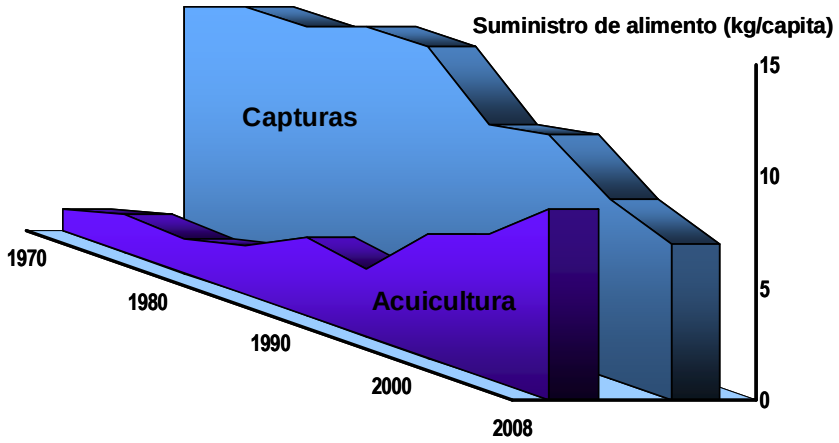

Figura 9.1 Contribución relativa global de la acuicultura y de la pesca en el consumo de pescado desde 1970 a 2008 (adaptado de la FAO, 2010).

Durante muchos años, el consumo de pescado ha dependido en gran proporción de las capturas de la pesca extractiva. Sin embargo, la captura mundial proveniente de la pesca ha dejado de crecer desde mediados de los 80, especialmente aquellos recursos que han sido explotados al máximo o más allá de su nivel de sostenibilidad (FAO, 2010). Con el fin de aliviar la presión sobre los recursos pesqueros, la acuicultura se posiciona como un sector de rápido crecimiento que puede llegar a satisfacer la creciente demanda mundial de productos del mar. Según la FAO (2010), la producción mundial proveniente de la

acuicultura ha aumentado considerablemente en los últimos 50 años, pasando a representar en relación al total de producción acuícola desde un 3,9% en 1950 a un 47% en 2008 (Figura 9.1). En contraste con la producción pesquera, el sector de la acuicultura ha mantenido una tasa media de crecimiento anual del 8,3% entre 1970 y 2008. Dentro de éste ámbito, el cultivo de peces marinos representó en 2008 un 3,4% del total de la acuicultura (FAO, 2010).

9.1.2 LAS PROTEÍNAS EN DIETAS PARA PECES

En el contexto de la alimentación animal, la proteína es referida como el contenido de proteína cruda calculado a partir del contenido de nitrógeno multiplicado por 6,25, hecho basado en el supuesto de que el contenido de nitrógeno de la proteína que se encuentra en la mayoría de los animales es de aproximadamente el 16% (NCR, 1993). Los aminoácidos son las unidades básicas de las proteínas, las cuales están compuestas por un máximo de 20 α-aminoácidos ligados entre sí por enlaces peptídicos. Algunos de ellos son esenciales ya que los peces no pueden sintetizarlos *de novo* y deben ser suministrados en la dieta (Laird y Needham, 1988).

Los peces consumen proteínas para obtener aminoácidos los cuales una vez absorbidos por el tracto gastrointestinal, son transportados a los diferentes tejidos y órganos para ser utilizados en el crecimiento de los peces y/o en la síntesis de otros compuestos o incluso para producir energía a través de procesos catabólicos (NRC, 1993). Por lo tanto, los peces como cualquier otro animal requieren una cantidad mínima necesaria de proteínas para satisfacer las necesidades de aminoácidos y lograr un crecimiento óptimo. Estas necesidades mínimas se conocen como los requerimientos cuantitativos de proteínas en la dieta, cuyo valor ha sido establecido en numerosas especies (NCR, 1993). En el caso de las especies carnívoras varía entre un 35-55% de la dieta, siendo este requerimiento marcadamente superior a los de las dietas de los animales terrestres de sangre caliente (Merzt, 1972; Smith, 1989).

Además de su importancia como nutriente, la proteína de la dieta ejerce cierto efecto sobre la calidad del agua. De hecho, uno de los principales factores que afectan la excreción de nitrógeno es la cantidad y calidad de proteína en la dieta (Lied y Braaten, 1984; Liu *et al.*, 2009).

9.1.2.1 Importancia de las proteínas en piensos para peces de cultivo

El desarrollo y la intensificación de la acuicultura pasa por la correcta formulación de los piensos destinados a alimentar a los peces. La alimentación es todavía una de las actividades con mayor costo operativo en la mayoría de las granjas de peces (Deutsch *et al.*, 2007), representando normalmente entre un 30 y un 60% del coste total de la actividad. Las proteínas son el constituyente más caro de los piensos formulados (Tacon y Metian, 2008; FAO, 2010).

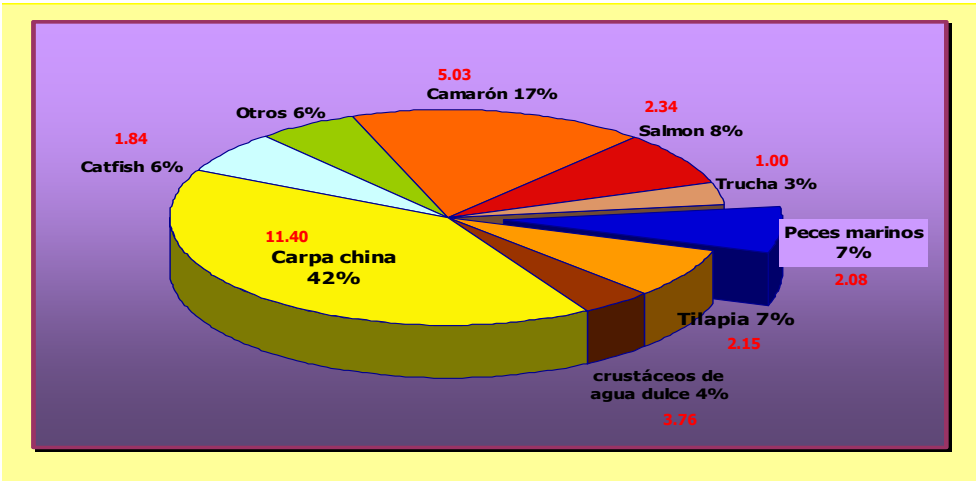

Figura 9.2 Estimación total de la producción de piensos (millones de toneladas) para las principales especies de cultivo en el 2009 (adaptado de la FAO 2004-2009).

En el 2006 la producción de piensos para acuicultura fue de 22,73 millones de toneladas (Tacon y Metián, 2008). Con el incremento de la tasa de crecimiento de la acuicultura se estimó que esta producción alcanzó los 29,3 millones de toneladas en el 2009 (Figura 9.2). La producción de carpa china consume la mayor cantidad de alimento peletizado, unos con 11,40 millones de toneladas, lo que representa el 47% de la producción total. Mientras que el grupo de los peces marinos alcanzó los 2,08 millones, representando un 7% de la producción total. En una estimación global, las necesidades de harina de pescado serían de 3,72 millones de toneladas, lo cual equivale a 16,6 millones de toneladas de recurso pelágico (Tacon y Metian, 2008; Hardy, 2010).

Resulta por tanto necesario considerar la relevancia de las proteínas no solo como nutriente sino como un factor económico importante para la sostenibilidad de la acuicultura como actividad emergente.

9.1.2.2 La harina de pescado fuente principal de proteínas en piensos formulados para peces de cultivo

La harina de pescado posee una alta calidad nutricional basada en su alto contenido proteico, adecuado perfil de aminoácidos y ácidos grasos así como la ausencia de anti-nutrientes, siendo junto a esto altamente digerible. Además, la calidad de la materia prima utilizada se refleja en algunos parámetros que la definen tales como una baja concentración de aminas biogénicas (máximos de 500 ppm de histamina).

El contenido crudo de proteína de la harina de pescado de tipo estándar oscila entre un 64% y un 67%, mientras que su contenido en grasa suele ser de un máximo del 12%. Debido a su alto contenido proteico de alta calidad, la harina de pescado ha sido utilizada como fuente principal de proteínas en la formulación de piensos granulados para alimentación animal, no sólo de acuicultura sino también de origen terrestre.

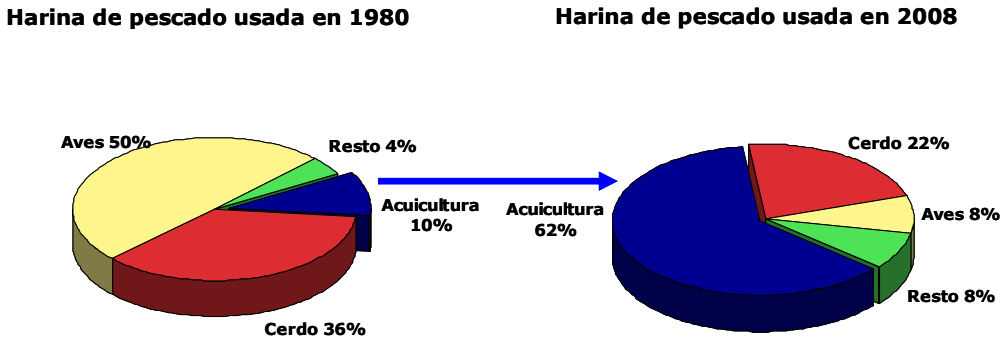

Figura 9.3 Harina de pescado usada en los diferentes sectores de cultivo animal desde 1980 hasta el 2008.

Tradicionalmente su uso se centraba en la alimentación de aves de corral, cerdos y algunos rumiantes. Sin embargo, la reciente expansión de la acuicultura ha contribuido en gran parte en la creciente demanda mundial de este producto (IFFO, 2009). La demanda de harina de pescado por el sector de la acuicultura se ha incrementado en casi un 50% desde 1980 al 2008, y de acuerdo con New (2002) se estima que pueda llegar al 70% para el año 2012. Por su parte, en los otros sectores de la alimentación animal, su uso ha disminuido considerablemente (IFFO, 2009) (Figura 9.3). Entre los grupos de especies acuícolas, los mayores

consumidores de harina de pescado en el 2006 fueron los camarones, seguidos del salmón, la carpa china, la trucha, la anguila y la tilapia (Hardy, 2010).

9.1.2.3 Situación actual de la producción mundial de harina de pescado

Desde el año 1950 el incremento de la demanda de harina de pescado, principalmente para su uso en alimentación animal, ha hecho que ésta se convierta en un producto de alto valor. Sin embargo, los recursos de anchoveta (*Engraulis ringens*), de la que mayoritariamente proviene la harina de pescado, no son estables en el tiempo. La población de esta especie decrece notablemente cuando ocurren fenómenos climáticos como "*el Niño*". Por otra parte, la pesca de esta especie es estacional, regulada por una férrea legislación administrativa encargada de controlar las épocas de veda con el fin de salvaguardar la salud y abundancia de los stocks. Como resultado de todo ello, la producción global de harina de pescado ha experimentado una notable reducción en los últimos años, pasando de 2,3 millones de toneladas en el año 2000 a 1,34 millones en el 2009, lo cual supone una reducción del 58% (FAO Fish Globe, 2010). Esta reducción se debe principalmente al descenso de las capturas de Perú, un 70% menores que las obtenidas en el año 2000 (FAO Fish Globe, 2010).

El principal país productor y exportador de harina de pescado es Perú seguido de Chile, mientras que el mayor consumidor es China seguido de Japón, Taiwán y la Unión Europea (FAO Fish Globe, 2010). Con respecto a Chile, éste país incrementó su producción en el 2009 con el objetivo de cubrir la fuerte demanda por parte de China. Sin embargo, los graves daños producidos por el terremoto ocurrido en el 2010 han provocado un corte en la producción y exportaciones chilenas. Por otra parte, la posible reducción de las capturas peruanas para los próximos años junto con el impacto producido por el fenómeno climático "*el Niño*" reducirá aún más la disponibilidad de la harina de pescado en el mercado (FAO Fish Globe, 2010).

Teniendo en cuenta este escenario, las predicciones futuras en la producción de harina de pescado no podrían ser más pesimistas e inciertas. Con el continuo incremento de la demanda, los precios en el mercado subirán notablemente, lo que unido a su dependencia, va a hacer que la acuicultura se convierta en una actividad cada vez más costosa y menos sostenible. Es por ello

que su uso en la alimentación para peces será cada vez menor, estimándose una reducción del 31,3% desde el 2006 al 2020 (Gatlin *et al.*, 2007; Tacon *et al.*, 2006), Esto obligará a remplazar esa harina de pescado por otras fuentes proteicas que resulten mas baratas y disponibles (Drew *et al.*, 2007).

9.1.3 INGREDIENTES PROTEICOS ALTERNATIVOS A LA HARINA DE PESCADO EN DIETAS PARA PECES.

9.1.3.1 Fuentes de proteínas alternativas de origen terrestre

Con el fin de aliviar los problemas relacionados por el incremento de los precios de la harina de pescado, han sido realizados numerosos estudios sobre la sustitución de la harina de pescado por otros ingredientes proteicos alternativos en los piensos para la acuicultura.

En este sentido, el efecto de la sustitución parcial o total de la harina de pescado por fuentes proteicas de origen vegetal ha sido estudiada en varias especies de peces con diferentes grados de éxito en los resultados de crecimiento, utilización del alimento así como en la calidad final del filete (Moyano *et al.*, 1992; Gomes *et al.*, 1995; Robaina *et al.*, 1997, 1999; Hardy, 1996; Kaushik *et al.*, 2004; Francesco *et al.*, 2007; Shafaeipour *et al.*, 2008). La harina proveniente de la soja es una de las fuentes proteicas de origen vegetal más ampliamente utilizadas (Robaina *et al.*, 1997, 1999; Mambrini *et al.*, 1999; Refstie *et al.*, 2000; Wang *et al.*, 2006; Venou *et al.*, 2006; Overland *et al.*, 2009), aunque también se han realizado sustituciones con harina de guisantes (Hardy *et al.*, 1996; Gouveia *et al.*, 2000; Borlongan *et al.*, 2003; Sánchez-Lozano *et al.*, 2009; Davies *et al.*, 2010), diferentes especies de lupinos (Robaina *et al.*, 1995; De La Higuera *et al.*, 1988; Glencross *et al.*, 2003; Serrano *et al.*, 2011), así como otras plantas (Watanabe *et al*, 1997; Davies *et al.*, 1997; Mente *et al.*, 2003; Palmegiano *et al.*, 2006; Sánchez-Lozano *et al.*, 2007; 2009). A pesar de los buenos resultados obtenidos, se ha comprobado que altos niveles de inclusión de proteínas vegetales producen problemas relacionados con el déficit de amino-ácidos, presencia de anti-nutrientes, baja palatabilidad, pobre digestibilidad así como déficit de ácidos grasos esenciales (Francis *et al.*, 2001). También se han descrito lesiones en el epitelio gastrointestinal (Caballero *et al.*, 2003; Penn *et al.*, 2011). De los

resultados en los diferentes estudios se ha comprobado que las especies omnívoras toleran mayores dosis de inclusión de proteínas vegetales (Kaushik *et al.*, 1993; Robinson y Li, 1994; El-Saidy y Gaber, 2002).

A pesar de que la mejora en la tecnología del procesado de la materia prima y del alimento ha solventado numerosos problemas de las fuentes proteicas vegetales (Drew *et al.*, 2007), algunos autores como Tacon y Metian (2008), argumentan que si bien se intenta hacer dietas más económicas, el uso de fuentes proteicas de origen vegetal podría incrementar aún más el costo de los piensos ya que sería necesario tratar la materia prima para eliminar anti-nutrientes o añadir ciertos amino-ácidos para mejorar el perfil nutricional de la dieta.

Con respecto las fuentes proteicas de origen animal provenientes de sub-productos, éstas han demostrado tener un gran potencial en la sustitución de la harina de pescado, especialmente en especies carnívoras (Bureau *et al.*, 2000). Sin embargo, el uso de este tipo ingredientes promueve resultados variables de crecimiento y una reducción en la digestibilidad de los nutrientes debido a su alto contenido en cenizas y en lípidos y fibra poco digeribles (Robinson y Li, 1996; Kureshy *et al.*, 2000). Además, un excesivo reemplazo de la harina de pescado por harina de subproductos de origen animal en dietas para peces afectaría de forma adversa la comercialización debido a una peor percepción del consumidor, ya sea por cambios sensoriales o por los menores beneficios para la salud al reducir el contenido en los ácidos grasos omega-3 (Pigott, 1989; Williams *et al.*, 2003).

9.1.3.2 Productos y sub-productos de origen marino como ingredientes novel en piensos para la acuicultura

Extendiendo el rango de las materias primas desde las cuales se pueden obtener ingredientes idóneos y disponibles con los que sustituir la harina de pescado en dietas para la acuicultura, los recursos provenientes del medio marino han recibido un creciente interés en los últimos años. Un perfil de ácidos grasos y amino-ácidos más semejante a la alimentación de las especies de acuicultura en su medio salvaje, repercutiría positivamente en la salud de los animales alimentados con harinas de origen marino más que si lo hacen con fuentes proteicas tanto vegetales como animales de origen terrestre.

Estos recursos incluyen los llamados by-catch o pescas incidentales

(especies no objetivo de pesca más las que si son objetivo pero están por debajo de la talla mínima permitida), descartes (serían los by-catch más las especies deformes o dañadas) y los desechos del procesado de los productos marinos (restos de partes comestibles como no comestibles de las especies). Estos recursos, al ser de origen marino, poseen un extraordinario potencial nutricional basado en su alto contenido en bio-componentes de alto valor biológico (minerales, lípidos, amino ácidos, polisacáridos, vitaminas, pigmentos…etc.), pudiendo ser utilizados en diversas aplicaciones en vez de acabar en su mayoría como residuos.

Se estima que mundialmente los descartes de la pesca representan un 25% de las capturas totales (Rustad, 2003; Ferraro *et al.*, 2010), mientras que la cantidad de subproductos varía dependiendo de las tallas de los peces, temporada estacional y zonas de pesca, pudiendo alcanzar en muchas ocasiones hasta un 50% de los alimentos marinos que son procesados (Falch *et al.*, 2006; Rustad *et al.*, 2011). En la Unión Europea estos descartes y sub-productos representan un total de 5,2 millones de toneladas anuales (AWARENET, 2004; Mahro y Timm, 2007; Ferrearo *et al.*, 2010), siendo el fileteado y ahumado de los peces el que genera la mayor cantidad (50–75% del pez) con un total de 3,17 millones (AWARENET, 2004; Ferrearo *et al.*, 2010). Se estima que el actual incremento en el consumo de productos de la pesca junto a cambios en los hábitos alimenticios hacia alimentos listos para consumir, aumentará más los desechos de este tipo en el futuro. Por lo tanto, la utilización de estos desechos como recursos nace en parte de la necesidad de gestionar de forma adecuada y útil estos residuos, los cuales hasta ahora constituyen un serio problema de contaminación del medio ambiente (Arvanitoyannis y Kassaveti, 2008). Además, la Unión Europea ha establecido el 2015 como fecha límite en el que se hará obligatorio la recuperación y reciclaje de los subproductos de la pesca y acuicultura (Rustad *et al.*, 2011).

El uso de este tipo de subproductos no es nuevo, siendo ampliamente investigados en diferentes aplicaciones tales como fertilizantes (Thingoc y Kyun;, 2011), en alimentación animal (Naylor *et al.*, 2008), alimentos para consumo humano (Falch *et al.*, 2006; Rustad *et al.*, 2011), así como para la extracción de bio-componentes activos (Kim *et al.*, 2006; Ferraro *et al.*, 2010). En acuicultura, el uso de ingredientes provenientes de los descartes, incluidos los by-catch y desechos del procesado de los productos provenientes tanto de la pesca como de la

propia acuicultura, reducirían la dependencia de la cara harina de pescado en la formulación de piensos y ayudaría a reciclar estos recursos.

Entre la gran mayoría de material proteico producido a partir de este tipo de materia prima se encuentra harina de pescado y concentrado proteico de pescado tanto como silage (los restos son tratados con diferentes ácidos como el ácido láctico proveniente de la fermentación de carbohidratos) o bien como hidrolizados (tratados enzimáticamente). El uso experimental de silage de pescado como fuente de proteínas ha sido probado en algunas especies como el salmón *Salmo salar* (Jackson *et al.*, 1984; Lie *et al.*, 1988; Parrish *et al.*, 1991; Heras *et al.*, 1994; Espe *et al.*, 1999; Hevrry *et al.*, 2005), la trucha arco iris *Oncorhynchus mykiss* (Rungruangsak y Utne, 1981; Hardy *et al.*, 1983; Stone *et al.*, 1989), la tilapia *Oreochromis niloticus* (Fagbenro, 1994; Fagbenro y Jauncey, 1994) e incluso el abalón *Halotis fulgens* (Viana *et al.*, 1996). Los resultados generalmente muestran la idoneidad de estos ingredientes en su uso como fuentes proteicas, con algunos estudios demostrando su alta digestibilidad incluso con reemplazos de hasta un 75% de la harina de pescado en la dieta (Vidotti *et al.*, 2002; Goddard *et al.*, 2003).

A pesar de las diferentes calidades de las harinas, éstas van a ser el producto mas valioso obtenido a partir de los subproductos marinos (Ferraro *et al*, 2010). Su producción global varía de entre 5,5 y 7,5 millones de toneladas al año (Hardy y Tacon, 2002), siendo su composición un reflejo la materia prima con la que ha sido producida, normalmente con un contenido en proteínas del 56-66%, en lípidos del 6.3-8.0% y en cenizas del 19-23% (Rathbone *et al.*, 2001). Evidentemente, su calidad referida a dicha composición es inferior a la harina de pescado convencional (Tabla 9.1). Este tipo de harinas de pescado ha sido evaluadas en dietas para la dorada *Sparus aurata* (Kotzamanis *et al.*, 2001), salmón *Oncorhynchus kisutch* (Rathbone *et al.*, 2001), trucha arco iris (Hardy *et al.*, 2005), tambor rojo *Scianops ocellatus* (Li *et al.*, 2004; Whiteman y Gatlin III, 2005) e incluso la tilapia (Goddard *et al.*, 2008; Saidi *et al.*, 2010). Los resultados demuestran que las harinas de especies de descartes y de subproductos del procesado son efectivas para el reemplazo total o parcial de la harina de pescado en dietas para especies de acuicultura.

Sin embargo, el uso de estas posee una serie de limitaciones relacionadas

con las fluctuaciones en la abundancia de la materia prima de la que están hechas, lo cual repercute en la calidad final de la harina. De esta forma, el producto final es extremadamente variable en composición. El alto contenido en cenizas (debido al gran contenido en huesos y cartílagos de la materia prima) de estos ingredientes constituye también otra limitación en su utilización, ya que excede en la mayoría de los casos las regulaciones impuestas por algunos países para prevenir las descargas contaminantes de fósforo al medio (Goddard *et al.*, 2008). Más aún, las harinas provenientes de los subproductos de la acuicultura no deberían utilizarse para alimentar a la misma especie (reciclaje intra-específico), como medida de bio-seguridad para prevenir la posibilidad de extender ciertas enfermedades (Tacon y Metian, 2008).

Tabla 9.1 Perfil nutricional de harinas provenientes de by-catch y de restos del procesado (adaptada de Goddad *et al.*, 2008).

	COM	MBM	SPM	CAM	TCW
Composición proximal (g kg^{-1})					
Poteína	672	662	629	565	643
Lipidos	114	63	68	72	79
Cenizas	130	161	224	244	173
Humedad	71	82	59	84	70
Amino ácidos esenciales (g 100 g^{-1})					
Arginina	5.49	6.04	5.52	6.38	5.62
Histidina	1.86	2.36	2.37	2.52	2.71
Isoleucina	3.12	3.73	3.32	3.75	3.49
Leucina	6.75	7.70	6.94	8.10	7.01
Lysina	6.66	8.06	7.15	8.44	6.84
Metionina	3.55	4.10	3.75	3.49	3.64
Phenilalanina	6.20	7.39	6.70	7.50	6.77
Threonina	4.17	4.73	4.21	4.86	4.39
Tritofáno	1.04	1.29	1.15	1.29	1.17
Valina	3.96	4.32	3.92	4.37	4.05
Composición minela (% peso seco))					
Calcio	1.29	3.61	2.56	2.74	2.94
Fosfóro	0.88	1.85	1.32	1.48	1.54

COM, harina comercial de anchoveta; MBM, harina de mezcla de especies bentónicas; SPM, harina pequeñas especies pelágicas; CAM, harina de subproductos de pez gato; TCW, harina de subproducto del procesado del atún.

9.1.3.2.1 Productos y subproductos de cangrejos

Entre todos los descartes y sub-productos generados por la industria del procesado, los provenientes de especies de crustáceos constituyen una fuente rica

en todo tipo de nutrientes de alta calidad, especialmente proteínas, minerales, pigmentos carotenoides y quitina (Lee, 1990; Negro *et al.*, 2000; Cremades *et al.*, 2003; Pérez-Gálvez *et al.*, 2008). Los cangrejos representan un 20% de todos los crustáceos capturados y cultivados en el mundo (FAO, 2009). Generalmente, los by-catch y las especies fuera del talla objetivo son descartados. Durante el procesado el caparazón y las vísceras también son descartes y pueden llegar a suponer el 50% del volumen total de la materia prima (Islam *et al.*, 2004).

Los desechos de cangrejos son procesados para obtener componentes de alto valor añadido como la quitina, enzimas y pigmentos, especialmente astaxantina (Stewart y Noyes-Hull, 2010), los cuales pueden servir como excelentes fuentes de materia prima en la generación de ingredientes para la alimentación animal (Shahidi y Synowiecki, 1991). En esta línea, la composición de harinas provenientes de sub-productos de cangrejo es altamente variable en función del origen de su materia prima (animal entero o despojos), de la especie y del método de procesado. Generalmente tienen una gran cantidad de proteínas (25-50%), carotenoides (17-37%) y cenizas (15-35%), (Lee *et al.*, 1982; Sachindra *et al.*, 2005).

En acuicultura, las harinas de cangrejo han sido utilizadas para sustituir la harina de pescado pero también como fuentes de pigmentos para colorear la piel o el filete de algunas especies. La astaxantina y cataxantina sintéticas eran comúnmente los carotenoides más ampliamente utilizados en las dietas para la pigmentación de las especies de acuicultura (Torrisen, 1990; Metusalach *et al.*, 1996; Higuera-Ciapara *et al.*, 2006). Sin embargo, la inclusión de este tipo de pigmentos sintéticos en dietas para la alimentación animal no es muy recomendable debido al alto coste de éstos, motivo por el cual en estos últimos años se vienen utilizando fuentes naturales de carotenoides para reemplazar a las sintéticas. Se ha comprobado que el contenido de carotenoides de harina de cangrejo mejora notablemente la pigmentación de especies de salmónidos (Satio y Regier, 1971; Spinelli y Mahnken, 1978; Coral-Hinostroza *et al.*, 1997; 1998; Lyons *et al.*, 2001) y en platija *Paralichthys lethostigma* (González *et al.*, 2006).

Por otro lado, el reemplazo parcial de la harina de pescado por harina de cangrejo de especies como *Pleuroncodes planipes* y *Cancer pagurus* en dietas para camarón *Litopenaeus vannamei* y bacalao Atlántico *Gadus morhua*

respectivamente, ha dado buenos resultados de crecimiento en ambas especies (Goytortua-Bores *et al.*, 2006; Toppe *et al.*, 2006; Villarreal *et al.*, 2006). En ambos estudios fue constatado que la inclusión de harina de cangrejo en la dieta potencia la ingesta de la misma, demostrando uno de los efectos positivos de estos ingredientes como atractantes y altamente palatables. Aún más, una mejor eficiencia alimenticia fue observada en el caso del camarón, sugiriendo la presencia de promotores del crecimiento que provocan una mejor utilización proteica de la dieta.

A pesar de que el alto contenido de cenizas y quitina limita la inclusión de este tipo de ingredientes al reducir la digestibilidad de otros nutrientes, algunos estudios han demostrado buenos valores de digestibilidad para varios tipos de harina de cangrejo en diferentes especies como el abadejo *Melanogrammus aeglefinus* (82%) (Tibbetts *et al.*, 2004), el bacalao (89%), el fletan Atlántico *Hippoglossus hippoglossus* (88%) (Tibbetts *et al.*, 2006) y la cobia *Rachycentron canadum* (89%) (Fines y Holt, 2010). De acuerdo con distintos autores, algunos peces muestran habilidades biológicas específicas para digerir debidamente este tipo de ingredientes sin ningún efecto adverso sobre la digestibilidad de los nutrientes, diferencias relacionadas con las secreciones ácidas digestivas adecuadas y complejo enzimático específico (Danulat y Kausch, 1984; Wood *et al.*, 1992; Gutowska *et al.*, 2004; Toppe *et al.*, 2006). Las nuevas tecnologías en el procesado de los ingredientes podrían mejorar aún más el valor nutricional de estos recursos y así proveer nuevos ingredientes de gran interés en el desarrollo de acuicultura.

Existe poca información sobre las especies de cangrejos capturadas incidentalmente y su cantidad. Algunos autores como Pingguo (2005) han reportado que cantidades de 767.000 kg de la especie *Neolithodes grimaldii* son capturados y descartados durante la pesquería de rodaballo en aguas de Canadá, ejemplo de que las capturas incidentales ocurren muy frecuentemente, a pesar de lo cual los suministros no son regulares o las cantidades son insuficientes para mantener una producción rentable en las plantas de procesado, limitándose la utilización de estos recursos como fuentes de proteína. A pesar de ello, su aprovechamiento de forma local podría generar pequeñas industrias y sería una forma eficiente de utilizar los by-catch a pequeña escala (Match *et al.*, 2010).

9.1.3.2.1.1 La quitina, polisacárido de cadena larga, como uno de los biocomponentes con mayor potencial encontrado en los crustáceos

El exoesqueleto de los crustáceos constituye una de las principales fuentes naturales del polisacárido quitina (Healy *et al.*, 2003). Químicamente, la estructura de la quitina consiste en unidades de acetil-2-amino-2-deoxy-D glucopyranosa ($C_8H_{15}O_6N$) ligados por enlaces glucosídicos (1→4) (Díaz-Rojas *et al.*, 2006). El mayor contenido de quitina sobre peso seco lo presenta el caparazón de los camarones (30-40%) seguido del de los cangrejos (15-30%) (Qin y Aboh, 1997). Comercialmente, los caparazones o partes duras de estos crustáceos, provenientes en su mayoría del procesado de los mismos, son utilizados como materia prima para producir quitosano o chitosán en una cantidad de 37.000 toneladas al año (Ferraro *et al.*, 2010). El chitosán es obtenido tras el procesado químico de la quitina, previa (1) trituración de los exoesqueletos de crustáceos, (2) descalcificación o eliminación de la materia inorgánica (metales, sales y carbonato cálcico) mediante tratamiento ácido y (3) desproteinización mediante la extracción de la materia proteica en medio alcalino y proteasas derivadas de bacterias (Díaz-Rojas *et al.*, 2006).

En acuicultura, la quitina y el quitosano han sido probados en algunas especies como inmuno-estimulantes, mostrando su capacidad protectora hacia algunas enfermedades bacterianas (Anderson y Siwicki, 1994; Esteban *et al.*, 2000; Ortuno *et al.*, 2000; Esteban *et al.*, 2001; Cuesta *et al.*, 2003; Wang y Chen 2005; Gopalakannan y Venkatesan, 2006).

Con respecto a sus efectos sobre los parámetros de crecimiento, se ha encontrado que la adición de quitina y quitosano en la dieta provoca resultados antagónicos según la especie. Así, Shiau y Yu (1999) observaron una reducción del crecimiento en tilapia *Oreochromis niloticus* × *O. auratus* después de alimentar a los peces con dietas suplementadas al 2%, 5% y 10% de quitina y quitosano, sugiriendo una interferencia de estos componentes con la absorción de los nutrientes. En carpa común *Cyprinus carpio*, menores contenidos de quitosano (1%) en la dieta han mejorado el crecimiento de los animales (Gopalakannan y Venkatesan, 2006), especulando que el quitosano podría ejercer un rol importante en la mejora de la digestión y absorción de los nutrientes cuando es añadido a bajos niveles. Sin embargo, en periodos largos de alimentación aun con bajos

niveles de inclusión, puede reducirse el crecimiento debido al desarrollo de alguna intolerancia a la quitina. En otras especies, como dorada japonesa *Pagrus major*, anguila japonesa *Anguilla japonica*, o seriola *Seriola quiqueradiata,* la alimentación con dietas conteniendo niveles de quitina o chitosán de hasta un 10% no afectó al crecimiento de ninguna de ellas (Kono *et al.*, 1987).

Destacar que un estudio reciente realizado con cobia (Fines y Holt, 2010) se ha constatado que las actividades de las enzimas quitasa y quitobiasa así como la digestibilidad aparente de la quitina (67-78%) eran altas. Además, no se encontraron diferencias entre la digestibilidad de las proteínas, lípidos o energía de las harinas de crustáceos y la harina de pescado. Con un contenido calórico para la quitina de 17,1 kJ g^{-1} (Gutoska *et al.*, 2004), ésta debe de constituir un gran porcentaje de la energía digerida en aquellas especies cuya alimentación en el medio salvaje está basada en crustáceos y por tanto la ingesta de quitina es considerable (Fines y Holt, 2010). Estos autores sugieren que los ingredientes ricos en quitina, como son las harinas de crustáceos, pueden ser utilizados para reemplazar carbohidratos y harina de pescado en la dieta. Además, también se plantea la importancia de realizar pruebas en otras especies de hábitos alimenticios similares para conocer el potencial de uso de este tipo de ingredientes en dietas para la acuicultura.

9.1.3.2.1.2 Inclusión de harina de cangrejo y su efecto de la calidad final del filete

La calidad del pescado envuelve conceptos diferentes dependiendo de la industria que lo procesa o el conocimiento del consumidor. De esta forma, las propiedades organolépticas, valor nutricional y vida útil del filete son una serie de características que condicionan la percepción de la calidad del pescado desde el punto de vista del consumidor. Todas estas características mencionadas dependen fuertemente de la composición del filete de pescado, la cual a su vez está condicionada por otros aspectos cualitativos entre los cuales destaca la composición de la dieta (Lie *et al.*, 2001; Grigorakis, 2007). Parece ser que todos los atributos cualitativos del filete de pescado están correlacionados con la cantidad y calidad de los lípidos depositados en el mismo (Waagbo *et al.*, 1993; Guillou *et al.*, 1995; Morris *et al.*, 1995). Ha sido demostrado que el metabolismo y acumulación de lípidos en los peces están influidos por el tipo de proteínas de la

dieta (Lupatsch *et al.*, 2002; Francesco *et al.*, 2007), y ello podría afectar significativamente a parámetros de la calidad del filete tales como los atributos sensoriales (Williams *et al.*, 2003b; Karlsen *et al.*, 2006; Suontama *et al.*, 2007b) y la textura (Hernández *et al.*, 2007).

Son pocos los estudios sobre el efecto que la inclusión de harina de crustáceos en la dieta ejerce en la calidad final del filete. Fernández (2006) reporta un incremento significativo de la relación ω-3/ω-6 en músculo de besugo *Pagellus bogaraveo* alimentado con un 30% de inclusión de harina de crustáceos (10% de harina de krill y 20% de harina de caparazón de langostino). Por otro lado, Suontama *et al.* (2007a), encontraron que la sustitución de un 20% de harina de pescado por harina de krill *Thysanoessa inermes* no modificó el perfil de ácidos grasos del filete de salmón Atlántico ni del fletán mientras que en el mismo trabajo mayores sustituciones de la harina de pescado tanto con krill (60%) como con harina del amphipodo *Themso libellula* (40%), produjeron cambios significativos en el perfil de ácidos grasos. En platija *Paralichthys lethostigma* alimentada con una dieta que contenía un 5% de harina de cangrejo (González *et al.*, 2006), los resultados obtenidos evidenciaron una mejora de la calidad nutritiva del filete debido a un incremento significativo en el contenido de los ácidos grasos ARA (20:4n-6), adrénico (22:4n-6) y DHA (22:6n-3) junto a una mayor ratio n-3/n-6. Esto sugiere que la utilización de harinas de cangrejo en la dieta, incluso a baja concentración, podría mejorar la calidad final de producto.

9.1.3.2.2 Otros ingredientes potenciales de origen marino: sub-productos provenientes de la industria del procesado de equinodermos

Algunos trabajos han sido realizados para estudiar el posible uso potencial de recursos marinos provenientes de los sub-productos de la industria del procesado de los equinodermos, tanto los erizos como las holoturias. Durante el procesado comercial de los erizos sólo las gónadas tienen valor comercial, mientras que de las holoturias sólo se eligen las bandas musculares. El resto de las partes del cuerpo de estos animales son desechadas junto con los que no cumplen las características de talla o de calidad para ser debidamente comercializados. En el caso de la especie holoturia Atlántica *Cucumaria frondosa* y del erizo verde de mar *Strongylocentrotus droebachiensis*, se estima que las

cantidades descartadas de estos animales durante su procesado pueden llegar a alcanzar hasta un 50% y 80% respectivamente (Mamelona *et al.*, 2010). La composición nutricional de la materia prima constituida por los descartes de estos animales revela un contenido rico en muchos nutrientes tales como proteínas (4,5-14,5% sobre peso húmedo y un 35% de amino ácidos esenciales) y lípidos (1,9-4,6% y un 44% de ácidos grasos poliinsaturados, 17% de EPA). En términos de vitaminas, estos recursos han mostrado ser ricos principalmente en alpha-tocoferol o vitamina E (Zhong *et al.*, 2007; Mamelona *et al.*, 2010a;b). Pero a pesar de ser destacado en la bibliografía su potencial nutritivo (De La Cruz-García *et al.*, 2000; Zhong *et al.*, 2007; Mamelona *et al.*, 2010a;b), apenas han sido probados en dietas experimentales.

9.1.4 El BOCINEGRO (*Pagrus Pagrus*; Linnaeus, 1758)

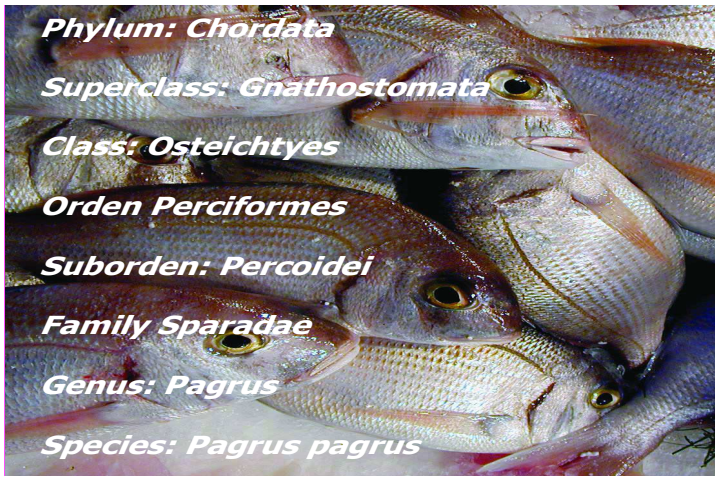

Figura 9.4 Clasificación taxonómica del bocinegro.

9.1.4.1 Apariencia externa, distribución geográfica, hábitat natural y reproducción

El bocinegro (Figura 9.4) es un pez marino perteneciente a la familia de los espáridos cuya distribución geográfica es bastante amplia. Se le puede encontrar a lo largo tanto de la costa oeste del Atlántico (desde el Norte de California hasta Argentina) como en la del este, desde las Islas Británicas hasta el sur de Senegal, incluyendo los archipiélagos de Madeira, Canarias y Cabo Verde. También habita en el mar Mediterráneo (Manooch y Hassler, 1976; Bauchot *et al.*, 1981; Pajuelo *et al.*, 1996).

El bocinegro es una especie demersal que habita en los fondos de aguas templadas (Afonso *et al.*, 2008). Los individuos juveniles son encontrados con frecuencia en fondos arenosos, a profundidades de entre 20-50 metros, mientras que los de mayor talla permanecen sobre fondos rocosos a profundidades de alrededor de 50 metros. De acuerdo con Manooch (1976) y Alekseev (1983), desde el punto de vista reproductivo el bocinegro es una especie hermafrodita, desarrollando el tejido de la gónada correspondiente al ovario en su primera estación reproductiva, para en segunda y posteriores desarrollar el tejido testicular. Este cambio de sexo ocurre cuando los individuos alcanzan aproximadamente un tamaño comprendido entre los 32,5 y los 42,5 cm. La maduración de las gónadas en el medio natural se produce cuando tienen unos 3-4 años de vida (Manoocher y Alekseev, 1990), pero en cautividad llegan a presentar una primera maduración sexual como hembra entre los 250-300 gramos de peso total. La temporada de desove va desde enero hasta abril, cuando la temperatura del agua se encuentra entre unos 16 y 21ºC.

9.1.4.2 Dieta natural del bocinegro

Los hábitos alimenticios de los bocinegros son similares a pesar de su amplia distribución geográfica (Manooch, 1977; Chakroun-Marzouk y Kartas, 1987; Labropoulou *et al.*, 1999). En un estudio detallado sobre los mismos (Castriota *et al.*, 2006), se reveló que de unas 78 especies epibentónicas consumidas por el bocinegro, los crustáceos eran el grupo dominante seguido por moluscos y algunos peces. También se encontraron erizos marinos aunque consumidos en una menor proporción. Además, a pesar del gran número de especies identificadas, solo algunas de ellas estaban presentes en una proporción importante de la dieta, indicando una alimentación selectiva de estos animales basada principalmente en especies como los cangrejos ermitaños, cangrejos del orden braquiura y gasterópodos.

El conocimiento de la dieta natural de esta especie ayudará a entender los mecanismos nutricionales envueltos en la biología de su alimentación, lo que redundará positivamente a la hora de formular dietas para su cultivo.

9.1.4.3 Cultivo del bocinegro

La diversificación de la acuicultura a través de la producción de nuevas especies de cultivo es una de las iniciativas más importantes para la estabilización del sector productivo, actualmente caracterizado por altas producciones de unas pocas especies. A la hora de seleccionar una especie nueva en acuicultura, algunos aspectos importantes son una buena tasa de crecimiento bajo las condiciones inherentes al cultivo, alto valor en el mercado y una esperada demanda en su consumo. Además, los aspectos técnicos y biológicos de su cultivo deben ser posibles y estar superados (Suquet *et al.*, 2002).

En este sentido, el bocinegro es apreciado fundamentalmente por la alta calidad de su carne, caracterizada por su delicado sabor, firmeza y su alto contenido en proteína y bajo en grasa. Esta especie, que posee una fuerte demanda en el mercado de la región mediterránea (Kokokiris *et al.*, 2006), debido a la prolongada sobrepesca, ha sufrido notables reducciones en algunas poblaciones (Haimovici, 1998: Vaughan *et al.*, 2002; Afonso *et al.*, 2008), no siendo suficientes las capturas salvajes para cubrir la actual demanda del mercado. Estudios preliminares llevados acabo con esta especie bajo condiciones de cultivo han mostrado buena adaptabilidad y crecimiento, desoves espontáneos, buenas tasas de crecimiento y no serios problemas de enfermedad o mortalidad (Kentouri *et al.*, 1994; Kokokiris, 1998; Cejas *et al.*, 1999). Estos logros hacen que el bocinegro se convierta en una de las especies de peces marinos prioritarios para la diversificación de la acuicultura (Kentouri *et al.*, 1995; Hernández-Cruz *et al.*, 1999).

9.1.4.3.1 Requerimientos dietarios de proteínas y pigmentos carotenoides

Una de las mayores limitaciones en el cultivo de esta especie consiste en la pérdida de su característica coloración rojiza bajo condiciones de cultivo (Kentouri *et al.*, 1995; Klios *et al.*, 1997; Basurco y Abellán, 1999). La conservación de su color natural y la similitud en apariencia externa a su homólogo salvaje son de gran importancia desde el punto de vista comercial ya que de ello dependerá la aceptación o rechazo del producto por parte del consumidor (Shahidi *et al.*, 1998; TECAM, 1999). Los peces no son capaces de sintetizar carotenoides *de novo* (Goodwin, 1984) y por tanto, bajo condiciones de cultivo, los carotenoides deben

ser suministrados en la dieta. De esta forma, la mayoría de los estudios realizados en esta especie han estado principalmente enfocados a conseguir una coloración de la piel similar a la de los individuos salvajes, probando diferentes fuentes y niveles de carotenoides en la dieta. De los diferentes carotenoides testados, solo la astaxantina en su forma esterificada ha dado los resultados deseados (Cejas *et al.*, 2003; Chatzifotis *et al.*, 2005; Kalinowski *et al.*, 2005; Tejera *et al.*, 2007). En el medio salvaje, el bocinegro obtiene la astaxantina mediante la ingesta de crustáceos, los cuales constituyen el alimento natural de esta especie (Labropoulou *et al.*, 1999; Castriota *et al.*, 2005). Por ello se vienen empleando harinas de crustáceos como fuente principal de astaxantina esterificada (Cejas *et al.*, 2003; Kalinowski *et al.*, 2005; 2007).

La segunda gran limitación de esta especie, desde el punto de vista nutritivo, es su alto requerimiento de proteínas en la dieta, alrededor del 50% en peso seco (Schuchart *et al.*, 2008). Algunos trabajos han sido realizados en bocinegro sustituyendo la harina de pescado por otra fuente de proteínas adecuada para la especie. En esta línea, Schchardt (2005) estudió el efecto de la sustitución de la harina de pescado por harina de krill hidrolizado. En general, una buena utilización del alimento, un buen crecimiento y una mejora de la coloración de la piel del animal fueron obtenidos con la dieta que contenía un 20% de inclusión de harina de krill (25% de sustitución de la proteína de pescado) cuando el contenido en lípidos era bajo (15%). En el mismo estudio se intentó reducir el contenido de proteínas junto a una subida del contenido de lípidos, provocando una reducción del crecimiento de los animales.

El bocinegro es considerado un pescado magro ya que su contenido en grasa no supera el 5% (Ruedas *et al.*, 1997). En esta línea, tanto la cantidad como la calidad de la proteína de la dieta para esta especie son consideradas más importantes que otros ingredientes energéticos menos caros como los lípidos. Consecuentemente, para que el bocinegro sea una especie competitiva en el mercado no basta con conseguir una coloración óptima, sino que también es conveniente reducir la harina de pescado en su dieta, ya que ello permitiría obtener piensos más económicos. De esta forma, el desarrollo de nuevas fuentes de proteínas y pigmentos naturales en dietas de engorde para el bocinegro ayudaría a la consolidación del cultivo comercial de esta especie.

Bajo estas premisas, el presente estudio está enfocado en la identificación de ingredientes potenciales, especialmente de origen marino, alternativos a la harina de pescado, que contengan pigmentos naturales adecuados para dietas en la especie bocinegro. Los resultados de este estudio ayudarán a mejorar el conocimiento básico nutricional y metabólico de la especie. Todos los aspectos estudiados contribuirán a la producción de una dieta de engorde para el bocinegro más semejante a su dieta del medio natural.

9.2 OBJETIVOS

El objetivo general de esta tesis es evaluar ingredientes potenciales que ayuden a reducir la cantidad de harina de pescado en dietas de engorde para el bocinegro. Todos los estudios fueron propuestos para elucidar la respuesta dietaria del crecimiento, utilización de los nutrientes y la calidad final del filete, así como la coloración de la piel similar a la de los individuos salvajes. Además, algunos de los estudios fueron llevados a cabo para obtener más conocimientos sobre la respuesta metabólica del bocinegro.

Para alcanzar esta premisa se abordan los siguientes objetivos:

1. Evaluar el efecto del reemplazo parcial de la harina de pescado con dos harinas de cangrejos de diferente origen, de río o de mar, sobre la utilización del alimento, rendimiento final del crecimiento y coloración de la piel del bocinegro hasta llegar a talla comercial.

2. Determinar la digestibilidad de los nutrientes de las dos harinas de cangrejo así como valorar su efecto sobre la excreción nitrogenada y la deposición de calcio y fósforo.

3. Estudiar el efecto de la inclusión de las harinas de cangrejo sobre la composición y calidad final del filete y sobre la oxidación lipídica del músculo durante su almacenamiento.

4. Determinar el efecto de la inclusión de dos ingredientes de origen marino, harina de cangrejo y harina de erizo, sobre la utilización del alimento, rendimiento del crecimiento, coloración de la piel, excreción de amonio, composición y calidad final del filete y oxidación lipídica del músculo durante su almacenamiento.

9.3 MATERIAL Y MÉTODOS GENERALES

En este apartado se explica de forma general todo el material y métodos utilizados en todos los experimentos que constituyen la presente tesis doctoral. Todos los experimentos se han desarrollado en el marco del proyecto titulado "Harinas de algas y de cangrejo y subproductos de las mismas como ingredientes alternativos a la harina de pescado en dietas para bocinegro (*Pagrus pagrus*): efectos digestivos y metabólicos y repercusiones" (**AGL2006-12888/ACU**), concedido por el Ministerio de Ciencia e Innovación del Gobierno de España.

9.3.1 ANIMALES DE EXPERIMENTACIÓN

Los juveniles de bocinegro utilizados en todos los experimentos de engorde se obtuvieron a partir de puestas naturales procedentes del stock de reproductores con los que cuenta la planta de Cultivos Marinos del Instituto Canario de Ciencias Marinas (ICCM) (Fig. 9.5), ubicado en la localidad de Taliarte en Gran Canaria. Para el reclutamiento de los animales, se seleccionaron peces de la población original dentro del rango de talla elegido con el fin de evitar diferencias significativas de peso medio entre grupo de peces al inicio de cada experiencia. Tras ser anestesiados con una solución de 2-fenoxil-etanol (10 ml L^{-1}) al 10% en agua salada, los peces fueron medidos, pesados y distribuidos al azar en los diferentes tanques experimentales.

Figura 9.5 Instituto Canario de Ciencias Marinas (ICCM).

9.3.2 CONDICIONES DE CULTIVO

9.3.2.1 Instalaciones

Las experiencias desarrolladas en el presente trabajo fueron realizadas en las instalaciones de la Planta Experimental del área de Cultivos Marinos que posee el Instituto Canario de Ciencias Marinas (ICCM), institución que perteneciente a la Agencia Canaria de Investigación, Innovación y Sociedad de la Información del Gobierno de Canarias.

9.3.2.2 Tanques experimentales

Los tanques utilizados estaban fabricados con fibra de vidrio, en color gris, con fondo troncocónico y una capacidad de 500 litros. A lo largo de las experiencias, todos los tanques estuvieron provistos con un flujo continuo de agua de mar y una aireación constante. El foto-período utilizado fue natural (latitud 28º 10' Norte). Cada uno de los tanques fue cubierto con una red para evitar escapes.

9.3.2.3 Condiciones experimentales

Los animales fueron alimentados hasta saciedad aparente, 2 veces al día (9:00 y 15:00 horas) de lunes a sábado. Diariamente se anotaban las mortalidades en su caso así como el alimento suministrado y el alimento sobrante de cada tanque para determinar la ingesta. Por otra parte, las medidas de temperatura y concentración de oxígeno disuelto del agua se tomaron utilizando un Oxímetro YSI 95 Disolved Oxygen, modelo 95/10 FT CE.

9.3.2.4 Muestreos

En todos los experimentos se realizaron muestreos al inicio, a intervalos periódicos de tiempo según lo exigiera cada desarrollo experimental y final de cada experiencia, con en el fin de determinar las variables directas objeto de estudio (parámetros de color *L*, *a* y *b*, peso y longitud).

9.3.3 ELABORACIÓN Y OBTENCIÓN DE LOS INGREDIENTES EXPERIMENTALES

9.3.3.1 Harina de cangrejo rojo de río *Procambarus clarkii*

Figura 9.6 Cangrejo de río *Procambarus clarkii*.

El cangrejo *Procambarus clarkii* (Fig. 9.6) es un cangrejo de agua dulce nativo del noroeste de Méjico y del suroeste de los Estados Unidos. Sin embargo, puede ser encontrado en un amplio rango de lugares debido principalmente a su introducción en muchos países. Este cangrejo llegó a España en 1974 con el objetivo de su producción comercial. La primera introducción se llevó a cabo en el río Guadalquivir, al sur de la Península Ibérica (Hadsburgo-Lorena, 1979; Algarin 1980; Ocete y López, 1983). Su expansión ha sido tal que España es en uno de los principales países productores y comercializadores de esta especie (Laurent, 1990).

La harina procedente del cangrejo rojo de río *Procambarus clarkii* fue suministrada por la compañía SEA FOOD Sevilla S.L., empresa ubicada en el sur de España que se dedica fundamentalmente a la comercialización de esta especie para consumo humano. Esta harina está compuesta fundamentalmente de los desechos del procesado, tales como los exoesqueletos de los animales separados de la carne y los descartes de animales deformes o fuera de la talla de comercialización. Estos subproductos son procesados por la propia empresa hasta obtener la harina utilizada en el presente estudio.

9.3.3.2 Harina de cangrejos marinos *Chaceon affinis* y *Paramola cuvieri*

Figura 9.7 Cangrejo marino (*Chaceon affinis*).

El cangrejo rey *Chaceon affinis* (Fig. 9.7) es el braquiura epibentónico más largo que habita en aguas del este del Océano Atlántico, hasta la latitud de Senegal y también alrededor de la región de la Macaronesia (López Abellán *et al.*, 2002). En las Islas Canarias, esta especie fue descrita por primera vez durante una expedición científica en julio de 1985 (Lozano *et al.*, 1992). Se captura a profundidades que van desde los 550 a los 1200 metros (Pinho *et al.*, 2001; López *et al.*, 2002), y a pesar de su potencial económico como alimento destinado al consumo humano, todavía no hay un mercado desarrollado para esta especie.

Por su parte, el cangrejo rojo *Paramola cuvieri* (Fig. 9.8) está ampliamente distribuido por el Atlántico occidental, desde el sur de Islandia (63 ºN) y Noruega hasta el sur de África (36 ºS), incluyendo las islas Azores, Madeira, Canarias y Cabo Verde, y también algunos sectores del Mediterráneo. En Canarias se captura ocasionalmente a profundidades de entre 120 y 860 metros. Tampoco existe un mercado para esta especie y constituye mas bien un by-catch en las pesquerías locales (Sanchez *et al.*, 2004), apareciendo en trampas de fondo como especie secundaria, especialmente del camarón (*Plesionika edwardsii*) a profundidades de entre 250 y 350 m o del cangrejo rey entre 600 y 900 metros.

Figura.9.8 Cangrejo marino (*Paramola cuvieri*).

Los individuos de ambas especies utilizados en este trabajo fueron capturados por pesadores locales de la zona del muelle de Taliarte mediante artes de pesca tradicionales. Ya en las instalaciones del ICCM fueron debidamente procesados siguiendo una metodología adaptada de Sudaryono *et al.* (1996) hasta obtener harina a partir de individuos enteros. Primeramente, los cangrejos fueron pesados individualmente y dispuestos en vasos grandes de precipitado para ser autoclavados durante 15 minutos a alta presión. Tras ello se enfriaron inmediatamente durante 10 minutos a -20ºC y posteriormente troceados separando la parte blanda del caparazón para su mayor facilidad durante el secado. Para dicho secado las piezas se introdujeron en una estufa con sistema de aireación a 40ºC durante 12 horas para conservar la calidad de la harina. Una vez retiradas de la estufa, fueron molidas y almacenadas al vacío a 4ºC hasta su utilización. En este punto, los diferentes lotes de harina fueron mezclados hasta conseguir un único producto homogéneo (Fig. 9.9).

Figura 9.9 Harina de cangrejo de río y de cangrejo de mar usadas para las dietas experimentales.

9.3.3.3. Harina de erizo de mar *Diadema antillarum*

Figura 9.10 Erizo de mar (*Diadema antillarum*)

El erizo de mar *Diadema antillarum* (Fig. 9.10) es una especie herbívora de aguas templadas del Atlántico centro-oriental con un fuerte "boom demográfico", llegando a provocar la desaparición de la cobertura de plantas acuáticas en las zonas donde se asienta, los llamados blanquizales (Sala *et al.*, 1998; Pinnegar *et al.*, 2000). Los erizos de mar utilizados en este trabajo fueron capturados en la zona del litoral del Confital, al norte de la isla de Gran Canaria. La harina se obtuvo siguiendo la misma metodología empleada en las harinas de cangrejo anteriormente descrita, en este caso a partir de individuos enteros tras desechar las púas del animal.

9.3.3.5 Otros ingredientes

Tanto la harina de pescado como el aceite de pescado fueron suministrados por la empresa Proaqua Nutrición S.A, España. Por su parte, el almidón de maíz pre-gelatinizado merigel 100 y la vitamina C (*Stay-C*) fueron donados por la empresa Especialidades Puma, S.A. (Amylum Group), España y DMS-Nutrición S.A, España respectivamente.

9.3.4 ELABORACIÓN DE LAS DIETAS EXPERIMENTALES

9.3.4.1 Formulación

Una vez obtenidos todos los ingredientes y materias primas necesarias, éstos fueron analizados para poder formular en base a su composición proximal. Todas las dietas se formularon de manera que fueran isopreteicas e isolipídicas a razón de aproximadamente 50% de proteínas y 15% de lípidos, expresado en peso seco, de acuerdo con los requerimientos de la especie (Schuchardt *et al,.* 2008). En todos los experimentos se formuló una dieta control de referencia con harina de pescado de alta calidad como fuente de proteínas, mientras que las demás dietas fueron formuladas reemplazando la proteína de la dieta control por la proteína del ingrediente alternativo a un determinado nivel de sustitución ajustando las cantidades a expensas del almidón. El aceite de pescado fue la única fuente principal de lípidos en todas las dietas experimentales.

9.3.4.2 Elaboración de las dietas

Las dietas fueron fabricadas en las instalaciones del área de Cultivos Marinos del ICCM. Antes de su elaboración, se prepararon las mezclas de vitaminas hidrosolubles (excepto el cloruro de colina y el ácido ascórbico o vitamina C) y de minerales según la tabla 9.1, en la que se muestran los requerimientos estándar de vitaminas y minerales para peces marinos de cultivo. Para ambas mezclas se utilizó la α-celulosa como base o transportador de los componentes, conservándolas en nevera a 4ºC. Por otra parte, la mezcla de vitaminas liposolubles (Tabla 9.1), se preparó en el momento de la elaboración cada dieta, utilizando la etoxiquina como antioxidante.

Todos los ingredientes secos fueron mezclados por orden. Las vitaminas liposolubles se diluyeron en el aceite y se añadieron hasta conseguir una masa homogénea. La vitamina C fue la última en añadirse para minimizar sus pérdidas por oxidación. Por último se incluyó el agua con el cloruro de colina disuelto. La mezcla resultante fue pasada por una granuladora (CPM Mod. CL3, USA) a través de una matriz de granulado de 5 mm de diámetro. Posteriormente, los granos obtenidos fueron repartidos en diferentes bandejas y secados en estufa de aireación a una temperatura de 30ºC durante 12 horas y conservados a 4ºC.

Tabla 9.1 Mezcla de vitaminas hidrosolubles, liposolubles y minerales utilizados en la elaboración de las dietas experimentes.

Vitaminas (g/ kg dieta)		Minerales (g/ kg dieta)	
Hidrosoluble			
Cianocobalamina (B_{12})	0,5	$(H_2PO_4)Ca$	1,605
Biotina (B_8)	0,001	$CaCO_3$	4,0
Acido Fólico (B_9)	0,01	$FeSO_4.7H_2O$	1,5
Pyridoxina (B_6)	0,04	$MgSO_4.7H_2O$	1,605
Riboflavina (B_2)	0,05	K_2HPO_4	2,8
Tiamina (B_1)	0,04	$Na_2PO_4H_2O$	1
Colina	2,7	$Al(SO_4)_3.6H_2O$	0,02
Acido pantoténico	0,12	$ZnSO_4.7H_2O$	0,24
Niacina (B_3)	0,2	$CuSO_4.5H_2O$	0,12
Acido ascórbico (C)	0,7	KI	0,02
Mio-inositol	2	$CoSO_4.7H_2O$	0,08
Liposoluble		$MnSO_4H_2O$	0,08
∝-tocoferol (E)	0,25		
Retinol acetato (A)	0,025		
Medianona (K_3)	0,02		
calciferol (D_3)	0,005		

9.3.5 PARÁMETROS BIOLÓGICOS Y DE UTILIZACIÓN DEL ALIMENTO

Para evaluar el efecto de la inclusión de los diferentes ingredientes utilizados en este estudio sobre el crecimiento y utilización del alimento en el bocinegro se utilizaron los siguientes índices:

9.3.5.1 Crecimiento relativo

Representa el peso como porcentaje del peso inicial.

Crecimiento (%) = [(peso final (g) – peso inicial (g))/ peso inicial (g)] x 100

9.3.5.2 Factor de condición (K)

Relaciona el peso con la longitud del animal.

$K = peso\ (g)/ (longitud\ (cm))^3$

9.3.5.3 Tasa de crecimiento específico (SGR)

Ofrece información sobre la variación del crecimiento, en tanto por ciento, del animal por día de experimento.

SGR= [(Ln peso final-Ln peso inicial)/nº días experimento)] x 100

9.3.5.4 Índice de conversión del alimento (FCR)

Se define como la relación entre el alimento ingerido y el peso ganado.

FCR= alimento ingerido (g)/incremento de peso (g)

9.3.5.5 Índice hepatosomático (IHS)

Se define como la relación que existe entre el peso del hígado con respecto al peso total del animal.

IHS = [peso hígado (g)/ peso pez (g)] x 100

9.3.5.6 Índice visceral (IVS)

Es el índice que relaciona el peso de las vísceras con el peso total del animal.

IVS = [peso vísceras (g)/ peso pez (g)] x 100

9.3.5.7 Utilización de la Proteína (PER)

Es el índice que relaciona el peso total del animal (g) y la proteína ingerida (g), representando una medida tanto de la capacidad del pez para utilizar la proteína dietética como de la propia calidad de la proteína.

PER= incremento de peso (g) / proteína ingerida (g)

9.3.6 DETERMINACIÓN INTRUMENTAL DEL COLOR DE LA PIEL

Para la determinación instrumental del color de la piel se utilizó un colorímetro portátil (Hunter Lab MiniScan™ XE, USA). Una vez calibrado el aparato, las medidas se tomaron directamente colocando la lente sobre la piel del animal en la zona ubicada justo debajo de la inserción anterior de la aleta dorsal del lado izquierdo. Las lecturas del colorímetro proporcionaron directamente los valores de los parámetros *L**, *a** y *b** del sistema Hunter (Fig. 9.11).

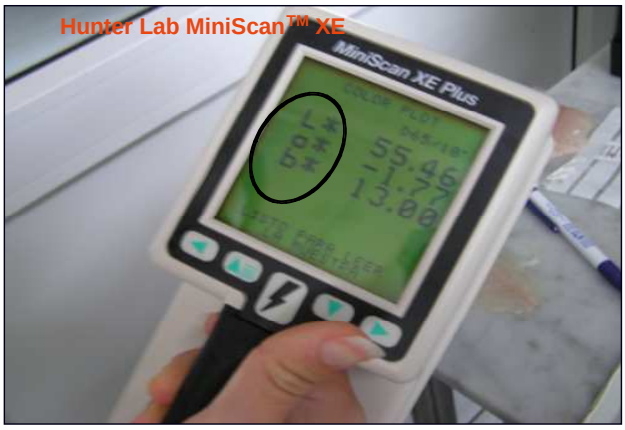

Figura 9.11 Medidas de color tomadas con el colorímetro portátil.

*L** es el parámetro de luminosidad, con un rango de valores que va desde el 0 para el negro hasta 100 que representa el blanco. El parámetro *a** asigna las tonalidades que van del verde (valores negativos) al rojo (valores positivos), mientras que *b** lo hace para los tonos azules (valores negativos) y amarillos (para valores positivos), de acuerdo con las recomendaciones de la Comisión Internacional de Iluminación CIE (1976).

A partir de los parámetros *a** y *b**, fueron calculados el tono (Hue: H_{ab}) y la saturación del color (Chroma: C_{ab}). El parámetro *Hue*, el tono observado (rojo, amarillo, verde o azul), es una variable angular cuyas medidas van desde 0º para tono rojo, 90º para amarillo y 270º que corresponde al verde. Mientras que el parámetro *Chroma* representa la saturación o intensidad del tono observable. Ambos parámetros fueron calculados aplicando las siguientes fórmulas: $H_{ab}=\arctan(b^*/a^*)$ para el tono, y $C_{ab}=((a^*)^2+(b^*)^2)^{1/2}$ para la intensidad (Hunt, 1977).

9.3.7 ANALISIS PROXIMAL Y DE LOS ACIDOS GRASOS

Todas las muestras destinadas a analizar fueron debidamente recolectadas y conservadas a -80ºC, bajo atmósfera de nitrógeno, hasta su posterior análisis. Todos los análisis fueron realizados por triplicado a partir de un pool homogéneo de un determinado número de individuos, según experimento.

9.3.7.1 Humedad

La humedad de las muestras se realizó por desecación en estufa a 110ºC hasta obtener un peso constante (AOAC, 1995).

9.3.7.2 Cenizas

Para la determinación del contenido en cenizas de las muestras, se incineraron las mismas en un horno Mufla a una temperatura de 450ºC, hasta alcanzar un peso constante (AOAC, 1995).

9.3.7.3 Proteínas

El contenido de proteínas de las muestras se obtuvo mediante el método Kjeldhal (AOAC, 1995). Para ello, se realiza la digestión de las muestras con ácido sulfúrico concentrado (10 ml) a 420ºC añadiéndole un catalizador de cobre, seguido de una destilación con Na(OH) al 40% con ácido bórico la como sustancia receptora, en una unidad de destilación (Kjeltec System 1003, Hoganas, Suecia). Finalmente se valora con HCL a una concentración de 0,1N. Para el cálculo del porcentaje de proteína bruta fue utilizado un factor de conversión de nitrógeno de 6,25.

9.3.7.4 Lípidos

Para la extracción de lípidos de las muestras se aplicó el método descrito por Folch *et al.* (1957). El método consiste en la homogenización de la muestra con la mezcla de solventes cloroformo:metanol (2:1, v:v), posteriormente se saponifica con una solución de KCL al 0,88% recuperándose la fase orgánica. Finalmente se procede a la evaporación total del solvente en una corriente continua de nitrógeno gaseoso. Para luego pesar los lípidos en seco.

9.3.7.5 Perfil de ácidos grasos

El perfil de ácidos grasos fue obtenido mediante el método de Christie (1982). Para ello, los lípidos extraídos por el método de Folch se transesterificaron con ácido sulfúrico al 1% en metanol y se añadió tolueno para la disolución de los lípidos neutros. Los metil esteres de ácidos grasos (FAMEs) así obtenidos se diluyen en hexano a una concentración de 20 mg/ml. Para la identificación y

cuantificación se utilizó un cromatógrafo de gases (Mod. Shimadzu GC-14A; Analytical instrument division, Kyoto, Japon) bajo las condiciones operativas descritas por Izquierdo *et al.* (1992). Cada metil éster fue identificado y comparado con un estándar externo EPA 28, Nippai, Ltd. Tokio, Japan.

La calidad lípidica de las muestras fue evaluada mediante los índices aterogénicos (**IA**) y trombogénicos (**IT**), los cuales fueron determinados de acuerdo con Ulbricht y Southgate (1991):

$$IA = [(12{:}00) + (4 \times 14{:}00) + (16{:}00)] \times [(PUFA\ n\text{-}6\ and\ n\text{-}3) + MUFA]^{-1}$$

$$IT = [(14{:}00) + (16{:}00) + (18{:}00)] \times [(0.5 \times MUFA) + (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3/n\text{-}6)]^{-1}$$

9.3.8 ANÁLISIS DE CAROTENOIDES

9.3.8.1 Extracción de carotenoides

La extracción de pigmentos carotenoides de los ingredientes y las dietas experimentales se realizó siguiendo el método de Barua *et al.* (1993). Para ello la muestra se homogeniza con una mezcla de los solventes etil acetato:metanol (1:1), seguida de etil acetato solo y por ultimo hexano. En muestras de piel, debido a su alto contenido en agua, el método utilizado para la extracción de carotenoides fue el descrito por Schiedt y Liaaen-Jensen (1995). En este caso, en la fase de extracción sólo se utiliza un solvente orgánico, acetona, a razón de 5 ml por cada gramo de muestra, repitiéndose hasta no observar color en la muestra inicial. Al solvente extraído se añade un volumen igual de hexano seguido de 2 ml de agua, la mezcla se mueve cuidadosamente y se deja reposar hasta que la fase acuosa se separa claramente de la orgánica. Posteriormente se selecciona la fase orgánica (superior), la cual contiene a los carotenoides disueltos.

Todos los supernadantes recolectados en ambos tipos de extracciones, son filtrados y evaporados en atmósfera de nitrógeno hasta la eliminación total de los restos de agua. Los carotenoides secos son finalmente disueltos en un volumen conocido de hexano cuya absorbancia esté comprendida en un rango de entre 0,2 y 0,8 y leídos en espectrofotómetro (Genesys UV10, Thermo Fisher Scientific Inc.

Waltham, USA) a la longitud de onda de máxima absorbancia (λ_{max}). Para la cuantificación de los carotenoides en las muestras se utiliza la siguiente ecuación:

$$Carotenoides\ totales\ (\mu g/g\ muestra) = 10000 \times V \times A/peso \times E_{1\%,1cm}$$

donde:

V= Volumen de hexano en el que se diluyó los carotenoides secos.

Peso= Peso de la muestra en gramos.

A= absorbancia

$E_{1\%,1cm}$= coeficiente de extinción, el cual corresponde a la absorbancia de una solución al 1% leída en una cubeta de 1cm. Cuando la λ_{max} fue 470 nm, los carotenoides fueron reportados como astaxantina y el valor utilizado para dicho coeficiente fue de 2100. Mientras que para una λ_{max} distinta de 470 nm, el valor utilizado fue de 2500 (Britton, 1995).

9.3.8.2 Separación y cuantificación relativa (%) de carotenoides por cromatografía de capa fina (TLC)

Para la cuantificación relativa de pigmentos rojos y amarillos contenidos en muestras de piel se utilizó la metodología de TLC. El solvente utilizado como fase móvil correspondió a una mezcla de hexano:dietileter (20:1) mientras que para la fase estacionaria se utilizaron placas de vidrio de 20x20 cm revestidas de silicagel (G60 Merk glass plates). Primeramente se prepara la cámara donde se desarrolla la cromatografía. Para proteger los carotenoides de la luz se cubre bien toda la cámara exteriormente con papel de aluminio. Luego se vierte el solvente y se coloca un papel de filtro rodeando el interior de la cámara, dejándolo reposar 30 minutos con el fin de crear una atmósfera saturada y homogénea propicia para el desarrollo de la cromatografía. Los extractos de carotenoides fueron aplicados sobre la fase estacionaria usando capilares de cristal a una altura de 2 cm del borde inferior de la placa en forma de finas barritas separadas una de otra. Una vez aplicada la muestra, la placa se coloca en la cámara de desarrollo y se deja correr la cromatografía hasta que el solvente llegue aproximadamente a 1 cm por debajo del borde superior de la placa. Para la cuantificación relativa se raspó la parte de la sílica conteniendo el carotenoide y se diluyó varias veces con acetona

hasta no observar color en la sílica. Posteriormente el volumen total de acetona es filtrado y evaporado bajo atmósfera de nitrógeno y diluido en un volumen conocido de hexano para ser leído en el espectrofotómetro. De esta forma se estiman las cantidades relativas (%) de pigmento rojo y amarillo (Fig. 9.12).

Figura 9.12 Pigmentos rojos y amarillos de la piel de bocinegro separados por TLC.

9.3.9 DETERMINACIÓN DE LA EXCRECIÓN DE AMONIO EN AGUA

9.3.9.1 Recogida de muestras

Para la determinación de amonio en el medio de cultivo los peces fueron alimentados una sola vez al día a primera hora de la mañana (8:00), tras lo cual se recogieron muestras de agua de cada tanque en recipientes oscuros y conservados a 4ºC. La toma de muestras se repitió cada dos horas entre las 8:30 y las 20:30 horas, completándose tres días de muestreos en tres semanas seguidas a razón de un día cada semana. Como blanco se utilizó un tanque de peces no alimentados. El flujo se mantuvo controlado y constante en todo momento.

9.3.9.1 Método analítico

Las muestras fueron analizadas siguiendo el método calorimétrico del indofenol azul (Koroleff *et al.*, 1983). El método se basa en el principio de que el fenol alcalino y el cloruro reaccionan con amonio para formar indofenol azul, el cual es proporcional a la concentración de amonio (Fig. 9.13).

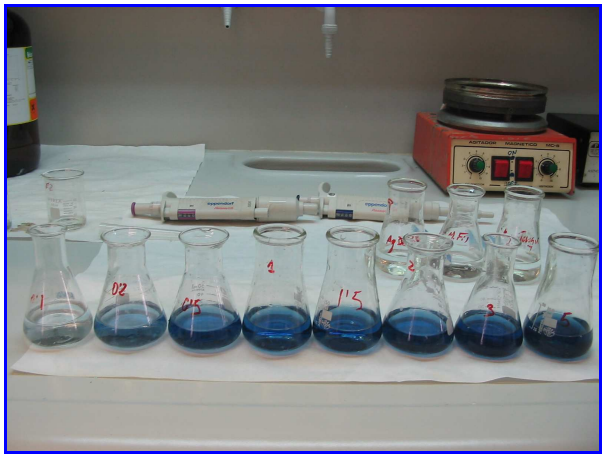

Figura 9.2.9 Método colorimétrico del indofenol azul.

9.3.9.1.1 Reactivos y soluciones

Para la realización del método fue necesario la preparación de las siguientes soluciones y reactivos:

- Solución patrón: Se pesan 2,360 g de sulfato de amonio seco (calentado previamente a 100ºC durante 24 horas) y se disuelven en 500 ml de agua.
- Solución stock: A partir de 10 ml de solución patrón anterior se le añade 500 ml de agua.
- Reactivo fenol: Se pesan 17,5 g de fenol cristalizado y 0,2 g de nitroprusside de sodio y se diluyen en 500 ml de agua.
- Reactivo citrato de sodio: Se pesan 170 g de citrato de trisodio y 10 g de sosa y se disuelven en 400 ml de agua destilada. La mezcla se calienta hasta hervir durante 20 min. Se enfría y se añaden 2 g de ácido diclorocianúrico más 500 ml de agua.

9.3.9.1.2 Estádares

Primeramente se realiza una curva de calibrado a partir de soluciones patrón. A partir de la solución stock se preparan los estándares con los siguientes volúmenes: 0,5, 1, 1,5, 2 y 3 ml, llevándolos a 100 ml con agua de mar proveniente de la entrada del sistema. Los estándares de trabajo tendrán así unas concentraciones de 50, 100, 150, 200 y 300 ppm de amonio, respectivamente. Con estos patrones se calibrará el espectrofotómetro y se determinará la curva trabajo.

9.3.9.1.3 Procedimiento

Se vierten 5 ml de las muestras en tubos de ensayo y se mezclan con 0,25 ml del reactivo fenol. Luego se agitan en el vortex y se añaden otros 0,25 ml de un segundo reactivo de citrato de sodio. Se vuelven a agitar y se calienta a 100ºC durante 30 minutos. Por último se deja enfriar en la campana y se lee en el espectrofotómetro frente a un blanco a la longitud de onda de 625 nm.

9.3.10 DIGESTIBILIDAD

9.3.10.1 Tanques de digestibilidad y recogida de muestras de heces

Para el estudio de la digestibilidad de los ingredientes experimentales se utilizaron tanques modificados del tipo Sistema Guelph (CYAQ-2) propuesto por Cho *et al.* (1975, 1982). Los tanques eran de forma cilíndrica y fondo troncocónico invertido, de 125 l de capacidad y desagües centrales hacia una columna de decantación de donde se recolectaban las heces diariamente (Robaina *et al.*, 1995). Cada día y después de alimentar, las muestras de heces fueron recogidas de la columna de decantación en tubos de centrifugación de 50 ml de volumen. Así, las muestras fueron centrifugadas a 10.000 rpm durante 20 minutos. Posteriormente se seleccionó la fase sólida, desechando los supernadantes, y se almacenó a -20ºC hasta su posterior análisis.

9.3.10.2 Determinación del óxido crómico (Cr_2O_3)

Para la determinación de la digestibilidad se utilizó Cr_2O_3 al 0.5% en las dietas como marcador inerte. El porcentaje de Cr_2O_3 fue establecido mediante la técnica de Furukawa y Tsukahara (1966). Las dietas y las heces son expuestas a una digestión con ácido nítrico seguido de ácido perclórico concentrado, para luego medir en el espectrofotómetro a una longitud de onda de 350 nm.

9.3.10.3 Determinación del coeficiente de digestibilidad aparente

El coeficiente de digestibilidad total de las dietas fue calculado aplicando la siguiente fórmula:

$$\textit{Digestibilidad total (\%)} = 100\,[1 - (Cr_2O_3 \textit{ en dieta} / Cr_2O_3 \textit{ in heces})]$$

El coeficiente de digestibilidad aparente (CDA) para la materia seca, proteína cruda, lípidos crudos y energía bruta fue calculado según las fórmulas descritas por Maynard y Loosli (1979):

$$CDA\ (\%) = 100\ [1 - (Cr_2O_3 \text{ en dieta} / Cr_2O_3 \text{ en heces}) \times (\text{contenido de nutriente en heces} / \text{contenido de nutriente en dieta})]$$

El coeficiente de digestibilidad aparente de un nutriente en un ingrediente (CDA_{ing}) incorporado a la dieta de referencia fue calculado por diferencia, asumiendo efectos no asociativos entre el ingrediente agregado y la dieta de referencia. El CDA_{ing} es calculado según Forster (1999) y Hardy *et al.* (2002) de la siguiente forma:

$$CDA_{ing}\ (\%) = [(Nutr_{td} \times AD_{td}) - (0.7 \times Nutr_{bd} \times AD_{bd})] / (0.3 \times Nutr_{td})$$

donde:

$Nutr_{td}$ = concentración de nutriente en la dieta que contiene el ingrediente

AD_{td} = CDA (%) del nutriente en la dieta que contiene el ingrediente

AD_{bd} = CDA (%) del nutriente en la dieta referencia

$Nutr_{ing}$ = concentración de nutriente en el ingrediente en la dieta que contiene el ingrediente

9.3.11 ANALISIS DEL PERFIL DE TEXTURA DEL FILETE

Para el análisis de la textura del filete crudo se utilizó un analizador de textura modelo Stable Micro System Texture Analyzer (TA.XT2, Haslemere, Surrey, Reino Unido). La medida de la textura se realizó siguiendo el procedimiento descrito por Gines *et al.* (2004). Las mediciones se realizaron sobre tres fragmentos (craneal, central y caudal) de dimensiones 2x2x1,2 cm, correspondientes al músculo del filete izquierdo tras retirar la piel. Los fragmentos fueron mantenidos sobre bloques de hielo durante todo el proceso. Para determinar el perfil de textura fueron calculados siete parámetros mecánicos de textura (Tabla 9.2).

Tabla 9.2 Descripción de los parámetros mecánicos del análisis del perfil de textura.

Parámetros mecánicos	Descripción	Variable (unidades)
Dureza	Fuerza mecánica para comprimir la muestra hasta un nivel determinado	Fuerza (N)= ($Kg \times m \times s^{-2}$)
Fracturabilidad	Fuerza a la que se produce la pérdida de la estructura del músculo	Fuerza (N)= ($Kg \times m \times s^{-2}$)
Cohesividad	Es el punto límite hasta el cual puede deformarse la muestra antes de romperse	Adimensional
Elasticidad	Recuperación total o parcial de la muestra a su forma original al cesar la fuerza de compresión	Distancia m
Adhesividad	Trabajo requerido para separar la muestra del cilindro de compresión	Trabajo (J)= ($Kg \times m^2 \times s^{-2}$)
Gomosidad	Energía requerida para la rotura de la muestra	Fuerza (N)= ($Kg \times m \times s^{-2}$)
Masticabilidad	La energía requerida para disgregar la muestra	Trabajo (J)= ($Kg \times m^2 \times s^{-2}$)

9.3.12 ANALISIS SENSORIAL

Para el análisis sensorial se contó con un panel de evaluadores entrenado. De cara a familiarizarlos con la especie de trabajo se realizaron varias sesiones preparatorias que concluyeron con el perfil más adecuado para la valoración.

9.3.12.2 Perfil sensorial

El cocinado se realizó introduciendo los fragmentos (3x3 cm) del filete, con piel, en envases de aluminio alimentario cerrados y debidamente identificados mediante códigos numéricos. Las piezas fueron cocinadas en un horno de convención a 115ºC durante 10 min y ofrecidas al panel. La cata se desarrolló en una sala dividida en cabinas individuales y diseñada para la realización de esta actividad de manera aislada, con ambiente e iluminación estandarizada siguiendo las indicaciones de la ISO 8589 (2007). En la evaluación se tuvieron en cuenta un total de 17 atributos agrupados según el tipo de percepción sensorial, esto es, olor (marino, aceitoso, anormal), aspecto (blancura, brillo), textura (cohesividad, jugosidad, dureza, adherencia), sabor (marino, aceitoso, anormal) y persistencia del sabor o el gusto residual (persistente, aceitoso). El panel evaluó cada uno de

los atributos sensoriales en una escala continua desde el 0 (ausencia) hasta el 100 (intensidad máxima).

9.3.13 DETERMINACIÓN DE LA OXIDACIÓN LIPÍDICA DEL FILETE

La oxidación lípidica se determinó en muestras de músculo del animal mediante la medida de las sustancias reactivas al ácido tribarbitúrico (TBARS) según la técnica modificada de Shahidi y Hong (1991). La muestra fue homogenizada en frío (Ultraturrax T25, Jane & Kunkel GMBH, Alemania) durante 1 minuto en ácido tricloroácetico al 10% (2:1 ácido: muestra, v/v) y, centrifugada a 4.000 rpm, y 4ºC durante 30 minutos. El extracto así obtenido se filtra y a 2 ml del mismo se añaden otros 2 ml de una solución ácido 2-tribarbitúrico 0,02M, calentándose a 100ºC durante 20 minutos en una manta calefactora. Transcurrido este tiempo, las muestras fueron enfriadas bajo un flujo continuo de agua y leídas a 532 nm en un espectrofotómetro (Genesys UV10, Thermo Fisher Scientific Inc. Waltham, USA). Los resultados fueron calculados a partir de una curva patrón de calibrado con 1,1,3,3 tetrametoxipropano (TMP), expresándose como mg de malonaldehido/kg de muestra.

9.3.1.4 ANÁLISIS ESTADÍSTICO

Para determinar las diferencias entre tratamientos se realizó un análisis de la varianza (ANOVA), usando el test de Tukey para las comparaciones múltiples. En el caso de que los datos no cumpliesen los supuestos del ANOVA, es decir, homogeneidad de varianzas y distribución homogénea de los datos, fue aplicada la prueba no paramétrica de múltiples rangos de Kruskal-Wallis (Sokal y Rolf, 1995). En aquellas variables en las que se pudo incluir el efecto del factor tiempo, éste se consideró junto con la dieta. Todos los análisis fueron realizados con el programa estadístico SPSS (Versión 13.1,Inc, IL,USA) contrastando las hipótesis a un 5% de nivel de significación (*P-valor < 0.05*). Para determinar las diferencias estadísticas entre tratamientos de la variable de color HUE, se realizó una prueba de estadística circular debido a la naturaleza angular de la misma. La estimación de la media y la desviación estándar fueron calculadas aplicando las fórmulas de los estadísticos para distribuciones circulares. Para comprobar la uniformidad de la distribución circular se utilizó el test de Rayleigh (Zar 1999).

Para los datos que resultaron ser no unimodal, se aplicó el test no paramétrico de Watson U_2 (Zar 1999). El paquete estadístico utilizado para realizar la estadística circular fue el Oriana (Versión 3, stadistical sofware, Kovach Computing Services, Pentach, Wales, UK)

9.4 RESÚMENES DE LOS EXPERIMENTOS

Estudio 1. Harina de cangrejo de río y harina de cangrejo de mar en dietas para bocinegro (*Pagrus pagrus*): efecto sobre el crecimiento, composición corporal y coloración de la piel.

Dos tipos de harinas de cangrejo de diferente origen, harina de cangrejo de río *Procambarus clarkii* (CR) y harina de cangrejo de mar *Chaceon affinis* (CM), fueron usadas para sustituir la harina de pescado en dietas para bocinegro *Pagrus pagrus* y estudiar su efecto sobre el rendimiento del crecimiento, composición proximal del pez entero y coloración de la piel. Los bocinegros fueron alimentados durante 165 días con cinco dietas. Una dieta control (DC), basada en harina de pescado de alta calidad como fuente de proteínas, y otras cuatro dietas donde un 10% y 20% de la proteína de la dieta control fue sustituida por la proteína proveniente de harina de cangrejo de río *Procambarus clarkii* (CR10 y CR20) y por la de harina de cangrejo de mar *Chaceon affinis* (CM10 y CM20). En todas ellas la fuente de lípidos utilizada fue aceite de pescado. La inclusión al 20% de harina de cangrejo de mar en la dieta aumentó significativamente la ingesta de alimento, peso final y crecimiento (%) de los peces. No se encontraron diferencias significativas entre tratamientos para los valores del factor de conversión del alimento (FCR) y la utilización de la proteína (PER). La inclusión de ambos tipos de harinas de cangrejo disminuyó significativamente el contenido en lípidos del animal entero con respecto a los alimentados con la dieta control. Por otro lado, la alimentación de los peces con las dietas de sustitución produjo una coloración más adecuada de la piel comparada con la de los de la dieta control, siendo los mejores valores de *Hue* los del grupo de peces alimentados con la harina de cangrejo de río. En conclusión, el presente trabajo sugiere que es posible sustituir la proteína de la harina de pescado en piensos para bocinegros por harina de cangrejo de ambas especies aquí probadas hasta sus mayores niveles de inclusión sin afectar al crecimiento de los animales y mejorando la coloración de la piel. Atendiendo al crecimiento, la máxima inclusión de harina de cangrejo de mar obtuvo mejores resultados incluso que la dieta control, mientras que desde el punto de vista de la

coloración del animal los mejores resultados se obtuvieron con la dieta de máxima inclusión de harina de cangrejo de río.

Estudio 2. Harina de cangrejo de mar y harina de cangrejo de río en dietas para la especie bocinegro (*Pagrus pagrus*): digestibilidad de las harinas y efecto sobre la excreción de amonio, tasa de retención de fósforo y calcio.

Dos experimentos fueron realizados para evaluar la digestibilidad de los nutrientes la harina de cangrejo de río *Procambarus clarkii* (CR) y harina de cangrejo de mar *Chaceon affinis* (CM) en la especie bocinegro *Pagrus pagrus* y el efecto de la inclusión de ambas harinas en dietas sobre la excreción de amonio, retención de P y Ca. En el primer experimento, fue determinado el coeficiente de digestibilidad aparente (ADC) de las proteínas, lípidos, cenizas, materia seca y energía contenida en la harina CR y la harina CM. Los resultados indicaron que ambas harinas de cangrejo fueron eficientemente digeridas por el bocinegro a pesar de su alto contenido en calcio y quitina, obteniéndose valores de ADC de la proteína, lípidos, cenizas, materia seca y energía significativamente mayores para la harina CM comparados con los de la harina CR.

En el segundo experimento los bocinegros fueron alimentados durante 6 meses con 5 dietas. Una dieta control (DC), basada en harina de pescado de alta calidad como fuente de proteínas, y otras cuatro dietas donde un 10% y 20% de la proteína de la dieta control fue sustituida por la proteína proveniente de harina de cangrejo de río *Procambarus clarkii* (CR10 y CR20) y por la de harina de cangrejo de mar *Chaceon affinis* (CM10 y CM20). La sustitución de la proteína de la harina de pescado por la proteína tanto de la harina CR como la de la harina CM, a cualquier nivel de inclusión, no afectó a la utilización proteica medida como excreción de amonio. Los valores obtenidos para dicha de excreción de amonio estuvieron en el rango de 105 a 119 mg $N\text{-}NH_4^+$ kg^{-1} $día^{-1}$ para todas las dietas. Con respecto a la retención mineral, La relación Ca/P de las dietas aumentó a medida que aumentó el incremento de la proporción de ambas harinas, resultando en una reducción en el contenido de P (desde 2,27% en el grupo DC a 1,71 y 1,61% en los grupos CR20 y CM20 respectivamente) y Ca (desde 3,72 en el grupo DC a 2,76 y 2,56% en los grupos CR20 y CM20 respectivamente) del animal. Las tasas

de retención de Ca y P se vieron significativamente reducidas por el incremento de la proporción de ambos tipos de harina de cangrejo. Además, se encontró una relación negativa entre la tasa de crecimiento del animal y su contenido total en P, Ca y cenizas. Sin embargo, no se encontraron diferencias entre tratamientos en la estimación de la excreción total de P (kg P t^{-1}).

Estudio 3. Harina de cangrejo de río y harina de cangrejo de mar en dietas para bocinegro (*Pagrus pagrus*): efecto sobre el perfil de ácidos grasos y parámetros de calidad final del filete.

El presente estudio fue realizado para evaluar el uso de la harina de cangrejo de río *Procambarus clarkii* (CR) y de la harina de cangrejo de mar *Chaceon affinis* (CM) sobre la composición proximal, perfil de ácidos grasos y calidad final del filete de bocinegro *Pagrus pagrus*. Para ello, los bocinegros fueron alimentados durante 165 días con 5 dietas: una dieta control (DC), basada en harina de pescado de alta calidad como fuente de proteínas, y otras cuatro dietas donde un 10% y 20% de la proteína de la dieta control fue sustituida por la proteína proveniente de harina de cangrejo de río *Procambarus clarkii* (CR10 y CR20) y por la de harina de cangrejo de mar *Chaceon affinis* (CM10 y CM20).. El contenido de grasa del filete resultó significativamente mayor en el caso de los peces alimentados con la dieta CM20. Alimentar a los peces con las dietas basadas con ambas harinas de cangrejos incrementó ligeramente por un lado el contenido muscular de los ácidos grasos poliinsaturados, 20:4n-6 (ARA), 20:5n-3 (EPA) y 22:6n-3 (DHA) así como la ratio n-3/n-6, mientras que por otro disminuyó el de ácidos grasos n-9, Sin embargo, las diferencias no fueron significativas excepto para el contenido de ARA, el cual fue menor en los peces de la dieta control. Respecto a los valores de los índices de calidad lipídica, el índice de aterogenesis (IA) fue similar para todos los tratamientos, mientras que el índice trombogénico (IT) de los animales alimentados con las dietas basadas en ambos tipos de cangrejo fue un 13% menor (aunque no significativamente diferente) que el encontrado para los animales alimentados con la dieta control, reflejo del mayor contenido de ácidos grasos n-3. El análisis sensorial mostró que los filetes de bocinegro fueron bien apreciados por los panelistas con algunas diferencias

encontradas sólo para el caso de los filetes de la dieta CM20, los cuales tuvieron mayores puntuaciones en los atributos de olor y sabor aceitoso, así como mayor cohesividad y firmeza. La textura del filete medida de forma instrumental no se vio afectada ni por la inclusión en la dieta de harina de CR ni por la de CM. Los valores obtenidos de oxidación lipídica en el músculo mostraron que la inclusión de ambas harinas de cangrejo retrasa la oxidación del filete en el tiempo comparados con los filetes de la dieta control. De esta forma, para el día 4 y 7 de almacenamiento los filetes de los peces alimentados con las dietas conteniendo CR o CM, incluso a sus niveles más bajos (10%), mostraron menores valores de TBARS que los alimentados con la dieta control. El menor valor de TBARS fue encontrado para la dieta CR20, seguido por las dietas CM20, CR10 y CM10, las cuales no fueron diferentes entre ellas. En resumen, los resultados demuestran claramente la posibilidad de usar ambas harinas de cangrejo en dietas para bocinegro, mejorando en la calidad nutricional del filete y retrasando la oxidación lipídica durante su almacenamiento.

Estudio 4. Harina de cangrejo araña (*Paramola cuvieri*) y harina de erizo de mar (*Diadema antillarum*) en dietas para bocinegros (*Pagrus pagrus*): efecto sobre el crecimiento, excreción de amonio, coloración de la piel y calidad final y oxidación del filete

El presente estudio fue realizado para evaluar el efecto del uso de la harina de cangrejo de mar *Paramola cuvierii* y harina de erizo de mar *Diadema antillarum* sobre el crecimiento, la coloración de la piel, excreción de amonio y calidad final y oxidación lipídica del filete en dietas de engorde para bocinegro *Pagrus pagrus*. Para ello, los peces fueron alimentados durante 180 días con 5 dietas isoproteícas (13%) e isolípidicas (48%): una dieta control (DC) basada en harina de pescado de alta calidad; dos dietas en las que la proteína de la harina de pescado de la dieta control fue sustituida en un 10% (SMC10) y 20% (SCM20) por la proteína de la harina de cangrejo marino araña y otras dos dietas donde la harina de erizó se incluyó a un 8% (SU8) y un 16% (SU16) del total de la dieta. Un incremento de la inclusión de ambas harinas en la dieta aumentó significativamente la apetencia por el alimento, lo cual repercutió positivamente en un mayor peso ganado y una mayor tasa de crecimiento específico con respecto

a la dieta control. Los resultados fueron aún mejores para el caso de los peces alimentados con dieta en la que se incluyó harina de erizo, los cuales obtuvieron una mejora significativa de la conversión del alimento y utilización proteica al compararlos con las otras dietas. Las tasas de excreción de amonio disminuyeron en los peces alimentados con las dieta SMC20, SU8 y SU16 respecto a la de los peces alimentados con la dieta DC y SMC10, a pesar de que solo SU16 resultó significativamente menor. La piel de los bocinegros mejoró significativamente con la suplementación de harina de cangrejo araña en la dieta, especialmente al 20% de sustitución, con unos mayores valores de rojo desde el día 45 de experimento, mientras que la harina de erizo promovió una coloración amarilla. Los contenidos en el músculo de ácidos grasos poliinsaturados (PUFAS), n-3, n-3 HUFAS y ARA (20:4n-6) aumentaron a medida que subió el porcentaje de inclusión de la harina de cangrejo araña, aunque sólo fue significativo para el ARA con respecto al control. La misma variación fue encontrada para el caso de los músculos de los peces alimentados con la harina de erizo, aunque con una tendencia menos acusada excepto para el contenido en ARA, con los mayores valores en los filetes del tratamiento SU16 seguido por los del SU8. Los valores de TBARS mostraron que la inclusión de harina de cangrejo araña retrasa la oxidación lipídica del filete de bocinegro durante su conservación (4ºC) comparado con la harina de pescado y la harina de erizo. De esta forma, los menores valores de TBARS a día 4 y 7 de conservación fueron los correspondientes a los peces alimentados con las dietas SCM10 y SMC20, sin diferencias entre ellos. Los resultados demuestran claramente la potencialidad de ambas harinas para mejorar la ingesta del alimento, utilización proteica y crecimiento al ser incluidas en dietas de engorde para bocinegro, mejorándose los resultados en la coloración de la piel y alargando la vida útil del filete para el caso de la harina de cangrejo araña a través de la disminución de la oxidación de los lípidos.

9.5 CONCLUSIONES

1. Todos los ingredientes de origen marino, harina de cangrejo *Chaceon affinis*, harina de cangrejo *Paramola cuvieri* y harina de erizo de mar *Diadema antillarum*, presentaron un gran potencial como atractantes, mayor incluso que la harina de pescado. De esta forma, se observó una mejor respuesta por el alimento y mayor ingesta del mismo.

2. La sustitución parcial de la harina de pescado por ambas harinas de cangrejos de origen marino, *Chaceon affinis* y *Paramola cuvieri,* afectó de forma positiva al rendimiento del crecimiento de los peces. Por el contrario, la inclusión de harina de cangrejo de río no mejoró el crecimiento con respecto a la dieta control.

3. La inclusión de harina de erizo de mar hasta un 16% de la dieta, afectó positivamente en el rendimiento del crecimiento así como mejoró significativamente la conversión alimenticia y la eficiencia proteica.

4. La inclusión de todas las harinas de cangrejo probadas, tanto las de origen marino como la de río, incrementó el contenido en PUFA, el ratio n-3/n-6 así como el contenido en ARA del filete de bocinegro, lo cual afectó de forma positiva a la calidad nutricional del mismo para el consumo humano. Mientras que la inclusión de harina de erizo de mar *Diadema antilarum*, a los dos niveles probados, produjo los mayores contenidos de ARA en el filete

5. La inclusión de la harina de cangrejo *Chaceon affinis* mejoró la coloración rojiza de la piel del bocinegro respecto a la piel e los alimentados con la dieta control. Mientras que la harina del del cangrejo de río *Procambarus clarkii* y la harina de cangrejo de mar *Paramola cuvieri* reveló ser más eficiente como fuente de carotenoides adecuados para esta especie.

6. La inclusión dietaria de la harina de erizo de mar *Diadema antillarum* incrementó significativamente el contenido en pigmentos amarillos de la piel del bocinegro.

7. Tanto la harina de cangrejo de río *Procambarus clarkii* como la harina de cangrejo de mar *Chaceon affinis* fueron digeridas eficientemente por el bocinegro. La harina de cangrejo de mar mostró valores de ADCs para todos los nutrientes ensayados significativamente mayores que los de la harina de cangrejo de río.

8. La relación Ca/P de la dieta no afectó a la relación Ca/P del animal entero, ya que este ratio se mantuvo constante, para todos los tratamientos.

9. La inclusión dietaria de la harina de cangrejo de río no influyó sobre los atributos sensoriales del filete de bocinegro, mientras que la inclusión de la harina de cangrejo de mar al 20%, *Chaceon affinis*, produjo significativamente mayor olor y sabor aceitoso y mayor firmeza y cohesividad en los filetes.

10. La inclusión de todos los tipos de harinas de cangrejos retardó de forma significativa la oxidación lipídica del filete durante su conservación en refrigeración (4ºC) comparado con la dieta control.

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