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NUTRITIONAL EVALUATION OF ANIMAL BY-PRODUCTS FOR THE PARTIAL REPLACEMENT OF FISHMEAL IN DIETS FOR GILTHEAD SEA BREAM (SPARUS AURATA L.)

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Nutritional evaluation of animal by-products for the partial replacement of fishmeal in diets for gilthead sea bream (*Sparus aurata* L.)

By

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ABSTRACT

As well as reducing the level of protein in feeds, the strategy of replacing fishmeal with alternative protein sources may be an effective approach towards reducing costs and offering more sustainable feeds for aquaculture. Within the framework of this study, four trials were conducted to evaluate the nutritional potential of selected animal by-products, namely: Poultry Meat Meal (PMM), steam Hydrolysed Feather Meal (HFM), Enzyme treated Feather Meal (EFM) and Spray Dried Haemoglobin (SDH), as partial substitutes for fishmeal in the diet of gilthead sea bream. The research strategy employed followed a two phase scheme which consisted of determining ingredient restrictions in the first place and validating subsequent formulations on the biological performances of the fish in the second place. Ingredient restrictions were related to nutrient specification, digestibility and palatability, whereas biological performances of the fish were assessed in terms of growth response, feed utilization, nutrient assimilation, tissue integrity and composition as well as basic health status.

Using a classical experimental design for the determination of apparent digestibility coefficients (ADCs), 8 diets (made with the individual ingredients plus 3 blends) were tested in trial 1. This trial demonstrated that protein and energy of PMM and SDH were highly digested by gilthead sea bream (80%) whilst protein ADC for the feather meals were much lower (22-23%). It was moreover observed that processing feather meal with an enzymatic treatment did not yield any significant benefit over the standard steam hydrolysed method, and that combining feather meals with blood meal was clearly not advantageous. This preliminary investigation also yielded valuable numerical ADC for essential amino acids (EAA), revealing in some cases significant discrepancies with regard to the overall protein digestibility (e.g. isoleucine: 54% and methionine: 60% in SDH).

In the second trial, six iso-energetic/iso-nitrogenous diets were formulated on a protein digestibility basis to test various inclusion rates of PMM, SDH and EFM over a period of 9 weeks. In comparison to the fishmeal reference diet, results indicated that diet with a 25% replacement of fishmeal by PMM was effective in supporting the growth of gilthead sea bream (SGR: 1.78%) and converting feed into body weight (FCR: 1.33) ($P<0.05$). Higher inclusion rates of PMM resulted in lower performance, but moderate inclusions of SDH and EFM were equally shown to be feasible without impairing fish productive values. These findings were further supported by histological and haematological assessments which provided evidence that such inclusions did not disrupt gut integrity, create anaemia conditions ($P<0.05$) or affect the physiological function of the liver. On the basis of trial 3 it was apparent that palatability of PMM could represent one of the main factors limiting the inclusion of this commodity in the diet for gilthead sea bream (daily feed intake/unit of time measured at 3.3g/min for fishmeal and 2.6g/min for PMM). Finally, in accordance with the measurements of lipid inclusion in hepatocytes (trial 2) and the fatty acid analysis of the carcass (trial 4), it is believed that the high lipid content of PMM could represent further argument toward the limitation of dietary PMM incorporation as long as a high quality product is desired.

From this study it is concluded that practical diets for gilthead sea bream would greatly benefit in terms of both nutrition and economics from adequate inclusions (considering specific ingredient restrictions) of animal by-products.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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The investigations reported in this thesis all conform to the UK 1986 Animal Scientific Procedures Act under the specific Project Licence Holder. This was also in compliance with the University of Plymouth Ethical Review Committee.

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Publications:

LAPORTE Jérôme, WOODGATE Stephen, DAVIES Simon, SERWATA Robert and GOUVEIA Antonio (2007). Combining Blood Meal with other animal protein sources in compound aqua-feeds: An alternative solution for the reduction of fishmeal in nutritionally balanced diets. Submitted to Aquafeed International.

DAVIES Simon, GOUVEIA Antonio, LAPORTE Jérôme WOODGATE Stephen and NATES Sergio. Evaluation of selected animal protein concentrates and blends with respect to their protein and amino acid digestibility profile in temperate marine fish species (European sea bass, gilthead sea bream and turbot). Submitted to Aquaculture.

LAPORTE Jérôme, WOODGATE Stephen, DAVIES Simon, SERWATA Robert, GOUVEIA Antonio and NATES Sergio (2007). Biotechnological process & Biological value of Feather Meal: Evaluation of a novel protein source for the partial replacement of fishmeal in aqua-feeds. Aquafeed International, Vol.10 Iss.4 (July/August), 16-23.

LAPORTE Jérôme, WOODGATE Stephen, DAVIES Simon, SERWATA Robert and GOUVEIA Antonio (2007). Poultry Meat Meal: A valuable source of protein for the partial replacement of fishmeal in aqua-feeds. Aquafeed International, Vol.10 Iss.2 (March/April), 12-17.

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List of Abbreviations

ADC	Apparent Digestibility Coefficient
ANF	Anti-Nutritional Factor
ANOVA	Analysis of Variance
ANPU	Apparent Net Protein Utilization
ArA	Arachidonic Acid
ABPR	Animal By-Products Regulation
BM	Blood Meal
BSE	Bovine Spongiform Encephalopathy
BV	Biological Value
CGM	Corn Gluten Meal
CPM	California Pellet Mill
DM	Dry Matter
DP/DE	Digestible Protein to Digestible Energy ratio
dPMM	Defatted Poultry Meat Meal
EAA	Essential Amino Acid
EFA	Essential Fatty Acid
EPA	Eicosapentaenoic Acid
EFM	Enzyme treated Feather Meal
FCR	Feed Conversion Rate
FM	Fishmeal
Hb	Haemoglobin
Hct	Haematocrit
HFM	Hydrolysed Feather Meal
HSI	Hepato-Somatic Index
HUFA	Highly Unsaturated Fatty Acid
KJ	Kilo Joules
MBM	Meat and Bone Meal
MCH	Mean Cellular Haemoglobin content
MCHC	Mean Cellular Haemoglobin Concentration
MCV	Mean Cellular Volume
MS222	Tricane Methyl Sulphonate
mmt	Million metric tonnes
MUFA	Mono Unsaturated Fatty Acid
PAP	Processed Animal Protein
PBM	Poultry By-product Meal
PER	Protein Efficiency Ratio
PMM	Poultry Meat Meal
PUFA	Poly Unsaturated Fatty Acid
RBCC	Red Blood Cell Count
RAS	Re-Circulating Aquaculture System
rpm	Revolution per minutes
SBM	SoyBean Meal
SDH	Spray Dried Haemoglobin
SEM	Scanning Electron Microscope
SGR	Specific Growth Rate
TAN	Total Amonia Nitrogen
TEM	Transmission Electron Microscope
TSE	Transmissible Spongiform Encephalopathies

CHAPTER 1

General introduction

▶ **Foreword**: With the development of new techniques and the rearing of new species, aquaculture gained a very broad audience in the press and the media from the 1970's. Although the farming of aquatic organisms is much older, the impressive growth it has undergone during the past three decades have attracted much attention and raised many questions (Hites *et al.*, 2004; The Economist, 2003; Naylor *et al.*, 2000). Depicting the current situation of the aquaculture and aqua-feed industry and providing fundamental information on fish nutritional requirement, the present chapter aims firstly to answer the question: Why is it necessary to look for alternative protein sources? In the second place, alternative protein sources will be presented and strategy to assess those potential alternatives described. Current knowledge on the use of animal by-products in fish feeds along with the capabilities of gilthead sea bream to utilise these alternative ingredient will be developed in the introduction of chapter 4.

1.1 KEY FIGURES OF THE AQUACULTURE INDUSTRY

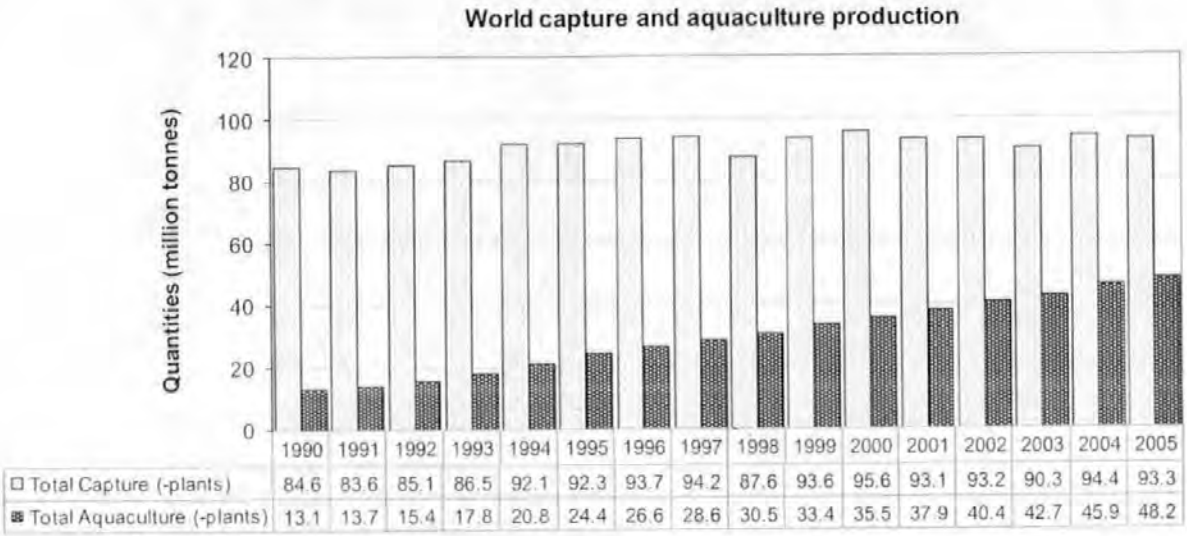
▶ **Growth in aquaculture production:** World aquaculture has grown tremendously during the last fifty years from a production of less than a million tonnes in the early 1950s to 63.0 million tonnes in 2005 (food fish and aquatic plants) (Figure 1.1). Nowadays, aquaculture represents one of the fastest growing food producing sectors of the world. This industry has effectively grown at an average annual rate of 8.8% since 1970 compared with 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period (FAO, 2006a).

1.1.1 Outlook on the aquaculture sector

▶ **Global aquaculture and capture fisheries:** Aquaculture production of fish, shellfish and crustaceans was estimated at about 47.8 million tonnes in 2005 (63.0 million tonnes with aquatic plants), while it provided 45.5 million tonnes in 2004 (59.4 million tonnes

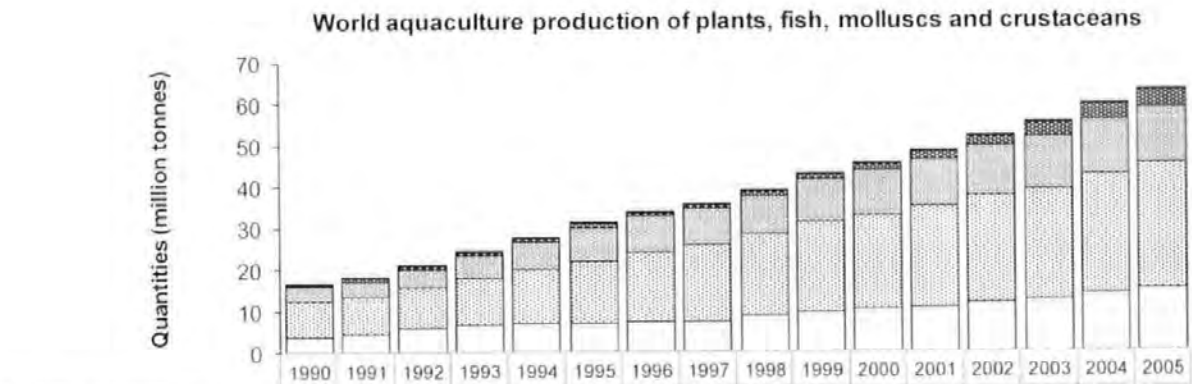
with aquatic plants). The contribution of aquaculture to the global supplies of fish, crustaceans, molluscs and other aquatic animals represented 33.7% of total fisheries production by weight (141.6 million tonnes) in 2005 (27.3% in 2000). Capture fisheries and aquaculture supplied the world with about 107.2 million tonnes of food fish in 2005. Of this total aquaculture accounted for 44.6%. As regards animal food supply, aquaculture produced the equivalent of 29.3 million tonnes of farmed aquatic meat products (after gutting and shelling) for direct human consumption in 2005, and ranked fourth in terms of global farmed meat supply after pig meat (104 million tonnes), poultry (82.2 million tonnes), and bovine meat (53.9 million tonnes) (Tacon, 2007).

▶ **Trends in Aquaculture production by environments:** Caution should be used in making conclusions on the current importance of each environment. First of all, figures on the contribution of each environment are different depending on whether we consider the production of animal organisms on their own (fish, crustaceans, molluscs...) or total animals plus plants. Furthermore the categorisation of specific aquaculture productions in brackish or seawater may be problematic and was reported in many cases to change from one country to another as there is no existing standard. This being said, in 2005 aquaculture production from marine waters was estimated at 31.4 million tonnes, representing 49.9% of the global total (FAO, 2006b, FAO FIGIS database, 2007).



	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Total Capture (-plants)	84.6	83.6	85.1	86.5	92.1	92.3	93.7	94.2	87.6	93.6	95.6	93.1	93.2	90.3	94.4	93.3
Total Aquaculture (-plants)	13.1	13.7	15.4	17.8	20.8	24.4	26.6	28.6	30.5	33.4	35.5	37.9	40.4	42.7	45.9	48.2

Figure 1.1: Evolution of world harvest and aquaculture production between 1990 and 2005 (FAO, FIGIS database, 2007).



	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Aquaculture miscellaneous	0.04	0.02	0.02	0.03	0.06	0.06	0.06	0.08	0.11	0.13	0.14	0.17	0.16	0.33	0.39	0.46
Aquaculture crustaceans	0.76	0.92	0.98	0.93	1.00	1.10	1.12	1.22	1.36	1.51	1.82	2.13	2.39	3.25	3.65	3.96
Aquaculture molluscs	3.61	3.80	4.50	5.62	6.72	8.23	8.50	8.56	9.14	10.1	10.7	11.4	12.0	12.6	13.1	13.4
Aquaculture fish	8.6	9.0	9.9	11.2	13.0	15.0	16.9	18.7	19.9	21.6	22.7	24.2	25.8	26.5	28.7	30.3
Aquaculture plants	3.7	4.5	5.8	6.6	6.9	6.8	7.2	7.2	8.6	9.6	10.2	10.6	11.6	12.5	13.9	14.8

Figure 1.2: Aquaculture production by major species groups between 1990 and 2005 (FAO, FIGIS database, 2007).

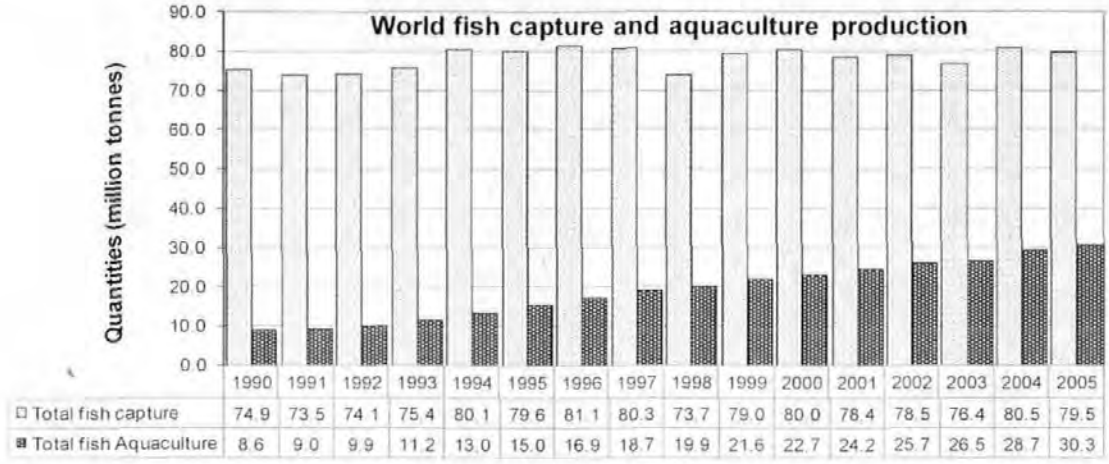
Freshwater aquaculture contributed 27.7 million tonnes, or 44.1%. The remaining 3.8 million tonnes or 6.0% came from production in brackish environments. On the other hand, most aquaculture production of fish, crustaceans and molluscs continues to derive from the freshwater environment with a share of 56.6% in quantity (data 2004). While much marine production (which accounts in this case for 36.0% of production quantity) consists of high-value finfish, there is also a large amount of relatively low-priced mussels and oysters. Although brackish-water production represented only 7.4% of production quantity in 2004, it contributed 16.3% of the total value, reflecting the prominence of high-value crustaceans and finfish.

▶ **Trends in Aquaculture, production by regions:** In 2004, countries in the Asian and the Pacific region accounted for 91.5% of the production quantity and 80.5% of the value. Europe came second contributing to nearly 4% (quantity), followed by Latin America and the Caribbean (2.26%), North America (1.27%) and Africa (around 1%). Of the world total China is reported to account for 69.6% of the total quantity and 51.2% of the total value of aquaculture production. Because of the importance of China and the uncertainty about its production statistics, China is generally discussed separately from the rest of the world; this way it does not distort the situation in certain analyses. With respect to economic grouping, the developing country share of global aquaculture production has increased from 42.4 percent (271,101 tonnes) in 1950 to over 93.3% (58.75 million tonnes) in 2005.

▶ **Diversity of cultured species:** Unlike terrestrial farming systems, where the bulk of global production is based on a limited number of animal and plant species, over 240 different farmed aquatic animal and plant species were reported in 2004, an increase of 20 species compared with the number reported in 2002. These 240 species represent 94 families; moreover this diversity is probably underestimated, as 8.9 million tonnes (15.1 percent) of global aquaculture production, including an additional 20 families was not reported to the species level in 2004, and this unspecified group is likely to include species

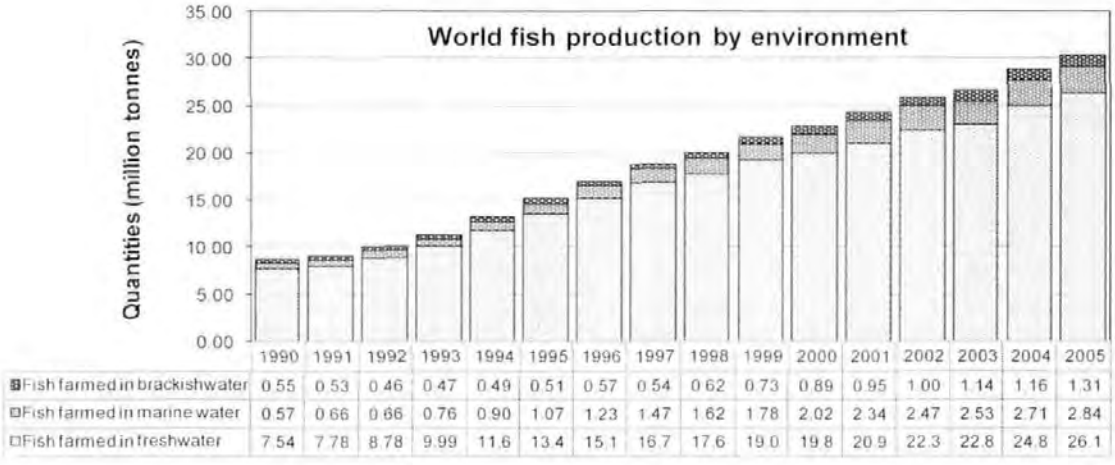
not yet recorded as being cultured. 60% of the total number of cultured species would be fish species, 22% mollusc species and 12.5% crustacean species. The cyprinids, with 18.2 million tonnes valued at 16.3 billion US$, emerge as the most important taxonomic family by quantity and by total value. By volume, Ostreidae (oysters) are a distant second at 4.6 million tonnes and are followed closely by Laminariaceae (kelps) at 4.5 million tonnes. Farmed fish species of commercial importance are: Atlantic salmon and Pacific salmon, sea trout, turbot, halibut and flounders, sea bass and sea bream... (marine species) ; rainbow trout and brown trout, European and Japanese eel, channel catfish and African catfish, sturgeon, carp and tilapia (freshwater species), (Figure 1.6).

▶ **Finfish production share of global aquaculture:** With 23.07 million tonnes produced in 2000 (representing 50.5% of total aquaculture production including plants) and 30.3 million tonnes in 2005 (representing 48.1 percent total production by weight) fish farming has become today a significant Agri-business industry (FAO, 2006b). In 2005, finfish were by far the largest cultured species group (Figure 1.2). Between 1970 and 2000, global finfish production expanded by an average rate of 10.4 percent per annum. The period 2000-2004 has actually seen a strong growth in production of crustaceans, in particular and of marine fish. Further information and trends in the fish aquaculture sector can be found in Figure 1.3, 1.4, 1.5 and 1.6.



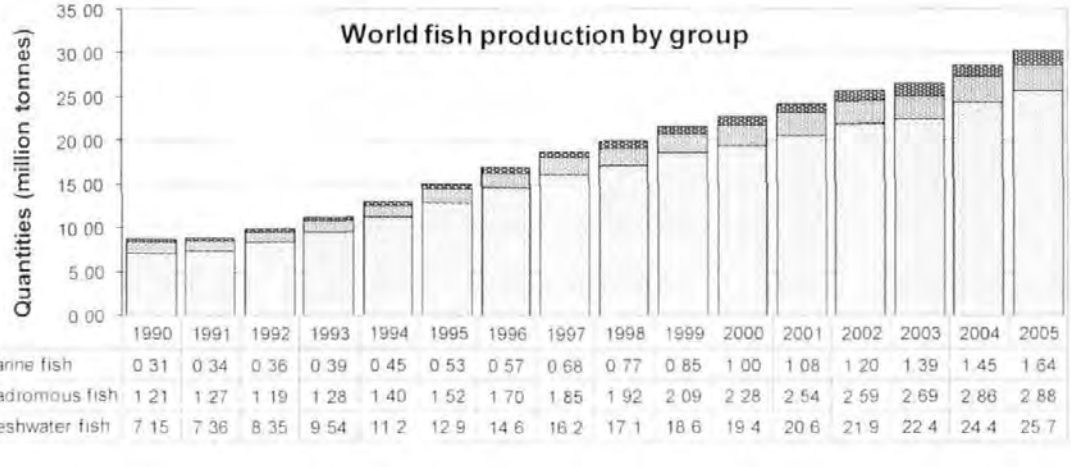
	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Total fish capture	74.9	73.5	74.1	75.4	80.1	79.6	81.1	80.3	73.7	79.0	80.0	78.4	78.5	76.4	80.5	79.5
Total fish Aquaculture	8.6	9.0	9.9	11.2	13.0	15.0	16.9	18.7	19.9	21.6	22.7	24.2	25.7	26.5	28.7	30.3

Figure 1.3: Comparison of world fish capture and production between 1990 and 2005 (FAO, FIGIS database, 2007).



	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Fish farmed in brackishwater	0.55	0.53	0.46	0.47	0.49	0.51	0.57	0.54	0.62	0.73	0.89	0.95	1.00	1.14	1.16	1.31
Fish farmed in marine water	0.57	0.66	0.66	0.76	0.90	1.07	1.23	1.47	1.62	1.78	2.02	2.34	2.47	2.53	2.71	2.84
Fish farmed in freshwater	7.54	7.78	8.78	9.99	11.6	13.4	15.1	16.7	17.6	19.0	19.8	20.9	22.3	22.8	24.8	26.1

Figure 1.4: World fish production by environment between 1990 and 2005 (FAO, FIGIS database, 2007).

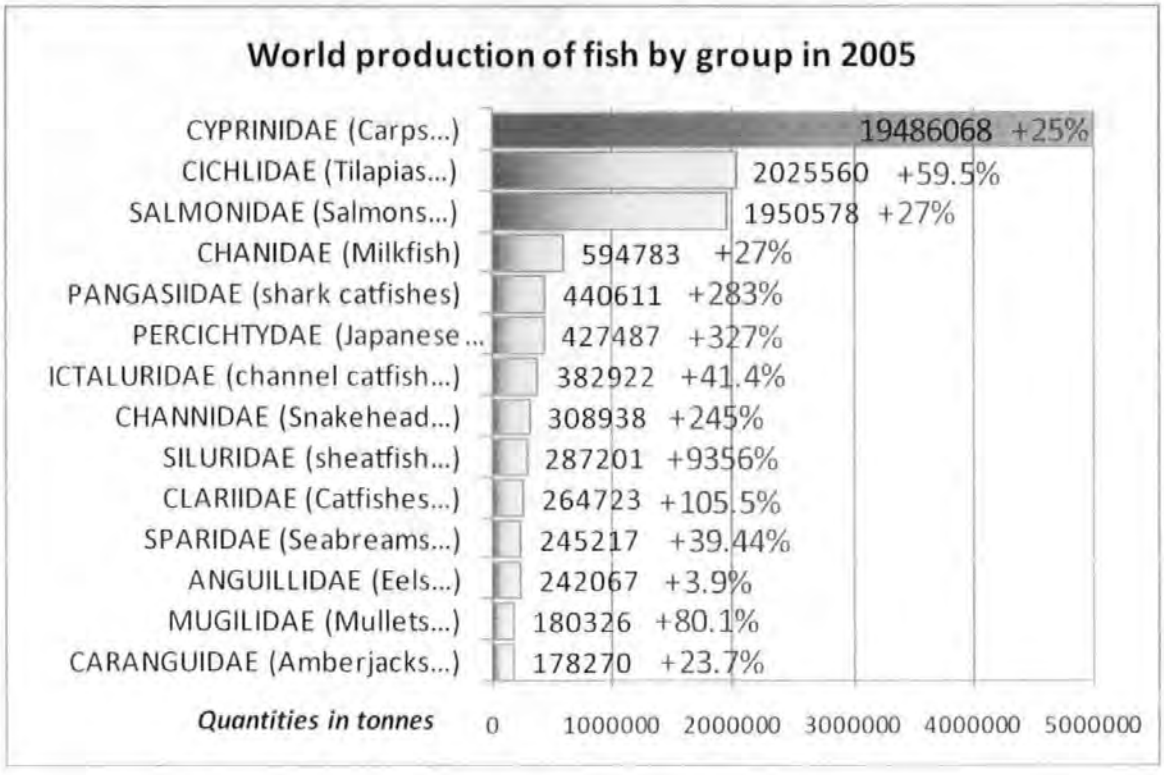


	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Marine fish	0.31	0.34	0.36	0.39	0.45	0.53	0.57	0.68	0.77	0.85	1.00	1.08	1.20	1.39	1.45	1.64
Diadromous fish	1.21	1.27	1.19	1.28	1.40	1.52	1.70	1.85	1.92	2.09	2.28	2.54	2.59	2.69	2.86	2.88
Freshwater fish	7.15	7.36	8.35	9.54	11.2	12.9	14.6	16.2	17.1	18.6	19.4	20.6	21.9	22.4	24.4	25.7

Figure 1.5: World fish production by group between 1990 and 2005 (FAO, FIGIS database, 2007).

► **Methods and culture systems in Aquaculture:** With the diversity of the species, sites and methods concerned, many forms of aquaculture currently coexist on the global scale: The huge production of Indian and Chinese carp corresponds almost exclusively to an extensive production, whereas the near total of rearing shrimps is produced with semi-intensive techniques, where the role of the natural food is significant. However, in industrialized countries as well as in the developing countries, the intensive production of marine fish especially with formulated diets is increasing.

During the past three decades, if aquaculture has started an important expansion and advanced technologically, it has also diversified and intensified. The increased intensification of culture methods is indeed another trend observed in aquaculture (Goddard, 1996). These methods are nowadays especially well established in the industrialized countries. The terms extensive, semi-extensive and intensive, which are commonly used in aquaculture to define culture methods, are generally linked to the level of input of feed and to the stocking densities of fish that can be supported. In intensive production fish are reared at high density in tanks or cages in which all the nutrients they consume are externally sourced (artificial/formulated diet): The water serves here as a physical support for the fish, provides oxygen, removes metabolic wastes and regulates temperature (Barnabé, 1994). All aquaculture production systems must obviously provide a suitable environment to promote optimal growth and health of the aquatic crop. Also, in intensive fish culture, whether of marine or fresh-water fish, with the maintenance of adequate environmental conditions, the provision of high quality diet (which is given in pre-determined quantities at precise intervals) is a problem of prime importance; and feed and feeding represent accordingly one of the major operating costs in this type of aquaculture.



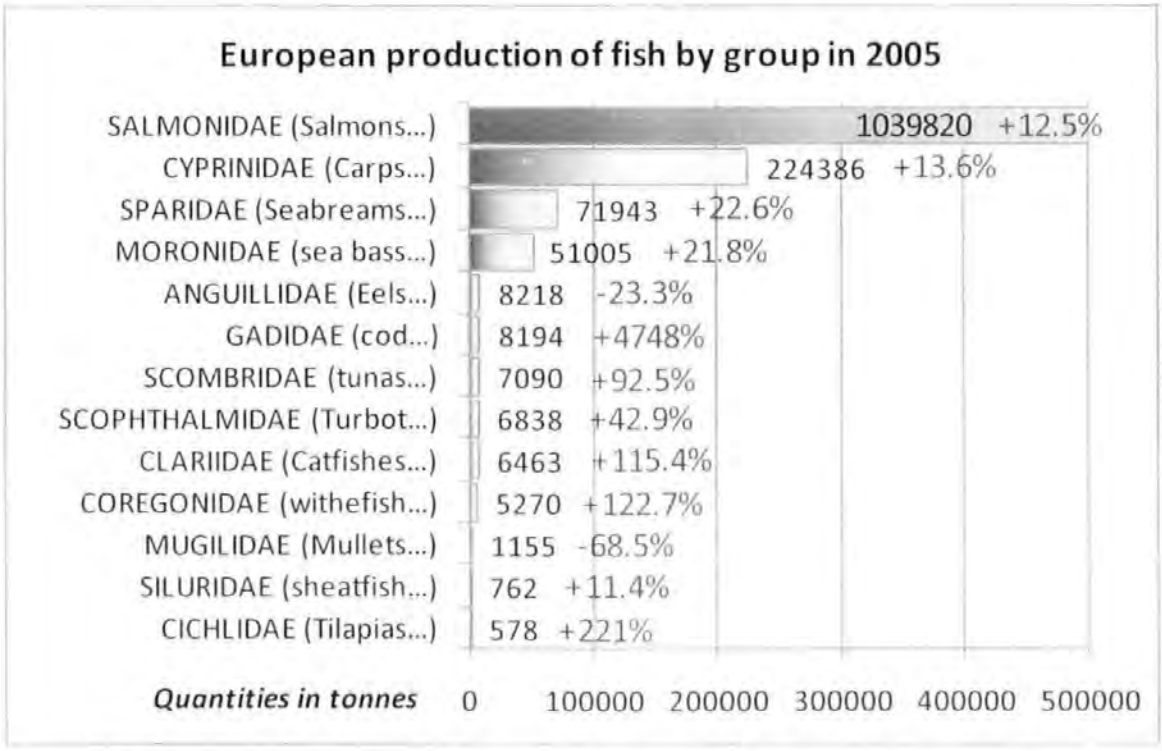


Figure 1.6: Major families of fish produced in the world and in Europe in 2005 (FAO, FISHSTAT PLUS, 2007). Growth rates between 2000 and 2005 are indicated in red.

Aside from this, a wide variety of culture systems are used around the world (earthen ponds, concrete tanks, raceways, pens, cages, vertical or horizontal line... etc.). The type of system used depends obviously on the rearing method and species reared; thus although there are no statistics on the share of the production by system or method, this may be inferred for a region and a species.

► **Projections and consequences on food supply:** The recent FAO report (FAO, 2006a), "State of World Aquaculture 2006", acknowledges the lack of potential growth in capture fisheries while estimating a need for additional 40 million tonnes of aquatic food by 2030. The total fish catch from the world's fishing grounds has indeed plateaued in the last decade. It is well recognized that over-fishing has already caused devastating effects on wild fish stock abundance on a world-wide scale, and sustainable harvest of exploitable fishery resources is unlikely to be surpassed and may even decline in future years. Wild capture fisheries landings decreased by 1.2 percent to 94.6 million tonnes in 2005. In contrast, aquaculture production seems to be responding to the increase in fish demand, having exclusively increased the world fish production by 20 million tonnes in the past decade. Although aquaculture production still remains at present insufficient compared to the estimated needs (deficit of 26 million tonnes in 2000 according to the FAO), its rapid expansion assists in meeting the increasing demand for seafood and related products. At the turn of the millennium it was stated that aquaculture will have to play a more definite role in meeting the dietary needs of an increasing world population while making up in the decline in natural marine resources (Hasan, 2001). However, as regards this challenge, questions rise about feed availability as a limiting growth factor for the aquaculture industry.

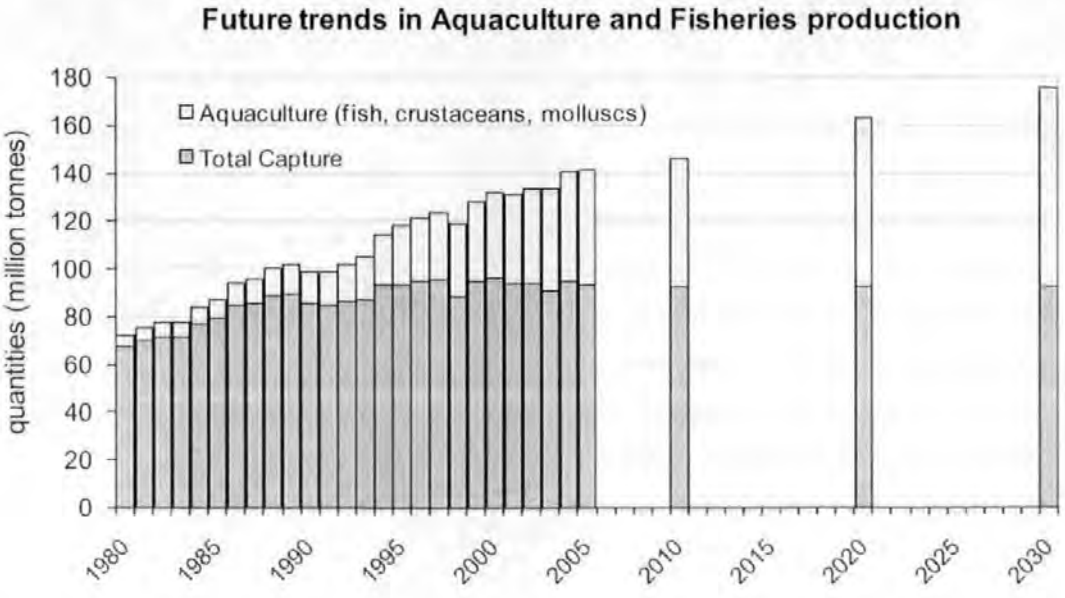


Figure 1.7: Fisheries and aquaculture production between 1980 and 2005, projection for 2010 and later (FAO, FISHSTAT PLUS, 2007; FAO, 2006b).

► **Challenges for Aquaculture:** Aquaculture will have to face numerous challenges over the next decades. These notably concern diseases and epizootics, brood stock improvement and domestication, development of appropriate feeds and feeding mechanisms, hatchery and grow-out technology, as well as water quality management and environmental issues. All these issues present considerable scope for biotechnological interventions.

At present, fish nutrition and feed technology are a focus of considerable research, stimulated by the requirement of an increasingly demanding intensive aquaculture. Given the economic importance of feeds and feeding in modern aquaculture along with the future expectations in terms of volume of production, nutrition research and the aqua-feed industry remain under pressure to provide cost efficient and nutritionally complete feeds and to ensure the sustainability of aqua-feed production. Additionally, this field of research remains widely involved with other important issues like product and environment quality, safety and human welfare and health.

1.1.2 Fish mariculture and marine fish farming

▶ **Introduction:** The dramatic increases in aquaculture production observed over the past 15 years have been made possible, to a large part, by gains in our understanding of fish biology and by technical advancements. Increased understanding of nutritional requirements of certain fish species, coupled with improvements in feed manufacturing technology and feeding techniques, have in particular been central to the expansion of modern aquaculture. However, it is unequivocally agreed that global aquaculture will continue to increase in the next decades (Hasan, 2001) (Figure 1.7). Aquaculture production of high value species has effectively the potential to increase much further, especially in marine locations where water is not as limiting compared to established freshwater sites.

▶ **Current status at the world scale:** In 2005, total production of marine fish species amounted to 1.6 million tonnes, while 2.8 million tonnes of fish were reported to be cultured in marine environments (Figure 1.4, 1.5). Although some marine species may be farmed in brackish waters, this overall difference is explained from the fact that a great quantity of fish classified as diadromous (not as marine fish) are actually cultured in seawater. Nevertheless, whatever the number considered, both productions (marine aquaculture and marine species) remain relatively small compared to the 30.3mmt of fish produced in 2005 (representing 9.2% and 5.3% respectively). Marine fish production, which really began to develop in the 80s, is indeed more recent than any other type of aquaculture, but is now rapidly expanding. During the period 1970-2004 global production of marine fish species showed the greatest average annual rate of growth after crustaceans (10.5% against 18.9% for crustaceans; 8.8 overall; FAO, 2006a) (Figure 1.8). According to the latest records, Japanese sea bass (*Lateolabrax japonica*), Japanese amberjacks (*Seriola quinqueradiata*) and red sea bream (*Chrysophrys major*) are some of the most important species on the worldwide scale (Figure 1.10). In addition, diversification of marine fish

species cultured is also more and more important; the number of cultured species has tripled since 1980.

▶ **Situation in Europe:** Stimulated by consumer demand and technological progress, mariculture has equally grown exponentially in Europe, which now contributes 9.3% of world marine fish production (FAO, FIGIS database, 2007) (Figure 1.9). The production increased from 28000 tonnes in 1980 to 152000 tonnes in 2005.

Gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) are the most farmed marine fish species in Europe and Mediterranean area (Figure 1.11). The two have been farmed side by side successfully for the last two decades and are accepted success stories for aquaculture, taking possibly the pressure off wild stocks of the species.

Overtaking the production of sea bass in 1994, gilthead sea bream has become the first marine farmed fish within Europe and the Mediterranean countries (Figure 1.11, 1.12). In 1993 the aquaculture production caught up with fisheries captures for the first time. In 2003 total production reached ~350 millions fry and ~85000 tonnes. For both species, intensive farming constitutes the majority of production, with floating cages and ponds as the preferred technology. Major producing countries are Greece, Spain and Italy, with expansion in Turkey and Cyprus.

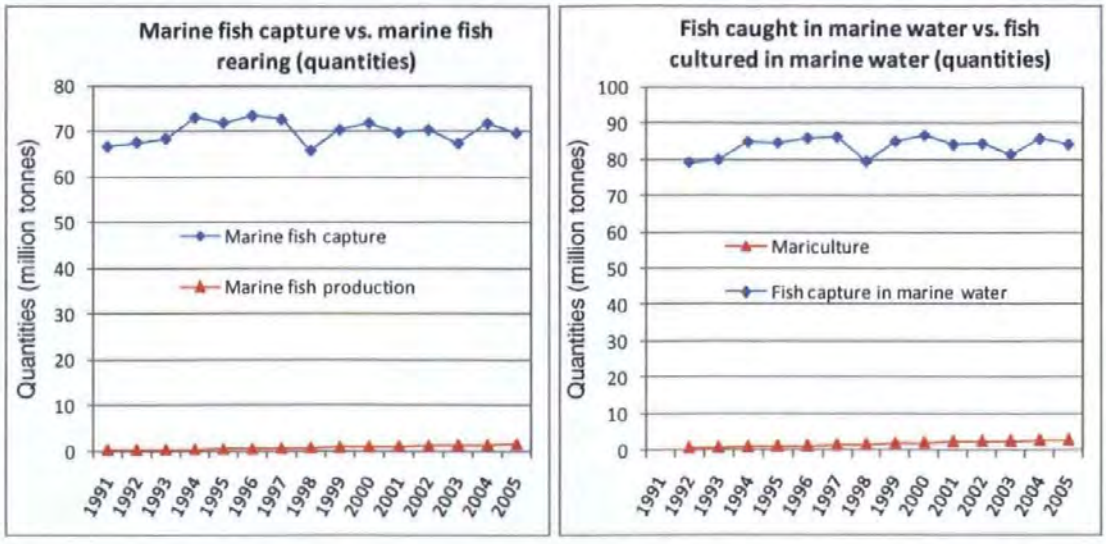


Figure 1.8: Total global marine finfish landing (left) or production of finfish in marine waters (right) through capture fisheries and aquaculture (FAO, FISHSTAT PLUS, 2007; FAO, Yearbook of fishery statistics, 2007).

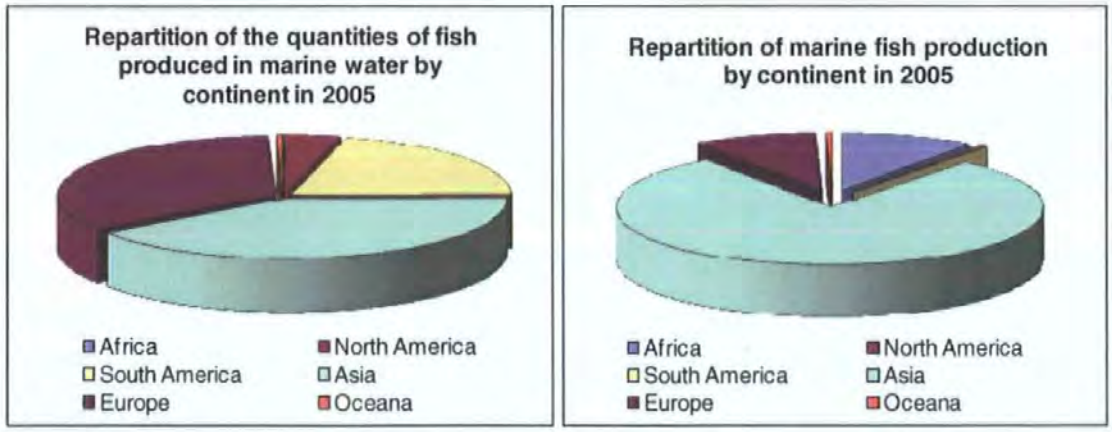


Figure 1.9: Geographic repartition of mariculture or marine fish production in 2005.

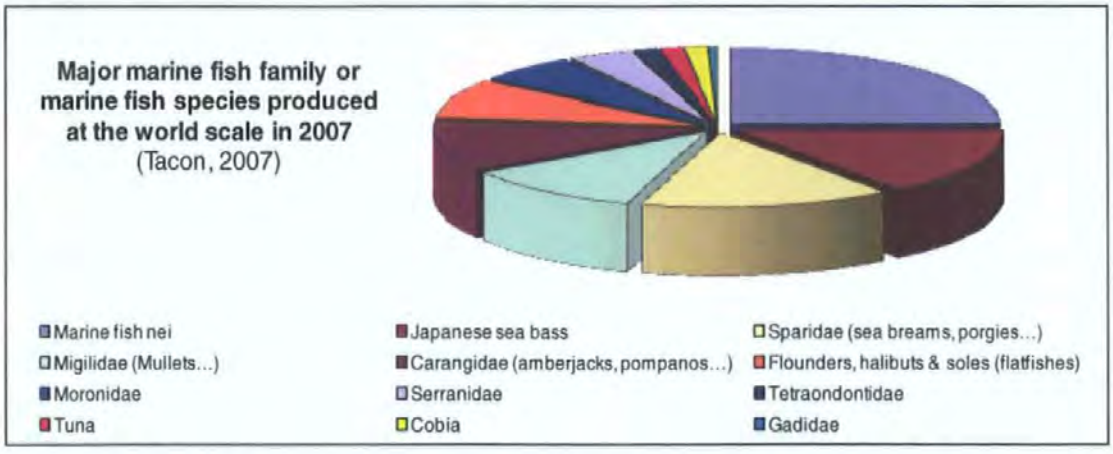


Figure 1.10: Main group or species of marine fish produced in 2007 in the world.

▶ **Evolution, rationale behind future expansion:** Success of hatcheries with mastery of modern technologies is a requisite for marine aquaculture development. If the industrial production of marketable size fish (marine fish such as sea bream and sea bass) has mainly been limited by difficulties in the hatching and larval rearing steps (especially in relation to the specific aspect of the larval nutrition), the knowledge and techniques in this field are now much more advanced. Nutrient enriched starter diets, and improved methods such as "mesocosm" and "pseudo green water technology" have effectively led to increased survival rates at the critical larval stages, allowing better control in marine finfish larval production, and consequently a superior and steady supply of fry (Divanach *et al.*, 1998). High growth rates observed for marine fish production in recent years are probably the result of the development achieved in hatchery techniques; also larviculture research currently conducted on new species might allow further expansion. Offshore aquaculture might be another important factor for future expansion.

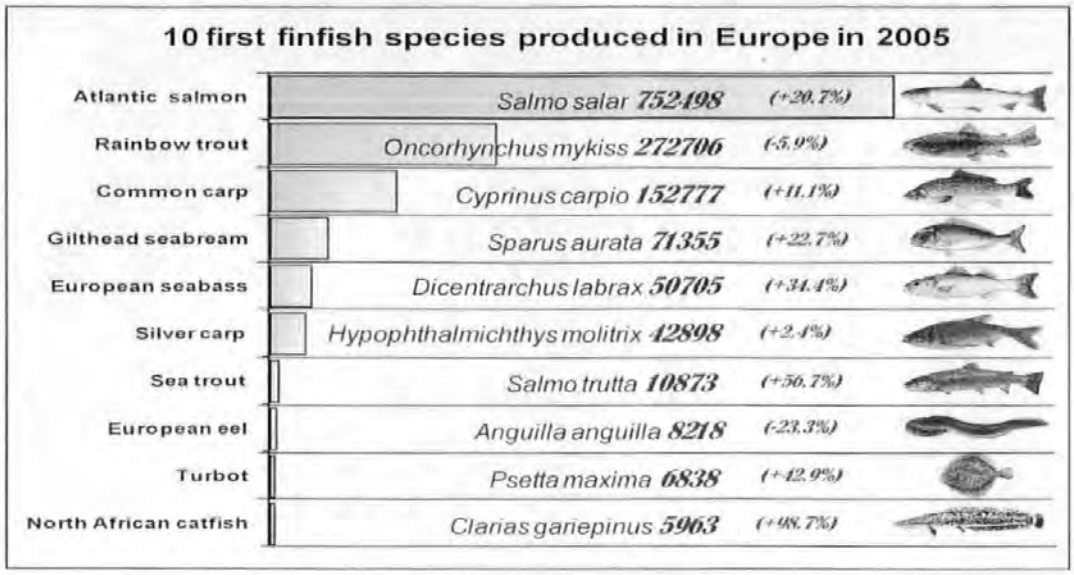


Figure 1.11: Major fish species produced in Europe in 2005 (FAO, FISHSTAT PLUS, 2007). Growth rate of each production for the period 2000-2005 is indicated in red.

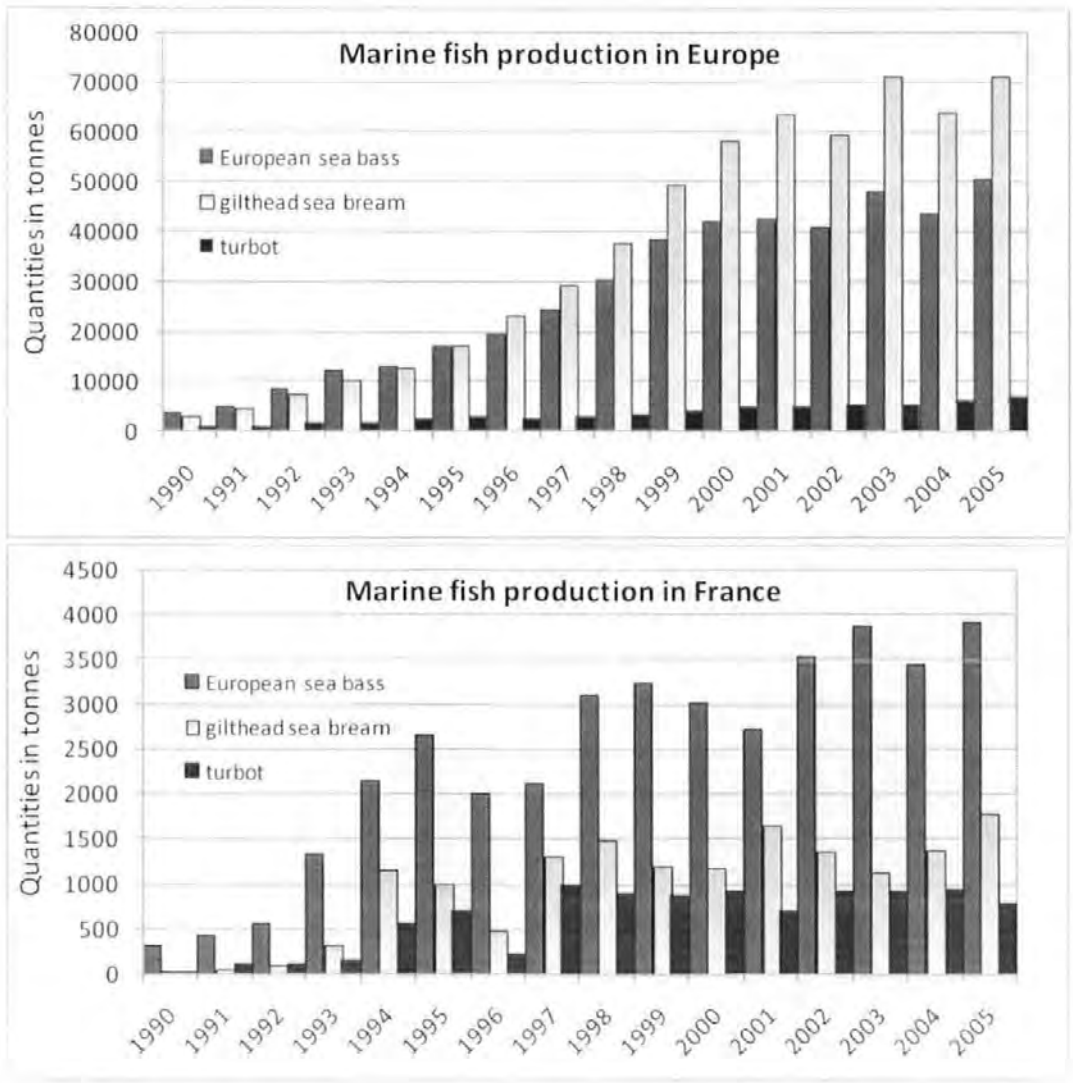


Figure 1.12: Production of European sea bass, gilthead sea bream and turbot in Europe (top) and France (bottom) between 2000 and 2005 (FAO, FISHSTAT PLUS, 2007).

1.2 NUTRITION AND FEEDING FOR SUSTAINABLE AQUACULTURE DEVELOPMENT

1.2.1 Nutritional characteristics and nutritional requirements of fish

Nutrition is the physiological science which studies the processes ensuring the supply of energy and nutrients to the organism for vital functions. It covers different stages such as ingestion, digestion, absorption, metabolisation and excretion.

While the nutrition of terrestrial animals like cattle and poultry is a quite advanced science, the nutrition of fish is a more recent field of study. In spite of some fast progress, the difficulties encountered by researchers in this field are numerous. Those are related to the great diversity of fish, their aquatic environment as well as specific biological characteristics.

The type and list of nutrients fish require to stay active, healthy, and grow, are the same as those required by other animals. However, due to their biological, physiological and ecological characteristics, those requirements can vary qualitatively and quantitatively when compared to other farmed animals.

1.2.1.1 The fish as a biological model for nutritional studies

From the anatomical and physiological point of view fish presents some particularities that deserve special attention when working in the nutritional area.

► **Physiology:** Fish are poïkilotherms (their blood temperature remains close to that of the environment in which they are living) and ammoniotelic (they release nitrogen wastes in the form of ammonia rather than urea and uric acid); both of these phenomena having of course an obvious influence on the energetic metabolism. Similarly, living in an aquatic medium is, from an energetic point of view, another advantage since fish do not have to consume energy to maintain position (floating). The consequences of living in an aquatic

medium also include a more frequent use of the anaerobic pathway since the partial pressure of oxygen in the water is relatively low (this results in high ventilation costs and hypertrophy of white muscles).

▶ **Anatomy:** Length of the digestive tract, oesophagus morphology, presence or absence of a stomach, number of pyloric caecae, and type of intestine are some of the factors that contribute to the great diversity of gut morphology in fish (Cahu, 2004). However, constitution of the intestine wall (made of three folds: mucosa, muscularis and serous membrane), enzymatic equipment (relatively similar to those of higher vertebrate), presence of pseudo-villae and relative differentiation of the intestine (as it appears in mammals or birds), can be cited as some of the anatomical characteristics that remain constant whatever the fish group considered (Guillaume *et al.*, 1998). Although fish can retain an ability to respond to new niches and food varieties, an important relationship between food habits and gut morphology (i.e. mouth anatomy, length of gut…) is generally found, reflecting a certain degree of adaptation (Cahu, 2004).

▶ **Ecology:** Unlike most domesticated farmed animals, most of the high value fish farmed are carnivorous. Also, if terrestrial animals draw their energy from starch or cellulose, in regard to the trophic chain structure in aquatic ecosystems, fish in general are much more adapted to the transformation of proteins and lipids.

1.2.1.2 Nutritional characteristics of fish, brief overview of fundamental requirements

Fish must have an energy source to maintain the body machinery (i.e., metabolism). They also require an adequate amount of protein, essential amino acids, fats and essential fatty acids plus vitamins and minerals to sustain life and to promote growth. In the following paragraphs fundamental principles of fish nutrition will be broadly developed in terms of protein/energy requirements; similar introductions on lipid, carbohydrate and mineral

nutrition in fish are not considered as essential here, but some information on these nutrients will be provided in sections 1.2.1.4 and chapter 5.

▶ **Proteins:** Proteins are polymers of amino acids arranged in poly- peptides which are involved in structural and many key physiological functions. Thereby, protein usually accounts for 70 to 85% of the dry matter component of a fish carcass and is represented by a variety of substances that have a fundamental role such as enzymes, hormones or antibodies. Dietary protein is utilized by fish in three different ways: i) maintenance (energy source, synthesis of carbohydrates, lipids, proteins, hormones, enzymes, antibodies), ii) repletion of depleted tissues and iii) growth (synthesis of new additional protein tissues). Those major tasks are, in fact, completed through the catabolism and anabolism of amino acids: although we do often refer to a dietary requirement for protein, it is indeed of utmost importance to note that fish do not have a protein requirement as such but a requirement for a certain level of well balanced and available amino acids. The requirement for dietary protein actually has two components: i) a need for indispensable amino acids that fish cannot synthesize *de novo* and ii) a supply of dispensable amino acids in the correct ratio.

Protein is often considered as the most important component of fish diet because of the influence of protein intake on growth, the high cost of proteins and the high level required per unit of feeds. The high protein requirement of fish (in term of dietary percentage) is generally attributed to their carnivorous feeding habit and their preferential use of protein over carbohydrates as a dietary energy source. However, absolute requirements (g/Kg body weight gain) end up being closer to those of warm blood animals as a result of differences in feed efficiency and absolute energy requirements in fish. The optimum level of protein in fish diet is generally influenced by fish species, protein quality, dietary protein to energy ratio, physiological status (age and size of fish), environmental parameters and feeding rates.

▶ **Protein & energy:** Protein is used for fish growth if adequate levels of fats and carbohydrates are present in the diet. If not, protein may be used for energy and life support rather than growth. This refers to the protein sparing effect of lipids/carbohydrate and means that dietary protein supply can be reduced, provided that diet formulation is based on digestible protein to digestible energy ratio (DP/DE). Moreover, increasing the non-protein energy content of the diet can prove to be beneficial in terms of feed efficiency (involving a better protein retention) and may lead to a significant reduction of nitrogen excretion (resulting from protein catabolism). On the other hand, excess energy relative to protein content in the diet may result in high lipid deposition. Properly formulated prepared feeds must have a well-balanced energy to protein ratio. Although this could not be confirmed by Velàsquez *et al.* (2006), the data obtained by Kentouri *et al.* (1995) and Lupatsch *et al.* (2001) show that gilthead sea bream are able to regulate their feed consumption on the basis of the protein and/or energy content of the diets, in order to fulfil their nutritional needs. Due to the fact fish feed to meet their energy requirements, diets with excessive energy levels may result in decreased feed intake and reduced feed efficiency. Similarly, a diet with inadequate energy content can result in reduced weight gain because the fish cannot eat enough feed to satisfy their energy requirements for growth.

1.2.1.3 Nutritional requirement of gilthead sea bream (Table 1.1)

▶ **Dietary protein, protein/lipid ratio:** As a result of their highly carnivorous nature, the protein requirement (dietary percentage) for marine fish is usually found to be higher than those of salmonids. The first dose-response investigation carried out by Sabaut and Luquet (1973), with semi synthetic diets, showed that gilthead sea bream juveniles require 40% protein in their diet. Testing feeds containing different protein levels (45/63% or 48/54%), Koening (1973) and Kissil (1981) reported that high protein diets were more effective in supporting the growth of 5g gilthead sea bream and suggested reducing the dietary protein

level beyond this size class of fish. It is now well known that protein requirements (as a proportion of the diet) decrease as fish approach maturity. More recently, levels of dietary protein for gilthead sea bream fry were established at 55% by Vergara *et al.* (1996a), while Santinha *et al.* (1996), working with juveniles, estimated that a minimum of 45% of protein was necessary to obtain high growth rate (2.3% per day) together with good feed efficiency.

Apart from that work, further investigations were performed on the optimal protein to lipid ratio since a protein sparing effect of lipids was observed in many fish. Marais & Kissil (1979) stated that 9% of fat in a diet containing 44% protein would represent the maximum amount needed for optimum growth of gilthead sea bream. However in this trial, the fact that the authors used soybean oil as a lipid supplement may have affected the conclusion. In another investigation on red sea bream, Takeuchi *et al.* (1991) found that suitable crude protein and crude lipid levels in the diet were around 52% and 15%, respectively. Vergara *et al.* (1996b) concluded that dietary protein level could be decreased from 58 to 46% when increasing the lipid content of dry matter from 9 to 15%. However, despite this protein sparing effect, these authors did not find any significant effects of the dietary lipid level on the protein efficiency ratio (PER). Santinha *et al.* (1999), obtained better feed efficiency and nitrogen retention values with a diet containing 21% lipid, regardless of dietary protein level tested (47/51%). Nevertheless, a diet with a level of 21% lipid did not improve fish growth and resulted in a significant increase in body fat content. Vergara *et al.* (1999) suggested that 22% would be the optimum level of lipid in diets based on high quality fishmeal, while an elevation of dietary lipid levels up to 28% would be necessary to promote best growth when standard or lower quality fishmeal is utilized. Similar work by Caballero *et al.*, (1999) led to the same conclusion with additional confirmation from histological observations. Company *et al.*, (1999a, 1999b) found no differences in growth rate of sea bream with diets including 9% lipid - 55% protein or 17% lipid - 47% protein when fed to satiety. This lack of protein sparing effect was equally observed by Velàsquez

et al., (2006). A few contradictions arise then from these studies regarding the protein sparing effect of lipids, and it may be argued that sea bream do not seem to use lipid as efficiently as salmonids for energy.

▶ **Protein/energy requirements:** As protein may function as an energy source apart from its essential role for growth, the optimal balance between the supply of dietary energy and protein should be examined with more attention. Most of the research effort is now indeed directed toward identification of the digestible protein to digestible energy ratio since the protein sparing effect appears to be influenced by the dietary digestible energy level rather than the nature of the non-protein energy sources (Kaushik, 1997). Work mentioned previously on the protein to lipid ratio indicated that the optimum protein to energy ratio was higher and the protein retention efficiency lower in marine fish compared to salmonids (Kaushik, 1997). Data from the literature on optimal P/E or DP/DE ratio for marine fish are also rather contradictory. Besides, comparisons may be inconclusive since the determination of the ideal ratio is affected by many factors (DP/DE value depends on fish size, digestibility coefficients, feeding rate and other aspects of methodology). Following a comprehensive investigation Lupatsch *et al.*, (2001) recommended using DP/DE ratios of 28.5, 21.6 and 19.3 (mg DP/ KJ DE) for fish weights of 10, 100 and 250g respectively. A decrease of DP/DE ratio with increasing fish weight was also indicated for gilthead sea bream in the study of Garcia-Alcázar *et al.*, (1994), where smaller fish up to 100g grow better on a 49% protein and 12% lipid diet while bigger fish up to 330g performed better on a 45% protein and 19% lipid diet. Using the factorial approach, Lupatsch *et al.*, (1998), also determined the protein and energy requirement of gilthead sea bream in terms of absolute daily feed intake per unit of weight. For instance, DE for maintenance and growth was estimated at 3.4 KJ $fish^{-1}$ day^{-1} for 10g fish and an assumed growth rate of 1.5% per day, and 69.9 KJ $fish^{-1}$ day^{-1} for 250g fish at an assumed growth rate of 1.0% per day.

▶ **Essential amino acids (EAA):** An absolute requirement for ten amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) has been demonstrated in all fish species examined so far (NRC, 1993). In gilthead sea bream, qualitative requirements were experimentally established for tryptophan, arginine and methionine (cystine), following the injection of radio labelled glucose (Sabaut & Luquet, 1973). The complete quantitative amino acid requirements have been determined for a few fish species only (e.g. Chinook salmon, Japanese eel, channel catfish, common carp, Nile tilapia), (NRC, 1993). Only a limited number of values are available on the quantitative requirements of gilthead sea bream. These were estimated by Sabaut and Luquet (1973) with a classic dose-response study, and concern arginine (5.0 g/16gN), lysine (5.0 g/16gN), methionine (4.0 g/16gN) and tryptophan (0.6 g/16gN). Since carcass amino acid patterns correlate well with quantitative amino acid requirements, and amino acid composition does not change significantly among fish species, available data on the amino acid requirement of other species or amino acid profile of the carcass may be used as a guideline for practical diet formulation when specific amino acid requirements of a given species are not known (Kaushik, 1998).

▶ **Fatty acids (FA):** Considerable information is available on the EFA requirements of gilthead sea bream larvae, especially with regard to the relative importance of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and Arachidonic acid (ArA) (Liu *et al.*, 2002; Rodriguez *et al.*, 1998). Investigations on the requirement of juveniles or grow-out stages are relatively scarcer and it is rather difficult to draw conclusions on the qualitative and quantitative change over life stage. The provision of EPA in broodstock proved to be beneficial in terms of reproductive performance and egg quality (Kaushik, 1997, Almansa *et al.*, 2001). While Harel *et al.* (1994) recommended a minimum of 0.42% highly unsaturated fatty acids (HUFA) for the production of good quality eggs, Fernandez-Palácios *et al.* (1995) estimated that spawning quality of sea bream may be improved with

the increase of dietary HUFA up to 1.6%. Apart from broodstock related studies, quantitative requirement for poly unsaturated fatty acids (PUFA) in larvae is generally expected (assumed) to be higher as larvae grow much more rapidly than juveniles (Sargent *et al.*, 1997).

Evidence for the qualitative requirements of EPA and DHA in marine fish such as gilthead sea bream can be found in the high level of those fatty acids in fish tissues (also related to the FA composition of prey), as well as in the impairment of the desaturase/elongase pathway that would allow the formation of those PUFA from C18 precursors. *In vivo* (Mourente and Tocher, 1993) and *in vitro* (Tocher and Ghioni, 1999) trials led to the observation that Δ5 desaturase activity was significantly lower, and that C18-C20 and C20-C22 elongase activities were substantial in sea bream.

The minimum levels of dietary n-3 HUFA including EPA and DHA required by gilthead sea bream for optimum growth and development have been reported as 1.5% of the diet dry weight for larvae (Rodriguez *et al.*, 1994, 1998), around 0.9-1% for fingerlings (1g fish) and juveniles (11.5g fish) (Kalogeropoulos *et al.*, 1992; Ibeas *et al.*, 1996). Furthermore, n-3 HUFA requirements might not only be a function of the total amount of these fatty acids in the diet, but also of the relative proportions of DHA, EPA, and ArA (as mentioned previously). Maintaining the amount of n-3 HUFA in the diet for juvenile sea bream at 1%, Ibeas *et al.*, 1997 obtained the best growth rate and lowest hepatosomatic index with EPA/DHA ratio of 2/1. Reduction in growth of gilthead sea bream juveniles has been reported with diets containing 5% n-3 HUFA in the form of triglycerides (Ibeas *et al.*, 2000).

An important role played by ArA has also been mentioned in connection with the formation of eicosanoids that are produced in response to stressful situations. Eicosanoids formed from EPA being less biologically active and competitively interacting with the formation of eicosanoids from ArA, the determination of the appropriate ArA/DPA ratio is

of importance (Sargent *et al.*, 1999a, 1999b; Bell and Sargent, 2003). Fountoulaki *et al.*, 2003 observed that the increase in ArA levels in diets did not differentiate growth of sea bream fingerlings.

▶ **Carbohydrates:** The ability of fish to use carbohydrates differs among species and appears to be associated with the complexity of the carbohydrate. As carnivorous fish, most marine farmed fish are generally not known to be efficient users of carbohydrate as an energy source. However, some commercial feeds can contain appreciable amounts of carbohydrates like starch (often incorporated for practical reasons such as water stability, texture and palatability enhancer) and a certain degree of assimilation can occur (Wilson, 1994). The utilization of carbohydrate as a protein sparing source of energy has effectively been reported for a few fish species (Shiau and Peng, 1993; Erfanullah and Jafri, 1995), but requires further validation, especially for marine species (Alvarez *et al.*, 1999; Peres and Oliva-Teles, 2002). Since carbohydrate is the least expensive source of dietary energy, the maximum tolerable dietary levels should be used with regard to these types of fish species.

Complexity of the molecule, concentration in the diet and technological treatment applied to the carbohydrate are some of the factors that may potentially affect carbohydrate digestibility in fish. For instance in sea bream, digestibility of wheat and corn carbohydrate was found to be significantly improved as a result of extrusion treatments (Venou *et al.*, 2003). Working with sea bream, Fountoulaki *et al.* (2005) obtained reduced carbohydrate digestibility value in relation to the increasing level of starch in the diets. Fernández *et al.* (1998) concluded from their results that gilthead sea bream could easily assimilate the gelatinized starch present in the diets (with proportion up to 21.4%) when high quality protein was used. Apart from those studies, the relative ability or inability of fish to utilize dietary carbohydrate can be reflected in digestive enzyme measurement, or illustrated by the glucose tolerance test. Providing comparative data on proteolytic and amylase activities in 6 fish species (including gilthead sea bream) Hidalgo *et al.*, (1999) showed

that omnivorous species had higher amylase activity than carnivores. Glucose tolerance tests (oral administration of glucose) conducted so far with various fish species resulted in most cases in a persistent hyper-glycemia. Although this was assumed to be linked to the poor response of plasma insulin levels, contradictory results have indicated that this response could be similar to a non-insulin dependant diabetes, Peres *et al.* (1999) observed that sea bream was able to restore plasma glucose levels within 12 hours after an intra peritoneal injection of 1g glucose per kg body weight. Also all basal values for the parameters analysed were restored within 24 hours after glucose injection.

Literature reports on the maximum level of dietary carbohydrate that can be tolerated by different species often appear to be contradictory; whereas the protein sparing effect of carbohydrate is controversial. Further data related to gilthead sea bream are needed in this area.

▶ **Vitamins and minerals:** Information is needed on the vitamin requirements of gilthead sea bream. Investigations carried out so far demonstrated the importance of vitamin C (Alexis *et al.*, 1997; Henrique *et al.*, 1998), but concentration has still to be determined for gilthead sea bream (in other marine species it is usually found to be within the range of 5-30mg/kg, Kaushik, 1997). Taking into consideration various criteria, the requirement for pyridoxine (vitamin B6) was quantified to be around 2mg/kg dry diet by Kissil *et al.* (1981). This latter author also investigated biotin requirement in a second series of experiments, and suggested dietary requirement between 0.21 and 0.37 mg/kg. Data available on mineral requirements are also poor. In this area, the obvious difficulty comes from the fact that mineral supply can be derived from water to satisfy fish requirements. However, due to its low concentration in the water and the contribution of its excretion to water pollution, phosphorus has received more attention. Pimentel-Rodriguez and Oliva-Teles (2001) estimated the phosphorus requirement of gilthead sea bream to be around 0.75%.

Table 1.1: Summary of known nutritional requirements of gilthead sea bream juveniles from the scientific literature and nutritional composition of a commercial diet formulated for gilthead sea bream.

Nutrients	Scientific literature		Commercial diet "Sparus"
Protein	**40%**.....	(Sabaut and Luquet, 1973)	**46%**
	45%.....	(Santinha *et al.*, 1996)	
Protein:Lipid ratio	**44% : 9%**.....	(Marais and Kissil, 1979)	
	46% : 15%.....	(Vergara *et al.*, 1996b)	
	47% : 21%.....	(Santinha *et al.*, 1999)	
	47% : 17%.....	(Company *et al.*, 1999b)	
Lipid	**22%**.....	(Vergara *et al.*, 1999)	**16%**
	22%.....	(Caballero *et al.*, 1999)	
Raw Energy			**19.5MJ**
Digestible Energy			**18.0MJ**
DP:DE ratio	**28.5**.....	(Lupatsch *et al.*, 2001)	**21.1**
Arginine	**5.0g/16gN**.....	(Sabaut and Luquet, 1973)	
Lysine	**5.0g/16gN**.....	(Sabaut and Luquet, 1973)	
Methionine	**4.0g/16gN**.....	(Sabaut and Luquet, 1973)	
Tryptophan	**0.6g/16gN**.....	(Sabaut and Luquet, 1973)	
n-3 HUFA	**1%**.....	(Ibeas *et al.*, 1996)	**4%**
EPA+DHA			**3.20%**
EPA:DHA ratio	**2:1**.....	(Ibeas *et al.*, 1997)	
Tocopherols (Vit. E)			**200mg/kg**
Ascorbic Acid (Vit. C)			**150mg/kg**
Pyridoxine (Vit. B6)	**2 mg/kg**.....	(Kissil *et al.*, 1981)	
Biotin (Vit. B8)	**0.21-0.37mg/kg**	(Kissil *et al.*, 1981)	
Phosphorus	**0.75%**.....	(Pimentel-Rodriguez and Oliva-Teles, 2001)	**1.26%**

1.2.2 The aqua-feed industry: present status, problems and perspectives (the use of fishmeal in aquaculture)

▶ **Introduction:** In 2003, global aqua-feed production was approximately 19.5 million tonnes. The total use of industrially compounded aqua-feeds in 2005 was estimated to be about 23.13 million tonnes, up 18% from 2003 and 7.8% from 2004 (Figure 1.13). 9.6 million tonnes were used for feeding carps, 1.8 million tonnes for salmon and 1.4 million tonnes for marine fish (Tacon, 2007). Development of intensive aquaculture, improvement of manufacturing technologies as well as new knowledge on feed ingredients and formulation strategies are major factors affecting the aqua-feed industry.

Diet formulation is the process of combining feed ingredients to form a mixture that will meet the specific goals of production. In intensive production systems, compound feeds provided must in particular support growth, promote health and lead to the production of desirable final products (attractive and safe), while being economical, palatable and with minimal effect on the surrounding environment (Goddard, 1996).

Nowadays, regulations established for consumer protection combined with projections concerning fishmeal utilization bring many additional constraints to the formulator who is led to eliminate or replace some raw materials; amongst those raw materials, meat and bone meal, fishmeal and fish oil are particularly concerned.

Of the macronutrients in the feed, protein has and continues to receive special attention because fish present high and specific requirements for this constituent. Traditionally, fishmeal has been used in commercial feeds as the main protein source due to its ideal nutritional profile and high palatability.

▶ **Aqua-feed dependence on fishmeal: fishmeal** (see section 2.3.1 for more details on this commodity and manufacturing process) is effectively not only an excellent source of highly digestible protein and energy, it provides essential amino acids and fatty acids, lipid soluble vitamins, phospholipids as well as trace and macro minerals. For those reasons fishmeal represents a key ingredient in aqua feeds and accounts for a large part of the diet. According to the fishmeal information network (FIN, 2007), fishmeal would constitute 35% on average of salmon feeds, 30% of trout feeds and 50% of marine fish feeds (Table 1.2). However, depending on fish species and formulations employed by manufacturers, fishmeal inclusion rates may reach in certain cases 60% of the total diet.

Catches for reduction into fishmeal and fish oil have remained relatively constant for the last 25 years at between 20 and 30 million tonnes (the lowest value being obtained years where El Niño occurred). Those catches lead to a production of fishmeal fluctuating from 5 to 7mmt per annum. Various studies have estimated that global aqua-feed manufacture was using between 35 and 45% of this global fishmeal volume in recent years (Figure 1.13, 1.14 right).

According to Naylor *et al.* (2000), to produce one kilogram of marine fish it would be necessary to catch 5kg of wild fish (Table 1.2). As well as illustrating the current dependence of the aqua-feed industry on fishmeal, this statement summarizes the huge challenge that aquaculture is facing. Besides, such a poor ratio seriously calls into question the ambition of the aquaculture industry presented as an alternative to fisheries and pretending to relieve pressure on ocean fisheries.

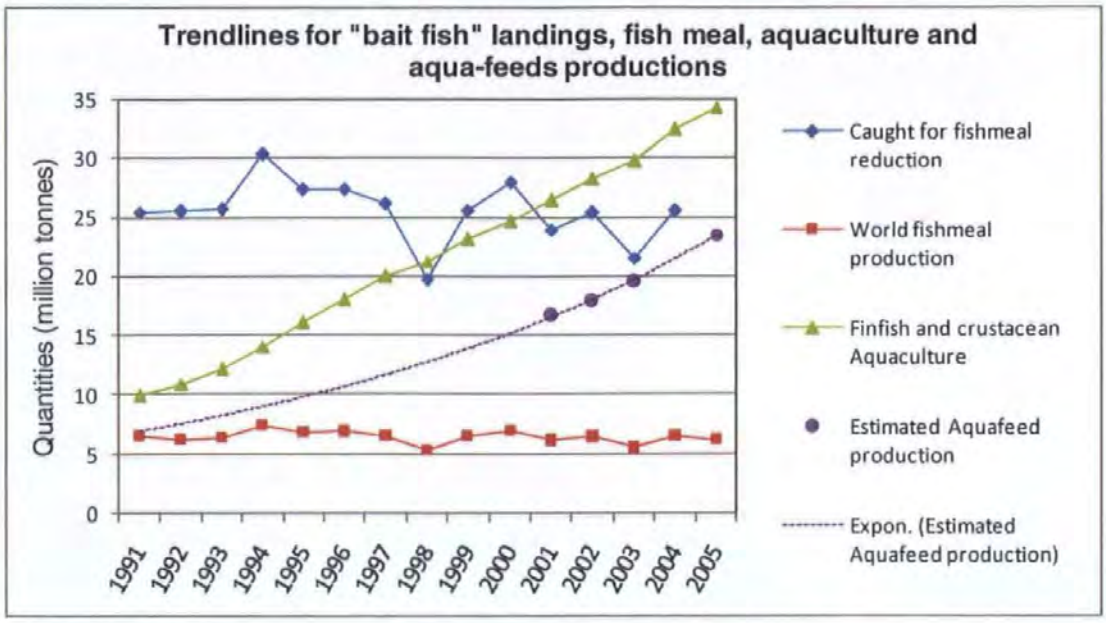


Figure 1.13: The relationship between world aquaculture production (only production of fish and crustacean are entered, FAO, FISHSTAT PLUS, 2007), total world fishery production for reduction (FAO, Yearbook of fishery statistics, 2007), total world fishmeal production (FAO, FISHSTAT PLUS, 2007) and aqua-feeds production (various sources: Tacon, 2007).

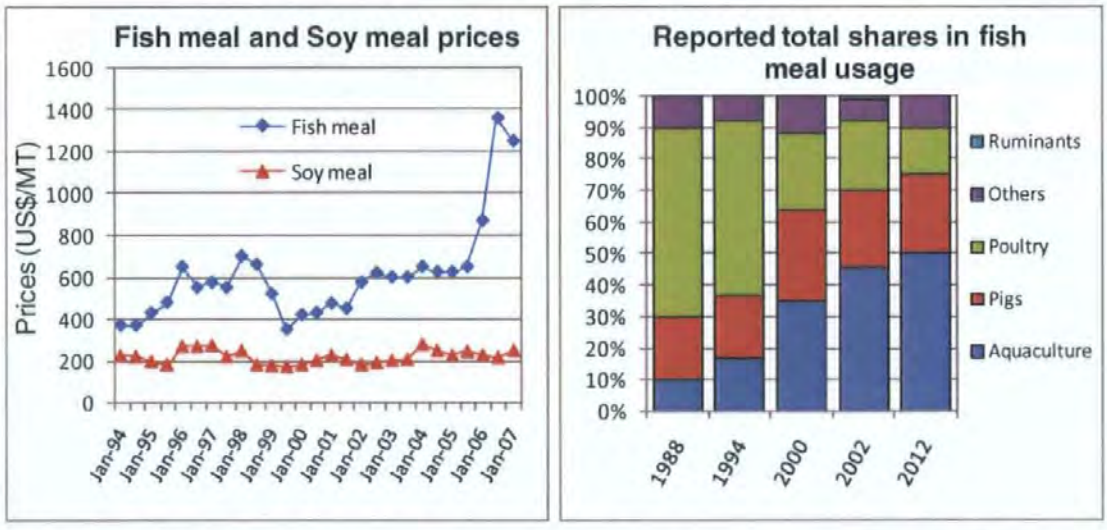


Figure 1.14: Left: Evolution of fishmeal and soy meal prices between 1994 and 2007 (FAO, Globefish, 2007). **Right**: fishmeal market for different years: 2002 and 2012: data from Tacon *et al.* (2006); 1988, 1994, 2000: data from Delgado *et al.* (2003).

Table 1.2: Global aquaculture production, aqua-feed production, use of fishmeal and wild fish input used in feeds established for major species groups in 2005: predictions for 2025.

2005	**1 Global production[a] (tonnes)**	**2 Percentage produced with compound feeds[b]**	**3 Production with compound feeds[c] (tonnes)**	**4 Estimation of compound feeds used[b] (tonnes)**	**5 Average feed conversion ratio[d]**	**6 Percentage of fishmeal in diet[h]**	**7 Estimation of fishmeal used[e] (tonnes)**	**8 Estimation of wild fish used (tonnes), to obtain the fish meal quantities required[f]**	**9 Ratio of wild fish: fed farmed fish[g]**	**10 Conversion efficiency of pelagic to farmed fish according to Naylor *et al.*, 2000**
Feeding Carps	11831342	45	5324103.9	9674000	1.82	4	386960	1547840	0.13	0.75
Marine Finfish	1643125	50	821562.5	1390000	1.69	45	625500	2502000	1.52	5.16
Tilapia	2025560	60	1215336	2188000	1.80	7	153160	612640	0.30	1.41
Shrimp	2675336	87	2327542.32	4189000	1.80	25	1047250	4189000	1.57	2.8
Salmon/Trout	1950578	100	1950578	2535000	1.30	35	887250	3549000	1.82	2.81
Total	20125941	-	11639122.7	19976000	-	-	3100120	12400480	-	-

[a]: *Data compiled from FAO FishStat Plus, 2007 (Feeding carps: Cyprinids without silver carp, bighead carp and calta; Tilapia: "Tilapia and other cichlidae"; Salmon/trout: Salmonidae)*

[b]: *Data taken from Tacon, 2007 (International Aquafeed volume10 issue2)*

[c]: *Calculated from 1 and 2;* [d]: *Calculated from 1 and 4;* [e]: *Calculated from 6 and 4*

[f]: *Calculated considering a 4:1 conversion ratio of fish (wet weights) to fish meal (based on the quantities of fish caught for reduction and fish meal production in 2004)*

[g]: *Calculated from 8 and 1;* [h]: *Data based on estimation made for 2002 (Pike and McDonnel Barlow, 2004)*

2025	**1 Estimation of Global production[a] (tonnes)**	**2 Percentage produced with compound feeds[b]**	**3 Production with compound feeds[c] (tonnes)**	**4 Estimation of compound feeds used[d] (tonnes)**	**5 Average feed conversion ratio[b]**	**6 Percentage of fishmeal in diet[b]**	**7 Estimation of fishmeal requirements[e] (tonnes)**	**8 Estimation of wild fish used (tonnes), to obtain the fish meal quantities required[f]**	**9 Ratio of wild fish: fed farmed fish[g]**
Feeding Carps	25923927	75	19442945.3	25275828.83	1.3	0	0	0	0.00
Marine Finfish	4249000	95	4036550	4843860	1.2	30	1453158	5812632	1.37
Tilapias	5251000	80	4200800	5461040	1.3	2.5	136526	546104	0.10
Shrimp	3501000	95	3325950	4656330	1.4	15	698449.5	2793798	0.80
Salmon/Trout	5284000	100	5284000	3698800	0.7	25	924700	3698800	0.70
Total	44208927	-	36290245.3	43935858.83	-	-	3212833.5	12851334	-

[a]: *Data for Feeding carps calculated from 2005 data considering an average annual percentage growth rate of 4; other data obtained from Hasan, 2001*

[b]: *Data taken from Hasan, 2001 (estimations)*

[c]: *Calculated from 1 and 2;* [d]: *Calculated from 3 and 5;* [e]: *Calculated from 4 and 6*

[f]: *Calculated considering a 4:1 conversion ratio of fish (wet weights) to fish meal (based on the quantities of fish caught for reduction and fish meal production in 2004)*

[g]: *Calculated from 8 and 1*

▶ **The sustainability issue:** Due to its high nutrient quality, costly manufacturing processes and high demand, fishmeal represents one of the most expensive types of feedstuff on the market (bearing in mind that fishmeal price appears however more competitive when considering its cost per unit of digestible protein or per unit of difficult EAA such as lysine or methionine). Moreover, fishmeal prices have risen in real terms in the past three decades and are likely to increase further with continued growth in demand (Figure 1.14 left).

In accordance with fish farming development, it is projected that global aqua-feed production must raise a target of 37mmt by 2010 and by an even larger figure towards 2025 (Hasan, 2001), (approximate production was estimated at 13 million tonnes in 2000 and 19.5 and 2003). To some extent this agrees with the figure that places the aquaculture share in the utilization of the total fishmeal volume over 70% by 2030.

At present, the high costs of aqua-feeds are effectively and mainly due to the extensive use of fishmeal. The additional technological process employed to manufacture the dry pellets and high protein requirements of fish also contribute to the fact that aqua-feeds are the most expensive type of animal feeds on the global market today. Thereby, in intensive aquaculture feeding may represent over 50% of the operational cost (Goddard, 1996). Increase in fishmeal (as well as fish oil) prices could well undermine the profitability of many aquaculture enterprises.

According to Pike and McDonnel Barlow (2004), who based their argument on fishmeal production figures, there is no evidence that aquaculture expansion impacted wild fish stocks. But can more fishmeal be produced? It seems that Peru, Chile, Denmark, Japan, Iceland, Norway, USA and South Africa, the major producer countries of good quality fishmeal, are already exploiting pelagic fish stocks to their sustainable limits. Production of fishmeal in the past decade has fluctuated but shown no significant trends, averaging around 6 to 7 million tonnes: (bearing in mind that 30 million tonnes of fish, approximately

one third of capture production, are used annually to produce fishmeals, intended, to a large extent, for aquaculture) (Figure 1.13, 1.14 right). Fish stocks used in fishmeal reduction actually appear to be in worldwide decline. In addition, El Nino is an important factor responsible for a certain inconsistency in the quantitative and qualitative supplies of fishmeal (Hardy, 2006).

The long term sustainability of carnivorous fish farming may be threatened by its current over-dependence on fishmeal as the main source of dietary protein. International demand for fishmeal may represent a future limitation in the growth of intensive aquaculture for carnivorous species: an industry based on fishmeal, such as the aqua-feed industry, is not viewed as sustainable. While future availability and price of this commodity remains unclear, it is generally accepted that a reduction in the fishmeal content of feeds is required if the present rate of aquaculture development is to be sustained and profitability improved (Hardy and Kissil, 1996; Hasan, 2001).

As well as reducing the level of protein in feeds, the strategy of replacing fishmeal with alternative protein sources may be an effective approach towards reducing costs and offering more sustainable feeds for aquaculture. Selecting and evaluating ingredients and their effect on the performance of targeted fish species (a routine topic) is a task which is widely accepted as a main focus for aquaculture research and which has recently received significant and renewed interest.

1.2.3 Alternative protein sources to fishmeal in marine fish diets

Complete replacement of fishmeal and fish oil in aquaculture feeds faces several barriers, especially for carnivorous marine fish, since all common alternatives are known to be of inferior quality, nutritionally speaking: (if alternative protein sources were equal or superior in their nutritional and economic value compared to fish meal, they would already be widely used in aqua-feeds). However, a move towards partial substitution of the

fishmeal component by plant and terrestrial animal proteins is widely accepted within the aquaculture industry and to a certain extent already applied. Although they do not represent an equal and realistic alternative for the total replacement of fishmeal in fish feeds, all feedstuffs traditionally used for feeding monogastric farm animals are believed to be suitable for feeding most fish. Nevertheless, the judicious use of those ingredients in fish diet formulation as secondary protein sources can be achieved provided we know what makes them inferior to fishmeal and what their exact nutritional characteristics are (see section 1.4). A wide range of feedstuffs have been tested in marine fish diets. These fall into three general categories: i) plant protein concentrates, ii) "non conventional" feedstuffs such as single cell proteins and iii) animal proteins from rendering or slaughter.

1.2.3.1 Plant proteins

Among alternative protein sources, plant feedstuffs have the advantage of greater availability and more competitive prices. However, although they may improve the physical characteristics of the pellets and may have a certain potential for addressing the problem of phosphorus pollution (Lall, 1991; Bergheim & Sveier, 1995), using plant feedstuffs in fish diets may present some major drawbacks due to fact that they may not be palatable and may contain anti-nutritional factors (ANFs), (Alarcón *et al.*, 1999). The processing of plant materials with techniques such as heat treatment, solvent extraction or starch removal is vital to inactivate ANFs and increase protein concentration; unfortunately, the result is a significantly increased cost of the plant based product. Broadly speaking, their lower nutritional value can largely be attributed to inherent EAA deficiencies and to the presence of complex often indigestible compounds. Amino acid profiles of plant protein sources do not match the dietary requirements of carnivorous fish species as well as the amino acid profile of fishmeal. Thereby, their inclusion in marine fish diets, even at medium to low levels, can sometimes impair fish performance and feed utilization. Cereal grains (corn/maize, rice, wheat...), oil seeds (soybean, sunflower,

linseed, rape...) and grain legumes (lentil, lupin, pea...) are the principal types of plants under investigation. The advantage of a relatively constant nutritional composition between these plant feedstuffs may be stressed, provided that raw materials and by-products are considered separately: while whole cereals, pulses or oilseeds have a crude protein content lower than 25%, oilseeds cake/meals, protein isolates or concentrates (such as corn gluten meal or soybean meal) are relatively rich sources of protein (25-50%).

Corn gluten meal (CGM) is the by-product of corn starch extraction rich in protein and low in fibre. The indispensable amino acid profile is generally said to be suitable for fish requirement excepted for arginine, lysine and to a lesser extent methionine (similar deficiencies are also found in wheat gluten meal). Protein digestibility of CGM is generally high with reported values of 90% (Nengas *et al.*, 1995) and 88.5-92.0% (Robaina *et al.*, 1997) in sea bream. Pereira and Oliva-Teles (2003) succeeded in replacing up to 60% of the fishmeal content by CGM, without negative effects on sea bream performance.

Much research has been done to evaluate the nutritional value of soybean meal (SBM) as a substitute for fishmeal. Of all the plant protein feedstuffs, soybean is indeed considered to be the most nutritious (relatively rich in protein) and is used as a major protein source in many fish diets. Market availability and low costs are other reasons explaining its popularity. Soybean meal is the by-product obtained after removal of oil (which constitutes the major food reserve within the plant seed). Various grades of soybean based products can be distinguished based on the raw material and treatment used (see section 2.3.7). Presence of trypsin inhibitors (in crude or inadequately heated soybean meal), suboptimal amino acid balance and relatively lower energy content are some of the nutritional factors that might limit inclusion levels. Apart from this soybean has also been shown to induce gut damage in salmonids (Krogdahl *et al.*, 2003). Nevertheless, protein ADCs of SBM are usually comparable to those obtained for fishmeal, and fluctuate from 87 to 91% in sea bream (Nengas *et al.*, 1995; Lupatsch *et al.*, 1997). The feeding trial of (Robaina *et al.*,

1995) indicates that SBM may replace up to 30% of fishmeal in sea bream diets without influencing feed intake and growth. Nengas *et al.* (1996) stressed the importance of soybean source and processing methods stating that properly heated full fat soybean might constitute the best protein source for gilthead sea bream compared to other soybean products (soybean meal, soy protein concentrate). SBM may also be associated with other plant feedstuffs in beneficial way.

Within the last category (legume seeds), lupin seed meal and pea seed meal have gained most attention and generated the greater number of promising investigations (Crevieu-Gabriel, 1999; Gouveia and Davies, 1998, 2000). Results obtained by Pereira and Oliva-Teles (2002) suggest that pea seed meal may replace up to 20% fishmeal protein in diets for gilthead sea bream juveniles without affecting fish performance. Robaina *et al.* (1995) indicate that properly treated lupin seed meal could be an important alternative dietary protein source for gilthead sea bream.

Blending different plant products would enable overcoming the amino acid limitations of individual plant proteins. Furthermore, replacing fishmeal with complex mixture of plant protein sources (fractionation of the plant component) may be an effective approach to reduce the exposure to individual anti-nutritional factors (Borgeson *et al.*, 2006).

1.2.3.2 Non-conventional (miscellaneous) feedstuffs

This category gathers together an assortment of industrial and agricultural wastes including materials of invertebrates (worm), single cell (bacteria, yeast), and plant (leaf, fruit, algae) origin. To date, few of these novel protein sources have been studied in fish feeds, and ranges of suitable replacement for fishmeal for major fish species have been estimated. Economically speaking, using bacteria or yeast grown in an industrial fermentation system as fishmeal substitutes is becoming more profitable / advantageous since fishmeal prices have reached higher levels (Hardy, 2006). Conversely to single cell material (that are associated with industrial and biotechnological processes), potential protein sources may

also be represented by locally available and cheap material originating from agriculture wastes. In a nutritional investigation, a 50% replacement of fishmeal with brewers yeast in diets for sea bass juveniles was shown to be possible without impairing biological performance of fish (Oliva-Teles and Goncalves, 2001). According to Ng *et al.* (2001), growth performance and feed utilization efficiency of catfish fed diets including 40% mealworm (*Tenebrio molitor*) at the expense of fishmeal, were statistically equal to those fed the control diet. Abdelghany (2003) states that 50% of the production cost, resulting from utilization of expensive herring fishmeal, could be saved using locally and easily available protein source such as gambusia fishmeal in diet of farmed tilapia. Finally, Olsen *et al.* (2006) found that Antarctic krill could fully substitute fishmeal in diets of Atlantic salmon without causing any major adverse effects on growth, feed utilization or fish health/welfare parameters.

1.2.3.3 Animal by-products

Animal by products, generated on a large scale by the rendering industry, represent one of the most important sources of feed ingredients; a renewable resource of protein, energy and minerals. These by-products are the part of a slaughter animal not directly consumed by humans and processed into high quality protein meals and fats (EFPRA, 2003a). In fact variable raw materials (offal, meat trimmings, blood, feathers, bones...) from different species, contribute to a great diversity of animal meals and may influence protein quantity and quality of the final product (Hertrampf and Piedad-Pascual, 2000; Bureau, 2006). Meat and Bone Meal (MBM) is produced from mammalian tissues including bone but excluding extraneous blood, hair, hoof, horn (as may occur), stomach and rumen contents. Meat meal is produced from the same mammalian tissue as MBM, except that it contains less bone. Hydrolysis of feathers under heat and pressure results in a rich protein feed supplement (hydrolysed feather meal: HFM). Pure hydrolyzed feather meal quality may however vary according to the processing method. Blood meal (BM) is produced from clean, fresh,

animal blood, exclusive of extraneous material. New drying processes guarantee blood meals to be relatively uniform in digestibility and nutrient content. Poultry by-products meal (PBM) and poultry meat meal (PMM) are materials obtained from the wastes of poultry production and processing plants. They are usually exclusive of feathers and intestines but may contain the feet and head in association with rendered meat from the carcass (Woodgate, 2001). All these meals may then vary in terms of nutritional value and composition depending on the processing method applied and the materials that are included in the meal (Woodgate, 2004a, 2004b).

Animal derived proteins are relatively free from any ANF's and represent a more natural source of available protein and nutrients for farmed carnivorous fish species. Compared to other alternatives, terrestrial animal by-products have an excellent biological value and offer accordingly a more realistic opportunity in aqua-feeds to reduce fishmeal dependency.

Within the frame of feed inclusion, their high protein content and good nutrient availability are two elements that bring them closer to the fishmeal standard. Apart from that, they are less expensive protein sources than fishmeal, are palatable, rich in vitamins and more importantly have a valuable EAA profile (although certain of these ingredients may be deficient in one or more of the essential amino acids) (Guillaume *et al.*, 1998, Bureau, 2006).

For these reasons the various animal protein ingredients derived from bovine, porcine or avian sources can be recognized as potentially the most suitable alternatives to fishmeal in diets for carnivorous species. Moreover, due to their quality, uniformity and "nutritional density" (along with their cost benefit), meat and bone meal, blood meal, as well as poultry meat meal and feather meal were, until recently, extensively used in commercial aquaculture diets in Europe. Following the BSE outbreak in the nineties, most of these

products are now subject to legislative constraints with regard to safety and use in Europe (see section 1.2.4).

From the nutritional point of view, high ash levels as well as high variability in quality may constitute some of the drawbacks attributed to these products (Hardy, 2006). Finally, it is important to note that techniques used to process the raw ingredients of the aqua-feed industry have evolved significantly over the years, and will continue to progress, positively affecting the quality of feedstuffs. Biotechnology offers opportunities for development of alternatives to fishmeal by enhancing production and processing techniques. Applied enzyme technologies can, for instance, be used to up-grade animal by-products (Woodgate, 2004a).

1.2.4 Origin, process and legislative status of Animal by-products: An introduction to the rendering industry

▶ **Origin:** When animals are slaughtered to produce meat for human consumption, by-products are also produced which humans cannot, or choose not to, consume. For instance, it is estimated that only 68% of a chicken, 62% of a pig, 54% of a bovine and 52% of a sheep are actually directly consumed (Woodgate and Van der Veen, 2004; NRA, 2006). Thereby the volume of by-products arising from the meat industry is not negligible: every year more than 14.5 million tonnes of meat not destined for direct human consumption and derived from healthy animals, are produced in the European Union (EFPRA, 2003a; Woodgate and Van der Veen, 2004). In 2005, the poultry industry produced 600,000 metric tonnes of by-products in UK. Besides, other perishable material like catering wastes may be added to these by-products but treated separately.

▶ **Role and outlets:** The rendering industry, often associated with slaughtering facilities, collects and processes these perishable materials (mainly abattoir waste, i.e.: by-products) into a variety of products used in cosmetics, pharmaceuticals, paints, varnishes, toothpaste, textiles and lubricants. In fact, the majority of these materials used to be returned to the

feed industry within the form of high quality protein meals and fats. In Europe, this traditional application was removed in 2000 as a consequence of the measures taken to prevent the amplification of Transmissible Spongiform Encephalopathies (TSE) and prion related-diseases. Currently the major outlets for the protein meals produced are then reduced to the pet food industry (category 3), or solid fuel replacement, fertilisers and renewable energy (category 2 & 1) (Woodgate, 2004b).

▶ **Process and methods:** Abattoir waste (i.e. by products) are turned / transformed into protein rich meals and high quality fats following a process normally called rendering that consists basically of grinding, heating and pressing (thanks to those operations, the material is sterilised, the water evaporated and meals and fats separated). However, this is rather an over simplification and in reality many types of process are in existence throughout the world (wet rendering, dry rendering, natural fat, added fat...etc.); besides many have been altered and adapted in accordance with technical advances and legislative changes over the years. Most rendering plants now utilise a continuous rendering process facility as described by Woodgate & Van der Veen (2004).

▶ **BSE issue:** Another important factor which deserves consideration while discussing the question of future fish feed ingredients and the utilization of processed animal proteins (PAP) in particular is the spread of mad-cow disease. It has been proven that the meat meals manufactured in the United Kingdom were responsible for transmission of Bovine Spongiform Encephalopathy (BSE), and this discredited animal by-products. Meals concerned / involved, would have been manufactured with a low temperature heating process (SIFCO, 2007).

The word prion (which stands for proteinaceous infectious agent) was originally coined in 1982 to name the presumed agent of the transmissible spongiform encephalopathies (TSE), a group of neurodegenerative diseases which affect the central nervous system of humans and other mammals (scrapie in sheep, bovine spongiform encephalopathy in cattle and

Creutzfeldt-Jakob diseases in human). Since that time, this word's meaning has widened enormously, so that it is now used to designate a vast group of divergent proteins (Joly *et al.*, 2001). Nevertheless all those prion proteins have in common the fact that they present two isoforms: a cellular or normal conformation (PrPc) which is apparently benign and a protease resistant conformation (PrPSc) that according to the "prion hypothesis" would have the capacity to transmit its abnormal conformation to PrPc resulting in an accumulation of PrPSc in neural cells and subsequently in the disease. TSE type diseases have never been observed in fish (Joly *et al.*, 2001). In addition, investigations carried out to identify PrP genes in fish have led to contradictory results to date. While few researchers have claimed to positively test fish samples with anti-PrP antibodies using techniques like ELISA, western blot, or immunohistochemistry (Gibbs and Bolis, 1997; Maddison *et al.*, 2005), others were unable to detect a sequence with similarities to known prions. From their negative results Joly *et al.* (2001) concluded that an eventual fish PrP gene is probably very divergent from those characterized in mammals; and that it would be extremely unlikely to share the pathological properties of these latter molecules.

▶ **Legislation and regulations:** As mentioned earlier, a prohibition on the use of PAP (except fishmeal) in feeds of animals farmed for food production (including fish then) was established in 2000 as a result of the EU applying additional security measures to prevent the amplification of prion related diseases. Nowadays, the situation in Europe with regard to animal by-products is controlled by the animal by-product regulation (ABPR 1774/2002) together with the TSE regulation (999/2001) which defines conditions and restrictions attached to the current and future use of PAP. This regulation lays down key points in the handling, processing and marketing of animal by-products like: "categorisation of raw material", "no intra-species recycling" and "traceability", the precautionary principle being of paramount importance (Woodgate, 2004b). Three categories of raw materials must be segregated according to the new ABPR: ***category 1***:

high risk materials (destroyed), ***category 2***: deadstock (processed separately and used for specific applications such as fertilisers and bio-energy), ***category 3***: material derived from animal declared fit for human consumption that can be use in a range of applications when processed to proscribed standards (including animal feeds provided species barrier is maintained). Species segregation is another key aspect for category 3 materials (that are being processed for inclusion in feeds), as there is a requirement in the ABPR not to recycle within species. HACCP (hazard analysis critical control points), traceability and, in some cases codes of practice are applied to provide optimal control of the rendering sequences so that the production of safe and high quality rendered products is ensured in an effective environment. Control practices as well as a fully traceable system are in place to ensure for instance that no cross contamination with category 3 materials is possible (Woodgate, 2004b).

▶ **Current situation and expectation:** These rules, which came into force in 2003, offer an opportunity to re-open the animal feed market to a significant portion of animal by-products. As a matter of fact, the animal by-products ban in aqua-feeds was already recently eased. Non-ruminant haemoglobin and blood meal have been permitted in fish feeds produced in the EU since 2003 (commission regulation 1234/2003 which is an amendment to the TSE regulation 999/2001). However, constraints imposed by European retailers (on the basis of perceived consumer fears and demand regarding safety) do not yet permit any re-introduction of these products into commercial fish diets. Despite the present outbreak of avian flu in South East Asia (which may compromise any decision in favour of the re-introduction of poultry by-products) other by-products are expected to re-enter the feed chain in the near future. In the USA, Canada, South America, Asia and Australia there are no legislative constraints and animal by-products are widely used in aquaculture with successful results (NRA, 2006).

1.3 RESEARCH STRATEGY IN FISH NUTRITION FOR TESTING NEW INGREDIENTS IN DIETS

Nutritional assessment of novel ingredients (protein sources) presents several key facets for which various experimental approaches and methodologies can be employed. Also, the importance of carrying out systematic characterisation of biological values of raw materials with more controlled and standardized conditions has often been underlined / stressed. Aside from a good understanding of the nutrient requirements of fish and detailed information on ingredient properties (which constitute an obvious starting point), determination of digestibility coefficients, palatability thresholds, and nutrient utilization data, are usually recognized as the most important knowledge in order to enable the judicious use of a particular ingredient in feed formulation (Figure 1.15) (Glencross *et al.*, 2007).

1.3.1 Determination of nutrient digestibility

The biological value of an ingredient is mainly a function of its biochemical composition and the bio-availability of its constituents. The nutritional value of a feed is indeed not only dependent upon its nutrient content, but also upon the ability of the fish to digest and absorb those nutrients from the feed. Providing estimations of nutrient availability in foods, the knowledge of digestibility coefficients, thus represent a basic requirement for the elaboration of well balanced and efficient diets (meeting the nutrient specification of targeted species). Moreover, since the digestible protein (DP) to digestible energy (DE) ratio has been found to significantly influence growth and feed utilization (Cho and Kaushik, 1990), data on dietary protein requirements are re-considered on a digestible protein basis.

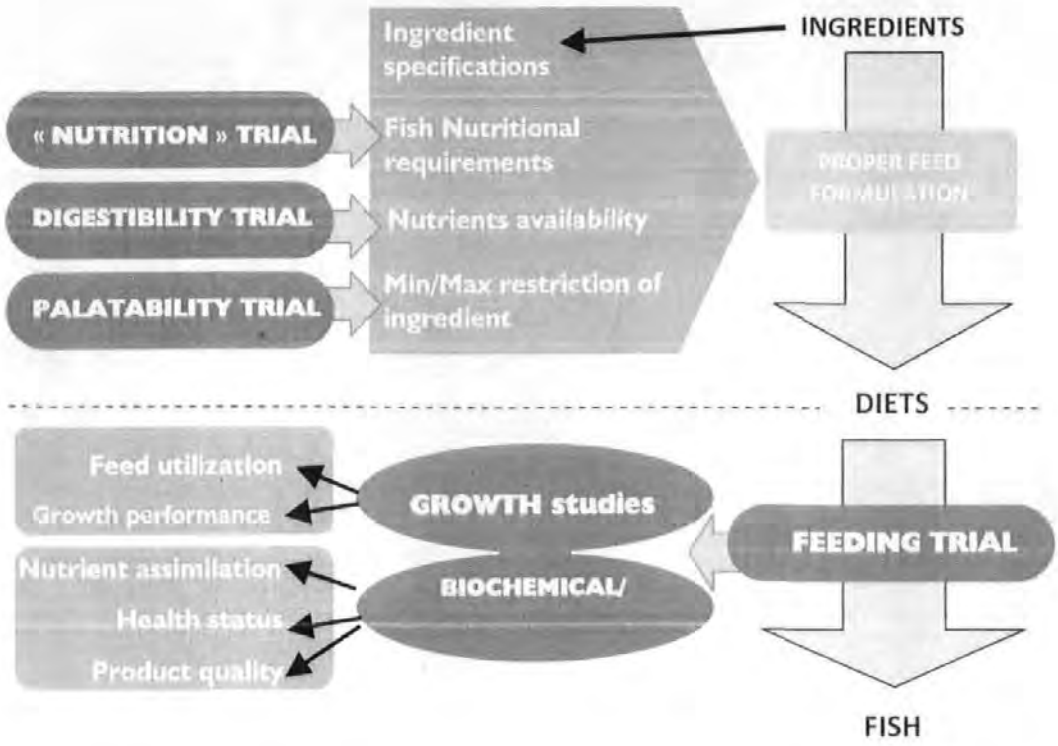


Figure 1.15: Research strategy in fish nutrition for testing new ingredients.

1.3.2 Palatability assessment

As indicated in Figure 1.15, the determination of palatability thresholds is another factor to take into consideration before designing new diets (Glencross *et al.*, 2007). Insuring that formulated diets are well accepted by fish, and that feed intake is not affecting optimal growth, is indeed important. Poor palatability performance of a specific ingredient must be indeed reflected in the inclusion rate or corrected with attractant supplementation (Guillaume *et al.*, 1998).

1.3.3 Evaluation of nutrient utilization

Once the variables of digestibility and palatability of an ingredient have been defined, the remaining key issue to resolve is based on the capacity of the animal to utilize the digested nutrient for growth. Basically, preliminary information obtained aims to help with the formulation of nutritionally balanced diets which must be validated through longer term

feeding trials, where growth and nutrient retention parameters are established (Glencross *et al.*, 2007).

1.3.4 Fish health and fish quality assessment

In intensive production systems, the diets provided must in particular support growth, promote health and lead to the production of desirable final products while being economical, palatable and with minimal effect on the surrounding environment. Since the expansion of aquaculture is dependent on finding alternative nutrient sources, the potential of these novel ingredients, investigated as fishmeal substitutes, must then be assessed in terms of growth, health and final product quality performance (Hasan, 2001).

Nutritional status is effectively recognized as an important factor determining muscular growth of juveniles with a potential impact on flesh characteristics of commercial size fish (Alami-durante and Rescan, 2003). Flesh quality can be determined through muscle fatty acid analysis and sensory evaluation using taste panels. Establishing consumer's preference would be important to erase the negative image of animal by-products and restore public and retailer confidence. Fish diet also has an obvious influence on the ability of the fish to resist diseases modelling the function of key tissues and organs. Apart from a direct influence on the immune system, animal feedstuffs might for example affect digestive structure and physiology with impact on nutrient utilization and health (Caballero *et al.*, 2003; Krogdahl *et al.*, 2003).

1.4 JUSTIFICATION AND AIMS OF THIS WORK

In the face of fish stock depletion, for the fastest expanding agri-business in the world, reducing fishmeal dependency appears as a major challenge to build the basis of a sustainable industry. Along with adjustment of the dietary protein-energy ratio (protein sparing effect), the reduction of dietary fishmeal level in the diet is another major

nutritional strategy. Therefore, the evaluation of alternative protein sources to fishmeal has been a research priority for many years, to reduce the constraints of availability and cost of feeds. The current situation, combined with various forecasts, stimulates research in this field: up-dated data and novel protein sources are of paramount importance to help formulators developing tomorrow's sustainable feeds.

To date, thanks to different sharing of this resource (reduction in land animal feeds) and minor replacements with vegetable materials, the supply of fishmeal has not been a limiting factor to aquaculture expansion. Although the forecast established on behalf of fishmeal organisations indicates that fishmeal supplies for aquaculture in the next decade will be "plentiful" (Pike and McDonnel Barlow, 2004), this situation does not appear to be sustainable on a longer term. As the demand from aquaculture continues putting pressure on fishmeal supplies and their prices, a renewed focus on the identification of novel proteins is necessary to ensure the future development especially of marine fish farming.

In spite of past studies (Nengas, 1991), our knowledge is still quite limited as regards replacement or reduction of fishmeal with animal by-product concentrates in diets for marine species. A paucity of information is available on animal protein "blending" for instance, and more work is necessary to identify complementary animal protein sources that have a synergetic effect in specific marine fish species.

Furthermore, although these types of material have proven to be effective substitutes and secondary protein sources to fishmeal in several fish, species there is currently a lack of data with regard to the effect of such replacements on health parameters as well as on tissue structure and composition.

Over the years, techniques used to process raw ingredients in the aqua-feed industry have evolved significantly and will continue to progress positively affecting the quality of feedstuffs. Processing conditions have already been shown to influence the biological value of ingredients of animal origin, like blood meal; besides, biotechnology tools

nowadays offer further opportunities to improve their nutritional qualities. New advanced methods like the treatment of ingredients with enzymes must, for instance, be considered (Woodgate, 2004a). It is also important to place this investigation in a post-BSE crisis context. As mentioned earlier, following the outbreak in 1991, renderers had to re-organise and apply new rules for the production of processed animal proteins. In the light of renewed information, nutritional investigations are now of utmost importance to evaluate this new generation of non mammalian animal proteins and restore public and retailers confidence. Terrestrial animal by-products represent, from a nutritional point of view, the most relevant fishmeal substitutes and, in spite of the current legislation, the investigations must go on as strategic research to cope with a probable easing of these regulations in the near future.

The commercial importance of gilthead sea bream in the Mediterranean area makes the development of nutritionally balanced and cost effective diets for this species of utmost importance. Also, to achieve this objective, it is essential to validate the effects of alternative ingredients on its biological performance. Given the assets of these raw materials, there is especially a growing need to obtain reliable information on up-graded animal by-products, in order to assess the feasibility of partially replacing fishmeal with these new protein sources in practical sea bream diets.

Therefore, there is a global strategy to assess the potential of a new generation of selected high quality animal proteins in diets of cultured marine fish species. Digestibility, feed utilization, growth performance, and health criteria will be the major key points investigated in order to draw a comprehensive picture and improve our understanding and efficacy of using animal protein sources in diets formulated for the intensive production of marine fish. More specifically, the main objectives of the current study may be formulated as follows:

a) Assess the biological value of selected animal by-products: i) Providing nutrient specification data. ii) Obtaining reliable digestibility coefficients for protein, amino acids, lipids and energy (trial 1).

b) Find optimal fishmeal replacement rate and validate preliminary data obtained on the biological performances of gilthead sea bream through a longer term feeding trial (trial 2): i) Formulating experimental diets based on the protein digestibility coefficients of the animal by-products selected. ii) Evaluating growth response and feed efficiency. iii) Establishing nutrient utilization parameters with fish carcass analysis. iv) Providing evidence that adequate inclusion of animal by-products in balanced diets does not impair general health status.

c) Formulate diets and conduct a short term preliminary trial with the view to determine the palatability threshold of products (trial 3).

d) Provide preliminary data related to the consequences of using animal by-products on the quality of the fish product (trial 4): i) Testing the influence of the high lipid content of PMM on the performance of this protein source. ii) Evaluating the effects of using significant inclusions of PMM on the quality of the final product through tissue fatty acid analysis.

e) Find ingredients combination that might work synergistically through digestibility or feeding trials (trial 1, trial 4).

f) Refine diet formulation and assess cost benefits of using animal by-products as fishmeal substitute in aqua-feeds using linear least-cost formulation system.

CHAPTER 2

The experimental approach in fish nutrition studies: general materials and methods

2.1 BIOTIC PARAMETERS

2.1.1 General presentation of the fish model: the gilthead sea bream

2.1.1.1 Systematic, distribution, and morphological description

Gilthead sea bream presents a tall and compressed body, with a broad head of convex profile and a spiny dorsal fin. A golden strip between the eyes (disappearing after death) and large black spot on the gill cover are two distinctive characters which usually allow an easy identification.

Table 2.1: General informations on the fish model (Fishbase, 2007)

Common and scientific names..	Gilthead sea bream, *Sparus aurata* Linnaeus 1758	
Taxonomy	Super class....	Osteichthyes (bony fishes)
	Class.....	Actinopterygii (ray-finned fishes)
	Infra class.....	Teleostei
	Order.....	Perciformes
	Family.....	Sparidae
Distribution.....	British Isles to Senegal all along the Mediterranean cost	
Length.....	20 to 50 cm (max. 70 cm)	
Weight.....	2 to 5 Kg	
Age.....	Max. 11 years	

2.1.1.2 Biology

Mainly carnivorous and accessorily herbivorous, gilthead sea bream can be characterised by diversified feeding habits. Larvae and sub-juveniles feed on plankton; whereas adults incorporate in their diet various benthic organisms. Within the Sparidae, this species is actually often described as a "conchyliphage" (shell feeder) due to the importance of the bivalve molluscs in its diet (mussel and oyster in particular) but crustaceans, polychaetes and fish are other prey commonly consumed (Pita *et al.*, 2002).

The gilthead sea bream is a sequential hermaphrodite fish, male during its first 2-3 years and then female (protandry). The spawning season is usually between January and March.

The spherical eggs of sea bream are just less than one millimetre in diameter and float in full strength sea water (pelagic spawner, pelagic eggs) (Ifremer, 2006, Fishbase, 2007).

2.1.1.3 Ecology

In the wild, sea bream inhabits rocky bottoms, seagrass beds as well as sandy bottoms. This demersal fish is also found in the surf zone, commonly to depths of about 30 m, but adults may occur to 150 m depth. Sea bream is well adapted to brackish water, but is cold sensitive. It migrates from the open sea to coastal areas (coastal lagoons, estuaries...) in springtime and returns in autumn. Despite this, it is often described as a sedentary fish, either solitary or in small aggregations. Like sea bass, this species is classified as euryhaline which means it has tolerance for large salinity fluctuations (Fishbase, 2007).

2.1.1.4 Interest, potential for domestication

Its gastronomical fame, its ability to reproduce easily in captivity, its robustness and capabilities to resist handling and stressful conditions as well as its fast growing rates, are some of the attributes that make it a popular and successful species for aquaculture. On the other hand drawbacks for sea bream farming may be related to: tricky larval rearing, pasteurellosis sensitivity, malformation susceptibility of juveniles, competing European market, limited production sites and poor transformation possibilities due to the relatively small size of the fish (Ifremer, 2006).

2.1.2 The culture of gilthead sea bream

Over the last decade Europe has witnessed an exponential growth of sea bream production (see details in section 1). This success is mainly due to significant progress achieved in hatchery techniques and research achieved in many Mediterranean countries.

2.1.2.1 Hatchery issues

In the Mediterranean area, sea bass and sea bream models were at the forefront for the development of finfish mariculture techniques. Within the hatchery stages, to date some of the problems solved concerned the development of enriched live prey, the utilization of skimmers to eliminate the oil layer on the water surface (to allow proper formation of fish swim bladder) as well as the development of micro-pellets to permit early weaning. Indeed, there is not yet any well developed technique which allows the rearing of marine finfish larvae with inert food from the beginning of their heterotrophic life. Although the current range of available techniques is quite diverse, all of them are based on the utilization of live prey (Divanach and Kentouri, 2000).

The hatchery must firstly organise mass production of various plankton to reproduce a simple food chain within the rearing systems. Phytoplankton (*Chlorella spp* for instance) is required to feed the zooplankton and then the zooplankton is used to feed the fish larvae. Rotifers (*Brachionus spp*) have proved to be an ideal food for these larvae (easy to reproduce and to enrich) and hatcheries produce it intensively (Benguoa-Ruigomez *et al.*, 1995). As the larvae grow they require larger feed animals, and the branchiopod crustacean *Artemia salina* replaces rotifers in the diet (the nauplii of Artemia are obtained from dried cysts collected and bought canned, they are not bred).

2.1.2.2 Life cycle

Under controlled conditions (temperature and photoperiod), a female sea bream can lay 1 million eggs per kilo each year in successive spawnings (which can be induced all year) (Zohar *et al.*, 1984). Hatching begins approximately 48 hours after spawning at 16/17°C. Newly hatched sea bream have closed eyes and mouths and must rely on their egg sac for nutrition. The pectoral fins develop two days after hatching. In the following 3 to 6 days depending on water temperature, body pigmentation increases, the mouth opens and most

of the yolk sac is consumed. At the larval stage (between 6-45 days) the eyes become functional, the mouth has opened, extensive body pigmentation appears, various fins develop and the swim bladder expands progressively to its final shape (AquaTT, 2003). The onset of feeding is a critical time in larval development. After 40-50 days the young fish are weaned off live feed and start feeding on commercially formulated fish food of a very fine particle size. When the fry are between 2 and 5 grams they are transported from the hatchery to the ongrowing producers in seawater tanks (AquaTT, 2003).

2.1.2.3 Rearing methods and facilities

As regards larval rearing different categories of techniques can be distinguished on the basis of parameters such as rearing density (intensive, mesocosms, extensive), water quality (clear water, green water, pseudo green water), type of feeding ("endogenous", "exogenous", both) and hydraulic circuit used (open or recycled, depurated or not) (Divanach *et al.*, 1998).

Despite a certain complexity, green water technology tends to predominate in commercial hatcheries nowadays. Reasons for this success are related to regularity and production quality. The combination with mesocosm technology results in a high performing larviculture technique which has the advantages of both intensive and extensive techniques without their inconveniences (Divanach and Kentouri, 2000). In mesocosms, larvae are reared at relatively low densities (2-8/ litre) in relatively large (30-100 m^3), deep (1.5-2.5m), well shaped tanks located in well organised installations (in intensive condition larval densities can reach 60-100/litre) (Divanach and Kentouri, 2000).

Fish leave the hatchery (where weaning has normally been initiated after 45 days) at a size of 1-5g. Before moving to the final rearing system, fish pass by the nursery. Within these facilities they will be exclusively fed on artificial feed (dried pellets). Rearing from juveniles to harvesting phase occurs mainly in marine cages; however enclosed ponds are

becoming increasingly popular as they allow for manipulation of environmental parameters. Intensive farming constitutes the majority of production (AquaTT, 2003; Ifremer, 2006).

2.1.2.4 Zoo-technical standards

Stocking densities applied in intensive production (post-hatchery) range from 10 to 40 kg/m^3, (densities usually rise with the rearing stage) (Canario *et al.*, 1998). In commercial operations feed conversion ratios (FCR) generally fluctuate from 1.3 to 2. Survival rates are also variable depending on the age of the fish. Cannibalism is a major problem encountered in the nursery. It takes 12-18 months (Malta, Turkey) to 24 months (France) to obtain fish of marketable size (300-500g) as a result of different climatic conditions (AquaTT, 2003; Ifremer, 2006). Reared to a range of sizes, the "portion" fish is usually 400-600 grams, provided fresh on ice to the market (Bendag, 1995).

2.1.3 Experimental fish obtained

Three different lots of fish were used to complete four trials. These were obtained from a local hatchery in Portugal for the trials conducted in Porto (TiMar – Culturas em Água, Lda., Tavira, Portugal) or imported from Brittany in France for the trials carried out in Plymouth (Aquastream). For this latter study, 1090 fish of an initial mean weight of 1.4g (total biomass of 1443g) were transported by van using the ferry. Techniques used for transportation involved utilization of eight 33L plastic containers filled with 20L of seawater and pure oxygen. The number of fish was then kept around 130 per container (biomass < 200g) to maximize survival rates. Transportation time did not exceed 24 hours, as fish were packed in the afternoon and delivered early the following morning. No mortality occurred during transportation and fish were distributed on arrival in four of the sixteen tanks of a new marine system. Fish were allowed to recover from transport in darkness for 24 hours, after which light level was slowly increased. Commercial pellets

(Biomar Ecostart 3) were obtained from the hatchery to ensure some continuity in feeding. Few meals were offered during this short adaptation period in response to the appetite of the fish, fish were then quickly fed to a fixed rate of body weight per day (2-3%). Veterinary certificate (certificat sanitaire n°56.04.AQ.1492) was established and provided to the authorities before importation in order to comply with UK regulations that only allow transfer of live fish between disease free zones.

Plate 2.1: Gilthead sea bream juveniles in the experimental system of the University of Plymouth during the adaptation period (before trial 2).

2.2 REARING FACILITIES AND ENVIRONMENTAL PARAMETERS

2.2.1 Introduction to the closed re-circulating technology

2.2.1.1 General characteristics and functioning

Re-circulating aquaculture systems (RAS) filter and clean the water for recycling back through fish culture tanks. They can be used as grow-out systems to produce food fish, as hatcheries to produce eggs and fingerlings, or as aquaria systems for ornamental fish. Despite their advantages, including reduced land and water requirement, high degree of environmental control and the feasibility of location in close proximity to prime markets, they are rather complex and attempts to advance these systems to larger commercial scale food fish production have not yet been really successful. Because of their convenience, such systems have nevertheless been used by fish researchers for decades in small scale configuration.

Functional parts of RAS include: growing tank, water circulation pump, and various elements to purify the water. There are innumerable designs for RAS; most will work effectively if they accomplish: aeration (oxygenation and removal of carbon dioxide), removal of particulate matter, oxidation of ammonia and nitrite nitrogen, buffering of pH and temperature control. These processes are usually achieved by interconnected components such as mechanical filters, charcoal filters, foam fractionators, sedimentation chambers, biological filters, air stone diffusers, ultraviolet light, buffering and thermostat system (Losordo *et al.*, 1998; Masser *et al.*, 1999).

2.2.1.2 Principles of filtration in closed recirculation aquaculture system

Ammonia nitrogen excreted by the fish through the gills is potentially toxic under certain pH conditions (within its unionized form) and can exert sublethal stress, resulting in poor growth and lower resistance to disease (Durborow *et al.*, 1997). To control ammonia levels in RAS, extensive surface area is provided for bacteria which biologically oxidize

ammonia to relatively harmless nitrate (NO_3^-). Bacterial nitrification is actually a two stage process resulting first in the transformation of ammonia to nitrite (NO_2^-), then a further oxidation of nitrite to nitrate. Nitrite is also toxic to fish at low concentration; hence, both reactions must occur for successful bio-filtration (Durborow *et al.*, 1997). To ensure that bacterial populations are sufficient to remove ammonia and nitrite at rates required during operation a bio-filter is typically conditioned for several weeks by adding ammonia and monitoring its breakdown prior to stocking fish (Durborow *et al.*, 1997).

Bacterial metabolisation of organic compounds such as faeces or uneaten pellets result in higher oxygen consumption while dissolved and fine suspended solids cause gill irritation and contribute to increase the oxygen demand in the system. In complement to mechanical filters, foam fractionators can be used to remove fine and dissolved solids, but those devices are efficient in sea water only. Subject to the chemical properties of the water, the process (which consists of introducing air bubbles at the bottom of a closed column of water) can indeed significantly reduce turbidity and oxygen demand in the RAS.

2.2.2 Experimental systems used in this study

Three different rearing systems were used in this project:

- System 1: digestibility system based in the marine station of the University of Porto
- System 2: marine system located in the west aquarium of the University of Plymouth
- System 3: marine system located in the marine station of the University of Porto.

These three rearing systems, all designed as closed recirculating systems, are briefly described in Table 2.2. Illustrations are also provided in Plate 2, Plate 3 and Plate 4 as well as in Figure 1 and 2.

Table 2.2: Brief description of the fish holding systems used within the course of this project.

	System 1 (plate 2)	System 2 (plate 3)	System 3 (plate 4)
Location of experimental facilities	Indoor, estação de zoologia maritime (Universidade do Porto)	Indoor, west aquarium (University of Plymouth)	Indoor, estação de zoologia maritime (Universidade do Porto)
Type of system	Small scale facilities for digestibility trial (modified Guelph system)	Small scale facilities for growth studies	Small scale facilities for growth studies
Hydraulic circuit	Closed (semi) re-circulating (95% re-circulated water)	Closed (semi) re-circulating (95% re-circulated water)	Closed (semi) re-circulating (95% re-circulated water)
System layout	15 tanks, 1 pump supplying 2 groups of tanks (waste water collected in two separated point, clean water distributed from 1 central point)	16 tanks, 2 pumps supplying 8 tanks. (waste water collected in 1 central point, clean water distributed from 2 separated points (treatment unit partitioned symmetrically)	
Tank characteristics	65L, fibreglass, rectangular (tilted floor)	104L, fibreglass, square	100L, fibreglass, cylindrical
Filtration components	Mechanical (sponge filters, pressurized sand filters) and biological (submerged biological filter bed)	Mechanical (sponge filters, skimmer) and biological (submerged biological filter bed)	Mechanical (sponge filters, pressurized sand filters) and biological (submerged biological filter bed)
Water supply	Natural sea water flow rate: 2-3L/min	Natural sea water flow rate: 10L/min, water exchande: 570%/h	Natural sea water 3L/min, 180%/h
Tank water flow	Inlet: tangential pipe, Outlet: bottom drain (perforated bar connected with settling column)	Inlet: tangential pipe, Outlet: central overflow (pipe with outer sleeve)	Inlet: tangential pipe, Outlet: screened bottom drain
Photoperiod	Natural	12L : 12D	Natural

[1]: One group of "interconnected" tanks were used only

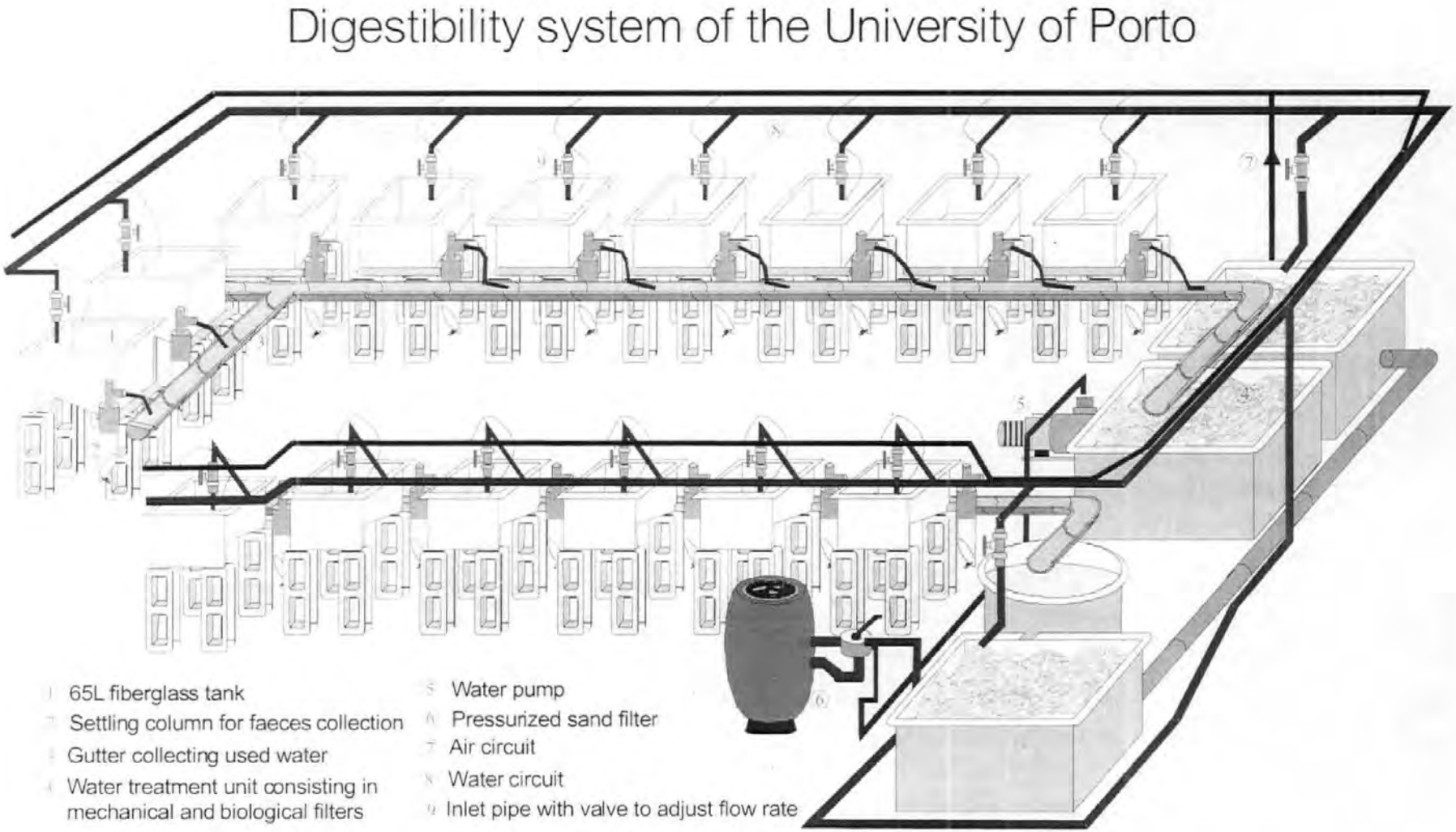


Figure 2.1: Re-circulating tank system located in the marine station of the University of Porto and used for trial 1 (after Sá, 2005)

Plate 2.2: Re-circulating tank systems located in the marine station of the University of Porto and used for digestibility trials

Plate 2.3: Re-circulating tank systems for marine fish located in the experimental aquarium of the University of Plymouth and used for trial 2 and 3

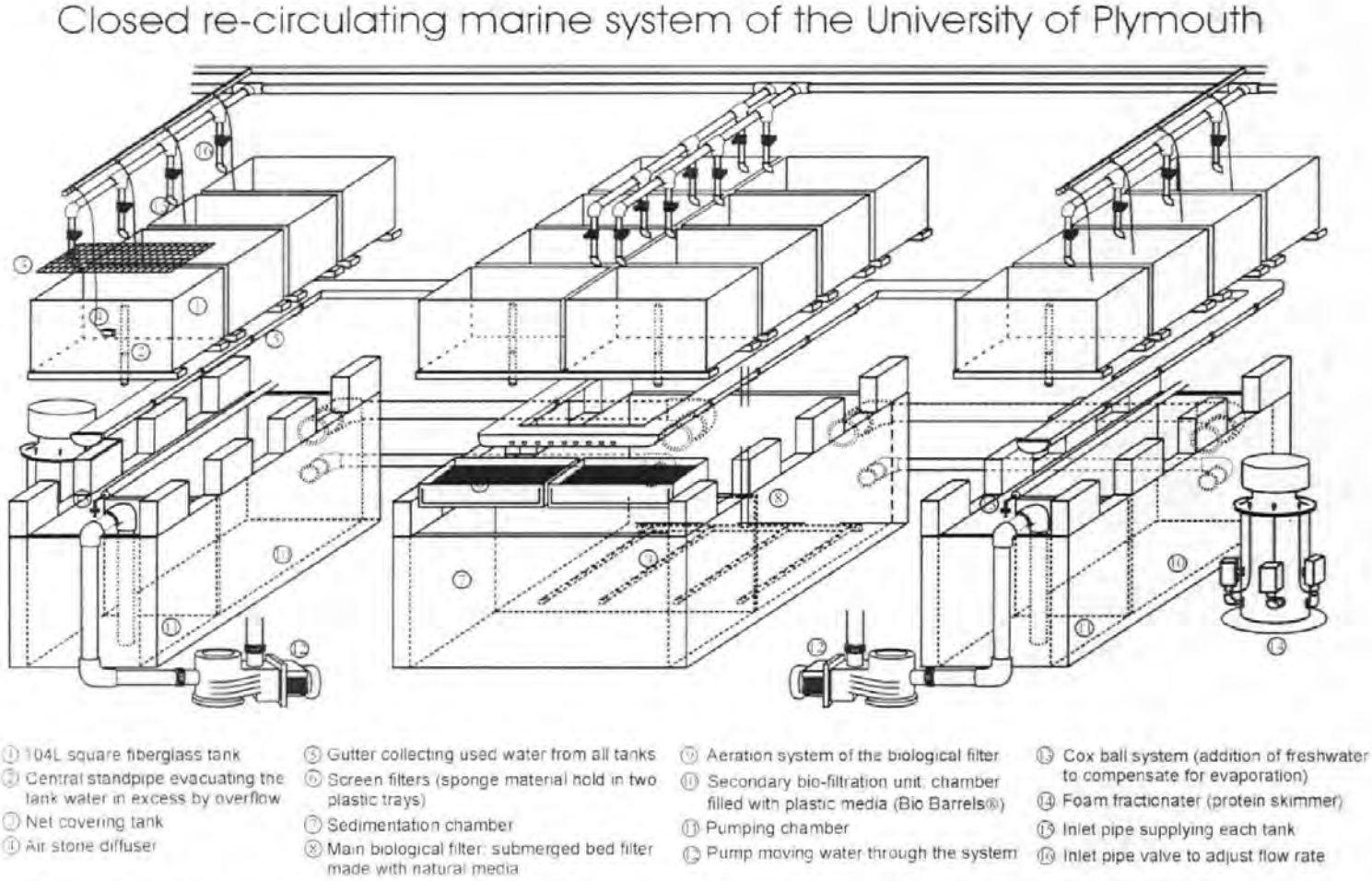


Figure 2.2: Marine re-circulation tank system located in the experimental aquarium of the University of Plymouth

Plate 2.4: Recirculation units for growth studies inside the marine station of the University of Porto.

2.2.3 Water quality and rearing conditions

In order to manage water quality, the same principles were used across all trials. All principal water quality parameters (dissolved oxygen, temperature, salinity, pH, total ammonia nitrogen (TAN), nitrite, nitrate) were monitored on a regular basis to ensure they remain at acceptable levels throughout experimental periods. Oxygen and temperature values were obtained with a portable meter (YSI model85 portable meter), pH was measured on water samples with a benchtop meter (Hanna pH210 meter) or directly with a multi-parameters portable meter. Levels of ammonia, nitrite and nitrate were determined using Hanna chemical (colorimetric) test kits and salinity was estimated using a salinity refractometer. Maintenance of good water quality involved cleaning tanks, sponge filters and foam fractionators regularly. Calcium carbonate ($CaCO_3$) or Calcium hydroxide ($CaOH_2$) was used to buffer the pH when necessary. Also, as a preventive measure and to remove nitrate, partial water exchange or continuous and low rate water renewal (semi-

closed system) was considered depending on the system used. Photoperiod followed natural (Porto) or artificial conditions (Plymouth).

2.3 SPECIFICATION OF ANIMAL BY-PRODUCTS TESTED

A brief description of the major ingredients tested during this work is provided within the following paragraphs. Processed animal proteins formed the basis for all investigations and were all obtained from UK rendering plants (Prosper de Mulder, Doncaster UK). All processed animal proteins provided are classified as category 3 as defined in the animal by-products regulation (ABPR) 1774/2002 (see section 1.2.4). Among the non ruminant animal by-products, poultry meat meal was particularly emphasised. Soya bean meal was also employed once in a blend to complement a source of animal protein (trial 4). From the abattoir waste to the final product, materials collected by the renderers undergo various physical, chemical or biotechnological treatments. Processing techniques used to manufacture those protein meals are also described since there is evidence that those treatments greatly affect ingredient quality (Crevieu-Gabriel, 1999; Bureau *et al.*, 1999; 2000; Allan and Booth, 2004).

2.3.1 Fishmeal (FM)

Fishmeals are typically manufactured from oily pelagic fish species such as sandeel, capelin, herring, smelt, cod, anchovy, pilchard, sardine, and menhaden. A distinction between fishmeals is usually made on the basis of the species used and area of production (Norwegian, American, Chilean... fishmeals). Of the various grades of fishmeals available, those used in aquaculture feeds are generally manufactured from whole fish ("brown fishmeal"), the others resulting from fisheries by-product processing (white fish offal: "white fishmeal") (Guillaume *et al.*, 1998). These fishmeals are generally obtained following a more or less complex manufacturing process, whose general principle consists of separating water and oil from the dry matter. Within the manufacturing processes,

cooking times, as well as cooking temperatures, are important factors determining fishmeal quality. Lower drying temperatures result in reduced protein damage and lipid peroxidation; and low temperature fishmeals are often used in aquaculture feeds since there is evidence that these meals are of superior quality in terms of nutrient availability. Thereby, in spite of a rather constant composition fishmeals may offer variable digestibility performances. Nevertheless the main factor controlling the quality of fishmeal remains the freshness of the raw material (Le Gouessant Aquaculture, 2007).

At present fishmeal represents the major component in aqua-feeds (FIN, 2007) (see section 1.2.2). It is indeed widely recognised that this key ingredient is a superior source of nutrients for fish, with especially and most importantly an excellent profile in essential amino acids and fatty acids (Guillaume *et al.*, 1998). A high quality grade, low temperature Norwegian fishmeal (LT-94) obtained from Skretting Aquaculture (a Nutreco company, Preston UK) was used as a reference in our control diets.

2.3.2 Poultry Meat Meal (PMM)

The product is a light brown meal derived from mixed poultry species including chickens, turkeys, ducks and geese. It consists of ground, rendered clean part of the carcass of poultry slaughtered fit for human consumption (category 3), such as necks, feet and viscera (but not feathers) (Woodgate, 2001). The PMM used typically contained 66% protein, 13% fat and 13.5% ash, other details on nutrient composition are shown in Table 2.3 (Woodgate, 2001). Despite a relatively good amino acid profile, in fish nutrition studies, lysine and methionine are often reported as the first limiting essential amino acids (EAA) (Hertrampf and Piedad-Pascual, 2000; Rawles *et al.*, 2006b).

Poultry material is reduced in size by mincing to less than 30 mm and then introduced into a continuous process (Rotadisc) that evaporates the water in presence of natural fat levels and sterilises the components. The residence time is approximately 90 minutes and the

maximum temperature reached is 125°C. On leaving the process, the dried components are separated into a protein fraction and fat by pressing in an expeller press. The protein fraction (poultry meat meal) is cooled, milled and treated with antioxidant (Woodgate, 2001).

2.3.3 Spray Dried Haemoglobin (SDH)

Despite essential amino acid deficiency, haemoglobin powder and blood meal are known as some of the most efficiently used supplement proteins in diets for various animals. It has been noted that non ruminant haemoglobin and blood meal have been permitted again in fish feeds produced in the EU since 2003. Spray Dried Haemoglobin (SDH), also termed AP 301 (trademark of the American Protein Corporation), is a rich protein ingredient (>80%) that is a fine and dark reddish powder. The raw material used is whole porcine blood obtained from animals slaughtered fit for human consumption. Once collected the blood is chilled and then separated into plasma and red blood cells by centrifugation. The red blood cell fraction (haemoglobin) is then dried by spray drying to produce a haemoglobin powder with moisture content <5%. The product is finally cooled and bagged prior to dispatch (Woodgate, 2001).

2.3.4 Feather Meal

Feathers are the complex derivatives of the integuments found in birds. Different types of feather (with nevertheless a relatively similar structure) are usually distinguished according to their location and function. They consist of several morphological parts (rachis, shaft, barbs...) that show some variation in term of chemical composition and amino acid profile.

Feather meal is a by-product of poultry meat processing (Hertrampf and Piedad-Pascual, 2000). Annually large quantities of feathers are made available. For instance, in the UK around 800 million broilers were slaughtered in 2003. A 2.0kg live chicken produces 180g of feathers, this gives a potential of 144 mmt of fresh feathers for this country in 2003

(Hertrampf and Piedad-Pascual, 2000). In reality, production volume is smaller: EFPRA (2003b) reported 750,000 mt of fresh feather per year in the EU (Which lead, from the same source, to 200,000 mt of hydrolysed feather protein). One of the most known outlets for feathers is the manufacture of duvet, but this material can also be return to the feed industry in the form of high quality meal.

Indeed, fresh feathers contain at least 80% crude protein (EFPRA, 2003b). However, the major protein component of feather meal is keratin which is not digested by mono-gastric animals and fish in its original structure. Proper processing methods must be employed to convert this raw material into a valuable protein feedstuff; final quality of the meal being greatly influenced by those methods. Besides the digestibility issue, it should also be mentioned that the utilization of feather meal as a protein source in aqua-feeds is also limited by amino acid deficiencies as a result of the dominant contribution of keratin within the protein spectrum of the ingredient (Hertrampf and Piedad-Pascual, 2000).

2.3.4.1 Steam Hydrolysed Feather Meal (HFM)

Hydrolysis of feathers under heat and pressure transforms the keratin to better digested peptides. Steam Hydrolysed Feather Meal (HFM) is derived by pressure cooking the clean, undecomposed feathers from slaughtered poultry. Mixed poultry feathers (including chickens, turkeys, ducks and geese) are steam hydrolysed at up to 5.5 bars (550 kPa) pressure for approximately 30 minutes in a continuous hydrolyser. The hydrolysed feathers are then dried in an indirect steam heated drier (rotadisc drier), to approximately 5% moisture, cooled, milled and stored. The final product is a light brown meal derived from poultry feathers only (Woodgate, 2001).

2.3.4.2 Enzyme treated Feather Meal (EFM)

Feathers can also be denaturated by enzymatic treatment. Enzyme treated Feather Meal (EFM) is obtained further to a specific digestion treatment that aims to improve the

breaking down process of keratin: Poultry feather material is heated to 50°C in the presence of an enzyme and cofactor mixture (Allzyme®), and continually mixed for 30 minutes. Following the enzyme treatment the feathers are pressure processed at 2 bars (200 kPa) for 15 minutes. The Enzyme Hydrolysed Feather Meal (EFM) is then dried in a rotadisc drier to approximately 5% moisture, cooled, milled and stored. Processes, which aim to improve nutrient availability, lead to a brown powder (Woodgate, 2001).

2.3.5 Defatted Poultry Meat Meal (dPMM)

Defatted poultry meat meal (dPMM) was obtained by using hexane extraction. PMM was soaked and mixed for 24 hours and then filtered through a 100 microns sieve to remove the fat and solvent mixture. The defatted sample was then air dried to remove traces of solvent.

2.3.6 Soybean Meal (SBM)

Soya bean is a south-east Asian annual leguminous plant (*Glycine max*) widely cultivated for forage, soil improvement as well as for its nutritious seeds. Its oilseed differs from cereal grains in that lipid replaces carbohydrate as the major food reserve within the plant seed. Soya beans can be used as animal feed in various forms including full fat soybean meal. There are several soybean feedstuffs in today's market that are produced using various techniques, including heat treatment to remove or deactivate anti-nutritional factors (ANF). Soybean meal is the by-product obtained after the removal of the oil from soya beans. With defatted Soybean flakes, soy protein concentrate, soy protein isolate or soy protein hydrolysate can also be produced further to additional treatments. Soya protein products (which are normally made from de-hulled soya beans) represent nowadays the most commonly used plant proteins (Guillaume *et al.*, 1998; Hertrampf and Piedad-Pascual, 2000).

Termed "Hi-pro Soya", the de-hulled soybean meal used in trial 3 was purchased from Skretting Aquaculture (a Nutreco company, Preston UK). The material obtained was

ground to a fine powder (with a Kenwood food processor) prior to incorporation in the diet mixture.

2.4 FEED PREPARATION AND FEEDING TECHNIQUES

2.4.1 Diet formulation

Since no ingredients contain all the nutrients required by fish, diet formulation must achieve the combination of feedstuffs that will meet the nutritional needs of fish as well as specific goals of production. Aside from the objective of balancing nutrients in the diet (protein and amino acids in particular), few formulation strategies (related to the reduction of the DP/DE ratio, or the reduction of poorly digested ingredients for instance) were developed as a consequence of economic and environmental concerns (Cho & Bureau, 2001). Furthermore the concept of "ideal" dietary protein where the minimum amount of protein is used to meet the essential amino acid requirement of the species (typically the animal's own carcass profile) is more and more applied in fish formulation (Rawles *et al.*, 2006b).

It must be noticed that mixtures should also facilitate the manufacturing process to produce a diet with the desired physical properties. When formulating a practical diet, it is in addition important to make sure that the diet is free of anti-nutritional factors that would impede the performance of the fish.

Table 2.3: Proximate composition of ingredients; FM: fishmeal Norse LT94, PMM: poultry meat meal, dPMM: defatted poultry meat meal, HFM: steam hydrolysed feather meal, EFM: enzyme treated feather meal, SDH: spray dried heamoglobin, SBM: soybean meal (Hi-Pro Soya). Data from analysis, a: Folch modified method, b: Soxlhet method. Data from literature, a: Guillaume *et al.* (1998), b: Fasakin *et al.* (2005).

Dry Matter (%)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Specification Manufacturer	**93.0**	**96.0**	-	**92.5**	-	**91.0**	-
Data from analysis	92.6	94.1	94.7		89.9	90.8	87.8
Data from literature	-	-	-	$93.0^{a,b}$	93.2^{b}	89.0^{b}-93.0^{a}	88.0^{a}-89.0^{b}
Crude Protein (%)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Specification Manufacturer	**71.0**	**66.0**	-	**80.0**	-	**92.0**	-
Data from analysis	67.1-73.0	60.4-64.7	70.0	77.2-85.8	71.9-91.2	86.2-95.6	56.4
Data from literature	-	-	-	80.0^{a}-85.8^{b}	80.0^{b}	84.0^{a}-92.0^{b}	48.0^{a}-48.5^{b}
Crude Lipid (%)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Specification Manufacturer	**11.0**	**13.0**	-	**6.0**	-	**2.0**	-
Data from analysis	10.0^{b}-11.9^{a}	12.4^{b}-16.6^{a}	5.7^{b}	10.5^{a}	2.4^{b}-6.3^{a}	0.01^{b}-2.8^{a}	0.8^{b}
Data from literature	-	-	-	3.5^{a}-5.0^{b}	6.0^{b}	1.0^{b}-1.1^{a}	1.0^{b}-1.9^{a}
Gross Energy (MJ/Kg)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Specification Manufacturer	-	-	-	-	-	-	-
Data from analysis	21.2	20.9	20.2	24.2	22.9	22.2	19.6
Data from literature	-	-	-	21.5^{b}-21.6^{a}	21.5^{b}	20.3^{a}-21.7^{b}	17.6^{a}-19.4^{b}
Ash (%)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Specification Manufacturer	**11.0**	**13.5**	-	**3.0**	-	**5.0**	-
Data from analysis	12.9-13.3	15.4-17.0	15.7	2.5	1.9-2.6	2.6-3.1	7.3
Data from literature	-	-	-	2.4^{b}-3.2^{a}	2.5^{b}	1.4^{b}-4.5^{a}	6.2^{a}

Table 2.4: Essential amino acid composition of test ingredients (g/100g DM, left or g/16gN, right).

Essential Amino Acids (% DM)	**FM** (LT94)	**PMM**	**dPMM**	**HFM**	**EFM**	**SDH** (AP301)	**SBM**
Arginine	4.26/3.02	4.17/2.75	-	6.13/4.90	3.63/2.90	3.64/3.35	3.59/1.80
Histidine	1.78/1.26	1.14/0.75	-	0.66/0.53	0.77/0.62	6.82/6.27	1.19/0.60
Isoleucine	2.63/1.87	1.82/1.20	-	3.98/3.18	1.83/1.46	0.54/0.50	2.42/1.21
Leucine	5.18/3.68	4.35/2.87	-	6.80/5.44	3.82/3.06	12.19/11.21	3.68/1.84
Lysine	5.39/3.83	3.83/2.53	-	1.73/1.38	2.49/1.99	8.19/7.53	3.05 1.53
Threonine	3.19/2.26	2.56/1.69	-	3.93/3.14	2.41/1.93	3.27/3.01	1.88 0.94
Tryptophan	0.71/0.50	0.55/0.36	-	0.35/0.28	0.40/0.32	1.09 1.00	0.65 0.33
Valine	3.26/2.31	2.86/1.89	-	6.48/5.18	2.59/2.07	8.37 7.70	2.46 1.23
Methionine	2.00/1.42	1.00/0.66	-	0.50/0.40	0.81/0.65	0.73 0.67	0.66 0.33
Phenylalanine	2.84/2.02	2.31/1.52	-	4.14/3.31	2.15/1.72	6.46 5.94	4.12 2.06

In preparing our laboratory research diets the following specifications were followed:

i) as far as possible diets were designed to be identical in all respects except for the variable being tested (with the exception of diets formulated for the specific purpose of digestibility or palatability studies, diets were made isonitrogeneous and isoenergetic) ii) diets were designed to be nutritionally complete and feedable (experimental diets were based on a control diet formulated with adequate level of high quality fishmeal in order to match the requirement of gilthead sea bream; size of the pellets were optimized in relation to fish size) iii) diets were made from purified ingredients, this excluding protein sources tested (in experimental formulation it is common practice to use purified/semi-purified diets in order to study the effect a nutrient, such as the amount or type of protein, may have on the growth or health of fish; dextrin, corn starch, αcellulose, carboxymethylcellulose were used as carbohydrate sources or binding agents).

2.4.2 Feed manufacturing

Raw material which generally comes in the form of flours or liquids will have to undergo binding by means of a technological process to obtain a food mixture (in the form of dry pellets) which is easy to use and preserve. Regarding manufacturing processes, two types of feeds are generally available on the market, namely pressed feeds and extruded feeds. A third type, designed as expanded feed is also marketed by some manufacturers; this type of feed is actually just a variant of extruded feeds. The main difference between a pressed and an extruded feed is the cooking of the feedstuff mixture that occurs in the case of extrusion. In fact, many types of extruders contribute to a great diversity of products (Guillaume *et al.*, 1998).

In this project, both California pellet mill (CPM) and Hobart A120 food processor (Hobart Manufacturing Company Ltd, London, England) were used to manufacture different sets of

diet. Considering the properties and functioning principle of these machines, the process employed within the framework of this study is often described as "cold extrusion".

Diets were prepared in batches of 2 to 8kg, depending on the food processor used. All the raw materials were first individually weighed in a bucket and transferred into a mixer (the stainless steel food mixer bowl of the Hobart machine, or a cement mixer when pellets were made with the CPM). Ingredient mixtures were uniformly mixed for approximately twenty minutes. After the initial blending period of the dry ingredients, marine fish oil was added very slowly in a continuous flow. After further mixing, hot distilled water was equally added to the ingredients until the mixture within the bowl reached a consistency that when gripped in the palm of the hand held firmly together without any sign of break up on release (amount of water typically added averaging 0.3 to 0.4L per kg of dry mix).

The pelleting process was achieved by using the extruder assembly of the Hobart processor or the CPM (fitted with the appropriate aperture die to obtain the desired pellet size). Strands produced by the former were carefully broken up and spread onto trays lined with tinfoil. Once filled, trays were placed into a warm air cabinet where they were left until moisture content was <10% (strands were completely reduced to pellets once dried only). Pellets produced by the CPM underwent the same drying treatment. Diets were all stored and conserved in opaque and airtight plastic containers prior to and during their use in the trial.

2.4.3 Feeding management and feeding methods

Feeding systems may be defined as all feeding standards and practices employed to deliver nutritionally balanced and adequate amounts of diets to animals. In practice different feeding methods and equipment can be employed such as hand feeding, demand feeders, automatic feed blower or automatic feed spreader. Depending on the equipment chosen, food can be provided in excess, to apparent satiation, or in restricted amounts. Feeding

rates may vary depending on whether we use automatic or demand feeders, while both options of feeding fish to satiation or to a fixed rate can be considered with hand feeding. Automatic and demand feeders save time, labour and money but at the expense of the vigilance that comes with hand feeding (Goddard, 1996). Since they do not allow the same control of feed intake, in research studies feeding methods must be selected carefully with regard to the objective and type of trial intended. To obtain useful FCRs (representative of the diet potential) it is for instance recommended to use a technique that allows knowledge of the exact amount of feed ingested.

Within the course of this project all fish were fed by hand either to satiation or to a fixed rate. In the latter case, feeding rates, expressed as % of body weight (%BW), were determined according to feed level charts considering water temperature and fish size. Fish were fed by the same person twice a day at regular times (meals usually consisted of two successive rounds) 6 days a week. Rations were re-adjusted accordingly, based on new weekly fish biomass or when mortality occurred. In the case of feeding to apparent satiety, pellets were distributed until the first feed refusal was visually observed (subsequent observations were made to ensure no further feed response).

2.5 EXPERIMENTAL PROTOCOLS AND STATISTICAL ANALYSIS

2.5.1 Sampling methods and handling techniques used during trial

In order to monitor growth and feed utilization parameters, each group of fish were weekly netted and batch weighed. Fish randomly sampled for carcass analysis, or tissue collection, were sacrificed by lethal anaesthesia with tricaine methane sulphonate (MS222). Prior to dissection, the vertebral column was sectioned behind the head and the cerebral system destroyed in order to comply with general fish husbandry policy.

2.5.2 Experimental design and statistics

When it comes to validating a hypothesis with statistical tools, the level of acceptance and number of replications are two important factors that determine the power of a test. Setting the level of acceptance at a more rigorous standard reduces type 1 error (rejecting a null hypothesis which is actually true), while increasing the number of data sampled may reduce type 2 errors (accepting a null hypothesis which should be rejected).

Unless otherwise stated mean values of replicate groups are reported together with the standard error of the mean. The group of fish contained in each tank was treated as the unit of replication, not individual fish themselves. Data treatment and results interpretation involved one way analysis of variance (ANOVA) in order to reveal significant differences between treatments at a probability of 5% (differences between means were tested with Tukey's pairwise comparison tests using Minitab 13). Non parametric testing was considered in a few cases as a result of a lack of normality in the data sets (Kruskal Wallis's test with post hoc multiple comparison testing). However attempts to transform the data were firstly considered. Values with different superscripts across treatments indicate significant differences.

2.5.3 Analytical chemistry methods

In fish nutritional experiments there are a number of laboratory techniques employed routinely. Gross chemical methods performed *in vitro* are commonly utilised to control and/or predict the nutritional characteristics of feedstuffs or other materials like faeces and carcasses. Proximate analyses, which consist of establishing the composition of all major nutrients (indicating their relative proportions) of a feed mixture or of an individual feed ingredient, constitute the basis of nutritional studies. Moisture, crude protein (nitrogen × 6.25), lipid (ether extracts), gross energy, ash, crude fibre and Nitrogen Free Extracts (NFE) are the nutrient components usually determined with specific and well standardized

protocols of analytical chemistry. The procedures used throughout this work are derived from AOAC (2003) and are described in the following paragraphs.

2.5.3.1 Moisture determination

In balancing the ration it is essential to know the water content of each component; also, moisture in prepared feed must be monitored because high levels of humidity favour the presence of insects and bring a risk of contamination by fungi and bacteria. Dry feeds (with less than 10% moisture content) are by far the most commonly used feeds in intensive aquaculture because they are easy to store, handle, and are more consistent in quality (Goddard, 1996).

The method is based on drying a sample and determining moisture content by the weight difference between dry and wet material. The samples, whether feeds or entire fish carcasses are firstly weighed and then fully dried using oven drying or freeze drying processes. Dried samples are finally re-weighed to find out water loss.

Oven drying was carried out in a Pickstone E 70F oven (R.E. Pickstone Ltd., Thetford, Norfolk, U.K) over a period of 12 hours (feed mixtures, feed ingredients) and at a temperature of 105°C. Protocols for carcasses were slightly different in that carcasses were dried to constant weight (further precautions must be taken with fish carcasses to ensure that sample is fully dried as the amount of water in this material is high, fish must be cut in several pieces for instance).

Freeze drying was achieved with a Flexi-Dry^{TM}, FTS Systems (USA) available at the University of Porto. Moisture measurements are normally made in triplicate for each sample. Materials like pelleted feeds were ground prior to analysis and kept in airtight plastic containers once dried for all other analyse (performed on a dry basis). Fish carcasses were ground with a blender and coffee grinder after drying and re-weighing, and

the resulting mixtures kept in airtight plastic containers for further analysis. The percentage moisture content in the sample was calculated using the following equation:

$$\text{Moisture content (\%)} = \frac{\Delta \text{ Weight (g)}}{\text{Initial sample weight (g)}} \times 100$$

Where **Δ weight** is the variation in weight between dry and wet sample

2.5.3.2 Determination of ash content

Ash is considered as the total mineral or inorganic content in the feedstuffs and determined after incineration of a sample. 450-550mg of dried material wer placed in a pre-weighed crucible and incinerated in a muffle furnace at 550°C for 12 hours. After a cooling period, where the samples were kept in a dessicator, crucibles were carefully re-weighed with the ash. Those results were then compared with initial weights of samples to calculate the ash content in percentage (for each sample the ash content was normally measured twice).

$$\text{Ash content (\%)} = \frac{\text{(weight of crucible + residue (g)) - weight of crucible (g)}}{\text{Initial sample weight (g)}} \times 100$$

2.5.3.3 Analysis of crude protein

Typically 100mg of each sample (dried feed, faeces or raw ingredients) were weighed into a weighing boat and transferred to a Kjeldahl digestion tube. A Kjeldahl catalyst tablet (3g K_2SO_4, 105mg $CuSo_4.5H2O$ and 105mg TiO_2 BDH LTD UK) and 10ml of concentrated H_2SO_4 (Sp.Gr. 1.84, BDH LTD UK) were then added to each tube prior to the digestion process. Digestion was performed on a 40 position Gerhardt Kjeldatherm digestion block (C. Gerhardt Laboratory instruments, Bonn, Germany) at 225°C for 40 min (pre-digestion) and at 380°C for the following hour. The tube rack was removed from the heating block and the samples allowed to cool down during an additional 30 minutes while the Turbosog scrubber unit was left on to eliminate the remaining fumes. After this digestion stage, all

tubes went to the distillation unit where the ammonia formed after the addition of NaOH (in alkaline condition) was steam distilled and trapped in boric acid solution (with 4.5 BDH indicator). Note that prior to the addition of NaOH (which leads to an extremely vigorous exothermic reaction), the sample was diluted with distilled water to make the acid weaker. Back titration was finally performed with HCl (0.1M) solution. A standard was normally include in each run.

$$\text{Crude protein (\%)} = \frac{(\text{Sample titre (ml)} - \text{blank titre (ml)}) \times 0.10 \times 14 \times 6.25}{\text{Sample weight (mg)}} \times 100$$

Where 0.1 is the molarity of the acid, 14 the relative atomic mass of nitrogen and 6.25 a constant relationship between N and the animal protein of the sample.

2.5.3.4 Total lipid extraction

Lipids are generally defined as food components that are soluble in organic solvents and insoluble in water. Therefore organic solvents can be used to extract fat from food products (lipids are isolated by using the difference in the components solubility); many methods are actually based on this principle for lipid determination. In order to measure the amount of lipid in our biological samples we used two distinctive extraction procedures throughout this work: a Folch modified technique and the automatic Soxhlet extraction method.

▶ The automatic Soxhlet extraction method: protocol and calculations

Typically 2 to 3 grams of ground and dried material were placed in a porous cellulose thimble lightly plugged with cotton wool. Thimbles were then inserted into the condensers and raised up into rinsing position making sure the magnet had connected to the thimble. Once the cups had been carefully weighed, filled with 40 ml of spirit and clamped into the condensers, the extraction knobs were moved into the boiling position so that the thimbles were immersed in the boiling solvent for 30 minutes. After this time, the thimbles were left hanging above the solvent for another 45 minutes by moving back the extraction knobs to the rinsing position. Solvent was then recovered, the cups released and placed in a fume

cupboard for 30 minutes to eliminate any trace of solvent. When the solvent in the cups had totally evaporated the mass of the remaining lipid was measured. The percentage of lipid in the initial sample could then be calculated as indicated below.

$$\text{Lipid (\%)} = \frac{\text{Weight gain of cup (g)}}{\text{Sample weight (g)}} \times 100$$

▶ The Folch modified method: protocol and calculations

The Folch method is another popular procedure for the preparation of total lipid extracts from various tissues. This method remains one of the best described and one of the most commonly used by lipidologists all over the world. Within the framework of our investigations we used a variant of this technique to determine total lipid in feed and carcasses. Modifications include the utilization of HCl in the first stage of the procedure (acid hydrolysis may allow yield of the extra lipid bound to the protein) and the use of dichloromethane/methanol to replace the chloroform/methanol mixture of the original protocol. The detailed protocol used is presented hereafter.

Firstly, a sample of 500mg to 2g (dried feed, raw material, faeces) was placed in a 50ml polypropylene centrifuge tube, to which 10ml of 6M HCl and 10ml of methanol were added. Then tubes were placed in the oven for 30 minutes at 70°C. After cooling of the samples to room temperature a further 20ml of dichloromethane (DCM) was added and the tubes were recapped to be shaken vigorously. Following this, the tubes were centrifuged for 10 minutes at 2800 rpm (MSE Mistral 3000). After centrifugation the upper-phase was carefully decanted. Pushing through the sample layer with a 5ml Hamilton gas tight syringe, 2ml of lower-phase was then sucked up and placed into an empty pre-weighed 4ml vial. This operation was repeated with all samples, washing, each time, the syringe with 1ml clean DCM. At the end of this process the solvent was left evaporating over night

in a fume cabinet. Finally after an additional time in the oven (1-2 hours at 70°C), the vials were re-weighed to measure the oil residue trapped in the vial.

$$\text{Lipid (\%)} = \frac{\text{Weight gain of vial (g)}}{\text{Sample weight (g)}} \times 10 \times 100$$

Where 10 is the dilution factor used (2ml of 20ml of DCM)

2.5.3.5 Gross energy measurement

The quantity of gross energy contained in a biological sample can be measured in a bomb calorimeter by determining the amount of heat dissipated by the sample following complete oxidation. In this study, all samples were analysed using the Parr Adiabatic Bomb Calorimeter model N° 6200 (Parr Instrument Company, 211 Fifty Third Street, Moline, Illinois, 61265-9984). Ground and dried samples were firstly pelleted using a tablet press and then weighed. The tablet obtained was placed in a nickel crucible itself positioned in the bomb crucible carrier. A piece of 10 cm fuse wire was attached to each electrode and adjusted in such way that it makes contact with the sample (the fuse wire then takes then the form of a "U" whose handle just touches the top of the sample and the two branches connect with each electrode). After having added 1 ml of distilled water to the bomb, this one was reconstituted and filled with oxygen to a pressure of 300 psi (20 bars). When this was done the bomb was placed inside the calorimeter in a stainless steel water bath filled with 2 litres of distilled water and connected to the electrical terminal of the calorimeter with two electrical leads. The run starts with a preliminary step necessary to equilibrate the water jacket temperature to the bucket temperature. When this was achieved the bomb fired and the temperature increase of the water bath was recorded until no further increase in the bucket temperature is detected. The resulting increase in temperature was used to calculate the energy content of the feed, faecal material, and carcass. For each sample, energy measurements were made in duplicate or triplicate.

Plate 2.5: Digestion block and distillation unit of the Kjeldahl system utilized (Gerhardt Laboratory instruments) at the University of Plymouth

Plate 2.6: Soxhlet system operated in the nutrition laboratory of the University of Plymouth

2.5.3.6 Quantification of chromic oxide

Chromic oxide is the substance most commonly used as a marker in evaluating the digestibility of experimental fish diets (see section 2.6.1). The following method is a modification of the one proposed by Furukawa and Tsukahara (1966) so as to handle micro-samples in determining the chromic oxide content of feed and faeces.

40 to 50 mg sample were weighed and transferred to a 100ml Kjeldahl flask. After the addition of 5ml concentrated nitric acid (HNO_3) all samples were then digested under an extraction hood, at a gentle boil, until 95% of the liquid was evaporated. This operation was repeated another time (adding 5ml more nitric acid and replacing the tubes on the digester). When the solutions were cooled (after 1-2 hours), 3ml of perchloric acid was carefully added down the flask sides. The flasks were replaced on the digester and allowed to boil for 10 minutes after the solutions turn from green to lemon yellow. Once cold, a dilution was then achieved, transferring all the liquid to a volumetric flask and making the level up to 25ml with distilled water. The absorbance of each sample was then read at 350nm after proper calibration.

2.5.3.7 Amino acid and fatty acid analysis

Amino acids and fatty acids were analysed by external and private laboratories (Eclipse Scientific group, Cambridgeshire; UK). Analyses were achieved using high performance liquid chromatography (HPLC) for AA and gas chromatography (GC) for FA. As replicates were pooled together, a single value per treatment were obtained and used.

2.6 PERFORMANCE MEASUREMENT IN NUTRITION TRIALS

2.6.1 Introduction

Information required to achieve successful diet formulation (see section 2.4.1) is usually obtained through a variety of chemical (detailed in section 2.5.3) and biological tests, whose control and set up can be quite complex (Nengas, 1991). According to Nengas

(1991), biological evaluation methods can be divided into 3 general categories: i) "retention studies" (in which the deposition of a nutrient in the carcass is measured over time), ii) "deficit studies" (in which losses of ingested food via faeces, urine and gill excretions are measured) and iii) "performance studies" (where growth response is used to evaluate and compare feeds). The following section deals with parameters established in experiments involving animals, focusing on digestibility and nutrient utilization (Glencross *et al.*, 2007).

2.6.2 Determination of digestibility coefficients

Although this point will be subject to a specific chapter, the following paragraph aims to introduce this important and complex issue and present some basic ideas about methodological aspects. Determination of nutrient digestibility is well recognized as an important preliminary step to evaluate the potential of an ingredient when dietary inclusion is intended (See figure 1.15) (Glencross *et al.*, 2007). Indeed, the biological value of a feedstuff is not only dependant upon its nutrient content, it also depends on the ability of a fish to digest and absorb those nutrients from the ingredient. In other words the Biological Value (BV) of an ingredient is both a function of its biochemical constitution and of the availability of its constituents for a certain fish species.

In digestibility studies there is no standardised method but a variety of techniques suited to each fish species. Thus interpretation of results from different trials is often complicated since various protocols can be applied. For aquatic animals an indirect method is usually preferred but inclusion rate of test ingredients, type of marker used, faecal collection technique and calculation of coefficients vary between researchers (Bureau and Cho, 2004; Glencross *et al.*, 2007). Practical reasons related to facilities, experimental design, characteristics of fish species, frequently influence the choice of methods.

The indirect methods do not require quantitative measurement of ingestion and faecal emission but involve utilization of an inert and non-digestible marker. Due to its

properties, the amount of marker included in the food remains theoretically the same in the faeces. Thereby the change (increase) in its concentration compared to a specific nutrient can be used to evaluate and quantify the disappearance of this nutrient (assimilated to the absorption of the nutrient) (Guillaume *et al.*, 1998).

When testing specific raw materials different approaches can be followed from the diet formulation point of view. In this project, the classical approach of incorporating the test ingredient as a fixed component of a basal diet formulation (along with the inclusion of an inert marker) was used. A reference diet was formulated to satisfy the nutritional requirements of the fish investigated using a high quality fishmeal (Norse LT-94). Test diets were based on this fishmeal reference diet and obtained by replacing 30 to 40% of the fishmeal component with each test ingredient.

As mentioned previously (see Table 2.2 and Figure 2.1) the digestibility system used was made with settling columns attached to the tanks. The faecal collection method utilized was based on the "Guelph-style" faecal settlement tank system developed by Cho and Slinger (1979). Chromic oxide (Cr_2O_3) was selected as the inert marker and incorporated in the diet at a rate of 0.5%. It is important to note that each group of fish were adapted to their experimental diet for three days prior to the start of faecal collection.

▶ Formulae to calculate dry matter digestibility and nutrient digestibility in the test diets

Equation 1: $ADC_{Dry\ Matter} = 100 - [(Marker_{diet} / Marker_{faeces}) \times 100]$

Equation 2: $ADCN_{diet} = 100 - [(Marker_{diet} / Marker_{faeces}) \times (Nutrient_{faeces} / Nutrient_{diet}) \times 100]$

▶ Formulae to calculate nutrient digestibility in the test ingredients:

Equation 3: $ADCN_{ingredient} = [ADCN_{test} - ((100 - i) \times ADCN_{basal})] \times i^{-1}$

Equation 4: $ADCN_{ingredient} = [((a + b) \times ADCN_{test}) - (a \times ADCN_{basal})] \times b^{-1}$

Where: **a** is the nutrient contribution of basal diet to nutrient content of test diet [level of nutrient in basal diet multiply by (100-i)]; **b** is the nutrient contribution of test ingredient to nutrient content of combined diet [level of nutrient in test ingredient multiply by i]; **i** is the level of test ingredient in test diet (%); and **(a+b)** represents the level of nutrient in test diet (%).

The formulae used allow determination of apparent digestibility based on the ratio of marker in the diet and faeces. Coefficients obtained are termed "apparent" digestibility coefficient because no corrections have been made for endogenous faecal excretion of nutrients. Apparent Dry Matter Digestibility was calculated according to equation 1; the digestibility of the nutrient components in diets was established with the equation 2 and the digestibility of nutrients for the respective ingredients obtained from formula 3 and 4. In the method described by Cho and Slinger (1979), extrapolation of the data provides ADC values on an ingredient specific basis (formula 3). However, this approach is subject to potential problems due to the relative contribution of different nutrient levels in different feeds and the interaction effects that may occur especially when the digestibility of ingredients is very low. Thereby more detailed formulae (formula 4) were proposed (Sugiura *et al.*, 1998; Forster, 1999) and adopted by many researchers. This latter formula was applied within the course of this study as often as data collected did permit it.

2.6.3 Growth and survival evaluation

With most nutrient utilization studies, the primary response variable is growth. Growth is often simply defined as the difference between initial and final live weights, which in most cases results in live weight gains. In growth studies it is important to insure that the final biomass is a least three times higher than the initial one and determined using the same equipment (Guillaume *et al.*, 1998). Biomass and mean weight for each tank were determined weekly by batch-weighing in containers of water on a tared balanced.

Growth rates are obtained when live weight gains are related to time intervals. Specific growth rate (SGR) was utilised to measure the average change of fish weight in percent per day. The calculation includes a natural logarithm transformation of the final and initial weight. Absolute growth rate (AGR), daily growth coefficient (DGC) and thermal growth rate (TGC) are other expressions of growth rates.

SGR (%/day) = [(LnWf – LnWi) / t] × 100

Where: **LnWf** is the natural logarithm of final weight, **LnWi** the natural logarithm of initial weight and **t** the duration of the trial in days.

Percentage survival was measured as the number of individuals surviving at the end of a study relative to the number included at the beginning, on a percentage basis.

Mortality (%) = [(initial Nb – final Nb) / initial Nb] × 100

Survival (%) = 100 – Mortality (%) = (final Nb / initial Nb) × 100

Where: **initial Nb** is the initial number of fish and **final Nb** the final number of fish

2.6.4 Diet and nutrient efficiency

Along with weight, feed intake is another parameter which is directly recorded and must be carefully monitored during feeding trials. Defined as the amount of feed each fish has ingested over a specific period of time (it is either reported as an amount or a rate), feed intake is theoretically difficult to established accurately. Feeding to satiation or with demand feeders allows feed intake to approximate feeding rate (the amount of feed given, which is assumed to be eaten). However this approximation might often result in over-estimations (especially with fish like gilthead sea bream, which can take the pellet in its mouth and break it down with its teeth without swallowing).

Feed efficiency relates to the ability of feeds to support weight gain and is usually reported as either feed conversion efficiency (FCE) or feed conversion ratio (FCR). FCR is defined as the ratio of dry feed consumed to the live weight gain. An FCR value of 1.5 means that every 1.5kg of feed produces 1kg of fish flesh. FCE is the reciprocal of FCR converted to a percentage value. Because these variables rely on both live weight gain and feed intake assessment, they assume the errors of both assessments.

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

The notion of feed efficiency can also be expressed on a nutrient basis. The protein efficiency ratio (PER), which is a measure of weight gain per unit of protein fed, evaluates the ability of fish to utilize dietary protein.

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

2.6.5 Nutrient retention

Nutrient composition of both feeds and fish carcasses on as fed or live weight basis is required to calculate nutrient retention parameters. Apparent net protein utilization (ANPU) represents the protein gain during an experimental period per unit of protein absorbed by the fish. Two variables were distinguished according to whether we calculate protein intake by multiplying the amount of feed consumed by the percentage of crude (ANPU) or digestible ("true" ANPU) protein in the diet.

ANPU = [increase in carcass protein (g) / amount of protein consumed (g)] × 100

Where: increase in carcass protein (g) = (Wf × % final carcass protein) – (Wi × initial carcass protein)
Amount of protein consumed (g) = feed consumed × % dietary crude protein

TrueANPU = [increase in carcass protein (g) / amount of protein digested (g)] × 100

Where: increase in carcass protein (g) = (Wf × % final carcass protein) – (Wi × initial carcass protein)
Amount of protein digested (g) = feed consumed × % dietary digestible protein

CHAPTER 3

Determination of apparent digestibility coefficients of selected rendered animal proteins in diets for gilthead sea bream

3.1 INTRODUCTION

The development of balanced diets using sustainable raw materials is the challenge and goal of nutritionists to meet the expansion of the industry and reduce costs. This was highlighted by Hardy and Kissil (1996) in a World Aquaculture report and is even more pertinent a decade later with the need to find sustainable alternatives to fishmeal.

Among alternative ingredients potentially suitable for fishmeal replacement, it is often stated that plant proteins offer less scope to match the nutritional requirement of high value marine carnivorous fish. This is mainly due to the fact that those feedstuffs have lower palatability, are deficient in certain EAA and contain anti-nutritional factors (ANF) (Alarcón *et al.*, 1999). On the other hand, animal derived proteins are relatively free from any ANF's and represent a more natural source of available protein and nutrients for farmed carnivorous fish species.

With the aim of reducing fishmeal dependence, some efforts have been directed toward establishment of the "feeding value" of animal proteins in fish such as salmonids or other freshwater species (Alexis *et al.*, 1985; Fowler, 1991; Gouveia, 1992; Steffens, 1994; El-Sayed, 1998; Abdel-Warith *et al.*, 2001; Li *et al.*, 2002; Yang *et al.*, 2004, 2006; Fasakin *et al.*, 2005). Likewise, other researchers focused on growth and feed utilization performance of marine fish fed with experimental diets formulated to test varying inclusion levels of animal by-products (Quartararo *et al.*, 1998; Nengas *et al.*, 1999; Millanema, 2002; Turker *et al.*, 2005; Yigit *et al.*, 2006). In these investigations test ingredients were included on the basis of their gross composition to reach a specific level of crude protein in the diet.

Nowadays, with the evolution of feed formulation strategy, a better knowledge of raw material properties is often required. Conduct of a digestibility trial as a preliminary investigation is, for instance, a well accepted practice in order to produce effective practical diets and reliable feed formulation. Determination of the biological value of a new ingredient enables a judicious and accurate use of this ingredient in the diets (section

1.4). The knowledge of apparent protein digestibility coefficients (ADC) helps to calculate the optimal and exact amount of ingredient to use in order to accurately match fish requirements when diets with multiple protein sources are formulated. Limiting the inclusion of a poorly digested ingredient can also reduce nitrogen waste. Besides, the optimization of the DP/DE ratio may contribute to reduce the level of protein in the diet (Cho & Bureau, 2001).

More and more nutritional studies are actually directly designed in "multiple phases" in order to assist the final formulation with pre-established information (e.g. digestibility coefficients of test ingredients). Also it should be noted that several workers investigated growth and feed efficiency effects of experimental diets formulated with various levels of animal by-products included on a digestible basis, using pre-determined or assumed coefficients (Bureau *et al.*, 2000; Fagbenro and Davies, 2001; Rawles *et al.*, 2006a; Wang *et al.*, 2006; Guo *et al.*, 2007; Goda *et al.*, 2007).

Digestibility trials have also been conducted separately to determine the biological value of a wide range of feedstuffs, including animal proteins, in various marine species such as: European sea bass *Dicentrarchus labrax* (Da Silva and Oliva-Teles, 1998), humpback grouper *Cromileptes altivelis* (Laining *et al.*, 2003), rockfish *Sebastes schlegeli* (Lee, 2002), red drum *Sciaenops ocellatus* (McGoogan and Reigh, 1996), haddock *Melanogrammus aeglefinus* (Tibbets *et al.*, 2004), Atlantic cod *Gadus morhua* (Tibbets *et al.*, 2006) and cobia *Rachycentron canadum* (Zhou *et al.*, 2004).

Digestibility coefficients of major nutrients within selected animal by-products are also available for salmonids including Atlantic salmon *Salmo salar* (Anderson *et al.*, 1992) and rainbow trout *Oncorhynchus mykiss* (Bureau *et al.*, 1999; Cheng and Hardy, 2002; Cheng *et al.*, 2004; Serwata, 2007), as well as for a wide range of other freshwater and diadromous species: silver perch *Bidyanus bidyanus* (Allan *et al.*, 2000; Stone *et al.*, 2000), Australian short finned eel *Anguilla australis australis* (Engin and Carter, 2002),

largemouth bass *Micropterus salmoides* (Portz and Cyrino, 2004) and hybrid striped bass *Morone saxatilis*♀ × *Morone chrysops*♂ (Sullivan and Reigh, 1995) or *Morone chrysops*♀ × *Morone saxatilis*♂ (Gaylord *et al.*, 2004; Rawles *et al.*, 2006a).

Similarly digestibility studies have been conducted for gilthead sea bream by Nengas *et al.* (1995) and Lupatsch *et al.* (1997). These authors reported ADC of diets containing various animal by-products for this European marine fish of commercial importance. Results obtained with poultry by-products and blood meal in particular indicated a good digestibility of nutrients. However it appears that the potential of feather meal must be confirmed within the light of new developments regarding technological process.

Over the years the techniques used to process the raw ingredients of the aqua-feed industry have evolved significantly and will continue to progress positively affecting the quality of feedstuffs. The influence of processing conditions on digestibility performance was especially well demonstrated for plant based proteins (Crevieu-Gabriel, 1999; Allan & Booth, 2004), but processing conditions have also been found to affect the biological value of other ingredients of animal origin like blood meal (Nengas *et al.*, 1995; Lupatsch *et al.*, 1997; Bureau *et al.*, 1999). Biotechnology offers opportunities for the development of fishmeal alternatives by enhancing production and processing techniques. Phytase is a good example of biotechnological manipulation (feed enzyme used as feed additive) to counteract anti-nutritional factors contained in plants and increase nutrient availability (Bransden & Carter, 1999; Paratryphon & Soares, 2001).

The new practices put in place within the European rendering industries along with continuous progress achieved in processing techniques justify, within the framework of this project, a systematic determination of nutrient digestibility in order to evaluate the new generation of animal by-products. Nutrient digestibility data are in fact characteristics of a well defined product used in a specific fish. The provision of these reliable digestibility

coefficients will enable nutritionists to formulate balanced diets accurately with the grade of animal protein currently available.

3.2 MATERIALS AND METHODS

3.2.1 Diet formulation and feed preparation

Diets were designed to primarily assess digestibility coefficients of the assigned animal by-products. A reference diet (diet A) was firstly formulated to satisfy the nutritional requirements of gilthead sea bream using a prime quality low temperature fishmeal LT94 (Skretting, UK). All experimental diets were then produced from the test materials provided by Prosper de Mulder Ltd (UK) by replacing 30 to 40% of the fishmeal component added in the control diet. Test ingredients included: steam hydrolysed feather meal (HFM), enzyme treated feather meal (EFM), poultry meat meal (PMM), spray dried haemoglobin (SDH) (APC, USA) as well as blends that consisted of the following: HFM/SDH (diet F), EFM/SDH (diet H), PMM/SDH (diet G). Those blends were obtained by mixing ingredients at the ratio of 75/25. The technical characteristic of the materials (based on specifications provided by the relative manufacturers) can be found in section 2.3; results of proximate analysis achieved in the framework of this trial are given later (Table 3.1). In the formulation (Table 3.2), the lipid levels were adjusted in order to achieve energetic balance (isoenergetic diets). Pellets were manufactured at a size of 4mm in diameter using a california pellet mill, as explained in section 2.4.2.

Plate 3.1: Control and test diets used in the digestibility trial

Table 3.1: Results of chemical analysis performed on the test ingredients (crude protein, lipid, energy and ash composition of all raw materials). Results are given as mean of replicated measurements ±SD.

	FM (LT94)	HFM[a]	EFM[b]	PMM[c]	SDH[d]
Crude Protein (%)	67.07±0.39	77.15±1.07	81.85±0.27	60.39±1.08	86.61±0.40
Crude Lipid (%)	11.95±0.07	10.46±0.19	6.34±0.59	16.57±0.65	2.86±0.33
Gross Energy (MJ/kg)	21.25±0.16	24.23±0.02	22.95±0.23	20.95±0.21	22.25±0.16
Ash (%)	12.95±0.18	2.52±0.07	1.95±0.07	15.41±0.04	3.08±0.11

[a] Steam Hydrolsysed Feather Meal (Prosper de Mulder Group, Market Harborough, UK).

[b] Enzyme Treated Feather Meal (Prosper de Mulder Group, Market Harborough, UK).

[c] Poultry Meat Meal (Prosper de Mulder Group, Market Harborough, UK).

[d] Spray Dried Haemoglobin (American Protein Corporation, Des Moines, Iowa, USA).

Table 3.2: Formulation of the experimental diets (amount of dietary ingredient in g/kg).

	Ref Diet	Test Diets						
	A	B	C	D	E	F	G	H
g/kg	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Fishmeal	600	200	200	200	250	250	200	200
HFM[a]	0	400	0	0	0	225	0	0
PMM[b]	0	0	0	400	0	0	300	0
SDH[c]	0	0	0	0	300	75	100	100
EFM[d]	0	0	400	0	0	0	0	300
Marine fish oil	100	120	120	100	150	137	100	100
Corn starch[e]	212	201	201	212	186	195	214	201
Dextrin[e]	68	59	59	68	94	98	66	59
Vitamins[f]	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Minerals[f]	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Chromic oxide	5	5	5	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000	1000	1000

[a]: Steam Hydrolysed Feather Meal.
[b]: Enzyme Treated Feather Meal.
[c]: Poultry Meat Meal.
[d]: Spray Dried Haemoglobin.
[e]: Purchased from Sigma chemical company, Poole, Dorset, UK.
[f]: Premixes obtained from Skretting aquaculture, Longridge Preston UK.

3.2.2 Stock fish

Although our interest remained focused on gilthead sea bream, digestibility trials were also conducted with two others Mediterranean fish of commercial importance: European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*). Data obtained (with the same diets and methodology utilized for the sea bream digestibility trial) were used within the framework of this chapter in order to draw comparisons (Figures 3.1; 3.2; 3.3).

Fish used for the experiments were purchased from commercial farms in Portugal with certified pathogen free stocks (gilthead sea breams (*Sparus aurata*) and European sea bass

were obtained from Aguarela-Sociedade de Piscicultura, Lda, Aveiro, Portugal, while turbots were provided by Piscultura rio Alto, Póvoa do Varzim, Portugal).

All digestibility trials were conducted indoor, in the marine station of the University of Porto (Matosinhos Portugal). On arrival, fish were firstly transferred to the quarantine unit of the research aquarium (100 L cylindrical tanks), and fed a medicated feed. Before the start of the trials, fish were acclimated to their experimental holding system for a period of two weeks and fed commercial marine fish diets. Fish were randomly distributed in the experimental system so that stocking density equals 15-17 fish per tank. The average weights of the species at the start of each trial were 106.3-108.2 g for sea bass; 35.9-36.1 g for sea bream and 131.8-135.5 g for turbot. In the following paragraphs, the sea bream trial is used as the reference trial to describe methodology, but same conditions, materials and methods were applied in the two others trials unless otherwise stated.

3.2.3 Holding facilities and water quality

Fish were held in eight 65 L fibreglass tanks (40 cm length, 17.5 cm width, and 27-38 cm depth) of a marine semi-closed re-circulating system (see section 2.2.2). These tanks were specially designed with a sloping floor so that faecal material could be voided and recovered in external conical transparent separation chambers fitted with a valve. Tanks were covered with plastic grids to prevent the fish from escaping.

Within the system, flow rates applied enabled a complete exchange of 3 to 5 volumes per hour. However, during experimentations, a low amount of fresh sea-water was continuously supplied to the system. This fresh sea-water flow was increased significantly during faeces collection and tank cleaning due to the significant amount of water removed. All principal water quality parameters were controlled on a regular basis during the course of the study to remain within acceptable limits (see section 2.2.3). Constant aeration was provided to each tank with air-stones connected to a central air compressor in order to

ensure that dissolved oxygen remained closed to saturation. Water temperature was held at 24±1 °C for sea bass, 25±1 °C for sea bream and 19±1 °C for turbot (temperatures fit for best growth) with a salinity of 33-34 ppt; for all trials photoperiod followed natural conditions.

3.2.4 Experimental conditions and feeding regime

Fish were hand fed to satiety twice a day throughout the experiments (with a "light" meal in the morning and a "normal" one in the evening). There were no obvious signs of stress or adverse effects of diets which were all well accepted by fish. Each diet was tested in triplicate. Also, as the experiment was originally designed with 8 tanks and 8 diets, three sequential feeding trials were carried out successively for each replication. Between each replication each diet was assigned to another tank. After the rotations fish were adapted to their new experimental diets for two days prior to the start of faeces collection. A 7 day average collection period allowed us to obtain more than 2g faecal material for each treatment (the E and F treatments resulting however in a significantly lower production of faeces).

3.2.5 Faecal collection technique and treatment of faecal material

The rearing tanks used for this investigation were designed to be based on the Guelph system. Developed by Cho *et al.* (1982) this method allows the measurement of digestibility parameters by collecting faecal material in a settling column (Plate 3.2). Faeces collection was performed every morning prior to the first meal, and tank cleaning took place every day following the evening ration (this last operation to ensure that no food remained in the system and in the faecal trap before the commencement of the next faecal collection). Faeces were first of all collected in large bucket and then transferred to beakers and centrifuge tubes, eliminating excess water after an adequate settling time. The material obtained after 5 min and 100×g centrifugation was dried overnight in an oven at 105°C.

cooled and ground to a fine powder. This faecal material was then stored in airtight plastic containers for further analysis (nutrient components and inert marker). During each replicate/rotation, the faecal material collected for each treatment/tank was pooled in the same container.

Plate 3.2: Fish faeces collection device

3.2.6 Analytical methods, diets and faeces analysis

All test ingredients, experimental diets and faecal samples were analysed for moisture, crude protein, energy and ash according to the protocols defined by the AOAC (moisture was established by drying the sample in an oven at 105°C for 24h; protein content (N× 6.25) was determined by the Kjeldahl method after acid digestion; energy level was

measured in an adiabatic bomb calorimeter (Parr 1356 Bomb Calorimeter) and ash quantity was calculated following incineration in a muffle furnace at 550°C for 12h. Lipid analysis was performed according to a modified Folch protocol. Chromic oxide was measured following nitric and perchloric acid wet acid digestion and colorimetric detection by visible spectroscopy according to the method of Furukawa and Tsukahara (1966). All protocols concerning analytical chemistry are detailed in section 2.5.3. Amino acid analysis was carried out by Eclipse Scientific Group: Cambridgeshire, UK.

Table 3.4 summarizes the analysis of the faecal material obtained from each of the diets fed to gilthead sea bream. This shows the levels of undigested protein, lipid and energy as well as the concentration of inert marker (chromic oxide) used to allow the measurement of the coefficients relative to the concentrations of marker and specific nutrients in diets (Table 3.3).

3.2.7 Calculations and statistical analysis

Calculation of digestibility was undertaken for dry matter and for each nutrient component. Nutrient digestibility of the diet and specific ingredients tested are distinguished using different equations. For the latter case, the original method of Cho and Slinger (1979) as well as the revised formulae given by Forster (1999) were both considered. All equations used to calculate apparent digestibility coefficients (ADC) are given in chapter 2 (section 2.6.2). For the blends tested a similarity index (SI) was calculated as followed:

SI = (predicted digestibility / measured digestibility) × 100.

Where appropriate, mean values of triplicate groups of fish are reported and standard errors included. Data analysis and results interpretation involved analysis of variance (ANOVA) in order to reveal significant differences between treatments at a probability of 95% (differences between means were tested with the Tukey's pairwise comparisons tests).

Table 3.3: Chemical composition of diets (values for nutrients are given as percentage of dry matter unless otherwise stated). Where appropriate, data are indicated as mean of 2 or 3 measurement values ± SE.

	Ref Diet	**Test Diets**						
	FM LT94	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Moisture	9.3	9.6	8.5	7.8	6.20	5.11	9.9	7.8
Crude Protein	41.53±0.13	45.06±0.57	42.12±0.42	39.47±1.46	46.32±0.44	47.87±0.79	40.38±0.36	42.56±0.56
Crude Lipid	17.17	18.57	16.92	19.02	18.83	19.24	17.65	14.58
Gross Energy (MJ/Kg)	22.1±0.13	23.1±0.35	23.1±0.3	21.9±0.06	22.5±0.01	21.7±0.04	22.0±0.03	22.9±0.06
Ash	9.19±0.05	4.73±0.05	5.68±0.02	8.34±0.25	5.56±0.02	5.24±0.05	7.36±0.04	5.60±0.04
Chromic Oxide	0.37	0.35	0.33	0.34	0.39	0.38	0.35	0.34
Arginine	2.37	2.44	2.50	2.27	1.69	1.98	2.28	2.45
Histidine	0.78	0.51	0.79	0.82	1.87	0.84	1.11	0.98
Isoleucine	1.59	1.93	2.01	1.55	0.73	1.33	1.54	1.87
Leucine	2.86	3.26	3.19	2.66	3.93	3.05	3.34	3.90
Lysine	3.26	1.87	1.55	2.06	3.09	2.03	2.05	1.96
Threonine	1.60	1.83	1.92	1.45	1.33	1.48	1.50	1.88
Tryptophan	0.49	0.30	0.33	0.41	0.58	0.34	0.41	0.42
Valine	1.68	2.40	2.39	1.67	2.21	2.01	2.09	2.72
Methionine	1.11	0.58	0.68	0.87	0.48	0.45	0.64	0.66
Phenylalanine	1.67	2.11	2.03	1.62	2.25	1.98	1.99	2.32
ΣEAA	17.41	17.23	17.39	15.38	18.16	15.49	16.95	19.16
Aspartic Acid	3.63	3.14	3.07	1.78	4.27	3.08	3.42	3.60
Serine	1.86	4.09	3.55	1.78	1.91	2.92	1.89	3.23
Glutamic Acid	5.54	5.17	5.06	5.13	4.42	4.31	5.01	5.04
Glycine	2.53	3.10	2.81	3.25	2.11	2.55	3.22	2.92
Alanine	2.43	2.11	2.14	2.33	2.93	2.11	2.65	2.50
Proline	1.55	3.13	2.98	2.08	1.50	2.31	1.94	2.84
Cystine	0.48	1.52	1.82	0.66	0.32	0.93	0.27	1.82
Tyrosine	1.37	1.32	1.29	1.17	1.40	1.47	1.23	1.29
ΣNEAA	19.39	23.58	22.72	18.18	18.86	19.68	19.63	23.24
ΣAA	36.8	40.81	40.11	33.56	37.02	35.17	36.58	42.4

Table 3.4: Chemical composition of faeces (all values are expressed as percentage of dry matter unless otherwise stated). Where appropriate data are indicated as mean of three replicates ± SE.

	Ref Diet	**Test Diets**						
	FM LT94	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Crude Protein	18.26±1.85	43.2±0.36	44.17±1.50	18.99±0.89	20.83±3.06	39.95±1.16	25.00±1.08	35.52±2.14
Crude Lipid	10.10±0.30	13.05±0.42	13.15±0.89	8.78±0.83	11.65±0.56	10.36±0.42	14.97±0.27	17.19±0.97
Crude Energy	13.51±0.35	17.86±0.23	17.97±0.27	13.82±0.22	15.63±0.29	17.12±0.17	16.78±0.10	19.12±0.23
Cr_2O_3	1.32±0.07	0.70±0.08	0.70±0.04	1.01±0.09	1.27±0.15	0.70±0.11	0.87±0.04	0.57±0.02
Arginine	0.70	2.22	2.20	1.03	0.92	2.16	1.37	1.90
Histidine	0.41	0.65	0.76	0.28	0.62	0.67	0.50	0.59
Isoleucine	0.73	1.89	1.86	0.81	0.66	1.62	0.99	1.44
Leucine	1.10	3.37	3.46	1.31	1.56	3.18	1.81	2.82
Lysine	0.85	1.13	0.98	0.57	0.97	0.96	0.78	0.86
Threonine	0.87	2.27	2.26	0.99	0.88	2.03	1.34	1.86
Tryptophan	0.21	0.38	0.39	0.21	0.23	0.36	0.25	0.31
Valine	0.88	2.86	2.92	0.96	1.13	2.51	1.41	2.20
Methionine	0.46	0.51	0.33	0.29	0.38	0.37	0.38	0.39
Phenylalanine	0.98	2.40	2.39	1.16	1.27	2.58	1.62	2.04
ΣEAA	7.19	17.68	17.55	7.61	8.62	16.44	10.45	14.41
Aspartic Acid	1.81	3.25	3.25	1.49	1.87	3.17	2.09	2.73
Serine	1.09	4.55	4.79	1.23	1.11	4.30	1.85	3.56
Glutamic Acid	2.49	5.62	5.65	2.68	2.60	5.31	3.32	4.57
Glycine	1.02	3.14	3.00	1.60	1.10	3.10	1.83	2.95
Alanine	1.00	2.34	2.36	1.13	1.22	2.17	1.40	1.88
Proline	0.84	3.53	3.65	1.09	0.81	3.17	1.56	2.70
Cystine	0.38	1.81	2.63	0.48	0.40	2.08	0.84	1.83
Tyrosine	0.73	1.40	1.33	0.71	0.62	1.36	1.01	1.17
ΣNEAA	9.36	25.64	26.66	10.41	9.73	24.66	13.9	21.39
ΣAA	16.55	43.32	44.21	18.02	18.35	41.1	24.35	35.8

3.3 RESULTS

Along with dry matter digestibility coefficients, Table 3.8 shows the apparent digestibility coefficient (ADC) profiles of nutrients calculated for each experimental diet mixture and the specific ingredients tested. These results were obtained for juvenile sea bream conditioned to the experimental diets for a defined period and they are representative of typical conditions for this species with respect to feeding and temperature conditions (Table 3.5). Data were obtained for all major nutrient components important for diet formulations.

3.3.1 Overview on the performance of the different test ingredients; Dry matter digestibility

The best results in terms of dry matter digestibility were obtained with fishmeal (71.8%), PMM (65.8%) and SDH (68.3%). Although the two blends including feather meals were found to be statistically different from the control, coefficients appear more or less homogeneous across treatments with values ranging from 40.24% to 71.8%.

Results of the feeding trial to assess digestibility of the selected animal by-products indicate very good digestibility for all components in the fishmeal of the reference diet with values ranging from 71.8% to 87.5%. Highest coefficients for alternate ingredients were obtained for SDH and PMM with values of 82.78% (protein/Forster) and 73.1% (lipid/Cho) in the former, and 79.23% (protein/Forster) and 85.74% (lipid/Cho) for the latter. Conversely, both feather meal sources (HFM and EFM) did not perform well in this evaluation for gilthead sea bream with most coefficients consistently and significantly lower compared with the control: coefficients calculated on the ingredient basis were no greater than 34.7%.

Table 3.5: Dry matter and nutrient apparent digestibility coefficients (ADC), calculated on a diet and ingredient basis, in gilthead sea bream. Values are means of 3 or 2 (*) replicates ± SE. In each row, values with same superscripts are not significantly different (Tukey's test, P>0.05). "nd" stands for no data (when more than one replicate gave aberrant result).

ADC of diets	A FM LT94	B HFM	C EFM	D PMM	E SDH	F HFM/SDH	G PMM/SDH	H EFM/SDH	Anderson-Darling	ANOVA
Dry matter	71.82±1.45^{c}	48.75±6.41^{abc}	52.57±2.63^{abc}	65.81±2.89^{bc}	68.33±3.63^{c}	42.50±10.9^{ab}	59.57±2.07^{abc}	40.24±1.82^{a}	P=0.48	F=5.82 P=0.002
Protein	87.52±1.71^{c}	50.93±5.85^{ab}	50.09±4.40^{a}	83.44±2.07^{c}	85.28±3.60^{c}	51.60±10.10^{ab}	75.05±0.80^{bc}	49.95±4.55^{a}	P=0.11	F=12.47 P=0.000
Lipid	83.44±0.82	63.91±5.04	63.43±0.54	84.36±1.36	80.32±2.68	68.91±6.23	65.63±2.40	29.79±1.98	P=0.01	
Energy	82.77±0.99^{d}	60.41±4.86^{ab}	63.11±2.05^{abc}	78.45±1.76^{cd}	77.96±2.90^{cd}	54.89±8.10^{ab}	69.14±1.75^{bcd}	50.09±1.72^{a}	P=0.39	F=10.16 P=0.000

ADC of ingredients	A FM LT94	B HFM	C EFM	D PMM	E SDH	F HFM/SDH	G PMM/SDH	H EFM/SDH	Anderson-Darling	ANOVA
Protein *(Foster)*	87.52±1.71^{c}	21.45±9.28^{a}	21.67±8.66^{a}	79.23±5.59^{bc}	82.78±9.47^{c}	*29.85±3.63^{ab}	63.48±1.93^{abc}	21.83±7.43^{a}	P=0.05	F=18.99 P=0.000
Protein *(Cho)*	87.52±1.71	*7.91±5.19	nd	77.31±7.23	77.20±13.40	nd	56.34±2.84	*3.89±2.58	P=0.02	
Lipid *(Foster)*	83.44±0.82	23.43±0.32	nd	86.11±3.63	nd	26.09±10.90	23.13±9.62	nd	P=0.01	
Lipid *(Cho)*	83.44±0.82^{c}	34.61±11.60^{a}	33.41±1.36^{a}	85.74±3.14^{c}	73.1±10.6^{bc}	54.94±6.14^{abc}	38.92±6.94^{ab}	nd	P=0.24	F=10.72 P=0.000
Energy *(Forster)*	82.76±0.99^{d}	29.80±10.4^{ab}	34.73±6.37^{abc}	71.63±5.35^{cd}	66.8±11.9^{cd}	*19.27±2.41^{ab}	47.90±5.82^{bcd}	*6.40±1.39^{a}	P=0.31	F=12.18 P=0.000
Energy *(Cho)*	82.76±0.99^{d}	26.9±10.9^{ab}	33.64±6.53^{abc}	71.98±5.16^{cd}	66.7±11.9^{cd}	*16.09±2.40^{ab}	48.69±5.67^{bcd}	*5.01±1.45^{a}	P=0.26	F=12.79 P=0.000

3.3.2 Macro-nutrient digestibility

Values for protein digestibility ranged from 49.9% to 87.5% for the mixed diets and from 21.4% to 87.5% for the respective raw ingredients (Table 3.5). The lowest protein digestibility coefficients were obtained with the feather meals. Proteins of both hydrolysed and enzyme treated feather meal (no significant differences were observed between both grades of feather meal) appear indeed to be poorly digested in sea bream. Moreover, inferior performances are equally found when these ingredients are blended with blood meals with values of 29.8% for HFM/SDH and 21.8% for EFM/SDH. All the remaining ingredients performed well with values greater than 63.5%. The results indicate a particularly good digestibility of protein for poultry meat meal and spray dried haemoglobin which were digested at a rate of 79.2% and 82.8% compared to 87.5% for the fishmeal in the control diet. Unexpectedly, the combination of poultry meat meal and spray dried haem resulted in a lower protein digestibility but still over 65%.

Similar variability was also found in the digestibility coefficients calculated for lipid and energy depending on the diet. Lipids ADCs calculated for the ingredients ranged between 23.13% (PMM/SDH, Forster) and 86.11% (PMM, Forster). As a consequence of negative values (which imply accumulation of lipid in the faeces) obtained for more than one replicate, coefficients were not calculated for several treatments (i.e. EFM, SDH, EFM/SDH). Digestible energy values calculated for the ingredients demonstrated the variation in protein and lipid digestibility that occurred in each ingredient, with values ranging between 5% (EFM/SDH) and 82.7% (FM). Energy digestibility coefficients for the complete diets showed relatively more uniform coefficients with nevertheless some variations still due to the animal protein ingredients. The lowest values were obtained for both feather meal spray dried haem blends (50.1% for EFM/SDH, 54.9% for HFM/SDH).

Table 3.6: Amino acid digestibility of diets fed to gilthead sea bream (%)

%	FM LT94	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Arginine	93.50	70.51	72.60	86.58	85.46	42.25	84.07	72.53
Histidine	88.42	58.70	70.05	89.90	91.14	57.77	88.06	78.68
Isoleucine	89.89	68.26	71.19	84.55	75.84	35.52	82.95	72.73
Leucine	91.53	66.50	66.23	85.44	89.39	44.80	85.63	74.39
Lysine	94.26	80.42	80.32	91.82	91.61	74.96	89.91	84.46
Thréonine	88.02	59.80	63.35	79.81	82.32	27.38	76.31	64.96
Tryptophan	90.56	58.95	63.21	84.86	89.41	43.94	83.83	73.86
Valine	88.46	61.38	61.96	83.00	86.34	33.89	82.11	71.35
Méthionine	90.87	71.50	84.89	90.14	78.85	56.47	84.26	79.07
Phénylalanine	87.08	63.14	63.35	78.83	84.92	31.02	78.41	68.86
ΣEAA	90.90	66.75	68.58	85.37	87.32	43.81	83.65	73.36
Aspartic acid	89.02	66.46	67.04	75.25	88.30	45.51	83.80	73.14
Serine	87.09	63.95	57.99	79.57	84.47	22.04	74.05	60.96
Glutamic acid	90.10	64.77	65.24	84.55	84.28	34.78	82.43	67.89
Glycine	91.12	67.17	66.76	85.44	86.07	35.64	84.93	64.22
Alanine	90.94	64.06	65.67	85.66	88.88	45.55	85.99	73.37
Proline	88.06	63.45	61.87	84.51	85.57	27.35	78.68	66.33
Cystine	82.56	61.41	55.01	78.50	66.60	-18.41	17.51	64.39
Tyrosine	88.26	65.63	67.90	82.06	88.17	51.02	78.23	67.88
ΣNEAA	89.37	64.76	63.47	83.07	86.22	33.66	81.22	67.40
ΣAA	90.10	65.60	65.69	84.12	86.76	38.13	82.35	70.10

Table 3.7: Amino acid digestibility of ingredients fed to gilthead sea bream (%)

%	FM LT94	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Arginine	93.50	36.04	41.27	76.22	66.70	-77.34	69.93	41.09
Histidine	88.42	14.11	42.49	92.13	97.48	-13.74	87.51	64.06
Isoleucine	89.89	35.83	43.15	76.54	43.07	-91.36	72.55	46.99
Leucine	91.53	28.95	28.29	76.31	84.41	-64.23	76.78	48.68
Lysine	94.26	59.66	59.40	88.16	85.44	29.94	83.39	69.76
Thréonine	88.02	17.46	26.35	67.50	69.02	-114.11	58.75	30.36
Tryptophan	90.56	11.53	22.18	76.30	86.71	-64.83	73.74	48.81
Valine	88.46	20.76	22.21	74.82	81.38	-93.45	72.58	45.69
Méthionine	90.87	42.45	75.92	89.05	50.79	-23.80	74.33	61.37
Phénylalanine	87.08	27.23	27.75	66.46	79.89	-99.79	65.42	41.53
ΣEAA	90.90	30.51	35.10	77.07	78.95	-66.07	72.78	47.05
Aspartic acid	89.02	32.62	34.08	54.60	86.62	-56.00	75.96	49.33
Serine	87.09	29.23	14.34	68.28	78.36	-129.76	54.47	21.77
Glutamic acid	90.10	26.78	27.94	76.23	70.71	-94.32	70.92	34.56
Glycine	91.12	31.25	30.23	76.93	74.29	-93.81	75.65	23.87
Alanine	90.94	23.75	27.76	77.75	84.07	-60.34	78.57	47.01
Proline	88.06	26.53	22.57	79.17	79.76	-114.32	64.60	33.73
Cystine	82.56	29.68	13.68	72.40	29.36	-254.00	-80.08	37.13
Tyrosine	88.26	31.67	37.36	72.75	87.94	-35.88	63.17	37.30
ΣNEAA	89.37	27.85	24.62	73.62	78.86	-96.32	69.01	34.45
ΣAA	90.10	28.86	29.07	75.17	78.97	-83.11	70.73	40.10

3.3.3 Essential Amino Acids (EAA) digestibility

Table 3.7 and 3.8 display the digestibility coefficients of the essential and non essential amino acids of the diets and test ingredients, respectively: and considerable differences are apparent for each of the animal by-products tested. EAA's within fishmeal were highly available with coefficients ranging from 87.08% to 94.26% for Phenylalanine and lysine respectively (fishmeal provided the best results as regard EAA digestibility coefficients). It was also observed that digestibility coefficients of PMM and PMM/SDH were consistently high for all important amino acids.

It is evident that in the main, trends occur that follow those seen for ADC of crude protein. Indeed, amino acids digestion pattern reflects the overall digestibility of protein. However such profiles can highlight fundamental variation with respect to specific amino acids. For instance, although spray dried haem provided ADC not surprisingly high for most of the EAA, ADC of isoleucine and methionine were found to be appreciably lower for this ingredient. Following the rule stated above, ADC calculated for the essential amino acids of all feather meal related diets were globally significantly lower. Among those diets, the most available EAA was lysine with coefficients ranging from 46% (HFM/SDH) to 69% (EFM/SDH). Those values remain however much reduced compared to the lysine digestibility of fishmeal (94.26%), PMM (88.16%), SDH (87.65%) and the SDH/PMM blend (83.39%). Lysine digestibility is generally regarded as fairly good indication of protein quality in terms of overall digestibility and degree of protein damage during processing.

Interestingly, methionine was very poorly digested within the standard feather meal but was appreciably better digested in the enzyme treated meal (EFM). This sulphur containing amino acid is especially associated with feather meal protein and is of importance in feed formulation since it is essential to fish. As far as concerned the comparison between EFM and HFM, the trend observed at the protein level was equally broken for histidine and

tryptophan: the digestibility of these EAA being improved as a result of the enzyme processing treatment applied to feather meal. With rates outperforming the one obtained in fishmeal, histidine was especially well digested from PMM and SDH (92.13% and 95.22% respectively).

Knowledge of individual amino acid availabilities provides a more refined approach in feed formulation and can produce more accurate EAA balance in final diet formulations. The overall protein digestibility for each of the animal by-products evaluated is an average of each EAA digestibility and masks the nutritional potential of the protein.

3.3.4 Digestibility results with respect to the methodological approach

Regarding the protein digestibility of ingredients, Cho and Forster calculations show close agreement in the case of PMM and SDH especially and to a lesser extent for PMM/SDH. Comparisons could not be established (EFM, HFM/SDH) or resulted in more pronounced differences (HFM, EFM/SDH) for the other treatments. Lipid digestibility established for the ingredients showed very close values between the two calculation approaches in the case of PMM. For this nutrient discrepancies were, however, observed for three other ingredients (HFM, HFM/SDH and PMM/SDH) with the higher values being for the Cho ratio. Energy digestibility values obtained with each method were comparable for all treatments.

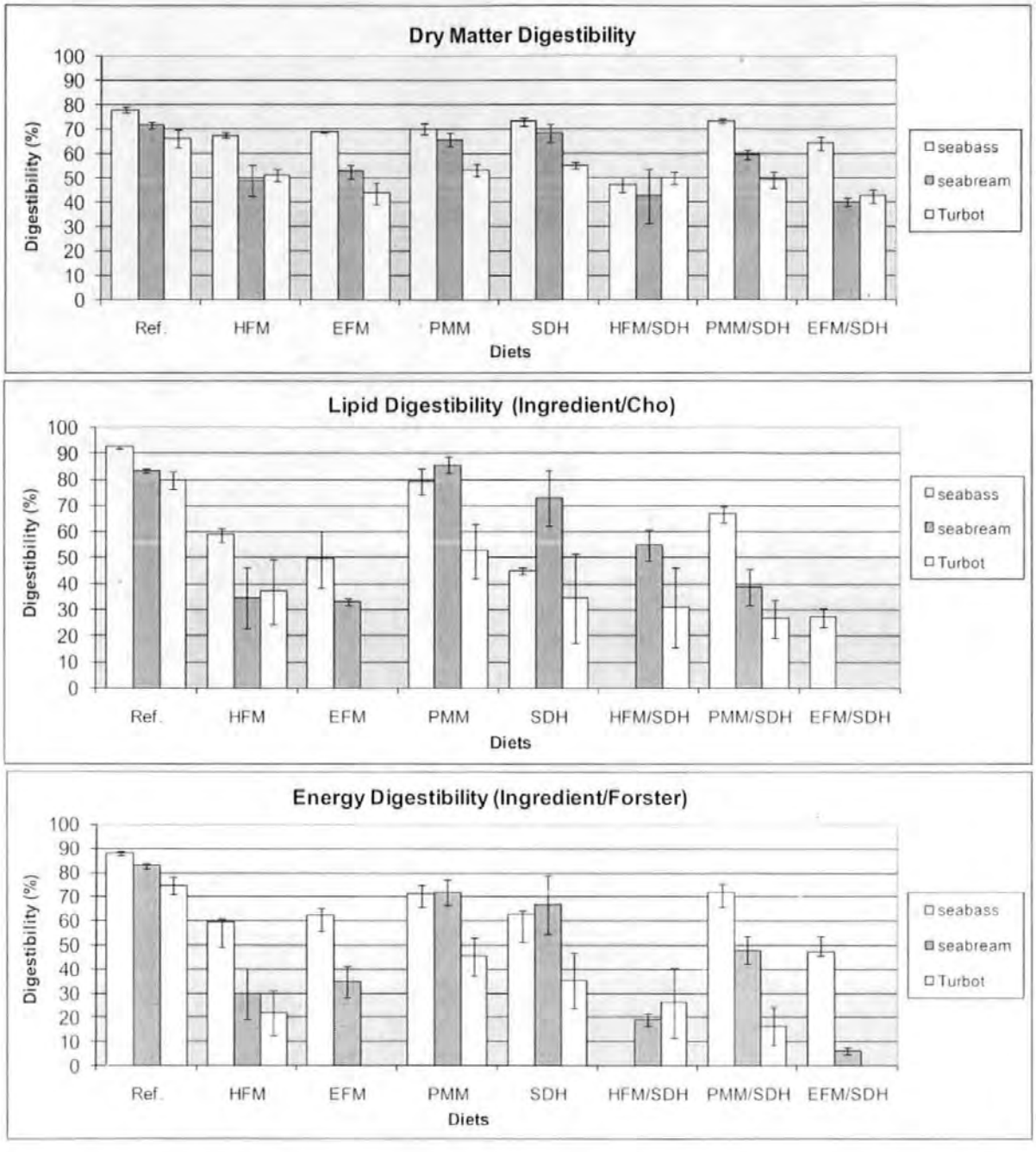


Figure 3.1: Comparison of dry matter, lipid and energy digestibility in animal by-products fed to three different marine fish species (sea bass, sea bream and turbot). Bars indicate SD.

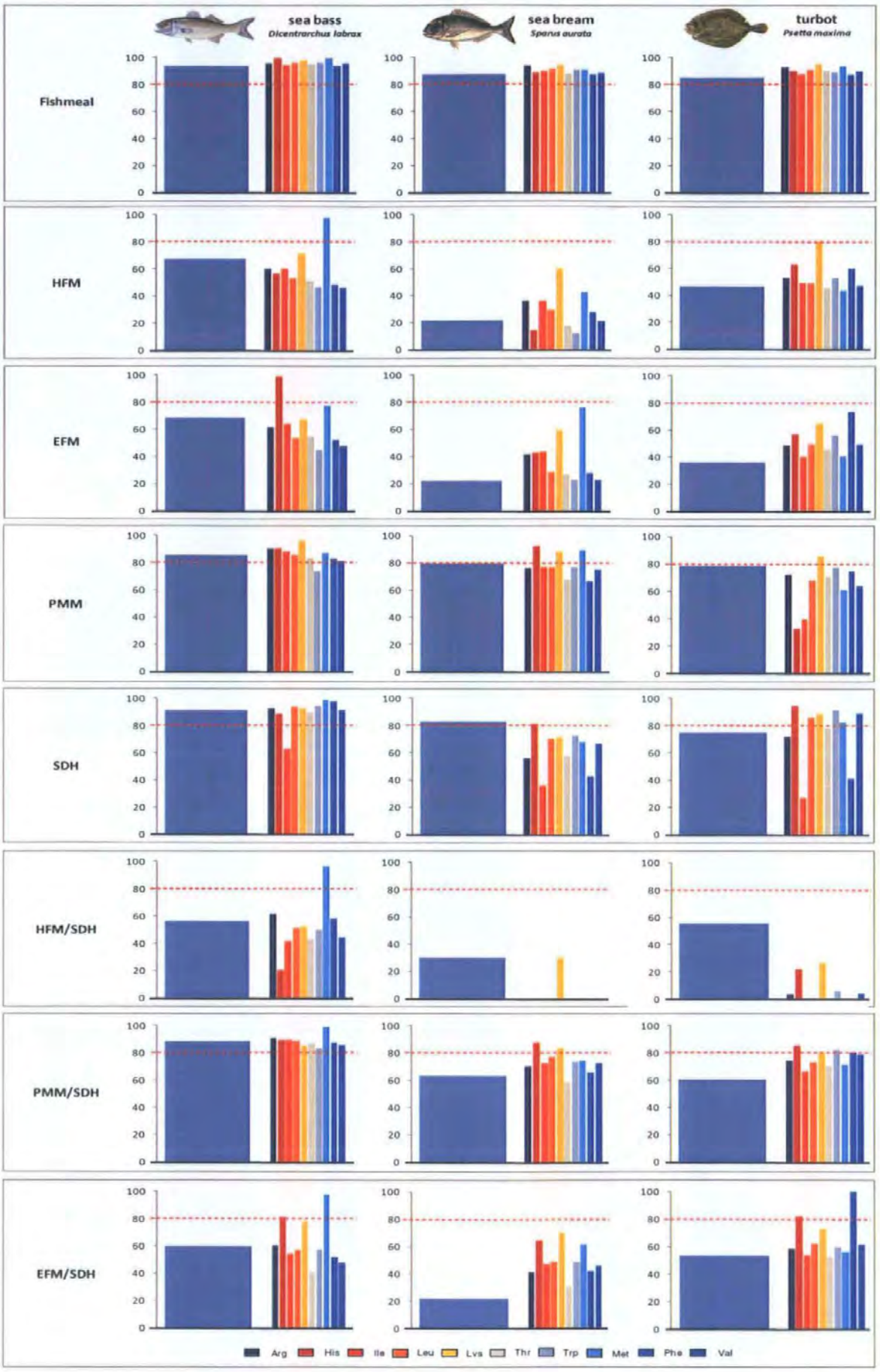


Figure 3.2: Comparison of EAA (small bars on the right) and protein (large bar on the left) ADC (%) of animal by-products fed to three marine fish species (sea bass, sea bream and turbot).

3.3.5 Drawing a comparison with digestibility coefficients obtained in two other temperate marine fish

Figure 3.1 compares the ADC measured in sea bream, sea bass and turbot, for dry matter, lipid and energy. Figure 3.2 gathers information related to protein and EAA digestibility coefficients in the three fish species. With the notable exception of feather meals (which appeared to be poorly digested in this fish), the gilthead sea bream broadly exhibited intermediate performances compared to sea bass and turbot. Apart from HFM and EFM, we observed relatively similar digestibility patterns for the three marine fish species investigated.

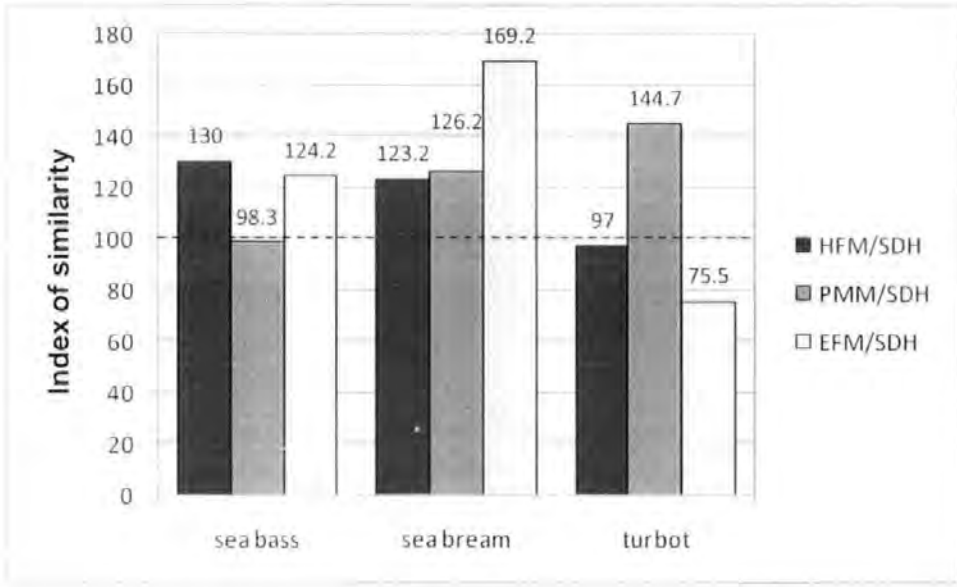


Figure 3.3: Index of similarity between predicted and measured digestibility values of protein in compound diets fed to European sea bass, gilthead sea bream and turbot.

3.3.6 Blends and predictability of digestibility coefficients

Digestibility values obtained for the blended mixture of feather meal (either HFM or EFM) and SDH appeared to be very poor based on each method employed. This was especially noticeable for the protein content of these blended ingredients. These inferior performances

obtained for both feather meal / SDH blends reflect the poor digestibility rates obtained in this fish when both grades of feather meals were used as single test ingredients. However, as demonstrated by the similarity index calculated, these ingredients did not prove to be particularly additive. Differences observed between predicted DC and measured DC in sea bream were also pronounced in the case of the PMM/SDH blend. Good indices of similarity between predicted (theorical) and measured DC were found for the HFM/SDH diet fed to turbot, and the PMM/SDH blend fed to sea bass (Figure 3.3).

With 33% (sea bass), 10% (sea bream) and 23% (turbot) of similarity indices included within the 95-105 window, comparisons between predicted and measured coefficient established for amino acids indicated that, in most cases, EAA ADC calculated for EFM, SDH and PMM could not be added to obtained the EAA ADC of the EFM/SDH, HFM/SDH and PMM/SDH blends.

3.4 DISCUSSION

3.4.1 General observations

Through the course of this trial the palatability of the diets was found to be good for sea bream, although some problems were encountered with diets E (SDH) and F (HFM/SDH). For those diets, the lower production of faeces observed might partially be related to a lower feed intake. The experimental trial demonstrated that specific classes of animal by-products were highly digested for sea bream juvenile fish within the scope of a limited feeding trial for evaluation of digestibility profiles of key nutrient components.

3.4.2 Biological value of tested ingredients; comparison of protein ADC obtained in the current investigation with the literature

► ***Fishmeal:*** The preference for fishmeal as the major protein source in compound aqua-feeds is supported by the high digestibility of its dry matter, energy and protein as well as the high digestibility of its amino acids that has been established for many fish. The present

trial with sea bream did not contradict this statement. Typically, a low temperature (LT) fishmeal proved superior in terms of overall protein and amino acid digestibility in sea bream as well as in the two other marine fish species we investigated. The advantages and merit of fishmeal for aqua-feeds has been extensively promoted and Pike *et al.* (1990) reviewed the beneficial role of fishmeal in diets for salmonid fish.

It should be noted here that some workers have evaluated the potential of alternative protein sources using inferior quality fishmeal (i.e. lower protein, high ash and higher processing temperature) in their control diet. This may lead to a situation where the test ingredient looks better than it really is when a comparison is established. Such effects of fishmeal quality in relation to its utilization as reference protein sources in control diets were highlighted by Gomes *et al.* (1995). In the current study, the utilization of LT fishmeal as the highest quality of reference protein might have resulted in accentuated differences with the test ingredients compared to other published data.

► ***Poultry meat meal (PMM):*** The ADC for protein obtained with poultry meat meal in our trial (79.23%) is slightly lower than the value previously reported by Nengas *et al.* (1995) (89.9%). These workers were able to show the effective use of a standard grade PMM for sea bream, reporting excellent digestibility and growth performances (Nengas *et al.*, 1999). The present result is, however, comparable to the value found by Lupatsch *et al.* (1997) who determined ADC for protein at 80% in "poultry by-product meal" (PBM) fed to sea bream (faecal sample collected by stripping). Using the same fixed ingredient level concept in mixed diets for their digestibility trials, different authors have revealed the excellent characteristics and potential of poultry materials for a number of important fish species. In salmonids, good performance was reported by Bureau *et al.* (1999) (87-91%), Pfeffer *et al.* (1995), who observed ADC for protein in three different poultry slaughter by-products ranging from 81% to 86% (faecal sample collected by stripping). Sugiura *et al.* (1998) who measured an ADC for protein of 96% (faecal sample collected with the Guelph

system) and Hajen *et al* (1993) who, sampling with a sedimentation system, reported an ADC of 85% for poultry by-products meal proteins. More recently, Cheng & Hardy (2002) and Serwata (2007), who used two different sampling methods, provided high protein ADC for various grades of poultry material fed to rainbow trout.

In tilapia, Sklan *et al.* (2004) reported an ADC for protein of 87.2% with their poultry meal diet (using chromic oxide as exogeneous marker and a siphoning technique for faecal collection).

In terms of digestibility performance, poultry material has equally been favourably reported for few marine fish species with ADC values ranging from 78.4% to 90.9% (Zhou *et al.*, 2004; Booth *et al.*, 2005; Tibbets *et al.*, 2006), and the present study support the potential of this by-product in aqua-feeds for gilthead sea bream with very promising results. Indeed, poultry meat meals are probably the most effective of the animal proteins currently available from both a nutritional standpoint and from bioethical considerations.

►***Spray dried haemoglobin:*** As a single ingredient, spray dried haemoglobin was very well digested by sea bream with respect to dry matter, crude protein and energy (68.33%, 85.28% and 77.96% respectively). SDH generated relatively high DC for protein in sea bass and turbot as well. These results are in accordance with the results of Lupatsch *et al.* (1997) who showed that protein digestibility for SDH fed to sea bream was over 90%. Using a lower grade of blood meal (dried in a steam-heated vessel), Nengas *et al.* (1995) reported value of 46.3% for proteins. For comparison, values for protein digestibility in similar grades of blood meal (spray-dried) for rainbow trout were reported to range from 93.2% to 100% (Bureau *et al.*, 1999; Serwata, 2007) while protein digestibility for spray dried blood meal was found to be around 94.9% in silver perch (Allan *et al.*, 2000) and 95.1% in Australian snapper (Booth *et al.*, 2005).

► ***Feather meals:*** Among marine fish investigated, proteins from feather meals were digested with a more or less good efficiency depending on the species considered (Figure 3.2). ADC of protein in both EFM and HFM averaged 75% and 60% for sea bass and turbot respectively, while proteins of hydrolysed and enzyme treated feather meal appeared to be poorly digested by sea bream with results no greater than 21.7%. Working with sea bream Nengas *et al.* (1995) reported two different results with regard to protein digestibility (24.9% and 57.5%) for two different sources of feather meal. Results reported for salmonids were promising with values for protein ADC of 81-87% (Bureau *et al.*, 1999), 82-84% (Sugiura *et al.*, 1998), 67%-84% (Pfeffer *et al.*, 1994; 1995) and 60.3-77% (Serwata, 2007). ADCp determined for Atlantic cod (62.4%) by Tibbets *et al.*, (2004) is also more in accordance with the values obtained in turbot and sea bass.

3.4.3 Processing techniques as a factor influencing digestibility performance in animal by-products

Numerous factors have been found to be involved in the digestibility of selected animal by-products. It is for instance known that the rate of digestion and nutrient assimilation in fish may be influenced by various physiological and abiotic factors including fish size, ration level and temperature (Windell *et al.*, 1978; Dos Santos & Jobling 1991; Watanabe *et al.*, 1996; Fernandez *et al.*, 1998; Kim *et al.*, 1998; Førde-Skjærvik *et al.*, 2006). Source and processing of any raw material are other important criteria governing quality and scope for inclusion in balanced diets (Deguara, 1997). Heating and drying treatments are particularly important. Indeed, if heating may improve the quality of native proteins and help to destroy anti nutritive factors in some cases, it may as well reduce the nutritive value of the ingredient further due to the modification of its nutrients. Digestibility of fishmeal is generally improved as a result of low temperatures applied during its drying process (LT fishmeal). On the other hand, as far as the feed manufacturing process is concerned,

contradictory results exist on the effect of high extrusion temperatures on protein digestibility (Deguara, 1997; Sorensen *et al.*, 2002; Sklan *et al.*, 2004a).

If various blood meals have proven effective for different fish species, most of the studies carried out so far have indicated that the digestibility of this material is equally subject to some variation depending on the processing treatment applied: spray dried and ring dried blood meal are usually found to be superior in terms of digestibility (Nengas *et al.*, 1995; Lupatsch *et al.*, 1997; Bureau *et al.*, 1999; Booth *et al.*, 2005). Recently El-Haroun and Bureau (2007) highlighted the importance of the processing/ drying technique on digestibility of lysine in blood meal.

Considering its unfavourable attributes (low biological value due to the nature its proteins), the utilization of biotechnological tools during the manufacturing process of feather meal is seen as a promising route to improving the quality of this ingredient. Enzymatic treatment can, for instance, be utilised in feathers with the aim to improve the breaking down of keratin and consequently the digestibility of nutrients. Processing conditions were already reported as a potential factor to explain discrepancies in feather meal digestibility (Nengas *et al.*, 1995).

In the context of modern processing techniques our results do not allow us to validate the hypothesis of any improvement related to the treatments employed, as no differences were observed between both grades of feather meal. In contrast to what was observed with rainbow trout (Serwata, 2007), processing feathers with an enzymatic treatment does not yield any improvement in terms of nutrient digestibility over the standard method in the three marine fish studied.

3.4.4 Methodological issues in digestibility trials

In digestibility studies there is no standardised method but a variety of techniques suited to each fish species (Glencross *et al.*, 2007). Thereby interpretation of results from different trials is often complicated since various protocols can be applied. In the present study, carried out with gilthead sea bream, a classical approach and rational design were adopted, and experimental conditions optimized according to the scientific literature to determine reliable digestibility coefficients.

Like in many investigations, the fixed ingredient level concept in mixed diets (where the test ingredient is substituted for a basal or reference diet also fed to fish in question) was employed for this trial. This technique is the basis for the Guelph method and the ratio of test ingredient to reference diet serves to allow the calculation of nutrient digestibility after collection of faeces from each treatment. There have been criticisms of the method by many researchers but the focal point of concerns is usually directed toward the choice of inert dietary marker or faecal collection technique. The properties and efficiency of specific markers have been discussed (Kabir *et al.*, 1998; Fernandez *et al.*, 1999; Austreng *et al.*, 2000; Goddard and McLean, 2001; Davies and Gouveia, 2006) while the variety of faecal collection techniques available led to several comparative studies with contrasting conclusions (Hajen *et al.*, 1993; Storebakken *et al.*, 1998; Fernandez *et al.*, 1998; Hemre *et al.*, 2003; Glencross *et al.*, 2005; Amirkolaie *et al.*, 2005; Førde-Skjærvik *et al.*, 2006). Practical reasons related to facilities, experimental design, characteristics of fish species, or any other restrictions frequently influence the choice of methods. For salmonid fish, the anatomical features are such that the manual stripping method is often used since this is ideal for rapid removal of faecal material and is not prone to the leaching losses that can result in the over-estimate of the ADC for each nutrient class. With sea bass, sea bream and turbot, since it is difficult to strip fish faeces by manual expression, the faecal material was obtained using the Guelph digestibility tank arrangement and daily collection of naturally

voided faeces in traps. In the European sea bass, Spyridakis *et al.* (1989) found that data obtained with the faecal collection method based on continuous screening was more comparable to that obtained by immediate siphoning. Despite the minor over-estimation reported when sedimentation techniques are employed, in the current study with sea bream, the data would nonetheless be expected to be of relative order for the absorption of crude protein, amino acids, lipid and energy.

3.4.5 Does sea bream exhibit specific abilities or inabilities as regard the digestion of animal by-products? Comparison of sea bream digestibility performances with those of two other Mediterranean marine fish

Although ADC tends to be lower in turbot, relatively similar patterns are observed with most of the test ingredients across the three species. Sea bream broadly exhibited intermediate performances. In fact, further to this series of trials, it appears that the inabilities of sea bream to utilise feather meal is the main notable difference between these marine fish. Despite the fact that they are all regarded as carnivores requiring a relatively high protein level in their diets, gilthead sea bream, European sea bass and turbot proved to be different in many respects (ecological niche, natural feeding habits, digestive characteristics, sexuality mode...etc), and some differences might be expected as far as their abilities to utilise certain feedstuffs and nutritional needs are concerned, justifying a proper investigation of all these important marine fish. Turbot tends to have a more defined feeding frequency and meal intake preferring larger pellet sizes, while sea bass may be expected to consume proportionally smaller meals. Moreover some diversity is found in the digestive tract of these fish in connection with their feeding habits. Length of digestive tract, number of pyloric caecae, and digestive enzyme capacity are obviously important factors to consider. Thus, the slight but systematic reduction in the digestibility performances of turbot compared to sea bass could be explained by a shorter digestive tract, which could involve a reduced residential time for digesta. Differences brought to

light demonstrate that caution must be made between extrapolating results from one species to another.

3.4.6 Can compound diets, with specific digestible protein level, be formulated on the basis of the nutrient digestibility of its individual ingredients for sea bream? On the additivity and predictability of ADC

Aside from the additive property of coefficients, in digestibility investigations "it is generally assumed that there are no interactions among ingredients that differentially affect digestibility, and that changing the inclusion content of a particular ingredient does not change its digestibility either. Neither of these assumptions holds true all the time" (Glencross *et al.*, 2007). According to Kim *et al.* (2006), varying the incorporation level of herring meal from 10 to 50% in the test diet does not affect the ADC value for protein and energy in juvenile haddock diets. Testing two levels of fish oil and different levels of protein sources (e.g. extruded wheat, poultry meal and meat meal), Booth *et al.*, 2005 arrived at the same conclusion in Australian snapper. In gilthead sea bream soy inclusion did not appear to have any effect on nutrient digestibility (Venou *et al.*, 2006) while Storebakken *et al.* (2000) reported a significant increase in ADC for crude protein and EAA with increasing proportion of dietary protein from wheat gluten in Atlantic salmon. Such variability in ADC with variable inclusion level of protein or lipid sources was also found by other investigators but their results usually showed that an increase in the incorporation level of the test ingredient in the test diet resulted in a decrease in ADC of the test ingredient (Nandeesha *et al.*, 1991; Appleford and Anderson, 1997). For the ADC measurement in gilthead sea bream it is interesting to note that Lupatsch *et al.* (1997) using a diet with test ingredients as the sole sources (91-98%), found ADC values of protein (83%) and energy (80%) for fishmeal lower than those (96% and 94%) determined by Nengas *et al.* (1995) who used a diet ratio of 50:50 reference diet to test feed ingredient. In

our investigation, the conventional 70:30 mixture of reference diet to test feed ingredient, widely used in fish digestibility studies, was applied.

Amounts of test material used for digestibility assessment, which obviously have to be identical across treatments, can be much higher than those which would be used in most formulations. As a result of this, the real nutrient absorption from a specific test ingredient might be miss-estimated compared to what would be observed with lower levels used in practical conditions. Since a variation in the dietary incorporation level may affect the digestibility of the test feed ingredient, it has been recommended that the ADC be evaluated based on the actual incorporation level into the diet (Tacon, 1995).

High ash content in diets was already reported as a potential reason explaining reduction in digestibility (Fernandez *et al.*, 1998) and some other studies described a relationship between ash content and digestibility of other dietary components (Hajen *et al.*, 1993; Da Silva and Oliva-Teles, 1998). There are also indications in fish that carbohydrate levels negatively affect the overall digestibility of diets (but mainly that of the carbohydrate itself) (Storebakken *et al.*, 1998; Fountoulaki *et al.*, 2005). Moreover, it was suggested that high lipid levels in feed ingredients would reduce protein digestibility in fish, which could be related to the formation of protein and oxidized fat complexes during the drying process (Sullivan and Reigh, 1995). In this study protein ADC does not appear to be correlated to the level of lipid ($r=0.199$; $p=0.75$) or ash ($r=0.69$; $p=0.20$) in our test ingredients.

Using a range of practical ingredients several researchers were able to test the assumption of additivity inherent to digestibility assessment and proposed that digestibility of major nutrients in a diet was predictable from the digestibility coefficients of these nutrients in its individual ingredients (Lupatsch *et al.*, 1997; Sklan *et al.*, 2004; Tibbets *et al.*, 2006).

Indices of similarity calculated in this work indicate that the differences between predicted and measured DC are relatively pronounced for protein and most of the amino acids. The different combination of ingredients tested in this trial did not allow us to obtain any

improvement on digestibility values. Indeed, according to this experiment, combining feather meal and blood meal in particular is clearly not advantageous, agreeing with the results found in the turbot and sea bass trial. A risk of creating interactions and antagonist effects exists when mixing various ingredients. It is speculated that a complex may have occurred with the proteins of the spray dried haemoglobin and/or feather meal resulting in a protein fraction quite unavailable to the digestive enzymes present in the sea bream intestinal tract.

3.4.7 Differences between two published equations to calculate ADC

Apart from marker and sampling issues, other workers have even questioned the validity of the calculation itself and suggested modifications to the digestibility equation suited to the common technique. Sugiura *et al.* (1998) indicated that the ADC calculation method for ingredients needed refinements when dealing with ingredients having extremely variable levels of a given nutrient. Forster (1999) claims that the classical equation outlined by Cho *et al.* (1982) is incorrect, but states that, in many cases, the values obtained using the two equations are very similar provided that the level of nutrient and digestibility in the test ingredient is similar to that of the reference diet. Such affirmations were supported by the findings of other scientists following further investigations (Tibbetts *et al.*, 2006; Kim *et al.*, 2006). Nevertheless, Bureau *et al.* (1999) argued that the equation of Forster is based on an assumption which is rarely verified (i. e. the nutrient level measured for a test diet is the same as what is predicted from the mash and test ingredient dry matter and nutrient levels (a+b)) due to the difference in the dry matter content of ingredient, reference diet and test diet or due to analytical or sampling errors. Having shown that this introduces significant bias in the estimation of ADC of test ingredient, these authors recommended the utilization of a simplified version of Forster/Suguira's equation.

3.5.8 Lipids and amino acids digestibility

ADC of lipids followed broadly the same pattern described for protein. It is noted that lipid digestibility in PMM outperformed the fishmeal diet (with 85% compared to 83% for the control). Lipids in SDH were reasonably well digested with a rate of 73% (obtained with formulae used by Cho *et al.*, 1982), while other coefficients were much reduced (33%-34% in feather meal). The relatively good coefficient obtained for SDH could be critically discussed regarding the lipid adjustments originally made in this diet (level of marine fish oil were kept higher to maintain the energetic balance as close as possible to the others). With the exception of feather meal, these values agree with the results of Portz and Cyrino (2004) who mention high ADC for the lipid fraction of several protein sources fed to carnivorous species.

This study is amongst the very few that have explored the detailed ADC patterns for all EAA in animal protein sources for marine fish. Limited information is available for Atlantic salmon (Anderson *et al.*, 1992), striped bass (Small *et al.*, 1999), Murray cod and Australian shortfin eel (De Silva *et al.*, 2000) as well as grouper (Lin *et al.*, 2004). In a recent study, the lower digestibility of EAA in poultry by-product fed to largemouth bass was attributed to the quality and processing of the feedstuffs, and in particular the method of extraction of lipids (Portz and Cyrino, 2004).

Amino acid composition and digestibility are prime factors when determining the nutritional value of test ingredients as a protein source. PMM is generally reported to possess a favourable profile of indispensable amino acids for fish production. However, when dietary inclusion is intended with this ingredient, amino acid supplementation might be necessary in order to maintain optimal performance of fish. Assessing poultry material in a feeding trial with sea bream, Nengas *et al.* (1999) indicated that the first limiting AA was methionine. Recently, a pet-food grade PBM was proven more efficient with Met and Lys supplementation in hybrid striped bass production (Gaylord and Rawles, 2005; Rawles

et al., 2006a). In our study, digestibility coefficients obtained for the EAA contained in PMM were close to those of fishmeal, reflecting the high digestibility performance of protein. Therefore, the biological value of PMM would rather be limited by slight deficiencies in certain EAA since good digestibility is generally described with high quality PMM. The digestibility of all EAA in PBM for largemouth bass was found to be included within the range of 83%-93% by Portz and Cyrino (2004) with the exception of methionine (71.3%) and tryptophan (51.5%). Different sources and processing conditions of poultry by-products may indeed result in lower EAA digestibility (Bureau *et al.*, 1999) or growth depression (Nengas *et al.*, 1999).

Blood meal products are known as a good source of histidine, leucine and lysine; the latter being however sensitive to heat and drying treatments (El-Haroun and Bureau, 2007). In our investigation, the determination of the digestible EAA profile enabled the variation of specific EAA to be highlighted for spray dried haemoglobin. Indeed, lower digestibility performances were found in the EAA profile of SDH for isoleucine and methionine (moreover, with the exception of Met in sea bass, it is interesting to note that those same variations were also visible in turbot and sea bass). El-Haroun and Bureau (2007) suggested that a deficiency in isoleucine could be responsible for the lower performances of rainbow trout fed a diet with 20% of flash dried bovine blood meal. Our results showed that treatments used to process feather meal equally affect the bio-availability of methionine.

CHAPTER 4A

Reduction of fishmeal in gilthead sea bream diets using selected animal by-products with respect to protein digestibility profile:

Effects on feed intake, growth and feed utilization

4A.1 INTRODUCTION *(The utilization of animal by-products in aqua-feeds)*

Animal protein meals from the rendering industry have been used in animal feeds since the middle of the 19th century (NRA, 2006). In Europe, this traditional application was removed in 2000 as a consequence of the measures taken to prevent the amplification of Transmissible Spongiform Encephalopathies (TSE) and prion related-diseases (section 1.2.4). Along with the prohibition of these products in the food chain, fish nutrition investigations considering animal derived proteins have equally widely been hampered. Following the outbreak, many research groups decided indeed to redirect or focus their effort toward the evaluation of plant based feedstuffs to partially replace fishmeal in diet of European important commercial fish species.

Nevertheless on a worldwide scale a great amount of information can still be found on the nutritional value of animal by-products, as research programmes continue to be developed in countries where PAP are not restricted. In fact, many fish nutritionists are well known for their contribution to improving our understanding and efficacy of using PAP type alternatives in specific fish models. Comprehensive nutritional assessment of animal by-products as potential protein sources were notably undertaken with tilapia *Oreochromis spp.* (El-Sayed, 1998; Fasakin *et al.*, 2005), silver perch *Bidyanus bidyanus* (Allan *et al.*, 2000; Stone *et al.*, 2000), rainbow trout *Oncorhynchus mykiss* (Bureau *et al.*, 1999; 2000; Serwata, 2007) and cuneate drum *Nibea miichthioides* (Wang *et al.*, 2006).

Broadly speaking, results obtained with animal by-products proved to be convincing since in most cases the feasibility of moderate to high inclusions were reported. A few cases of successful total replacement were described but the validity of such results often remain attached to specific conditions: the majority of studies showed that total replacement significantly reduces growth and feed efficiency. According to Forster & Domini (2006), rendered animal by-products can replace from 15 to75% of fishmeal in diet for shrimps. In

fish a 30% substitution rate of fishmeal by PAP is said to be suitable for most species although variation can occurred depending on the fish species and ingredient tested.

Most of the studies conducted have particularly stressed the potential of rendered products derived from poultry processing waste as secondary protein sources in fish diets. For instance some of the optimum rates of fishmeal replacement determined using PBM were: 40% for African catfish *Clarias gariepinus* (Abdel-Warith *et al.*, 2001), 50% for gibel carp *Carassius auratus gibelio* (Yang *et al.*, 2004), 25% in black sea turbot *Scophthalmus maeoticus* (Turker *et al.*, 2005) and 20% in Chinook salmon *Oncorhynchus tshawytscha* (Fowler, 1991). PBM has also been tested in diets for rainbow trout by Steffens (1994) and hybrid striped bass *Morone chrysops* × *Morone saxatilis* (Gaylord & Rawles, 2005; Rawles *et al.*, 2006b). Using PBM as a sole protein source or at high inclusion these latter authors stressed the need for amino acid supplementation.

Considering its direct implication in the BSE outbreak, the case of meat and bone meal (MBM) and its utilization in fish feed remains more controversial and will not be much developed as no feedstuffs of mammalian origin were tested in this project. For the record, this feedstuff continues nevertheless to be investigated as a potential alternative to fishmeal and its inclusion in diets for large yellow croaker *Pseudosciaena crocea* (Ai *et al.*, 2006), gibel carp *Carassius auratus gibelio* (Yang *et al.*, 2004), short finned eel *Anguilla australis australis* (Engin & Carter, 2005) and rainbow trout (Bureau *et al.*, 1999) resulted in recommendations ranging from 23 % to 50 % of fishmeal replacement.

Aside from this, blood meal and feather meal are often considered to be of inferior value because of their poor digestibility and/or unbalanced EAA profile. Due to these characteristics they proved to be rather inappropriate for fishmeal replacement in a few trials (Wang *et al.*, 2006; Fasakin *et al.*, 2005). However contradictory results exist here as well. Some researchers have indeed demonstrated the feasibility of using feather meal as a secondary protein source in aqua-feeds without any detrimental effect on the biological

performance of salmonid species especially (Koops *et al.*, 1982; Fowler, 1991; Bureau *et al.*, 2000). Ingredients such as blood meal are often tested and employed in blends in order to complement other protein sources synergistically. Indeed, the utilization of composite mixtures (including plant proteins or not) may also have beneficial effects when a reduction in the fishmeal content of the diet is intended (Quartararo *et al.*, 1998; Webster *et al.*, 2000; Millamena, 2002; Fasakin *et al.*, 2005).

Along with turbot and sea bass, sea bream has equally been utilised in several trials where the effect of fishmeal substitution with alternative protein sources was measured on growth and feed utilization performances. These investigations suggest that sea bream may tolerate substantial levels of fishmeal substitution, whether with animal, plant or other protein sources. Nevertheless a majority of these trials considered plant proteins, and information concerning the utilization of animal by-products in diets for gilthead sea bream is rather limited. Nengas et al. (1999) reported good results using PMM at high inclusion levels. Moreover their work showed that a combination of poultry meat meal and feather meal could serve as a useful protein source. In their evaluation, Robaina et al. (1997) found that growth of this particular marine fish was not significantly affected by the source of dietary protein tested (corn gluten and meat and bone meal).

Feeding trials form the basis of fish nutrition research with respect to the evaluation of new ingredients (see section 1.3). Once new and accurate feed formulations are obtained, they must be evaluated in terms of growth response and feed efficiency. The present work aims to validate diet formulations containing different processed animal proteins in gilthead sea bream. FCR and SGR obtained with optimal inclusions of PMM and realistic inclusion of EFM and SDH (calculated on the basis of the digestibility data pre-established) were compared to the same parameters obtained with a reference diet following a 9 weeks feeding trial.

4A.2 MATERIALS AND METHODS

4A.2.1 Growth trial (trial 2)

4A.2.1.1 Diets preparation:

Six iso-nitrogenous and iso-energetic diets were formulated for gilthead sea bream to test different inclusion rates of animal by-products. Using the digestibility coefficients pre-established (see chapter 3), PMM, SDH and EFM were included in the test diets to achieve different fishmeal replacement rates (table 4A.1) and so that total digestible protein level was 40%. All experimental diets were derived from a reference diet, firstly formulated to fulfil the requirement of gilthead sea bream (using high quality fishmeal as a sole protein source) and employed as a control during the course of this study.

Diets were prepared using the California pellet mill of the University of Stirling (Scotland) as described in section 2.4.2. For this trial pellets of 3 mm in diameter were manufactured.

Table 4A.1: Formulation (g/kg), composition (±SE) and nutritional value of experimental diets

Formulation (g/kg)	FM LT94	PMM 25	PMM 50	PMM 75	EFM 5	SDH 10
Fishmeal (LT-94)	640	480	320	160	608	576
Poultry Meat Meal	0	190	380	570	0	0
Enzyme Feather Meal	0	0	0	0	108	0
Spray Dried Haemoglobin	0	0	0	0	0	68
Marine Fish Oil	74	67.7	62.2	56.7	70	79.5
Starch[1]	113.3	113.3	113.3	113.3	113.3	113.3
Dextrin[2]	56.7	56.7	56.7	56.7	56.7	56.7
Vitamins[3]	5	5	5	5	5	5
Minerals[4]	5	5	5	5	5	5
αcellulose[5]	106	82.3	57.8	33.3	34	96.5
Total	1000	1000	1000	1000	1000	1000

[1]: Starch from corn (Sigma S4126); [2]: Dextrin typeII from corn (Sigma D2130); [3,4]: Skretting; [5]: αcellulose (Sigma C8002).

Composition (%)	FM LT94	PMM 25	PMM 50	PMM 75	EFM 5	SDH 10
Moisture	3.43	3.82	4.63	4.79	3.79	3.67
Crude protein	46.08±0.38	46.77±0.09	48.62±0.38	53.05±0.18	48.97±0.49	47.44±0.20
Crude lipid	12.15±0.40	11.41±0.11	12.65±0.15	14.06±0.03	14.40±0.16	8.01±0.07
Gross energy	20.44±0.02	20.57±0.06	20.61±0.16	20.82±0.05	21.80±0.35	20.92±0.07
Ash*	9.48±0.06	10.24±0.03	10.81±0.16	9.73±0.02	11.26±0.03	9.26±0.05

Values are means of 3 or 2 (*) replicates ± SE

4A.2.1.2 Fish and experimental design

One thousand and ninety six gilthead sea bream (*Sparus aurata L.*) juveniles were obtained from a commercial hatchery in France (Aquastream, Ploemeur) at an initial mean weight of 1.4g and acclimated to the laboratory for a period of 3 months. After their arrival, fish were firstly transferred to 4 of the 16 tanks that compose the rearing system, then redistributed to 8 tanks a few weeks later and finally randomly assigned to the 16 tanks so that the stocking density was 50 fish per tank at the start of the trial (initial fish weight was then averaging 22.7g). During this acclimation period, fish were fed with commercial pellets (Biomar Ecostart 3) at a rate of 3-2% body weight. After the fifth experimental week, stocking densities were re-adjusted to 30 fish per tank. To match the number of rearing tanks available, the reference diet (FM) and the diet with the lower inclusion of PMM (PMM25) were tested in duplicate while all other treatments (PMM50, PMM75, EFM5, and SDH10) were triplicated.

4A.2.1.3 Facilities and experimental conditions

The trial was conducted in the experimental facilities of the University of Plymouth (nutrition aquarium) in a closed marine system described in section 2.2.2. Despite the filtration systems mentioned in Table 2.2 to ensure water purity, partial water changes (amounting approximatively 20% of the system's volume) were carried out every week, while filters were cleaned daily to avoid any accumulation of waste products. Each tank (covered with a grid to prevent fish from escaping) was supplied with filtered sea water at a rate of 10L.min^{-1} (resulting in 6 water changes per hour).

All principal water quality parameters (pH, ammonia NH_3, nitrite NO_2^-, Nitrate NO_3^-, and dissolved oxygen) were monitored on a regular basis (Hanna pH210 meter, Hanna chemical test kits, YSI model85 portable meter) and remained at acceptable levels throughout the experimental period. Salinity was controlled within a range of 33-34 ppt,

and a 12/12h light/dark cycle was adopted. The water temperature was maintained at 22±1°C by a thermostatically controlled immersion heater. pH was buffered when necessary with calcium carbonate ($CaCO_3$) or Calcium Hydroxide ($Ca(OH)_2$).

All groups of fish were fed by hand twice a day (two successive rounds for each meal). Fish were fed to satiety (until the first feed refusal was visually observed) up to rates of 3% (week 1 to 5) and 2.8% (week 6 to 9) body weight. Fish were fasted prior to the weekly weighing being fed 6 days a week. Quantities of feed were adjusted based on new weekly fish biomass.

4A.2.1.4 Feed efficiency, growth and survival indices

During the experimental period, each group of fish were weighted weekly batch. Along with fish weight, feed intake was the other major parameter recorded. With the raw data collected, FCR and SGR were calculated with the equations given in section 2.6.3 and 2.6.4. All mortalities were recorded and taken into consideration to calculate the daily feed ration. At the beginning of the growth study, 15 fish were sampled for whole body composition and stored at -20°C until analyzed. At the end of the trial 3 fish were randomly collected from each tank for the same purpose. Prior to analysis those samples were oven dried for a night at 105°C (moisture contents were then determined), ground into a homogeneous mass and stored in air-tight plastic containers. Subsequent analyses enabled calculation of nutrient efficiency (PER) and nutrient retention (aNPU) indices with the equations detailed in section 2.6.4 and 2.6.5.

4A.2.1.5 Chemical analysis of the diets and fish carcasses

Ingredients, diets and fish carcasses (sampled before and after the feeding trial) were subject to proximate composition analyses. Fish sampled for whole body analysis (3×5 at the start and 3 fish per tank at the end of the trial) were oven dried, homogenized in a blender, and analysed so that nutritional values were available for each replication.

Moisture content (dry matter) was firstly determined according to the AOAC method. After dessication in an oven (105°C for 24h) all samples were then analyzed for ash (incineration at 550°C for 12h), crude protein (Gerhardt Kjeltech analyzer, %N × 6.25), total lipid (dichloromethane extraction by Soxlhet method) and gross energy (Parr Bomb Calorimeter) on a dry basis (section 2.5.3).

4A.2.1.6 Statistical analysis

All data are presented as mean plus or minus standard error to the mean (SE). Results were subject to one-way analysis of variance (differences between means were tested with Tukey's pairwise comparison test), but non parametric testing was considered as a result of a failure to get normal data (Kruskal Wallis's test with post hoc multiple comparison testing). To perform the analysis, treatment 1 and 2 (FM and PMM25) were entered with a missing value. Statistics were performed using Minitab 13 software.

4A.2.2 Palatability trial (trial 3)

4A.2.2.1 Diet preparation

For this second investigation, the same three ingredients tested in trial 2 (PMM, EFM and SDH) were incorporated as a fixed component (40%) of a basal diet with the view to assess their palatability characteristics. Both fishmeal and casein were used as reference protein sources and two different sets of 4 diets were then formulated (Table 4A.2).

Diets were manufactured with the cold extruder of the University of Plymouth: All dietary ingredients were firstly thoroughly mixed in a Hobart A120 pelleting and extruding machine (Hobart Manufacturing Ltd., London, England) to obtain a homogeneous mixture. Extruded diets were then passed through a 3 mm die to obtain strands which were placed in a drying cabinet for 48 h at 40°C. Dried strands of feed were later broken down into convenient sizes suitable for the experimental fish.

Table 4A.2: Formulation (g/kg) of experimental diets used in trial 3:

	Set 1 fishmeal based diets				Set 2 casein based diets			
(g/kg)	**FM**	**PMM40**	**EFM40**	**SDH40**	**casein**	**PMM40**	**EFM40**	**SDH40**
Fishmeal (LT-94)	640	240	240	240	0	0	0	0
Casein	0	0	0	0	608	208	208	208
Poultry Meat Meal	0	400	0	0	0	400	0	0
Enzyme Feather Meal	0	0	400	0	0	0	400	0
Spray Dried Haemoglobin	0	0	0	400	0	0	0	400
Marine Fish Oil	70	80	120	105	120	120	135	145
Starch[1]	140	140	140	140	140	140	140	140
Dextrin[2]	60	60	60	60	60	60	60	60
Vitamin[3]	5	5	5	5	15	15	15	15
Mineral[4]	5	5	5	5	30	30	30	30
αcellulose[5]	80	70	30	45	27	27	12	2
Total	1000	1000	1000	1000	1000	1000	1000	1000

[1]: Starch from corn (Sigma S4126); [2]: Dextrin typeII from corn (Sigma D2130); [3,4]: Skretting; [5]: αcellulose (Sigma C8002).

4A.2.2.2 Fish and experimental design

At the start of trial 3 (one month after the final sampling of trial 2), the remaining fish from trial 2 were re-allotted to 12 tanks to obtain a stocking density of 23 fish per tank (fish weight then averaged 97.9g).

The four diets of each set were fed to triplicate group of fish for a period of seven days (set1: fishmeal based diets during week 1; set 2: casein based diets during week 2). A 1 week gap was observed between the two feeding periods of the trial; during this week, a commercial "standard expanded" diet (Skretting) was used to feed the fish. Between the two experimental weeks, diets were rotated over the system so that a particular test ingredient was not fed twice to the same tank.

4A.2.2.3 Facilities and holding system

Apart from the addition of an aeration system (air stones connected to a central air compressor to provide each tank with constant aeration) and a second D-Deltec protein skimmer, the rearing system was run with the same equipment described before. Water quality was checked daily; pH found to range from 7-7.5 and dissolved oxygen from 6.5-7.2 $mg.L^{-1}$ (91-98% saturation). Temperature and salinity were controlled at 23.3±0.1°C and 35±0‰. Triplicate groups were fed by hand twice a day (two rounds per meal) to satiety. For this second trial, feed intake and feeding time were the prime parameters recorded; the latter consisted in measuring the time between the start and the end of the feeding with a stopwatch.

4A.3 RESULTS

4A.3.1 Growth trial (trial 2)

4A.3.1.1 Feed intake

Although feeding to a fixed rate of body weight was decided (3%BW during first 5 weeks, and 2.8% for the last 4 weeks), it appeared that certain diets were not as palatable as fishmeal and that the feeding level applied was too high for certain treatments, with fish not eating the entire ration offered. In order to obtain FCRs representative of diet quality, fish were fed to satiation up to the specified rate. Differences between intended ration and actual feed intake are presented in Figure 4A.1. In this figure, a trend in feed intake reduction was clearly observed with increasing inclusion of PMM. The PMM75 diet gave the lowest feed intake (55.9 g/fish); indeed, this feeding method resulted in a feeding level of 2.58% of BW per day over the experiment for the PMM75 diet, whereas a target of 2.91% of BW was attempted. Despite these variations, feed intakes recorded did not prove to be statistically different across the dietary treatments tested.

4A.3.1.2 Biological performance (survival, growth) and feed efficiency parameters

The evolution of individual weights over the 9 experimental weeks for all dietary regimes tested in trial 2 is represented in Figure 4A.2. It is evident that growth performance was uniformly high for all dietary treatments with the capacity to achieve a 3 fold increase in live weight gain over the 63 day trial period. As indicated by this figure, the growth was well sustained, the slopes of each curve being rather consistent and regular.

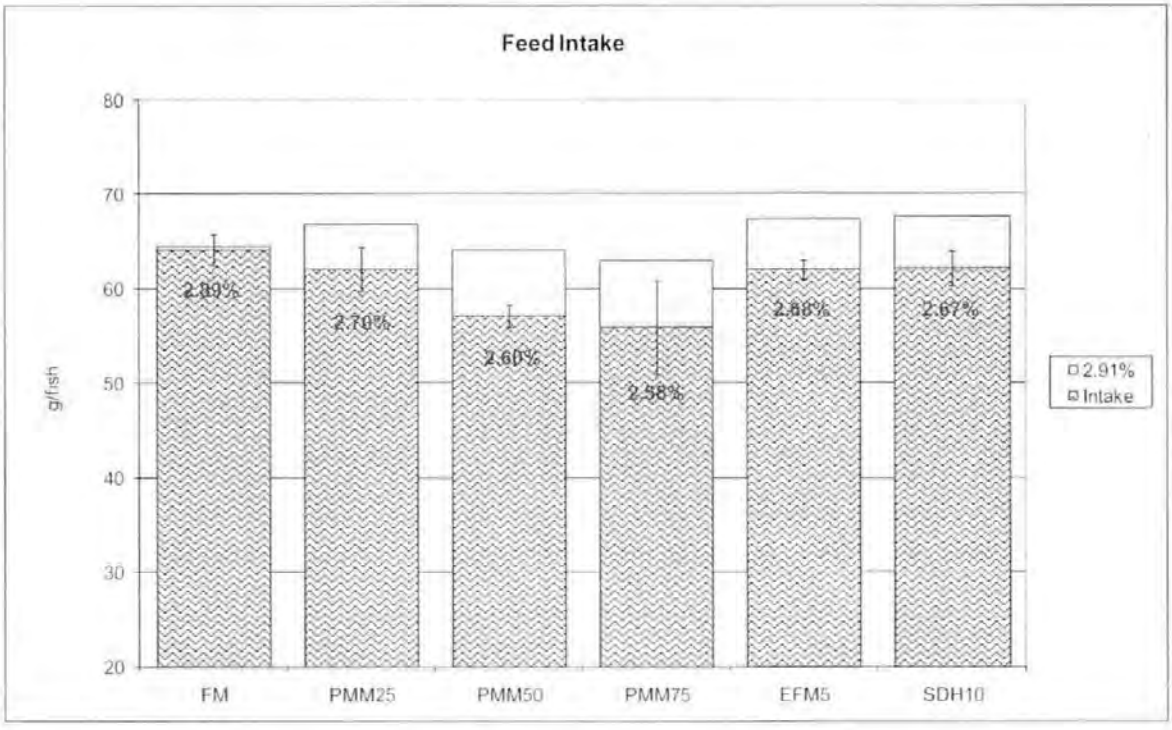


Figure 4A.1: Total feed intake (g/fish) determined for the whole trial period. Areas represent the part of the intended ration really fed to the fish (actual feed intake). Values are means of 2 or 3 replicates ± SD (bars).

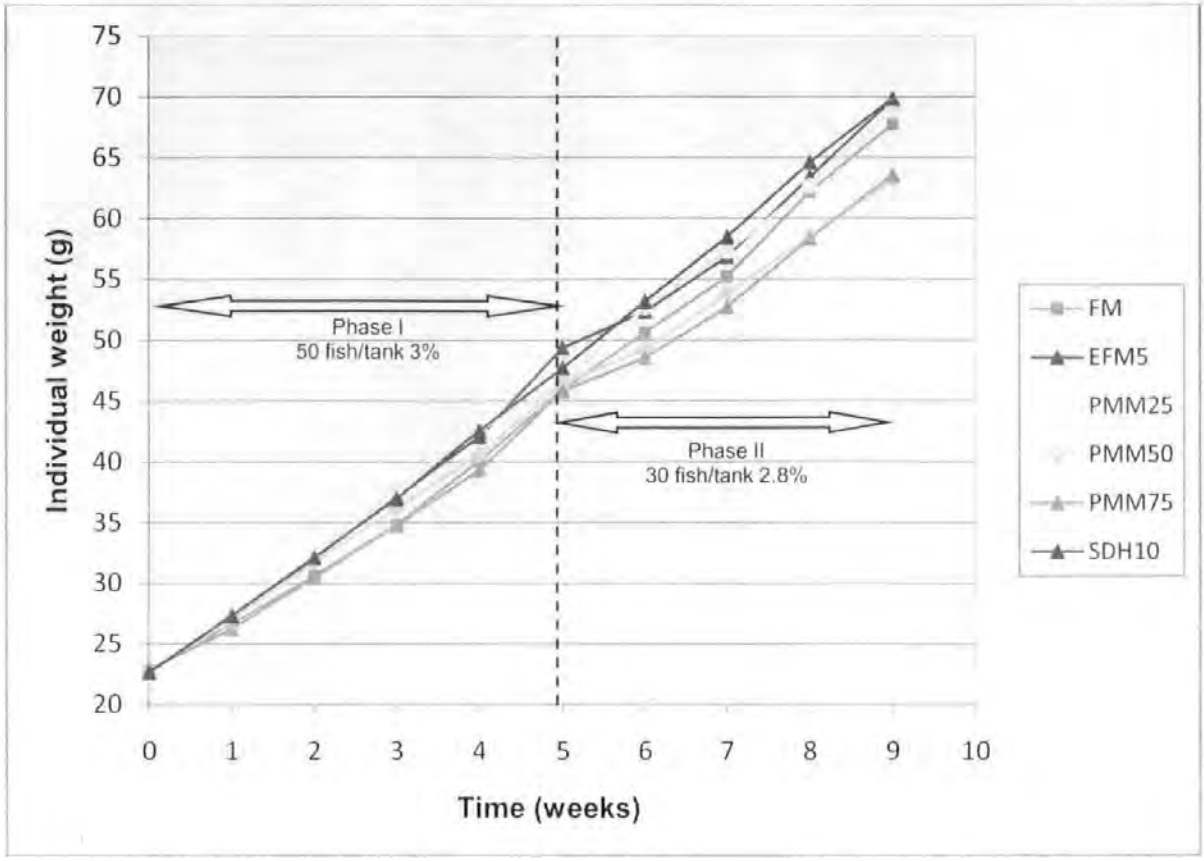


Figure 4A.2: Growth performance of gilthead sea bream fed 6 experimental diets.

It appears that PMM25, SDH10 and EFM5 were the most effective diets in supporting the growth of gilthead sea bream, whereas the diets with high inclusion of PMM (PMM50 and PMM75) resulted in growth performance visibly depressed, in comparison withfishmeal, after 8 weeks of feeding. Palatability issues may partially explain these growth reductions as a minor decrease in feed intake was also observed for PMM50 and PMM75 (Figure 4A.1).

Table 4A.3 gathers data related to survival, growth and feed efficiency established at the end of the trial. No major mortalities were recorded during the trial. Significant differences were found in final mean weight with PMM25, EFM5 and SDH10 proving to be statistically identical to the fishmeal control while PMM50 and PMM75 were appreciably lower. Weight gain and specific growth rate roughly followed the same trend. The inclusion of EFM, SDH and PMM (at the level of 25% of fishmeal replacement) led to the

highest SGR compared to the control (SGR was 1.73 for the fishmeal group, ranged from 1.78 to 1.81 for the best diets mentioned earlier, and reduced to 1.62/1.64 for the 75%/50% PMM). The best FCRs were obtained for SDH10 (1.30), EFM5 (1.32) and PMM25 (1.33) again (the first being significantly better than the control). With FCR of 1.38/1.39, fish fed high inclusion level of PMM were less efficient in converting food into body weight but performed still better than the fishmeal diet (1.43).

4A.3.1.3 Carcass composition and feed utilization (nutrient retention)

Carcass composition details, protein utilization and retention parameters are summarized in Table 4A.4. Fish fed SDH10 were more efficient in converting protein into wet weight gain (with a PER of 1.62 statistically outperforming fish fed the control diet). On the other hand PER of fish fed PMM75 (1.37) was significantly lower compared to all other experimental groups. Although net protein utilizations were found to be statistically similar for all treatments, the data appeared to be more or less correlated with PER results, following a comparable pattern. Highest aNPU was indeed obtained with the SDH10 diet, whereas the lowest percentage of protein retained in the carcass after 63 days of feeding was observed with PMM75. The percentage of protein in the whole fish carcass decreased in all treatments throughout the trial period (from initial to final fish), resulting in depressed aNPU. The percentage protein retention increased as a consequence of relating protein deposition to a protein intake calculated using the digestible protein content of diet (aNPU(t)) and not the crude protein content (aNPU). This did slightly modify results but did not induce any significant differences amongst treatments, agreeing with what was observed for aNPU. This would refer in a sense to the apparent biological value (ABV) as defined by Glencross *et al.* (2007).

Table 4A.3: Survival, growth performance and feed conversion of gilthead sea-bream juveniles used in trial 2. Values are means of 3 or 2 (treatment 1 and 2) replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Productivity index	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Survival (%)	100.00 ± 0.0^{a}	95.00 ± 4.99^{a}	97.11 ± 1.97^{a}	100.00 ± 0.0^{a}	95.78 ± 2.19^{a}	97.11 ± 1.55^{a}	P=0.536	F=0.92 P=0.507
Initial mean weight (g)	22.85 ± 0.54^{a}	22.42 ± 0.28^{a}	22.67 ± 0.29^{a}	22.91 ± 0.21^{a}	22.67 ± 0.32^{a}	22.66 ± 0.53^{a}	P=0.172	F=0.18 P=0.963
Final mean weight (g)	67.75 ± 0.55^{abc}	68.99 ± 1.16^{abc}	63.87 ± 0.14^{ab}	63.58 ± 2.18^{a}	69.77 ± 0.40^{bc}	70.69 ± 1.47^{c}	P=0.329	F=5.91 P=0.008
Weight gain (g)	44.88 ± 0.02^{abc}	46.57 ± 1.44^{abc}	41.20 ± 0.15^{ab}	40.67 ± 2.05^{a}	47.10 ± 0.26^{bc}	48.03 ± 1.52^{c}	P=0.340	F=6.53 P=0.006
Weight gain (%)	196.5 ± 4.57^{ab}	207.8 ± 9.02^{ab}	181.9 ± 3.00^{ab}	177.4 ± 8.10^{a}	207.9 ± 3.24^{ab}	212.2 ± 9.37^{b}	P=0.966	F=5.01 P=0.015
Feed intake (g)fish^{-1}	64.16 ± 1.14^{a}	62.07 ± 1.64^{a}	57.14 ± 0.67^{a}	55.92 ± 2.84^{a}	62.06 ± 0.59^{a}	62.17 ± 1.06^{a}	P=0.202	F=4.22 P=0.025
Feed intake (g)fish^{-1}day^{-1}	1.02 ± 0.02^{b}	0.98 ± 0.03^{ab}	0.91 ± 0.01^{ab}	0.89 ± 0.04^{a}	0.98 ± 0.01^{ab}	0.99 ± 0.02^{ab}	P=0.200	F=4.24 P=0.025
SGR (%/day)	1.72 ± 0.02^{ab}	1.78 ± 0.05^{ab}	1.64 ± 0.02^{ab}	1.62 ± 0.05^{a}	1.78 ± 0.02^{ab}	1.80 ± 0.05^{b}	P=0.927	F=5.18 P=0.013
FCR	1.43 ± 0.02^{c}	1.33 ± 0.01^{ab}	1.39 ± 0.02^{bc}	1.37 ± 0.01^{bc}	1.32 ± 0.02^{ab}	1.30 ± 0.02^{a}	P=0.992	F=8.06 P=0.003

Table 4A.4: Carcass composition of fish sampled at the start (initial fish) and end of trial 2. Values are means of 3 or 2 (diet 1 and 2) replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Carcass composition	Initial fish	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Moisture (%)	68.60±0.24	67.62±0.13^{a}	66.32±0.54^{a}	67.08±1.84^{a}	66.24±1.77^{a}	67.65±0.05^{a}	66.85±1.85^{a}	P=0.548	F=0.16 P=0.97
Crude protein *(% dry fish)*	52.24±0.08	42.01±0.84^{a}	39.76±1.35^{a}	40.95±1.11^{a}	41.23±1.55^{a}	41.54±1.11^{a}	40.74±2.43^{a}	P=0.584	F=0.18 P=0.96
Crude protein *(% wet fish)*	16.19±0.32	13.60±0.21^{a}	13.38±0.24^{a}	13.52±1.13^{a}	13.97±1.21^{a}	13.44±0.38^{a}	13.60±1.58^{a}	P=0.539	F=0.04 P=0.99
Crude Lipid *(% dry fish)*	33.64±0.65	28.59±0.96^{a}	30.47±0.39^{a}	29.32±1.48^{a}	29.03±0.50^{a}	27.45±0.48^{a}	29.08±0.66^{a}	P=0.259	F=1.16 P=0.39
Crude Lipid *(% wet fish)*	10.55±0.30	9.26±0.35^{a}	10.26±0.29^{a}	9.70±1.03^{a}	9.82±0.67^{a}	8.88±0.15^{a}	9.62±0.36^{a}	P=0.198	F=0.54 P=0.74
Ash *(% dry fish)*	10.5±0.14	8.37±0.03^{a}	8.27±0.21^{a}	8.42±0.30^{a}	8.65±0.47^{a}	8.39±0.40^{a}	8.41±0.53^{a}	P=0.119	F=0.09 P=0.99
Ash *(% wet fish)*	3.35±0.05	2.71±0.02^{a}	2.78±0.03^{a}	2.76±0.09^{a}	2.90±0.03^{a}	2.71±0.13^{a}	2.77±0.08^{a}	P=0.066	F=0.66 P=0.66
Gross Energy (MJ/kg) *dry fish*	25.03±0.00	20.04±0.99^{a}	20.92±0.34^{a}	20.44±0.43^{a}	19.95±0.23^{a}	19.99±0.38^{a}	20.34±0.70^{a}	P=0.112	F=0.43 P=0.81
Gross Energy (MJ/kg) *wet fish*	7.91±0.04	6.48±0.29^{a}	7.05±0.23^{a}	6.73±0.40^{a}	6.73±0.37^{a}	6.46±0.12^{a}	6.72±0.26^{a}	P=0.258	F=0.39 P=0.84

Protein efficiency/retention	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
PER	1.52±0.02^{b}	1.60±0.01^{bc}	1.48±0.02^{b}	1.37±0.01^{a}	1.55±0.02^{bc}	1.62±0.02^{c}	P=0.083	F=24.10 P=0.00
aNPU	18.57±0.56^{a}	18.98±0.54^{a}	17.62±2.38^{a}	17.29±2.73^{a}	18.61±0.93^{a}	19.92±3.52^{a}	P=0.599	F=0.17 P=0.97
aNPU(t)	21.40±0.65^{a}	22.19±0.63^{a}	21.42±2.89^{a}	22.43±3.62^{a}	22.78±1.14^{a}	23.62±4.17^{a}	P=0.599	F=0.09 P=0.99

4A.3.2 Palatability trial (trial 3)

Figure 4A.3 and Table 4A.5 gathers all raw data recorded or established further to one week feeding two different sets of diets. As expected, feed consumption (expressed in g/day) was notably reduced as a result of using casein as a carrier (Figure 4A.3). Feeding levels ranged from 1.62 (EFM) to 2.62 (FM) % of body weight for the fishmeal mixtures, and 0.69 (FM) and 1.45 (SDH) % of body weight for casein (Table 4A.5). Amongst tested ingredients, blood meal appeared as the most palatable feedstuff. Indeed, as regards feed intake parameters, SDH gave the best result during week 2 (with the casein mixture) and ranked second during week 1 (behind the fishmeal control). For the first week of the trial, the establishment of an appetite index (defined as the ratio of *ad libitum* food intake to feeding time) did not modify the pattern observed in raw feed consumption. According to this latter parameter blood meal still outperformed the fishmeal control (which comes second) during the second week of feeding (with the casein based diet).

Figure 4A.3: Daily feed intake (g) determined throughout week 1 (first set of diet) and week 2 (second set of diet) of the palatability trial. Bars indicate SD.

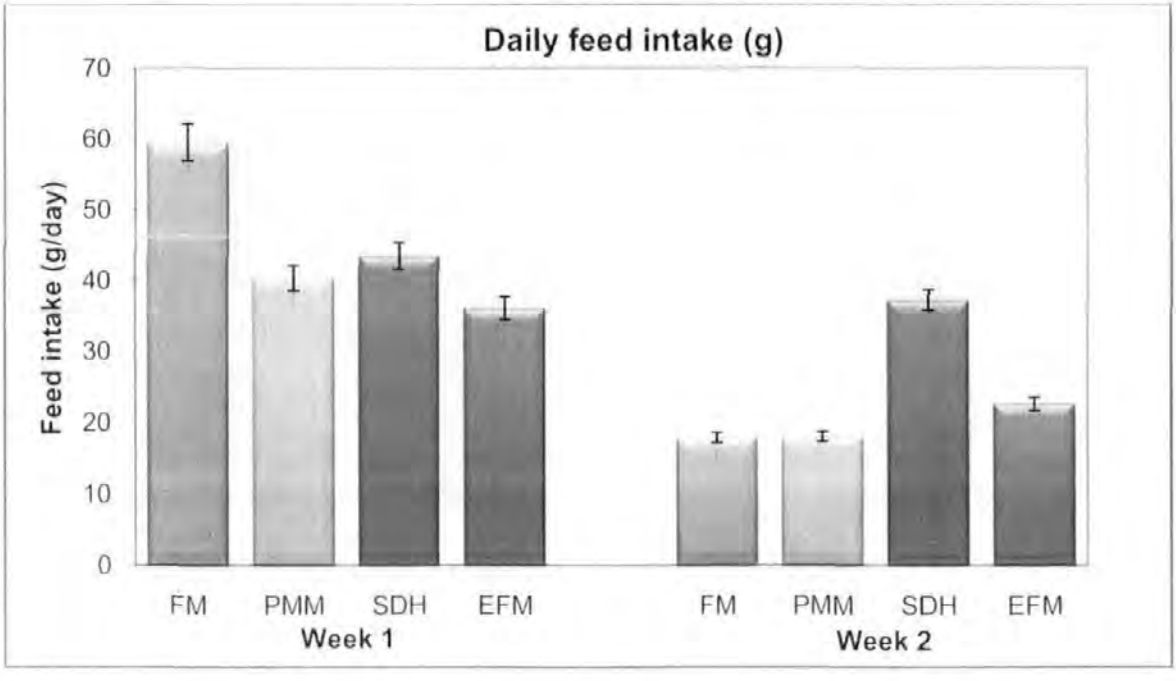
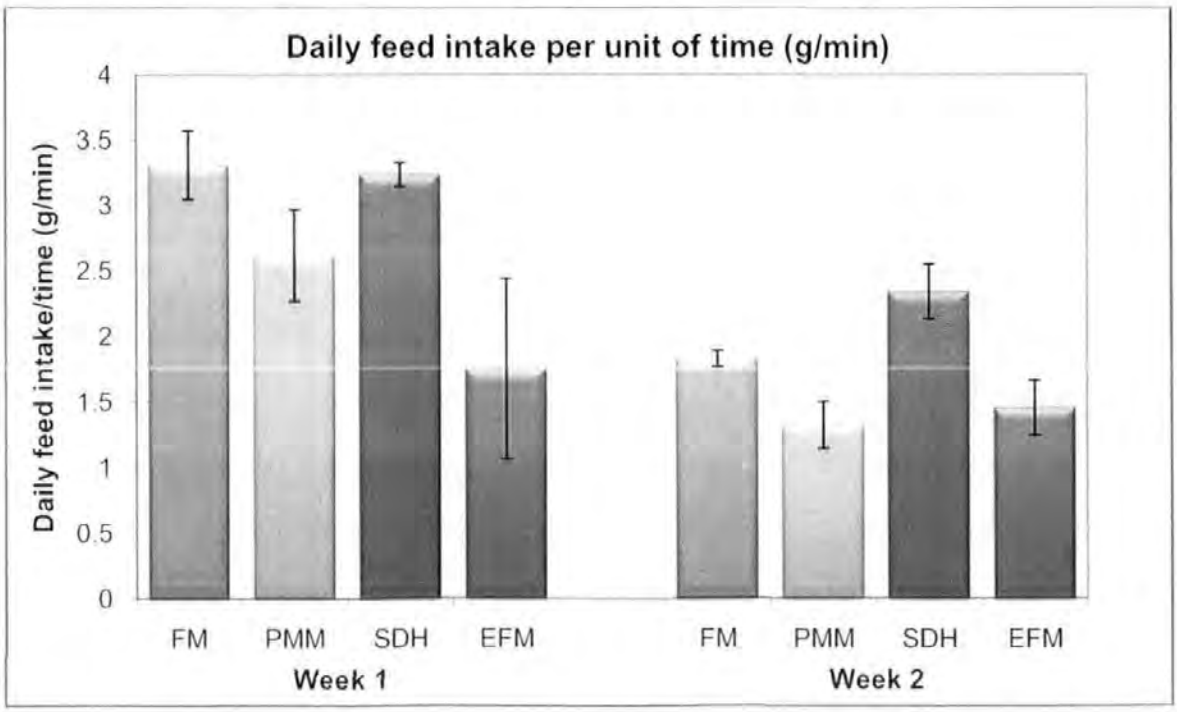


Table 4A.5: Feed intake and Feeding time (±SE) parameters established during trial 3.

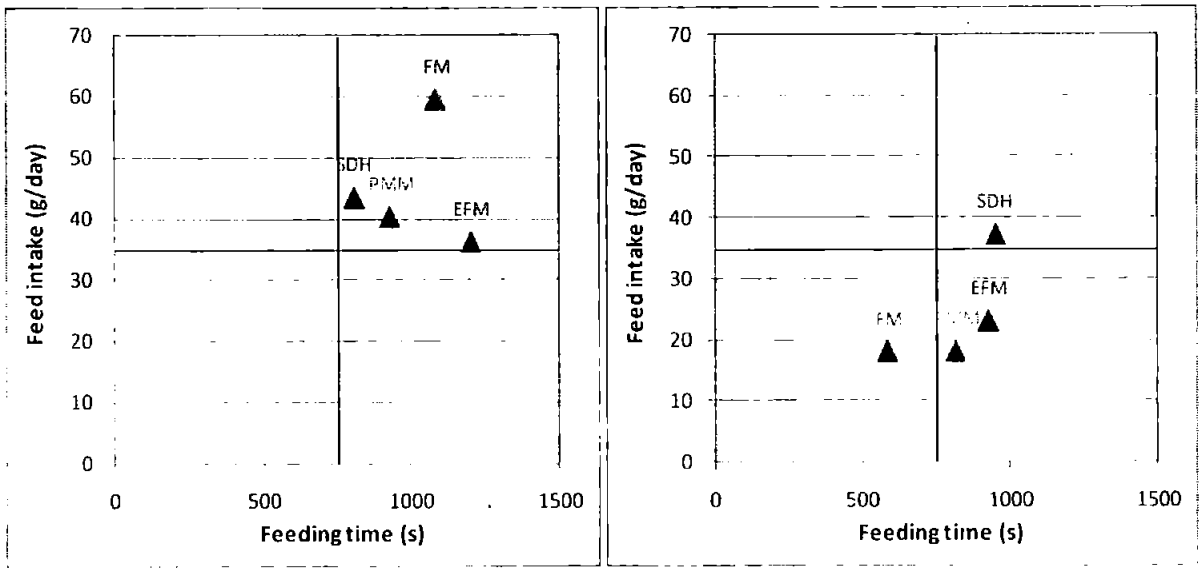
	FM	PMM	SDH	EFM	Anderson -Darling	ANOVA
Fishmeal based diets (Week 1)						
Feed Intake (g) $fish^{-1}day^{-1}$	2.58 ± 0.04^{b}	1.75 ± 0.01^{ab}	1.89 ± 0.04^{ab}	1.54 ± 0.42^{a}	P=0.324	F=4.6 P=0.038
Feed Intake (% BW)	2.62 ± 0.03^{b}	1.76 ± 0.04^{ab}	1.89 ± 0.03^{ab}	1.62 ± 0.40^{a}	P=0.259	F=4.8 P=0.033
Feeding Time (s)	1084 ± 59.3^{ab}	930 ± 71.5^{ab}	805.7 ± 29.0^{a}	1201 ± 74.4^{b}	P=0.538	F=7.7 P=0.009
Casein based diets (Week 2)						
Feed Intake (g) $fish^{-1}day^{-1}$	0.79 ± 0.01^{a}	0.79 ± 0.09^{a}	1.62 ± 0.14^{b}	0.99 ± 0.17^{a}	P=0.581	F=11.1 P=0.003
Feed Intake (% BW)	0.69 ± 0.02^{a}	0.70 ± 0.06^{a}	1.45 ± 0.13^{b}	0.94 ± 0.11^{a}	P=0.588	F=14.7 P=0.001
Feeding Time (s)	586 ± 9.8^{a}	816.7 ± 58.6^{ab}	949.3 ± 35.3^{b}	927.0 ± 89.7^{b}	P=0.607	F=8.6 P=0.007

***Figure* 4A.4**: Appetite measurement expressed as daily feed intake per unit of feeding time (g/min) and determined throughout week 1 and week 2 of the palatability trial. Bars indicate SD.



Relative palatability performance of the different diets as a function of feeding time (s) and feed intake (g/day) can be visualised in Figure 4A.4. As seen on this figure, the combinations of those data along with the comparison of the two weeks of palatability assessment tend to indicate that fish have a taste preference for fishmeal and blood meal.

Figure 4A.5: Palatability performance of the different diets, assessed through daily feed intake (g) and feeding time (s) for week 1 (left) and week 2 (right).



4A.4 DISCUSSION

In this study, palatability evaluation was based on the assumption that a short term response as regard feed consumption would reflect the attractiveness of the different diets and ingredients tested. To allow fish to demonstrate a feed intake response and to discriminate the diets with respect to flavour and texture, it is imperative to feed beyond apparent satiety. However, it must be noted that the feed intake values determined with this method remain estimations. In aquatic animals measuring feed ingestion remains a delicate task. Gilthead sea bream tend to break down pellets with their teeth, making accurate determination of the real quantity of feed ingested difficult without involving special techniques such as x-ray analysis of the digestive system. Formulation strategy and choice

of substance carrier to conduct proper palatability trials may also be subject to discussion (Kasumyan and Doving, 2003). For this work the basic approach described by Glencross *et al.* (2007) was adopted. As expected, type of basal diet used had an obvious influence on the apparent feed intake measured with an automatic reduction of FI for the casein mixtures. Besides, in comparison with the profile obtained with the fishmeal diets, discrepancies observed with the casein diets (fishmeal) might be related to the different quality of cold pressed pellets.

Productive values and biological performance established at the end of this 9 weeks feeding trial were typical of juvenile gilthead sea bream and were in accordance with conventional data for this species under intensive fish farming conditions. However, retention of nitrogen could be seen as slightly lower when compared to the normal range of values reported for gilthead sea bream of comparable size (Robaina *et al.*, 1995; 1997; Santinha *et al.*, 1996; 1999). This might be related to the relatively low amount of protein measured in the final carcass.

These studies confirm that PMM can be considered as one of the most effective animal protein source currently available, and this for both nutritional standpoint and bioethical considerations. With respect to final mean weight and SGR, PMM25 was indeed effective in supporting growth of gilthead sea bream. However, the growth trial demonstrated that when more than 50% of fishmeal was substituted a small depression in growth performance occurred. This gradual trend for reduced performance, as a result of increasing inclusion of PMM, was also found in most of the other parameters measured, although those did not always prove to be significantly different in comparison with the control. A longer feeding trial may have resulted in a more significant depression as far as growth performance was concerned in particular. Following our observations in trial 2 and 3 as regards feed intake, palatability is suspected to be partially involved in this growth reduction as all inclusion of PMM above 40% of fishmeal replacement led to lower feed

consumption. Palatability could thereby be considered as a factor that limits the value of PMM when high inclusion levels are intended. As far as FCR is concerned, although breams fed high inclusion levels of PMM were less efficient in converting food into body weight (FCR established for PMM50 and PMM75 were 1.37/1.39 respectively while PMM25, the best performing diet had a FCR value of 1.33) they were still performing better than the control group (1.43). Reference to protein utilization (PER and NPU) provided additional data that support a trend for reduced protein utilization: a significant reduction was found in PER for PMM75 but not for aNPU. According to those data, it would be thus reasonable to state that PMM could replace up to 25% of fishmeal without detriment to growth performance.

In a similar type of feeding trial carried out at the University of Plymouth with the same sources of animal by-products, tilapia fed diets where 66% of fishmeal was replaced with PMM showed the best productivity values in terms of weight gain, FCR, SGR, PER and daily feed intake (values statistically similar with those of the fishmeal based diet), indicating that a slight enhancement of the biological performance was possible with a high level of fishmeal replacement. These results confirmed that tilapia had an excellent potential for the utilization of rendered products of poultry processing waste as reported by El-Sayed (1998). With rainbow trout, a trial conducted in Plymouth to assess the rendered animal protein provided by Prosper de Mulder suggested that PMM could be a suitable protein ingredient for inclusion in diets below 20% of fishmeal replacement (Serwata, 2007).

The effective use of PMM with respect to growth performance of numerous fish species has already been reported by several authors (Fowler, 1991; Nengas *et al*, 1999; Abdel-Warith *et al.*, 2001; Wang *et al.*, 2006; Yigit *et al.* 2006). However, when PMM is used as a sole protein source or at high inclusion level in the diet of carnivorous fish, amino acid supplementation would be recommended. Several studies have indicated that results could

be improved when diets based on PMM as a primary protein source were supplemented with crystalline amino acids (Steffens, 1994; Gaylord & Rawles, 2005). Results reported by Alexis (1997) and Nengas *et al.* (1999) have also indicated that PMM was a good product for inclusion in gilthead sea bream diets with fish fed diets where 50% of white fishmeal was replaced by PMM performing as well as the control and fish fed diets with 75 to 100 % substitution of fishmeal giving just slightly lower performance. These latter authors stated that the first limiting EAA in poultry meals and related by-products was methionine. From the same series of trials, it was also shown that gilthead sea bream had lower performance with feather meal inclusion in the diets (this result was actually observed with a local product composed of a mixture of poultry by-products and feathers processed together). Also, it was nevertheless concluded that such a mixture (used at high substitution rate) could serve as a useful ingredient in feed formulation for sea bream (Nengas *et al.*, 1999; Alexis, 1997).

Feather meal is often considered as an inferior source of protein for fish because of its poor digestibility and essential amino acid profile (Davies *et al.*, 1991; Millamena, 2002; Fasakin *et al.*, 2005). Indeed, the proximate analysis of this test ingredient shows imbalances and deficiencies in a few EAA such as lysine methionine and histidine, whereas the nature of its protein (mainly keratin) explains its poor digestibility, contributing as well to the low biological value of the feedstuff. Due to these unfavourable characteristics feather meal might be regarded as an unsuitable feedstuff to replacefishmeals in aqua-feeds as verified by Wang *et al.* (2006). Conversely, successful trials have also been reported. Some researchers have indeed demonstrated the feasibility of using feather meal as a secondary protein source in aqua-feeds without any detrimental effects on the biological performance of salmonid species for instance (Fowler, 1991; Bureau *et al.*, 2000). Our investigations show that when formulations are based upon digestible protein/EAA and low amounts of ingredients are utilized in the diet, good

performance can be observed. Nowadays the utilization of biotechnological processes can be seen as a promising challenge that might help (offering scope) to improve the quality of such material (Woodgate, 2004b). Nutritional enhancement of feather meal has been obtained with the utilization of keratin degrading micro-organisms during the manufacturing process (bacterial fermentation), or when keratin degrading protease was used as a feed additive (Bertsch & Coello, 2005; Odetallah *et al.*, 2003); but further investigations are required to validate those findings in fish nutrition studies. The utilization of enzyme treatment during the manufacturing process of feather meal may improve the availability of nutrients in certain fish species as discussed in the previous chapter. Laboratory growth trials carried out with tilapia clearly indicated the superiority of EFM over HFM (Fasakin *et al.*, 2005).

In general, the response of different fish species to blood meal incorporated diets have been very variable, most fish species not being able to tolerate levels exceeding 20% in the diets. In an attempt to totally replace fishmeal with blood meal El-Sayed (1998) obtained significantly lower performance in Nile tilapia *Oreochromis niloticus*. Those poor results were recently confirmed by Fasakin *et al.* (2005) who tested in hybrid tilapia a fixed replacement rate of fishmeal of 66%. In Murray cod significant differences were reported with 8% of fishmeal replacement by blood meal (Abery *et al.*, 2002). Along with a certain unbalance in the EAA profile (arginine, isoleucine, methionine) it is thought that the high levels of iron and zinc that characterize blood meal are factors which would limit inclusion to moderate levels. For these reasons blood meal is usually employed as a complementary ingredient in blends of feedstuffs that are expected to combine synergistically (Millamena, 2002; Guo *et al.*, 2007).

In the current investigation, diets where SDH was used to replace 10% of the fishmeal component resulted in the highest final mean weight for sea bream (the value obtained did, however, not prove to be significantly different from the control). The excellent potential

of blood meal at the level of 10% of fishmeal substitution was also reflected in the other parameters established. If high inclusions of blood meal are likely to induce growth depression or pathological effects in fish for the reasons mentioned earlier, our growth trial confirmed the feasibility and benefit of using SDH in diet for gilthead sea bream in the case of a moderate substitution based on the digestibility value of the test ingredient (fish fed the SDH10 outperforming the control group in terms of SGR, FCR, PER and aNPU). This result is of major importance since non ruminant haemoglobin and blood meal have been permitted again in fish feeds produced in the EU since 2003. Indeed, unlike other animal by-products, there are indeed no legal restrictions on non-ruminant blood products at present, but constraints have been imposed by major retailers on the perceived consumer fears and demands regarding feed safety.

Ensuring fish health but also fish quality through muscle fatty acid analysis and sensory evaluation using taste panels in order to establish consumers' preference would be important to erase the negative image of those materials and restore public and retailer confidence. With histological and haematological analysis, this aspect was only approached during our trial. The preliminary data obtained concerning health related parameters are presented in the next chapter of this thesis.

CHAPTER 4B

Reduction of fishmeal in gilthead sea bream diets using selected animal by-products with respect to protein digestibility profile:

Histological observations in key digestive organs and consequences on haematological parameters

4B.1 INTRODUCTION

It is well established that the nutritional and physical characteristics of diets can modulate susceptibility of fish to infectious diseases (Tacon, 1992). Besides, under-nourished or malnourished animals cannot maintain health and growth, regardless of the quality of the environment (Lall, 2000). Quality diets may hasten recovery from infection, slow the progress of an idiopathic disease or overcome environmental stress (Verstraete *et al.*, 2000; Bjerkås and Sveier, 2004); whereas poor diet may cause nutrient imbalances, deficiency diseases and nutritional toxicoses leading in the worst case to high mortalities.

Reports on the nutritional requirements of fish generally do not place much emphasis on the possible effect that macro or micro nutrients might have on health status. However, work with nutrient deficient diets has been carried out in order to associate different pathological signs with the lack of specific nutrients (Richardson *et al.*, 1985; Tacon, 1992; Alexis *et al.*, 1997; Bell *et al.*, 2000; Sugiura *et al.*, 2004). The relationship between nutrition and health was eloquently detailed in a comprehensive review written by Lall (2000).

Many studies have been performed on the viability of replacing fishmeal in fish feed with alternative protein sources (see chapter 4A). Somewhat contradictory results have been found so far with fish feeds containing high levels of alternative protein sources depending notably on the biological variable assessed. Recommended inclusion rates of any fishmeal substitute should be based on more detailed analysis of the fish status, considering in particular health parameters (see section 1.3.4).

None of the potential alternatives for fishmeal is known to match its ideal amino acid profile. Certain raw materials may for instance be deficient in a specific EAA while presenting at the same time an excess in a particular micro-nutrient. Thereby inadequate utilization of those materials in fish diet formulation may result in specific nutritional

pathologies such as anaemia, cataract, fin erosion, liver steatosis, enteropathy, skin lesion, or oedema that may or may not be associated with growth depression (Robaina *et al.*, 1995; 1997; Zhou *et al.*, 2005). Results of feeding trials are often reported in terms of growth, feed efficiency and survival; but more subtle changes (as opposed to gross pathology) may also occur and impact animal health and welfare, as well as the final product quality. Along with EAA and trace elements (vitamins and minerals), modifications in the fatty acid profile of balanced diets based on fishmeal and fish oil, following the partial or total replacement of these prime ingredients, may also have consequences on health (Montero *et al.*, 2003; Wassef *et al.*, 2007).

Alongside its potential to meet the nutritional requirement of a fish species, one dietary ingredient may have a negative impact on fish health through the presence of undesirable components, specific agents or definite molecules, as illustrated by the wide variety of anti nutritional factors (ANFs) found in plant proteins that are fed to carnivorous fish (NRC, 1993). If the moderate utilization of plant protein to replace fishmeal in feeds for a wide variety of fish species were found to be feasible in terms of growth and feed performance, those substitutions have often been associated with physiological abnormalities due largely to the presence of ANFs such as protease inhibitors, lectins, antigenic proteins, oligosaccharides and phytates. Following an *in vitro* trial, Alarcón *et al.* (1999) showed that the inhibition produced by extracts of plant proteins on the activity of digestive protease of sea bream ranged from 25 to 50%, whereas that obtained using animal protein sources ranged from 1 to 20%. Anti-nutritional factors of soybean type protein are notably known to affect performance of salmonid fish, altering gut histology (Krogdahl *et al.*, 2003), inducing inflammations (Bakke-McKellep *et al.*, 2007) and leading to decreased digestion and reduced utilization of proteins (Kaushik *et al.*, 1995; Vielma *et al.*, 2000). However, in contrast with some of these latter results, other nutrition trials (where different fish species were fed soybean products as the major dietary protein source) were

completed without any signs of enteritis type changes in the intestine (Evans *et al.*, 2005, Morris *et al.*, 2005; Hansen *et al.*, 2006). Following a 6-month growth trial carried out with gilthead sea bream, Sitjà-Bobadilla *et al.* (2005) found that growth performance, plasma metabolites, gut integrity, liver structure and immune status remained unchanged in fish fed a mixture of five plant proteins substituting 75% of fishmeal. However, it may be argued that the utilization of a complex mixture of plant ingredients rather than one or two ingredients may have helped to reduce the exposure to individual ANFs in this latter case (Borgeson *et al.*, 2006).

Although some data suggest that certain animal by-products may be included in fish diets at relatively high levels without impairing fish growth, little is known about the effect of such substitution on fish health, fish welfare, or fish filet quality (Subhadra *et al.*, 2006a). With the variety of products currently tested to replace fishmeal it is imperative to ensure that nutritional unbalance or unwanted compounds (which may result from the inclusion of these novel ingredients) would not impair fish health, validating any new dietary formulae on the basis of the biochemical and physiological response of the fish.

A growing interest in fish health enhancers and immuno-stimulants that take the form of feed additives is also noticeable in recent years. Within this specific research area of "food sensitive diseases", other approaches were indeed developed to assess the capabilities of various products and molecules (like nucleotides, taurine, yeast, glucans or pro-biotics) to improve immunity and disease resistance of fish (Devresse *et al.*, 1997; Maita *et al.*, 2006; Takagi *et al.*, 2006a,b; Taoka *et al.*, 2006; Seung-Cheol *et al.*, 2007). All these efforts show that growth cannot be dissociated from health and that fish health is also becoming a topic of major concern in the aquaculture industry. Having witnessed the outbreak/emergence of alarming diseases in other food industries, fish farmers along with researchers are well aware that the challenge for the aquaculture industry will equally to respond to the threat of more and more resistant bacterial strains and viral syndromes. To

achieve this goal, the quality control of feedstuffs appears as another key aspect that must be considered to reduce the risk of cross contamination.

Blood tissue reflects physical and chemical changes occurring in an organism, therefore detailed information can be obtained on general metabolism and physiological status of different groups of fish. In recent years, haematological parameters have been commonly used to observe and follow fish health, and the haematological examination of intensively farmed fish is now an integral part of evaluating their health status (Omitoyin, 2006; Wassef *et al.*, 2007). Similarly histological examinations are widely utilized to monitor the dietary impact on fish regarding both structural and physiological type of change (Caballero *et al.*, 1999; 2004).

In this context the aim of this aspect of the study was to present the results of both haematological and histological evaluation for the previous trial. In this manner it was intended to provide evidence that adequate inclusion of animal by-products in balanced diets formulated for gilthead sea bream does not: disrupt gut integrity, create anaemia conditions and affect the physiological function of the liver.

4B.2 MATERIALS AND METHODS

4B.2.1 Hepato-somatic index and condition factor

Fish livers were weighed at the beginning, after 5 weeks and at the end of the trial (for the initial and intermediate sampling, eight to eighteen fish were dissected for this purpose; during the final sampling, livers weighed were those dissected for histological examination, see Table 4B.1). The information collected was used to calculate the Hepato-Somatic Index:

HSI(%) = (liver weight (g) / somatic weight (g)) × 100.

Morphometric data such as fork length and wet weight were recorded for all fish sampled and used to calculate the condition factor according to the following formulae:

$K = (\text{Weight (g)} \times 100) / \text{Length (cm)}^3$.

4B.2.2 Haematological analyses

4B.2.2.1 Blood collection and sample preparation

At the end of the trial (9 weeks) a total of five fish per tank were withdrawn for blood sampling. Fish were sacrificed by lethal anaesthesia with tricaine methane sulphonate (MS222) and blood collected by caudal sinus puncture with a 1ml heparinised syringes to prevent immediate coagulation. The quantity of blood obtained for each fish was used (as a unique aliquot) to prepare blood smears, realize erythrocyte counts and determine haematocrit values and total haemoglobin concentration.

4B.2.2.2 Haematocrit determination

Two haematocrit values were obtained for each of the five fish sampled. Heparinised capillary tubes were filled three quarters full, plugged with putty, and centrifuged for 3 minutes at 6000 rpm in a micro haematrocrit centrifuge. Packed cell volumes were read using a micro haematocrit reader. Data are expressed in percentage of the analysed volume.

4B.2.2.3 Haemoglobin concentration

Total blood haemoglobin concentration was measured by Drabkins's colorimetric assay on the 5 fish sampled in each tank. 20µl of fresh whole blood was added to 5 ml of Drabkins reagent, and vortexed immediately. The absorbance was read at 540nm on a Jasco Spectrophotometer a few hours later, and haemoglobin concentration of the blood samples calculated from a curve prepared from known standards (Sigma diagnostic kit N°525 A). Values obtained are reported in $g.dl^{-1}$.

4B.2.2.4 Erythrocyte and peripheral leukocyte differential counts

Erythrocyte counts were performed on diluted blood samples (1:100 dilution in Dacie's fluid) with a Neubauer haemocytometer (Dacie and Lewis, 2001). Using a glass pipette, and making sure the blood cells were re-suspended evenly, a small quantity of the blood cell suspension were introduced on the platform of the haemocytometer at the edge of the coverslip to be drawn into the counting area by capillary action. After a few minutes (allowing the cells to settle), five small squares in the centre of the grid were counted under a light microscope. Like erythrocyte count, blood smears were prepared immediately following blood collection. A drop of blood were smeared across a slide and allowed to air dry. Once dry, slides were fixed in methanol and cells were stained using Giemsa stain. One blood smear and one blood cell suspension were prepared for only three of the five fish sampled per tank. Publications of Lopez-Ruiz *et al.* (1992) and Hibiya (1982) were used to identify leukocytes.

4B.2.2.5 Haematological indices calculated

Measurement of total red blood cell count (RBCC), haemoglobin concentration (Hb), and haematocrit (Hct) enabled the mean cell volume (MCV), mean cellular haemoglobin content (MCH), and mean cell haemoglobin concentration (MCHC) to be calculated according to the following formulas (Dacie and Lewis, 2001):

①MCV = Hct(‰) / RBCC ($10^6 \mu l^{-1}$); ②MCH = [Hb ($g.dl^{-1}$) × 10] / RBCC ($10^6 \mu l^{-1}$); ③MCHC = [Hb ($g.dl^{-1}$) × 10] / Hct.

4B.2.3 Histological techniques

4B.2.3.1 Tissue processing and examination performed in light microscopy

For histological evaluation under the light microscope, 3 fish from the initial pool, and 2 fish per tank at the termination of the trial were used. All fish were sacrificed with an overdose of MS222 prior to dissection. Whole liver and gut were quickly fixed in buffered formol saline and stored until further processed. Dehydration (in a graded alcohol series), clearing (in xylene), and embedding (in fibrowax) were the following stages (before being dehydrated gut sections were divided in three parts to differentiate anterior mid and posterior intestine). Blocks of paraffin were then sectioned (7 μm) with a rotary microtome (short ribbons of sections were flattened into a heated water bath before to be transferred to slides to be dried) and sections stained according to Mallory's staining technique. Stained sections were mounted with a polystyrene resin dissolved in xylene (DPX) and examined on a Zeiss photomicroscope (images were captured using an Hitachi 3CCD colour camera) for any pathological signs. Photographs were, however, obtained with the view of assessing specific variables like: ①"Perimeter Ratio" (PR) to assess the "villae length", PR = internal gut perimeter (cm) / external gut perimeter (cm); ②number of mucus cells per villae; ③area of mucosal folds (widening and shortening of the intestinal folds), ④volume density of hepatic lipids (parameters ③ and ④ were part of the "qualitative" assessment).

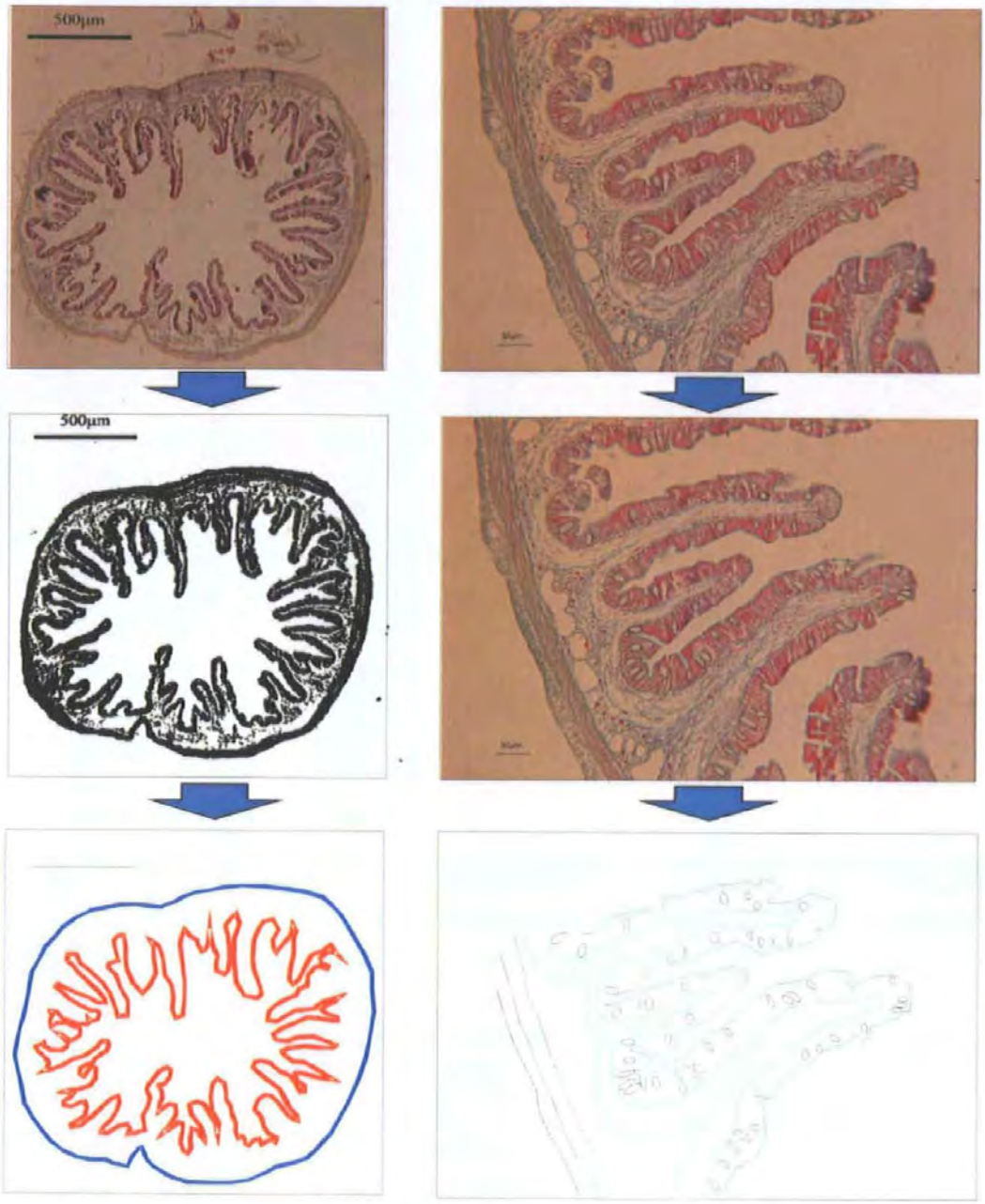


Plate 4B.1: Utilization of the photographs obtained in light microscopy (gut) to determine morpho-metric variables (perimeter ratio and number of mucus cell per villae).

4B.2.3.2 Transmission electron microscopy preparation and examination

The same sampling strategy and fish handling described in the previous paragraph were applied for electron microscopy. Liver and gut tissue collected (anterior and mid gut sections were considered) were dissected into small pieces and fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2, 3% NaCl). This fixed material was stored for several weeks prior to further preparative techniques. Pieces were then rinsed twice in cacodylate buffer, resized to get blocks of 1mm sides, and post-fixed for 1h in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.2, 3% NaCl). At this stage tissues were dehydrated in 30-100% ethanol solutions and gradually embedded in epoxy resin. After two days of infiltration, the resin was polymerized at 70°C to provide support for sectioning (for each sampled fish, 2-4 blocks were prepared). Resin blocks were trimmed to produce a small block face with relevant tissue exposure before semi-thin sections (0.5µm) were cut and stained with toluidine blue for a first examination under light microscope. When satisfied with the result, ultra-thin sections (70nm) were cut with an ultra-microtome using glass or diamond knife, mounted on the copper grids which support them on the TEM and stained with uranyl acetate and lead citrate. Observations were made on a JEOL 1200 TEM. Length of the microvillae① (for gut sections) and diameter and number of lipid droplets② (for liver sections) were determined on the micrographs obtained as a prime objective. However other parameters like nuclear displacement, cytoplasm vacuolization, leukocytes infiltration and number of mitochondria kept our attention within the frame of a qualitative evaluation.

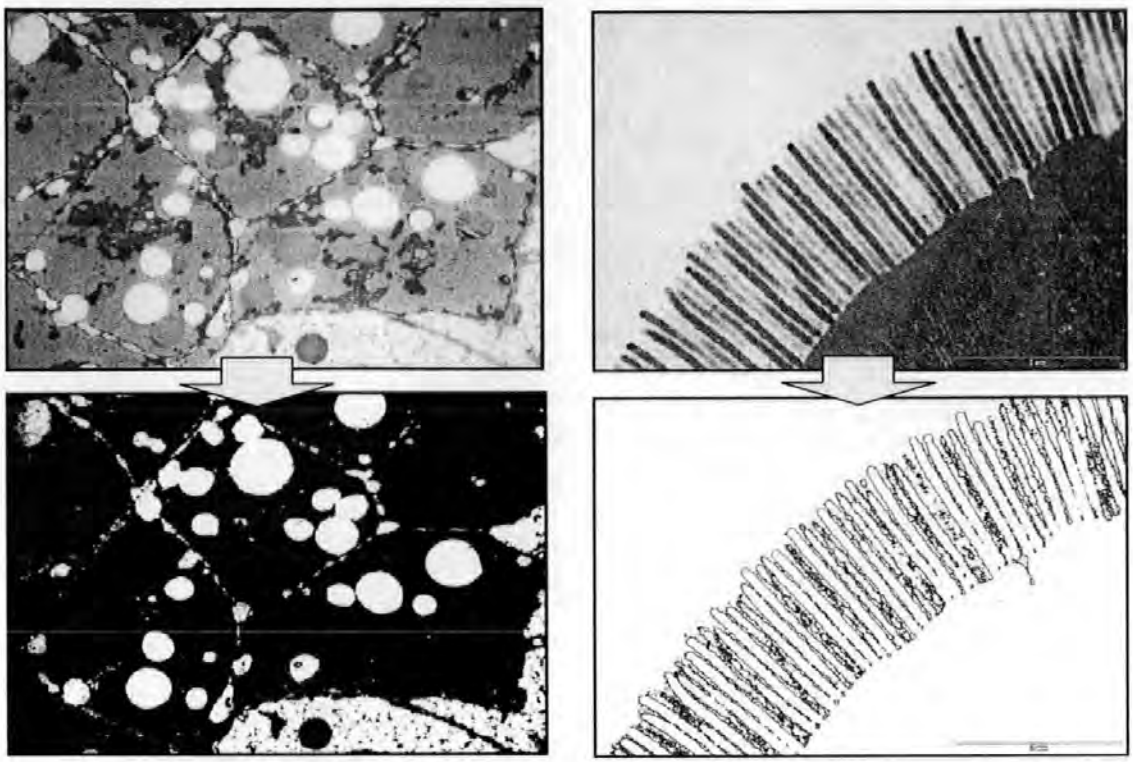


Plate 4B.2: Morpho-metric variables (size and number of lipid droplets and length of microvillae) measured on the microphotographs obtained with the TEM (liver, gut).

4B.2.3.3 Scanning electron microscopy preparation and examination

Scanning electron microscopy was also considered to supplement our observations. After the first fixation in 2.5% glutaraldehyde, tissues collected for TEM were basically used to generate another pool of tissue utilized for SEM preparation. Those were directly dehydrated, critical point dried, and coated after having been attached to support stubs with electrically conducting tape. Examination was carried out on a JEOL 5600 scanning microscope.

4B.2.3.4 Quantitation of the histological image

Microscopic images of interest were processed with imageJ (1.38x) in order to extract numerical data (morpho-metric measurements). Small sample size prevented the establishment of statistical trends in relation to diet (Table 4B.1).

Table 4B.1: Experimental design and sampling strategy for the establishment of the different health related parameters.

tissue	Parameters measured or evaluated	Sampling time	Sampling strategy† (number of fish per tank)
Whole wet fish	1 Condition factor (K) *(fork length, weight)*	Initial (W0) intermediate (W5) final (W9)	n=80‡ n=9 (20 in tank 1,4,5,6) n=12
Liver	2 Hepato-somatic index (HSI)	Initial (W0) intermediate (W5) final (W9)	n=18‡ n=8 (4 in tank 1,4,8,13) n=4
Blood	3 Haematocrit (Hct) 4 Haemoglobin (Hb) 5 Red Blood Cell Count (RBCC) 6 Differential Cell Count	Final (W9)	n=5 n=5 n=3 (4 in tank1,8,9,16) n=4
Liver (light microscopy)	7 Pathological changes*	Initial (W0) final (W9)	n=3‡ n=1
Liver (electron microscopy)	8 Size/number of lipid droplets 9 Ultra-structural changes*	Final (W9)	n=1
Gut (light microscopy)	10 Number of mucus cell per villae 11 Perimeter ratio 12 Pathological changes*	Initial (W0) final (W9)	n=3‡ n=1
Gut (electron microscopy)	13 Length/length variability of microvillae 14 Ultra-structural changes*	Final (W9)	n=1

†Number of fish per tank on which measurements or observations have been made. *Qualitative observation. ‡total number of fish sample in the initial pool.

4B.3 RESULTS

4B.3.1 General health parameters

Condition factors obtained throughout the experiment fluctuated from 1.79 to 2.11. Evolution of this index during the trial indicated that weight gain was relatively more important than length gain (Figure. 4B.1). As regards final sampling, no significant influence of dietary treatments could be observed on the condition factors (Table 4B.2).

The higher HSI value was measured for the fish fed PMM75 at the intermediate sampling (1.91, n=24) while the lower value (1.30, n=12) was obtained in the EFM5 group at the end of the trial. Relative to the body weight, liver weight proved to be lower at the end of the trial, with values ranging from 1.30 to 1.41 (Figure. 4B.2). After 9 weeks of feeding, it was observed that dietary treatments did not significantly influence HSI of fish (Table 4B.2).

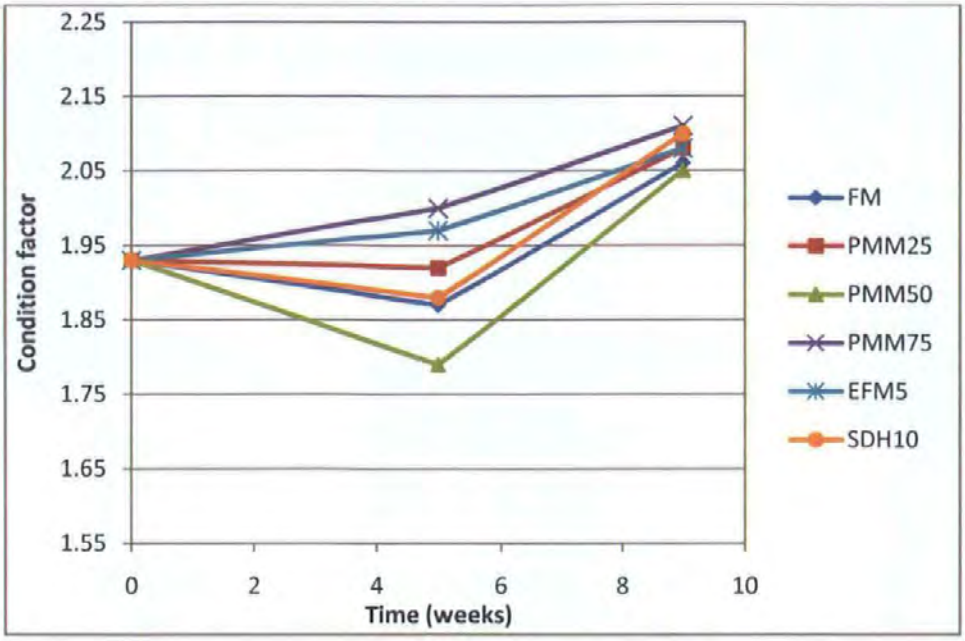


Figure 4B.1: Condition factor (K) of sea bream at initial, intermediate and final sampling for the six experimental diets used in trial 2.

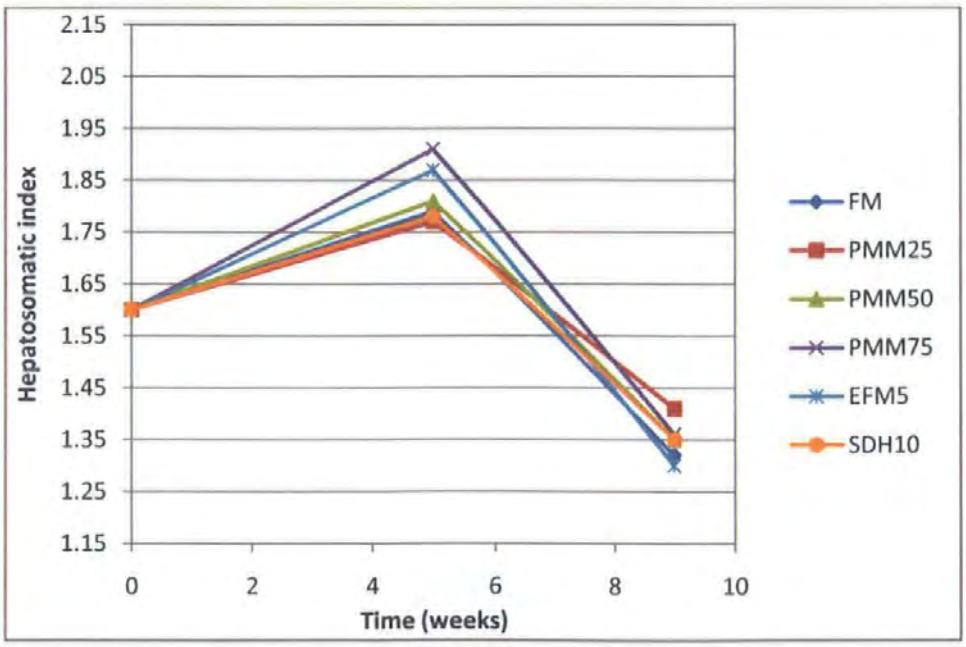


Figure 4B.2: Hepato-somatic index (HSI) of sea bream at initial, intermediate and final sampling for the six experimental diets used in trial 2.

Table 4B.2: General health parameters and haematology

Values are means of 3 or 2 replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Morphometry	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Condition Factor (K)	2.06±0.02[a]	2.08±0.00[a]	2.09±0.03[a]	2.11±0.03[a]	2.08±0.02[a]	2.10±0.03[a]	P=0.168	F=0.42 P=0.827
HSI (%)	1.32±0.28[a]	1.41±0.03[a]	1.35±0.03[a]	1.36±0.13[a]	1.30±0.08[a]	1.35±0.04[a]	P1=0.037 P2=0.055	F=0.13 P=0.983
Haematology	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Haematocrit (%)	39±1.80[a]	36.50±1.90[a]	38.53±3.71[a]	41.97±1.30[a]	39.33±2.76[a]	37.05±2.05[a]	P=0.845	F=0.57 P=0.719
Haemoglobin (g/dl)	7.65±0.60[a]	7.24±0.11[a]	7.72±0.99[a]	7.63±0.24[a]	7.74±0.12[a]	7.81±0.12[a]	P=0.167	F=0.13 P=0.983
RBCC ($\times 10^6$/mm^3)	2.40±0.20[a]	2.20±0.13[a]	2.73±0.44[a]	2.59±0.13[a]	2.71±0.14[a]	2.34±0.15[a]	P=0.076	F=0.69 P=0.641
MCV(fl)[1]	164.0±21.5[a]	166.7±18.8[a]	144.1±8.74[a]	162.6±3.60[a]	144.7±4.17[a]	158.3±3.40[a]	P=0.465	F=1.12 P=0.410
MCH(pg cell)[2]	32.28±5.27[a]	32.93±1.52[a]	28.91±3.47[a]	29.59±0.9[a]	28.67±1.30[a]	33.66±2.57[a]	P=0.523	F=0.72 P=0.626
MCHC(g/dl)[3]	19.60±0.65[a]	19.91±1.34[a]	20.12±2.22[a]	18.19±0.23[a]	19.85±1.21[a]	21.26±1.54[a]	P=0.318	F=0.51 P=0.765

[1] Mean Cellular Volume, [2] Mean Cellular Haemoglobin content, [3] Mean Cellular Haemoglobin Concentration

4B.3.2 Haematological parameters

Haematological measurements for the various groups of fish are given in Table 4B.2. No significant differences were found in the haematocrit value, haemoglobin concentration or number of circulating erythrocytes among blood samples from fish fed the different experimental diets. Values ranged from 36.5 to 41.9 (Hct, %), 7.24 to 7.81 (Hb, g/dl), 2.20 to 2.71 (RBCC, 10^6/mm^3) within the test groups, against 39.0 (Hct, %), 7.65 (Hb, g/dl) and 2.40 (RBCC, 10^6/mm^3) for the reference group. Determination of red blood cell indices enabled further description of the size and haemoglobin content of red blood cells. The average volume of a red blood cell (MCV) was ~144 in fish fed PMM50 and EFM5, and 164 in fish fed fishmeal. The average concentration of haemoglobin in a red blood cell (MCHC, g/dl) varied from 18.19 (PMM75) to 21.26 (SDH10), whereas the lowest and highest MCH (average content of haemoglobin in a red blood cell, pg/cell) characterized the EFM5 (28.91) and SDH10 (33.66) group respectively.

Ratio of erythrocytes to total leukocytes remained unaffected by any dietary treatment with values averaging 14% (Table 4B.3). Lymphocytes were by far the most common leukocyte cell type, and the only leukocyte cell type found on the blood smears of fish fed fishmeal. For the other treatments granulocytes and monocytes represented 0.1 to 0.2% of total white blood cell population.

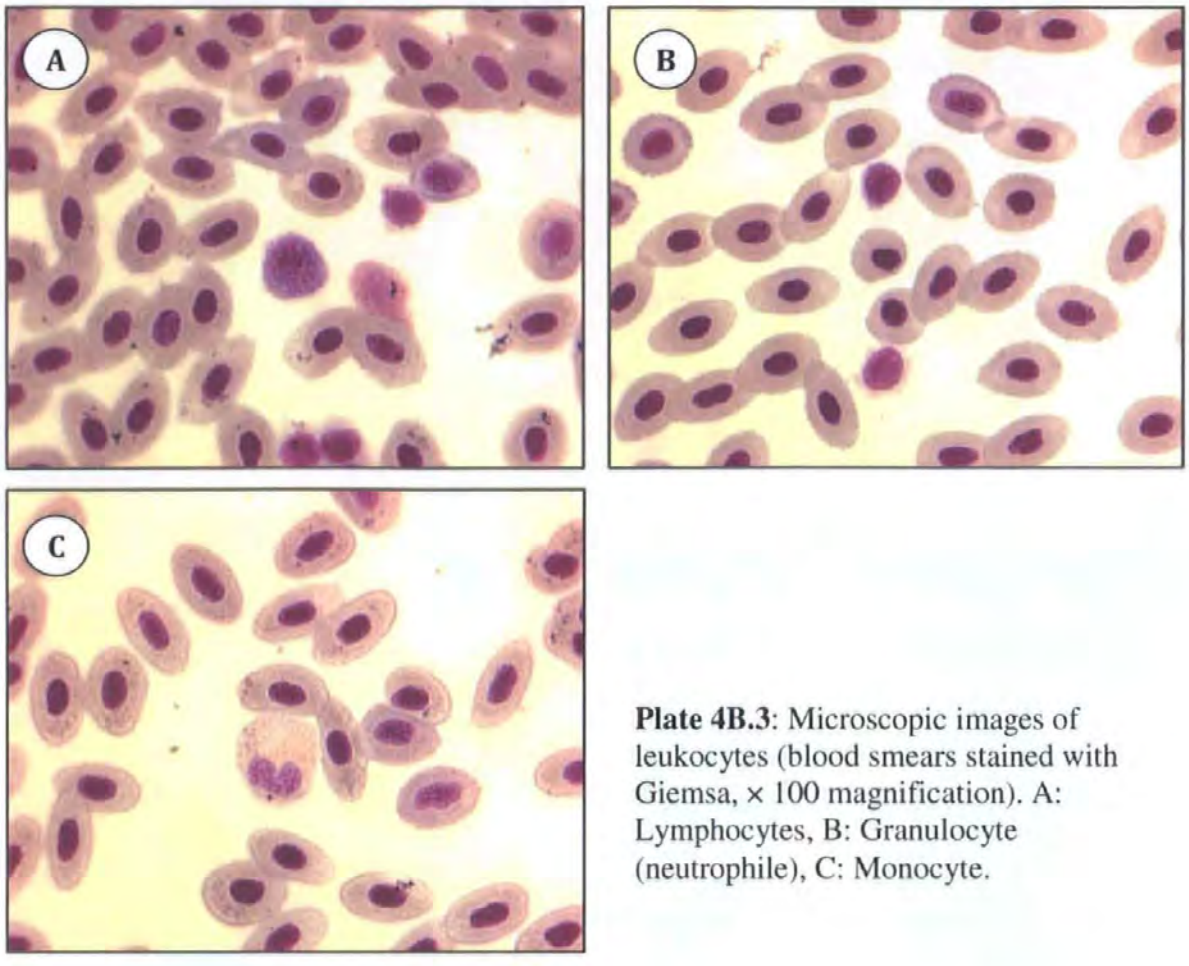


Plate 4B.3: Microscopic images of leukocytes (blood smears stained with Giemsa, × 100 magnification). A: Lymphocytes, B: Granulocyte (neutrophile), C: Monocyte.

Table 4B.3: Differential cell count in gilthead sea bream fed selected animal by-products

		Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Ratio RBC/WBC		12.64±1.29	17.18±1.18	13.21±1.37	15.67±1.71	13.80±1.21	13.77±1.61	P=0.096	P=1.76 F=0.2
Differential blood cell count	Erythrocyte	88.55±1.12	85.10±0.86	88.11±1.10	86.23±1.23	87.72±0.20	87.79±0.45	P=0.13	P=1.78 F=0.2
	Lymphocyte	11.18±1.00	14.60±0.83	11.60±1.07	13.42±1.24	12.07±0.17	12.05±0.47	P=0.13	P=1.75 F=0.2
	Granulocyte	0.00±0.00	0.02±0.02	0.00±0.00	0.03±0.02	0.01±0.01	0.02±0.02	P=0.005	-
	Monocyte	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01	P=0.005	-
	Thrombocyte	0.27±0.12	0.28±0.01	0.28±0.06	0.31±0.09	0.18±0.11	0.13±0.03	P=0.66	P=0.87 F=0.5
Differential leukocyte count	Lymphocyte	100±0.00	99.88±0.12	99.92±0.08	99.68±0.16	99.76±0.14	99.76±0.14	P=0.005	-
	Granulocyte	0.00±0.00	0.12±0.12	0.00±0.00	0.24±0.14	0.08±0.08	0.16±0.16	P=0.005	-
	Monocyte	0.00±0.00	0.00±0.00	0.08±0.08	0.08±0.08	0.16±0.08	0.08±0.08	P=0.005	-

4B.3.3 Gut histology

The intestinal wall of gilthead sea bream is typically composed of four layers represented by the mucosal epithelium (forming folds on the luminal side), the *lamina propria* (connective tissue supplied with blood capillaries), the muscularis (inner circular and outer longitudinal muscle layer) and the serous membrane as described in fish (Hibiya, 1982). The intestinal mucosal epithelium is arranged as a single layer of high columnar epithelial cells (Plate 4B.5). Hypertrophy and necrosis of the epithelial cells of the mucosa were commonly observed irrespectively of the dietary treatments (Plate 4B.4). Desquamation of mucosal epithelium or infiltration of leukocytes into the *lamina propria* was sometimes associated with those degenerative changes. Width of the *lamina propria* did not appear as a good indicator of pathological disturbances as shown with salmonids (Krogdahl *et al.*, 2003): it was found to be rather variable across all the numerous folds observed and not clearly linked to a specific level of cell infiltration. Although abundant deposits of fat were observed in the visceral cavity at the time of the dissection, no severe signs of intestinal steatosis were noted. Fatty changes in the intestinal epithelium could however be observed with the presence of supranuclear vacuoles in some enterocytes (Plate 4B.7 E).

In terms of structural changes, the gastro-intestinal tract presented a slightly longer absorptive area in fish fed PMM and SDH. This was particularly apparent in the perimeter ratio values established for the anterior and mid intestine but was not significant as regard statistics (Table 4B.3). Comparison of the perimeter ratios across the three different sections of the intestine (anterior, mid and posterior) indicated a tendency toward a reduction of the absorptive area moving from the proximal to distal part (Table 4B.3). Plate 4B.6 illustrates this trend with intestine sections showing longer and denser villi in the proximal intestine than in the distal area. Moreover it appeared that fold structure was much more complex in the anterior part of the intestine with a multi branch appearance.

The columnar epithelial cells lining the villi were predominantly enterocytes, interspersed with mucus containing goblet cells. In Mallory's stained sections, these latter cells were identified from the large and uncoloured droplet of mucus material they contain (Plate 4B.5). Counts of mucus cells in Mallory's stained sections did not permit to reveal any significant variation in relation with the different dietary treatments (Figure 4B.3). The average number of mucus cell per 100µm of villae was estimated around 7 (the density of mucus cells was found to be higher for all fish fed the experimental diets in comparison to the fish fed the reference diet). This evaluation was achieved considering sections from all origins since the number of pictures exploitable for a specific area of the intestine was too small.

Apart from the gilthead sea bream fed PMM50, microvilli of the anterior part of the intestine were found to be slightly longer in fish fed animal by-products in comparison to fish fed the reference diet. This finding corroborated the tendency of an increased absorptive surface in the anterior part of the intestine deducted from the measurement of perimeter ratios (Table 4B.3). This pattern was however different in the mid intestine with all experimental fish having shorter microvilli compared to the reference. Depending on the size, the shape and the length variation of the microvilli, some differences were observed in the brush border aspect of the fish sampled, but none of these variations could be clearly associated with a dietary treatment or a gut area (Plate 4B.7). From the data, it was not possible to observe a clear trend toward shorter or longer microvilli when sections of the anterior part were compared to sections of mid intestine. According to the measurement made in the anterior part of the intestine, it seems that inclusion of animal by-products induced a certain irregularity in the length of microvilli; but those differences in size variability were not deemed significant and not confirmed in the mid sections. With exception of the microvilli features, it was also difficult to highlight any significant ultra-structural changes in the enterocytes related to dietary treatment, considering sampling

strategy and pictures quality, but the inclusion of large lipid droplets were detected (Plate 4B.7 E). Transmission electron microscope (TEM) and scanning electron microscope (SEM) allowed visualization of: junctional complex (including desmosoms) located in the apical part of the cells (Plate 4B.7 D), exfoliating enterocytes in the apical part of villi (Plate 4B.8) and endocytotic vesicles (Plate 4B.7 F).

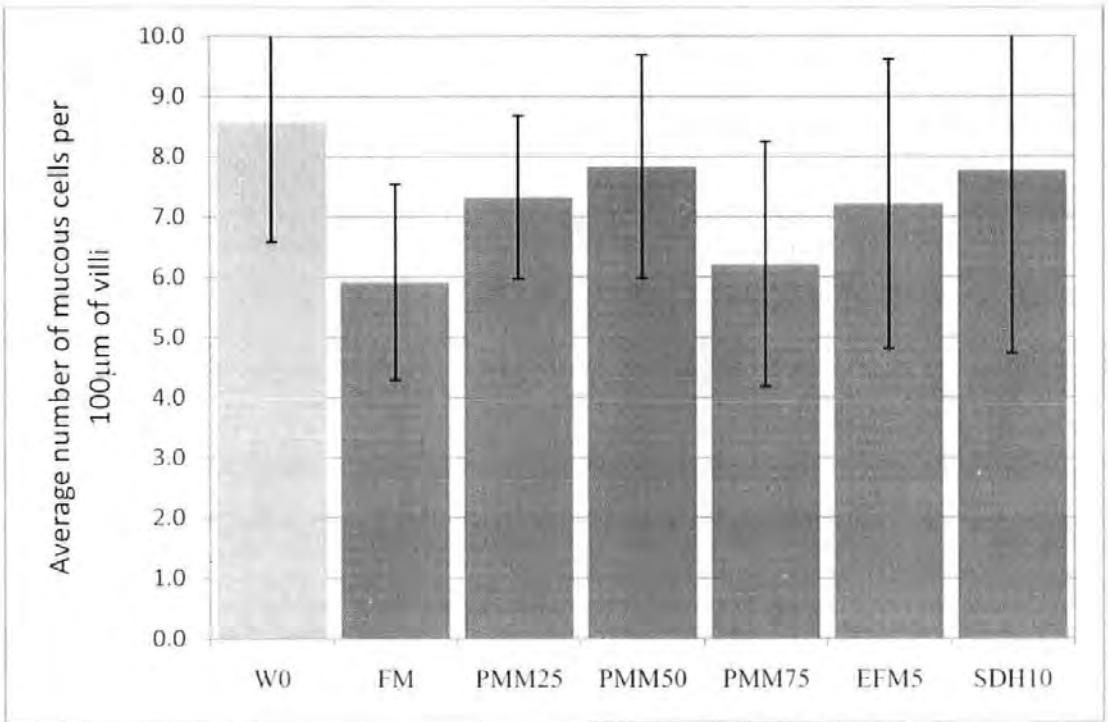


Figure 4B.3: Estimation of the average number of mucus cells per 100µm of villi (anterior, mid and posterior intestine undifferentiated). W0 (for Week 0) represents fish sampled at the start of the trial. Bars are SD.

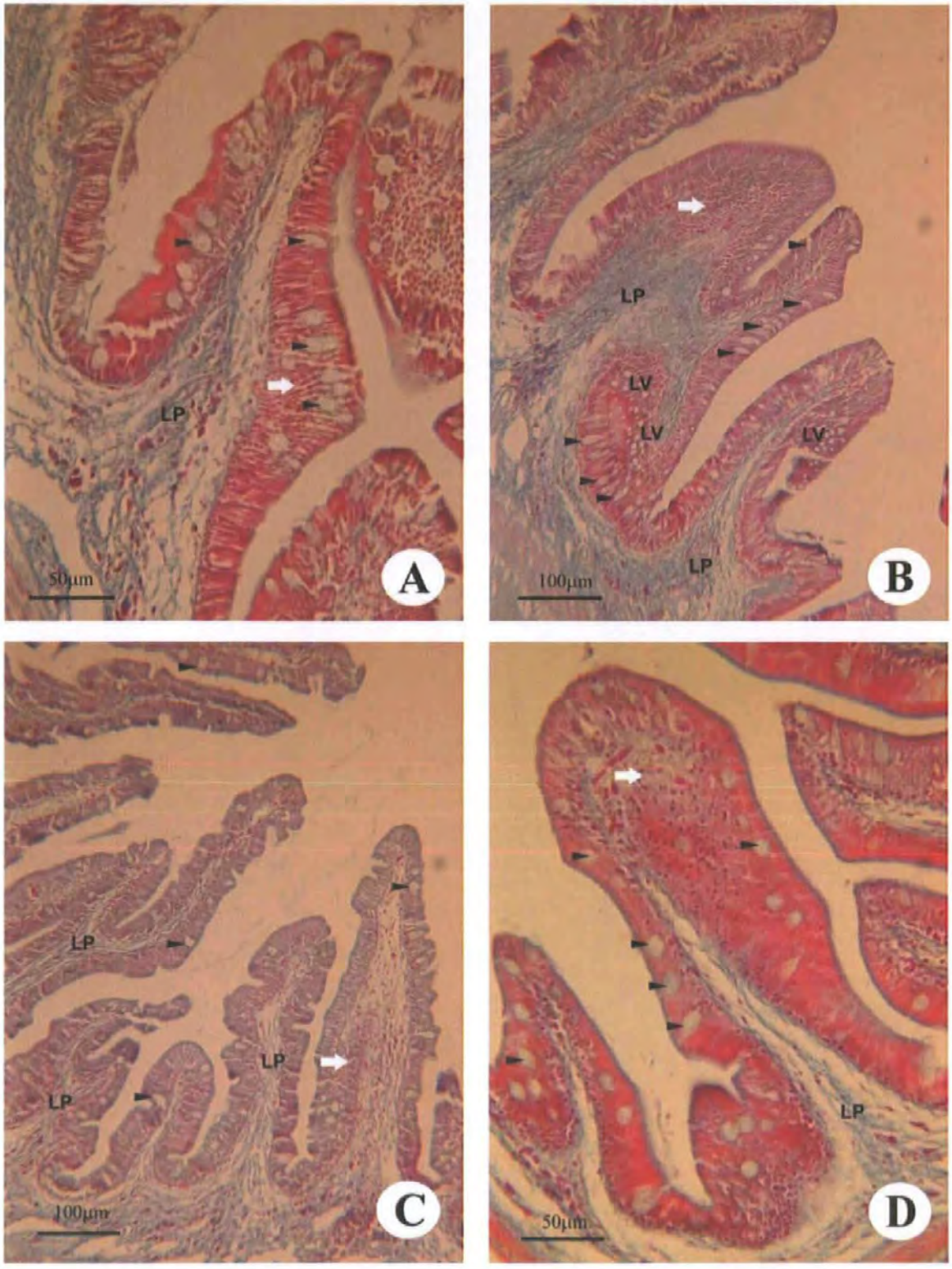


Plate 4B.4: Various sections showing degenerative changes (necrosis/hypertrophy) of the intestinal epithelium of gilthead sea bream (white arrows). Black arrows indicate goblet cells, LP: *lamina propria*; LV: lipid vacuoles. A: fish fed PMM50 (anterior intestine); B: fish fed EFM5 (posterior intestine); C: fish fed PMM75 (mid intestine) and D: initial fish (posterior intestine).

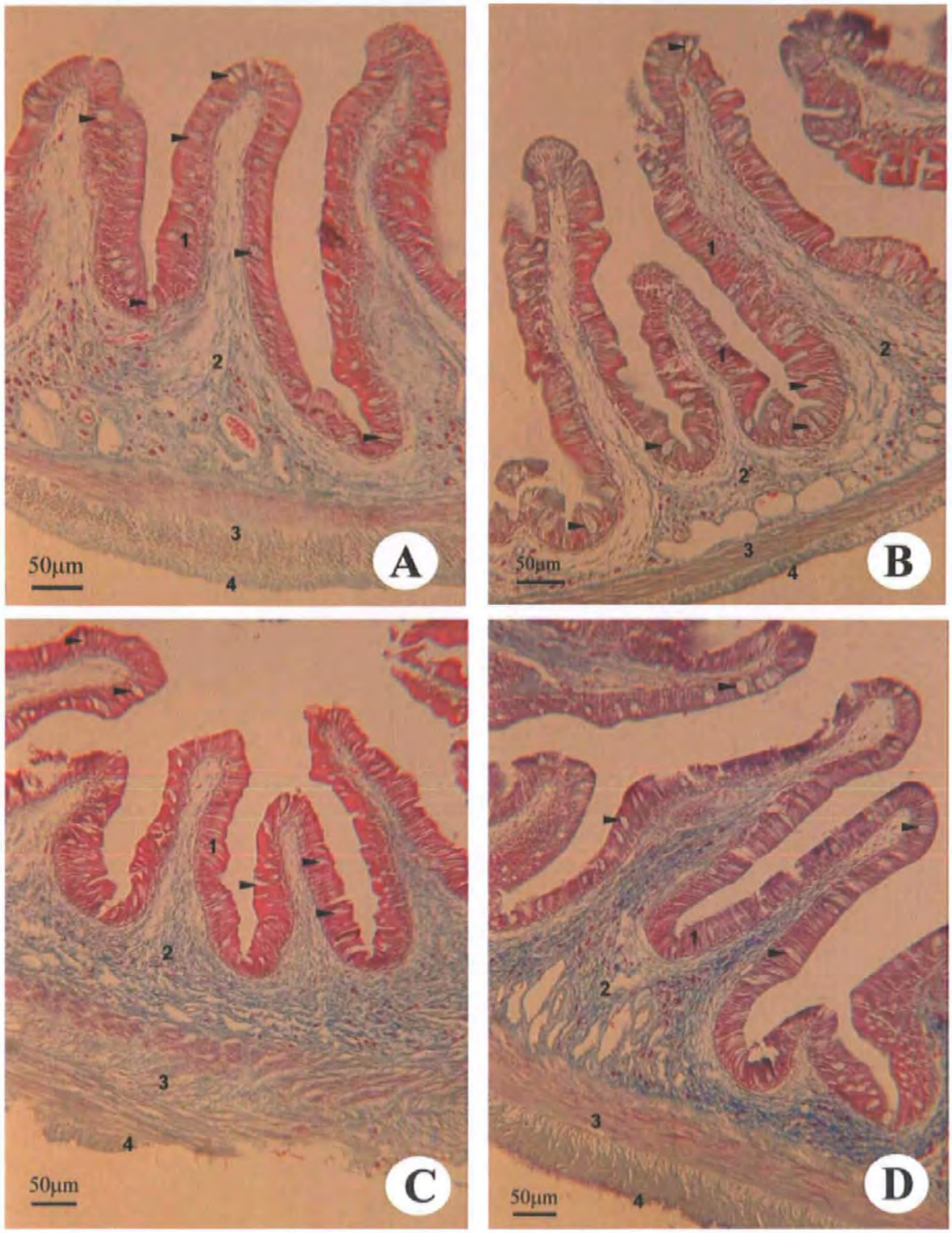


Plate 4B.5: Observation of intestinal folds and mucus cells in Mallory's stained section of the gut of gilthead sea bream (magnification ×20). 1: Mucosal epithelium, 2: *Lamina propria*, 3: *Muscularis*, 4: Serous membrane. A: fish fed EFM5 (mid intestine); B: fish fed fishmeal (mid intestine); C: fish fed PMM75 (posterior intestine) and D: fish fed SDH10 (posterior intestine).

Table 4B.4: Measurement obtained from the histological sections of gut.

Values are means of 3 or 2 replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Light Microscopy	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Perimeter Ratio (Anterior intestine)	4.98±0.77[a]	6.05±0.91[a]	6.22±0.66[a]	6.27±0.64[a]	4.96±0.5[a]	6.21±0.25[a]	P=0.712	F=1.07 P=0.431
Perimeter Ratio (Mid intestine)	3.81±0.8[a]	4.19±0.63[a]	4.31±0.87[a]	4.38±0.59[a]	3.35±0.6[a]	4.86±0.64[a]	P=0.869	F=0.58 P=0.717
Perimeter Ratio (Posterior intestine)	3.13±0.15[a]	4.07±1.15[a]	3.84±0.27[a]	3.54±0.65[a]	3.97±0.33[a]	3.52±0.24[a]	P=0.622	F=0.41 P=0.832

Electron Microscopy	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
Length of Microvillae (µm) (Average Anterior Int.)	2.32±0.19[a]	2.55±0.11[a]	2.12±0.03[a]	2.59±0.14[a]	2.77±0.23[a]	2.37±0.18[a]	P=0.227	F=2.17 P=0.139
Length of Microvillae (µm) (Average Mid Int.)	2.59±0.02[a]	2.13±0.04[a]	2.34±0.07[a]	2.24±0.14[a]	2.57±0.23[a]	2.58±0.16[a]	P=0.47	F=0.76 P=0.601
Length of Microvillae (SD Anterior Int.)	0.067±0.006[a]	0.089±0.028[a]	0.103±0.007[a]	0.119±0.010[a]	0.113±0.017[a]	0.120±0.036[a]	P=0.647	F=1.56 P=0.257
Length of Microvillae (SD Mid Int.)	0.145±0.064[a]	0.086±0.004[a]	0.102±0.016[a]	0.102±0.023[a]	0.088±0.010[a]	0.114±0.024[a]	P=0.000	P=0.905 (KW)

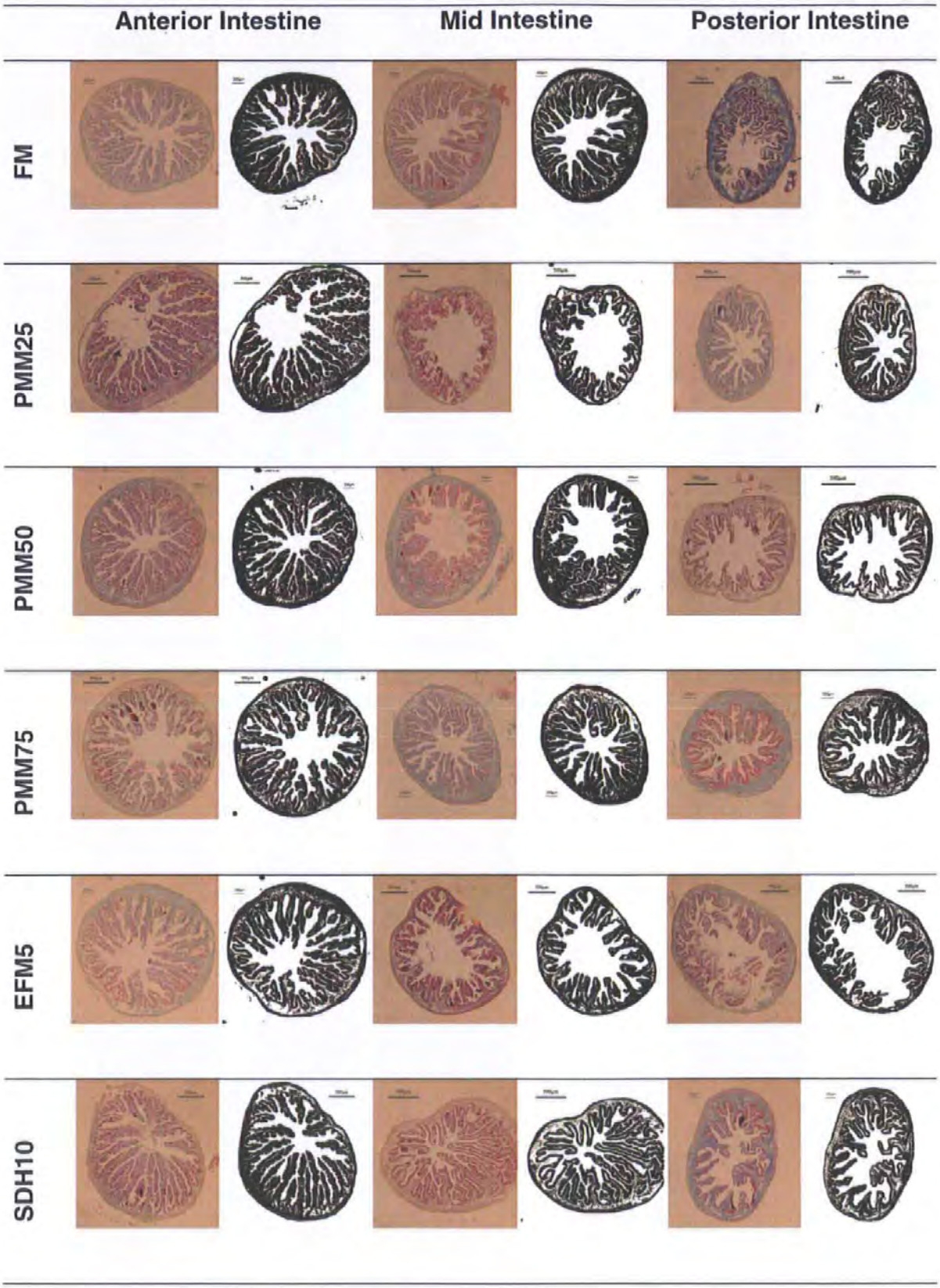


Plate 4B.6: Whole sections of gut from different parts of the intestine. Sample of light microscopic images of gut (magnification ×4) used to determine the perimeter ratio.

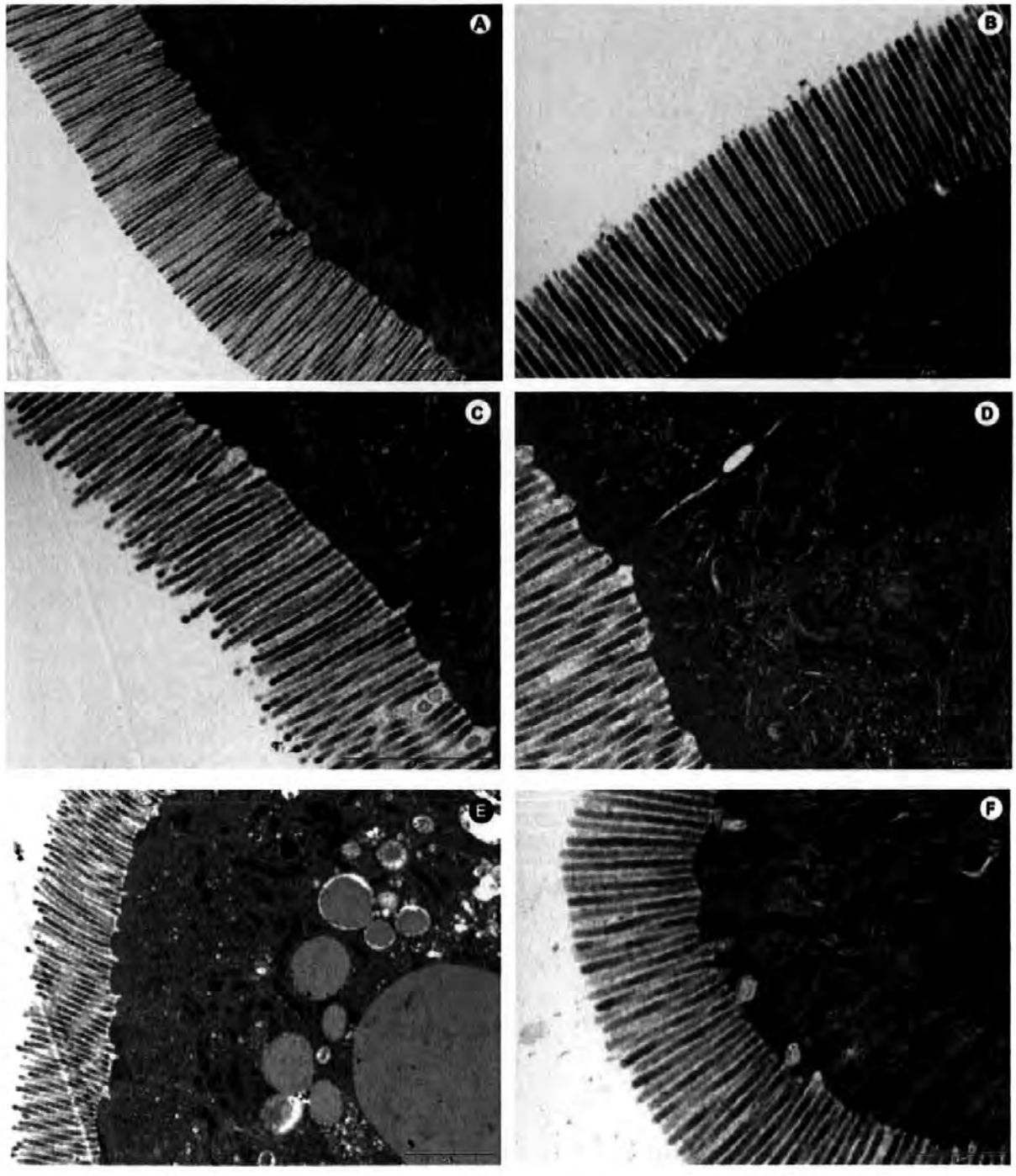


Plate 4B.7: Aspect of microvilli observed with the TEM and ultra-structure of enterocytes. Short/long regular microvilli in anterior (A) and mid intestine (B) of fish fed EFM5. Irregular microvilli observed in the anterior intestine of gilthead sea bream fed SDH10 (C). D: Tight junction observed between two enterocytes (EFM5). E: High vacuolization in an enterocyte of fish fed EFM5 (anterior intestine). F: Formation of endocytotic vesicles in the mid intestine of fish fed PMM25.

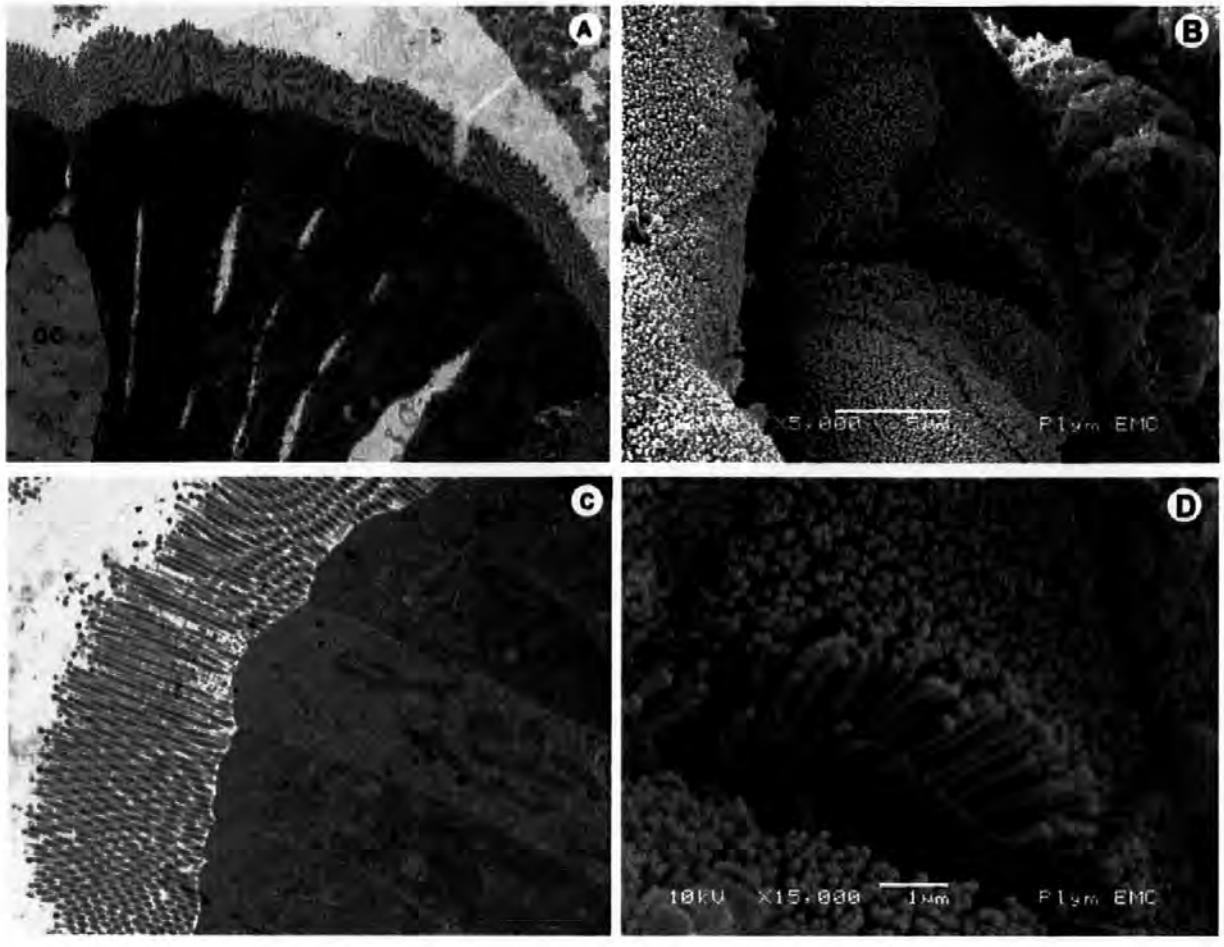


Plate 4B.8: SEM (B and D) and TEM (A and C) images of the gut of gilthead sea bream showing enterocytes and microvilli. A and C Fish fed PMM50, B and D fish sampled at the start of the trial. GC: goblet cell.

4B.3.4 Liver histology

From the light microscopy point of view, sections of liver of fish fed test diets showed normal histological characteristics, with regards to reference literature. Mallory's stained sections of representative samples from each treatment (Plate 4B.9) revealed homogenous-sized hepatocytes with vacuolated cytoplasm and centrally located nuclei. The cell outlines were easily distinguishable and similar features were noted after the visual evaluation of the different sections across treatments. As regards hepato-cellular vacuolation, minor differences were observable in accordance with the staining intensity (reference vs. all test diets).

Microscopic observations allowed the identification of the intra-hepatic exocrine pancreatic tissue (Plate 4B.9, G). The exocrine pancreatic cells were distributed around a blood vessel with zymogen material appearing more intensively stained at one periphery.

From the ultra-structural point of view, sea bream hepatocytes could be described as roundish polygonal cell body containing a rather dark and irregular nucleus. Cells generally presented a relatively high electron density cytoplasm, poor in organelles and of homogeneous aspect (Plate 4B.10).

Observations achieved by electron microscopy also evidenced intra-hepatic pancreatic cells which were ultra-structurally well differentiated from the other cellular types. Pancreatic cells typically included an important number of secretion granules, a round and regular nucleus, a low density cytoplasm appearing granulated, the presence of organelles like mitochondria and a well developed rough endoplasmic reticulum (Plate 4B.11).

Different sized vacuoles (lipid droplets) were distributed throughout the cytoplasm in varying number (Table 4B.4). In terms of liver steatosis, analysis of hepatocytes at the TEM scale revealed a somewhat different pattern than the one observed at the light microscopic scale. Estimation of vacuolization level of the hepatocytes after measurement and counting of the droplets indicated a trend toward an increase with incrementing level of PMM in the diet. Although important variations were visually observed (Plate 4B.4), average size of the lipid droplets (area in μm^2) did not vary more than a factor 2.6 between treatments. With values of 6.76, 4.13 and 5.11, lipid droplets were bigger in the hepatocytes of fish fed PMM25, PMM75 and EFM5 with comparison to those of the fish fed the reference diet (3.35). Average number of lipid droplets per cell was found to be similar for the fish fed FM, EFM5 and SDH10 (at about 10 droplets per cell); while increasing the level of PMM in the diet resulted in a gradual augmentation of this variable from 5.59 (PMM25) to 21.06 (PMM75).

4B.4 DISCUSSION

4B.4.1 General health parameters

In this trial, after a two month feeding period, the dietary conditions did not significantly change the condition factor (K), or hepato-somatic index (HSI). Values obtained for the condition factor were all supportive of good conformation of the sea bream after 9 weeks of feeding. The condition factor is a frequently used index by fish population biologists that refers to the curvilinear relationship between fish weight and fish length (i.e. the general shape of the fish). Based on the principle that individuals of a given length exhibiting higher weight are in a better condition, it indicates fish welfare in the habitat and furnishes important information related to fish physiological state (including its reproductive capacity). The HSI values obtained at the end of this trial (1.30-1.41) were comparable with the value established by Laiz-Carrión *et al.* (2005) for 134.2g gilthead sea bream raised in sea water (for those fish the condition factors reported were between 1.99 and 2.17); and were not correlated to the values of lipid density made from the electron micro-photographs ($r=0.052$; $p=0.922$). The hepato-somatic index (HSI%), which refers to the liver weight as a percentage of the whole body weight, is presumed to reflect tradeoffs in energy used by fish.

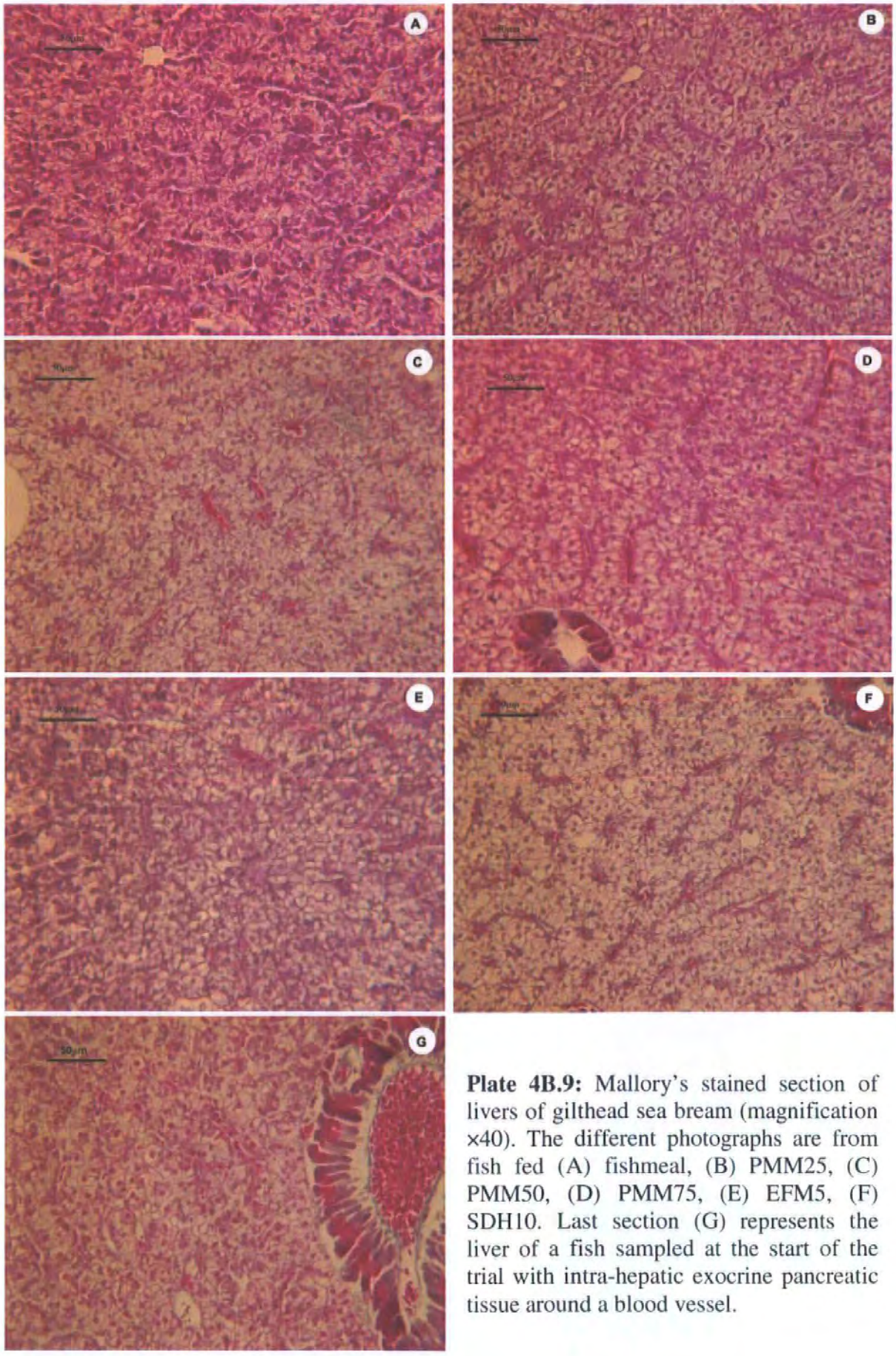


Plate 4B.9: Mallory's stained section of livers of gilthead sea bream (magnification ×40). The different photographs are from fish fed (A) fishmeal, (B) PMM25, (C) PMM50, (D) PMM75, (E) EFM5, (F) SDH10. Last section (G) represents the liver of a fish sampled at the start of the trial with intra-hepatic exocrine pancreatic tissue around a blood vessel.

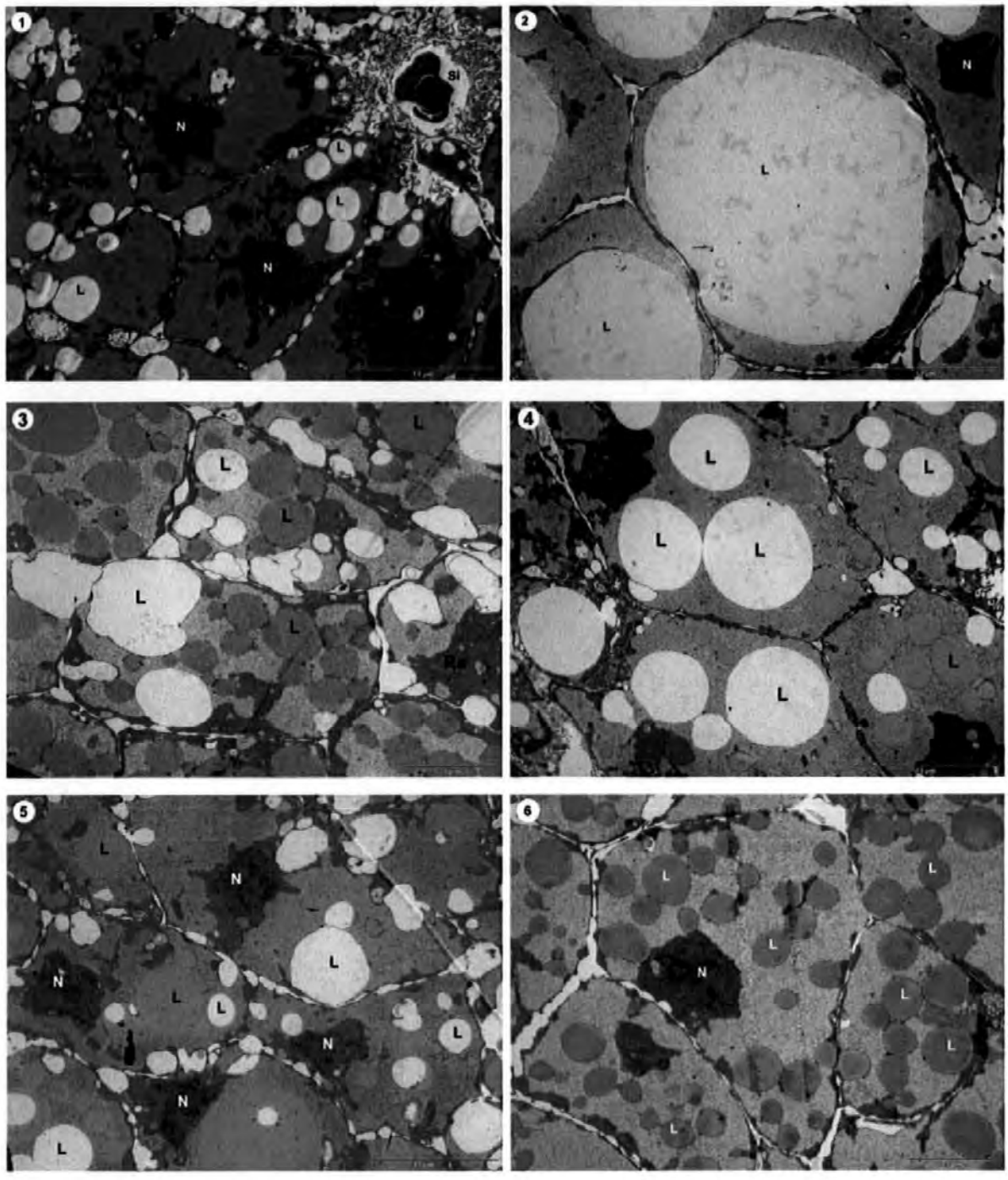


Plate 4B.10: Electron micrograph of hepatocytes of gilthead sea bream showing different level of vacuolization after 9 weeks of feeding with selected animal by-products: fishmeal (1), PMM25 (2), PMM75 (3, 4), EFM5 (5) and SDH10 (6). Nucleus (N), Lipid vacuoles (L), endoplasmic Reticulum (Re) and Sinusoid (Si) are indicated.

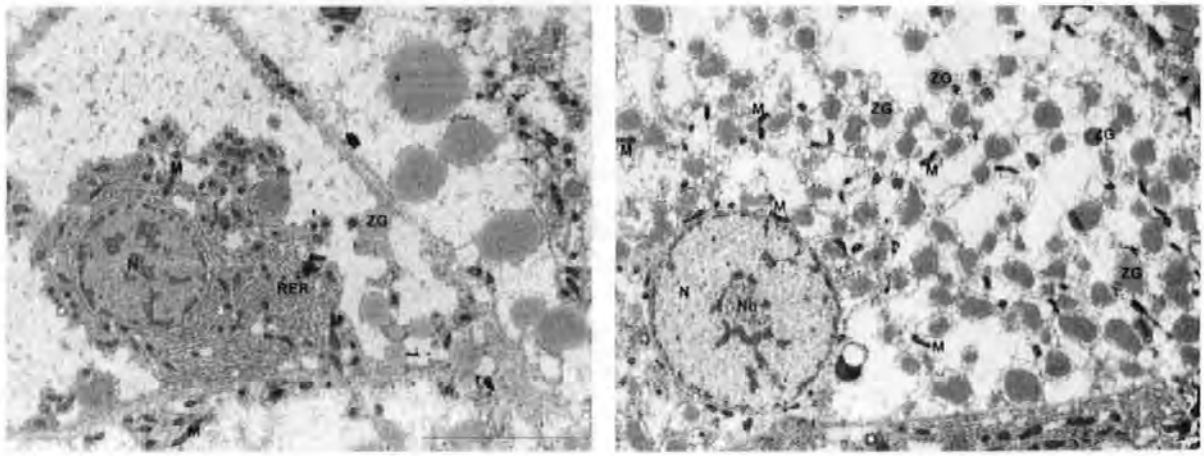


Plate 4B.11: Electron micrograph of intra-hepatic exocrine pancreatic cells. Typical attributes of secreting cells are well visible: Rough endoplasmic Reticulum (RER), Mitochondria (M) and zymogen granules (ZG). Cells also exhibit round nucleus (N) with discernable nucleolus (Nu).

Table 4B.5: Evaluation of lipid inclusion in the hepatocytes of gilthead sea bream from the morpho-metric measurements achieved on electron micrographs.

Electron Microscopy	Diet 1 FM LT94	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10
Mean size of lipid droplets (μm^2)	3.35±3.23	6.76±22.85	2.60±3.33	4.13±5.98	5.11±7.80	3.67±5.05
Average number of lipid droplets per cell	10.6±5.96	5.59±4.20	16.0±10.36	21.06±9.66	9.23±5.36	10.48±6.84
Volume density of hepatic lipid (μm^2/cell)	35.55	37.84	41.63	87.10	47.23	38.45

4B.4.2 Haematology and blood smears

Considering blood function, haematological parameters are also often used to detect any abnormalities indicative of physiological adaptations or disease. The volume of packed cells in the blood (haematocrit) varies depending on the health and physiological condition of individual fish (Moyle and Cech, 2004). Haemoglobin (Hb) is a red pigmented protein occurring in blood cells which is responsible for transferring oxygen through the blood

system. Erythrocytes (or red blood cells RBC) are the nucleated blood cells which carry the haemoglobin protein. Their primary function is then the transport of gases throughout the body. Anaemia is notably reported as a nutritional pathology and may occur as a consequence of folic acid, inositol, niacin, pyridoxine, riboflavin and different vitamins deficiency. Reduced haematocrit and low haemoglobin are generally associated with deficiencies in iron, vitamin C, E and B12 (Tacon, 1992). Nutritional toxicity may be another cause of anaemia. Osuigwe *et al.* (2005) suggested that the reduced haematological values of hybrid catfish was caused by the anti-nutritional factors present in jackbean seed meal used. Similar haematological response was found in *Clarias gariepinus* fed poultry litter (Omitoyin, 2006). Conversely, several trials have recently demonstrated the feasibility of including alternative protein sources or varying the quality of different dietary ingredient without affecting the haematological parameters (Montero *et al.*, 2003; Subhadra *et al.*, 2006a,b; Kumar *et al.*, 2007; Wassef *et al.*, 2007).

Variations in haematological parameters may also be attributed to many factors, both biotic (age, sex...) and abiotic (water temperature, oxygen content...), and in particular to stress (Örün and Erdemli, 2002; Silveira-Coffigny *et al.*, 2004). In our trial, biotic and abiotic factors remained under control with equal conditions for all experimental units, whereas stress conditions from rearing and handling were kept as low as possible and comparable for all fish so that possible differences may be associated only with the dietary regime.

In this study, none of the haematological parameters assessed indicated a reduction of the carrying oxygen capacity in relation with dietary treatments. Haematocrit values, haemoglobin concentration and erythrocyte count established for ~80g fish remained unaffected by dietary conditions and in accordance with other values reported for gilthead sea bream in the literature (Tort *et al.*, 2002; Montero *et al.*, 2003; Pavlidis *et al.*, 2007).

Leukocyte count is considered as an indicator of health status of fish because of its role in nonspecific or innate immunity (Lall, 2000). Leukocytes (or white blood cells WBC) are

non-pigmented, nucleated blood cells whose primary function is indeed to combat infection and in some cases to phagocytose and digest debris. Those cells also differ from erythrocytes, in that they can leave the vascular system to carry out their tasks by passing through the walls of small blood vessels. Various leukocyte types with different functions may be distinguished and results of differential leukocyte count are then widely used as an indicator of the immune status of fish. Measurements of change in total leukocytic concentration or in the percentages of the various WBC types often can lead to a better understanding of the physiological or pathological state of the animal. Stress conditions such as crowding conditions (Ortuño *et al.*, 2001) or dietary imbalance effect mechanisms of the immune system of cultured fish and may notably result in a WBC count increase (acute stress). In gilthead sea bream, immuno-suppression (which would appear as a more common response in the case of chronic stress) was also reported in fish affected by the winter syndrome (Tort *et al.*, 1998; Contessi *et al.*, 2006). Considering the proteinaceous origins of mitogenic and immunoglobulin based immune response, varying the protein quality of the diet has numerous potential repercussions on fish health. Lymphocyte proliferation, leukocytes count, macrophage aggregate, and IgM production are some factors which were shown to fluctuate as a result of a change in the dietary lipid or protein quality (Montero *et al.*, 1999; Krogdahl *et al.*, 2000; Mourente *et al.*, 2005; Subhadra *et al.*, 2006a,b). In our trial no differences were found in the percentages of the different leukocyte categories across the different treatments. Discrepancies observed (low level of granulocyte, thrombocyte and monocyte) with literature data (Pavlidis *et al.*, 2007), may be discussed in relation to the inexperience of the operator. Nonetheless all differential cell count patterns were established with the same accuracy, and no evidence of a dietary effect was observed.

4B.4.3 Gut integrity

Apart from a direct influence on the immune system, physical and chemical properties of diets might equally affect gut structure and digestive physiology which would subsequently impact nutrient utilization and health. Numerous histo-pathological alterations of the digestive tract and liver of fish species were reported in relation to the feeding regime and dietary treatments (Krogdahl *et al.*, 2003; Ostaszewska, *et al.*, 2005; Rodríguez *et al.*, 2005)

General observations made on the digestive tract of gilthead sea bream were generally concordant with the information found in the literature from the histological point of view (Cataldi *et al.*, 1987). Also there were no evidence of significant alteration resulting from the two month feeding trial with diets based on animal by-products.

In gilthead sea bream, a Y-shaped stomach is clearly distinguishable and precedes a relatively short intestine, as typically observed in carnivorous species. The pyloric region is characterized by four pyloric caecae, to the base of which the *ductus pancreaticus* and the *ductus hepaticus* discharge. The intestine is where the majority of food absorption takes place following the action of various digestive enzymes during the digestion process. Absorption is greatly facilitated by pseudovillae (folds) that multiply the tube surface by a factor of twenty. Absorption of nutrients across fish intestinal walls closely parallels the process described for mammals. The differentiation of the enterocyte membranes on the luminal side results in microvilli (that form the brush border), which multiply the absorbing surface by another factor of twenty. Nutrients may enter cells via diffusion (i.e. nonmediated), via mediated processes employing a membrane transporting protein or via endocytosis mechanisms. As regards histology, the intestine is composed of the mucosal epithelium, the *lamina propria* and the *muscularis*.

If the demarcation between the different parts of the intestine is often minimal in terms of gross anatomy, anterior, mid section and posterior part generally prove to be more readily

differentiated histologically (Guillaume *et al.*, 1998). According to our observations in gilthead sea bream, anterior and posterior intestines could firstly be distinguished by the shape of their respective mucosal folds. This reflects/corresponds to a bigger absorptive area in the anterior and mid intestine. It is usually recognized that the anterior portion of the intestine is functionally specialized for the absorption of small molecules. Besides it is also well established that the anterior section is involved in lipid absorption whereas enterocytes of the mid sections presenting endocytotic vacuoles would be responsible for protein transportation. Enterocytes located in the distal part of the intestine are often described with shorter microvillae and numerous mitochondria and play a role in mineral ion absorption. In this work, the intestinal location of the enterocytes did not result in any change as far as microvilli length was concerned. Moreover, in accordance with the result of Ostaszewska *et al.* (2005) who tested the inclusion of soybean type protein sources in diets for rainbow trout and pacu, no variation in the height of the microvilli could be associated with dietary regime. Comparing the absorptive intestinal surface areas in two species of tilapia with different eating habits Chakrabarti *et al.* (1995) did not find adaptative changes but showed that the microvilli height was affected. Nonetheless the visual assessment made in this study tended to confirm the general ultrastructural characteristics of the enterocytes in relation to their location and functional specialisation.

The density of globlet cells is another criterion that usually allows a distinction between the different parts of the intestine (Murray *et al.*, 1996). Higher numbers of globlet cells in the distal part of the intestine may allow better lubrication for faecal evacuation. Differing proportions of mucus secreting cells in the different area of tilapia intestine were reported by Sklan *et al.* (2004b). Because of the experimental design it was not possible to verify the variation of mucus cell density along the intestine of gilthead sea bream: however the average density determined in this study agrees with the data of Sklan *et al.* (2004) and did not prove to be affected by the diet quality. In rainbow trout it was demonstrated that fish fed soybean containing diet had a number of mucus cells per fold twice as large as the

number observed in the control fish (Ostaszewska *et al.*, 2005). In the case of soybean based diet, the modification in the number of mucus cells may also correspond to a decrease of many mucosal brush border enzyme activities as indicated by Krogdahl *et al.* (2003), which suggest a decrease in nutrient absorption.

Several authors have reported fatty change of the intestinal epithelium of various fish species as a result of lipid quality, energy to protein ratio or diet type modifications (Keembiyehetty and Wilson, 1998; Caballero *et al.*, 2003; Rodriguez *et al.*, 2005). Lipid droplets were observed in the cytoplasm of the intestinal epithelium of gilthead sea bream juvenile specimens by light and electron microscopy (Plate 4B.7 E). In sea bream the droplets resulting from the lipid absorption were defined as chylomicrons or very low density lipoproteins (VLDL) depending on the size of the inclusion. In spite of these lipid inclusions, the utilization of animal by-products in the diet of gilthead sea bream did not result in severe signs of intestinal steatosis.

Atrophy and necrosis of epithelial cells looked like a more common alteration in gilthead sea bream intestine. But leukocyte infiltration and widening or shortening of mucosal folds were also observed. Nevertheless, it was noticed that this degenerative change equally affected most of the fish regardless of the protein sources tested. Dietary treatments tested did not induce major pathological alterations of significant prevalence and intensity in the intestinal tract of gilthead sea bream.

4B.4.4 Liver histology

Histologically fish livers differ from those of mammals in many respects (e.g. *muralium* pattern, lobulars structure...etc.) but functions broadly similarly (Hibiya, 1982). The function of the liver as a digestive gland is to secrete bile, but hepatic cells have many other vital functions including a role in nutrient metabolism, nutrient storage, and detoxification. The liver also plays a key role in the synthesis and degradation of fatty

acids and glycogen storage. Additionally, the enzymes regulating these pathways showing varying affinities for different fatty acids, imbalances in the dietary fatty acids could modify the functioning and morphology of this organ. The hepatic tissue also functions as the main energy reservoir. In marine fish lipid deposition is reported to occur firstly in liver tissue and secondarily in viscera. When dietary lipid or energy exceeds the capacity of the hepatic cells to oxidize fatty acids, or when protein synthesis is impaired, the result is the synthesis and deposition of lipids in vacuoles, leading to a morphological pattern known as steatosis. Liver steatosis has been frequently observed associated with nutritional imbalances in cultured fish.

Hepatocyte features observed at the ultrastructural level (see 4B4.4) proved to be more or less in contradiction with what several authors have described in tilapia (Vicentini *et al.*, 2005) or gilthead sea bream liver (Wassef *et al.*, 2007). The structural modification of nuclei observed within the hepatocytes could reflect a nutritional pathology. Previous works suggested that the hepatonuclear size could be used as an indicator of the nutritional condition of fish (Mosconi-Bac, 1987; Strüssmann and Takashima, 1990). At the scale of the light microscopy, no visual differences could be observed in the degree of hepatocellular vacuolization among dietary treatments. However, on the basis of the morphometric measurement achieved at the ultrastructural level, total lipid content in the liver of sea bream proved to be affected by the inclusion of PMM. The poultry material tested contains a relatively high quantity of fat (13%) so that the final contribution of animal fat in the diet where 75% of the fishmeal was replaced by PMM normally reached 50% with a potential impact on fatty acid profile. Quality of the lipid provided was assumed to be rather consistent or gradually less modified in the other diets with a major contribution of fish oil. The effect of substituting fish oil with vegetable oils has already been the subject of much research. The result of these investigations suggested that the variation in the dietary fatty acid profile due to the inclusion of vegetable oil may alter fish metabolism (as indicated by the appearance of steatosis), which may affect fish health and

stress resistance. In sea bream steatosis has been observed as a result of an increase in the dietary lipid content (Caballero *et al.*, 1999), an essential fatty acid deficiency (Montero *et al.*, 2001), the use of artificial diet (Spisni *et al.*, 1998), and the inclusion of vegetable oils (Figueiredo-silva *et al.*, 2005). The issue relating to whether those changes should be considered as pathological alterations or as physiological adaptations to the diet may also be discussed since the steatosis signs were found to be reversible within certain conditions (Caballero *et al.*, 2004; Wassef *et al.*, 2007). Morpho-metric measurements made by Caballero *et al.* (1999) on gilthead sea bream hepatocytes using transmission electron microscopy revealed lipid droplets averaging 2.9 μm^2 in fish fed 15% lipid (fish oil) and ~15 μm^2 in fish fed 22 and 27% lipid (fish oil). In our trial where the dietary lipid level was fixed at 15%, the size of lipid droplet was found to be 3.35 μm^2 in the hepatocytes of fish fed the reference diet (100% fish oil) and 4.13 μm^2 in the hepatocytes of fish fed PMM at the highest fishmeal replacement rate (75%). Although we also observed large lipid vacuoles (Plate 4B.10, 2), it appears that the increased volume density of hepatic lipid was caused by an augmentation of the number of lipid droplets rather than an augmentation of their size.

The present study revealed that the practical inclusion of animal by-products did not induce major pathological changes in the alimentary system of gilthead sea bream. Most of the parameters established in this work indicate that administration of experimental diets containing poultry meat meal, enzyme treated feather meal and spray dried haemoglobin for a period of two months did not negatively impact the health of gilthead sea bream. Ideally longer term feeding trials should be conducted to validate these results and to ensure that more subtle changes (which may impact animal welfare as opposed to gross pathology) do not occur. Future research should also be directed toward assessing the effect of dietary formulation containing high amounts of animal by-products with respect to the modulation of immuno-competence in sea bream.

CHAPTER 5

Feasibility of Poultry Meat Meal (PMM) inclusion in diet for gilthead sea bream with respect to lipid origin:

Effects on biological performance and fatty acid composition of fish carcass

5.1 INTRODUCTION *(on the consequence of varying the nature of oil in fish diet)*

The quantity and quality of lipid in the diets of fish have been widely modified over recent years as a result of the different constraints affecting fishmeal and fish oil (Tacon, 2006). Escalating prices of fish oil on the market have encouraged the utilization of vegetable oils, whereas the necessity to reduce the level of protein in the diet combined with the known protein sparing effect of lipid has led to a trend to produce a more energetic diet (i.e. containing a higher level of lipid) (Kaushik, 1997).

Along with fishmeal, fish oil (FO) has traditionally constituted the main lipid source in marine fish diets due, notably, to its n-3 highly unsaturated fatty acid (HUFA) content which appears to be essential for the nutrition of stenohaline marine fish and beneficial to the health of consumers (NRC, 1993). Unfortunately, with the increase in the global demand for aqua-feed and the rather stable production of fish oil, the market price of fish oil has grown significantly since 1995, and this commodity is now subject to the same sustainability concern as fishmeal (Tacon, 2006).

In order to cope with this situation and reduce the dependency on fish oil, the inclusion of various vegetable oils has been considered in commercial aqua-feeds. Partial replacement of FO by certain vegetable oil has proved feasible in several species without affecting growth (Izquierdo *et al.*, 2003; Martínez-Llorens *et al.*, 2007). However, the effects of these oils on lipid metabolism and health of fish still remain unclear. Dietary inclusion of vegetable oils may lead to imbalances in the essential or non essential fatty acids, affecting tissue integrity and general health (Montero *et al.*, 2003; Caballero *et al.*, 2004; Mourente *et al.*, 2005).

The role of n-3 and n-6 fatty acids in the fish immune response is still unclear, with many contradictory results, there is, however, some evidence that the nature of lipid in the diet is reflected in the fish tissues through the fatty acid profile, and that diet can then be manipulated to obtain the high quality products desired (Sargent *et al.*, 1989; Caballero *et*

al., 2006). In chinook salmon, Silver *et al.* (1993) showed that absolute amounts of n-3 PUFA in muscle lipids could be enhanced by increasing amounts of the same fatty acids in the diets. Martínez-Llorens *et al.* (2007) observed that muscle fatty acid composition of gilthead sea bream varied with soybean oil concentration, higher concentrations leading notably to a significant increase in the level of both 18:2n-6 and 18:3n-3.

The inclusion of alternative ingredients as protein sources may also have some effects on the fatty acid or mineral balance of the diet depending on the nutrient profile of the ingredient. An excess or deficiency in specific components may represent a limitation to the utilization of certain feedstuffs as protein sources as illustrated by haem-iron in blood meal or animal fats in PMM and MBM. PMM and MBM are, for instance, known to possess a relatively high level of lipid (13 % in the case of PMM). In sea bream, Robaina *et al.* (1997) obtained good results with regard to growth, feed efficiency and protein efficiency ratio, but observed an increased deposition of lipids and a significant reduction in digestibility when meat and bone meal was used as a partial substitute for fishmeal. With the same ingredient, Alexis (1997) reported good performance with a fishmeal replacement level of 40%, indicating that the high ash content of MBM was one of the main limitations on its possible use within sea bream feeds.

The fatty acid profile of an animal fat is a function of the kind of species from which the fat was derived, as well as the breed, age, and diet of the animal. Animal fats are usually characterised by a high level of saturated (palmitic acid: C16:0 more specifically) and mono-unsaturated fatty acid (with a typical predominance of oleic acid: C18:1); poly-unsaturated fatty acid being mainly represented by linoleic (C18:2) and linolenic acids (C18:3). These oils contrast with marine types oil rich in HUFA like eicosapentaenoic acid (EPA, C20:5) and docosahexahenoic acid (DHA, C22:6).

Changes in the lipid quality of the diets resulting from the substitution of fish oil and fishmeal should be evaluated and controlled considering the potential impact on carcass

(fillets) quality, as well as fish health and fish welfare. Farmed fish are generally known for being fatter in comparison with their wild counterparts (Mnari *et al.*, 2007). Determination of the optimum protein to lipid ratio in fish diets (DP/DE) is a matter of prime importance which has led to much research (see section 1.2.1.3 and 1.2.1.4). In gilthead sea bream aquaculture, since the utilization of high energy diet (rich in lipid) has expanded, a good understanding of the biochemical response of the fish to quantitative variation of lipid in the diet (in relation with the nature of the lipid used) is also important (Kaushik, 1997).

Within the context of protein source variation, the present investigation was performed in order to gather more information on the effects of dietary crude fat on sea bream growth, feed efficiency, nutrient retention and carcass traits. More specifically, the aim of this study was to evaluate the feasibility of PMM inclusion with respect to the lipid origin in diet. Does the fatty acid composition of fish carcass become affected by the inclusion of a protein source such as PMM and to what extent the modification of the fatty acid profile induced by the inclusion of PMM modifies the performance of this protein source? May the utilization of a defatted grade of PMM lead to better performance in comparison with the full fat PMM?

5.2 MATERIALS AND METHODS

5.2.1 Diet preparation and experimental design

For the present investigation, five isonitrogenous and isoenergetic diets containing 40% digestible protein and 15% lipid were formulated for gilthead sea bream juveniles. The high grade Norwegian low temperature fishmeal (LT-94) used as the main source of protein in Diet 1 (control) was partially replaced by the following protein components: ①a Poultry Meat Meal (PMM), at the inclusion rate of 75% (Diet 2); ②a defatted grade of the same Poultry Meat Meal (dPMM) with the two substitution levels of 50% (Diet 3) and

75% (Diet 4); and ③a mixture of defatted PMM and de-hulled Soya Bean Meal (SBM), (the two ingredients were blended at a ratio of 1/1) at the replacement rate of 50% (Diet 5). Protein digestibility coefficients (DC) used to formulate the diets were those found in the literature (SBM) or determined in a previous study (PMM). The same DC was used for PMM and dPMM. The detailed formulation and composition of experimental diets for this trial are presented in Table 6.1. All diets were made in Plymouth with a cold extruder following the method described in section 2.4.2.

Table 5.1: Formulation (g/Kg) and composition (% ±SE) of experimental diets used

Formulation (g/Kg)	FM LT94	PMM 75	dPMM 50	dPMM 75	SBM/dPMM
Fishmeal (LT-94)	640	160	320	160	320
Poultry Meat Meal	0	570	0	0	0
Defatted Poultry Meat Meal	0	0	333	495	194
Soya Bean Meal (de-hulled)	0	0	0	0	19.4
Marine Fish Oil	73	57	100	110	100
Starch[1]	113	113	113	113	113
Dextrin[2]	57	57	57	57	57
Vitamin[3]	5	5	5	5	5
Mineral[4]	5	5	5	5	5
Additive (Vitamin C)	1	1	1	1	1
αcellulose[5]	106	32	66	54	11
Total	1000	1000	1000	1000	1000

[1]: Starch from corn (Sigma S4126); [2]: Dextrin typeII from corn (Sigma D2130); [3,4]: Skretting; [5]: αcellulose (Sigma C8002).

Composition (%)	FM LT94	PMM 75	dPMM 50	dPMM 75	SBM/dPMM
Moisture (%)	13.42	11.35	13.93	12.69	12.23
Crude protein (%)	45.67±0.33	47.65±0.12	46.08±0.08	45.44±0.26	45.48±0.06
Crude lipid (%)	11.47±0.22	14.82±0.40	11.41±1.30	13.13±0.09	13.30±0.12
Gross energy (MJ/kg)	16.86±0.00	17.54±0.00	16.60±0.02	17.14±0.07	17.32±0.00
Ash (%)	8.37±0.08	11.03±0.18	9.74±0.06	10.08±0.01	8.78±0.02

5.2.2 Fish stock and feeding management

Juvenile gilthead sea bream (*Sparus aurata* L.) were purchased from a commercial supplier in Portugal: TiMar (Cultivos em Agua) Lda. After the acclimation period fish were randomly distributed among 15 rearing tanks of the experimental system at a rate of 25 fish per tank. The weight of the fish (±SE) at the start of the trial averaged 10.08±0.04g. The 5 experimental diets were fed to triplicate groups of fish. Sea bream were hand fed to satiety twice a day throughout the experiment. Following one day of feed deprivation, fish were bulk weighed to establish tank biomass on a weekly basis.

5.2.3 Fish holding system and experimental conditions

The trial took place in the marine station of the University of Porto (Portugal) in a closed re-circulating seawater system, for a period of 5 weeks. The rearing system was composed of fifteen 100L cylindrical tanks connected in line with mechanical and biological filtration units (see Table 2.2). With a flow rate adjusted at 3L/min, natural seawater (36-38‰) was renewed in each tank at a rate of 180% per hour. Photoperiod followed the natural conditions. Dissolved oxygen, pH, total ammonia nitrogen (NH_4) and nitrite (NO_2) were measured on a regular basis to ensure the water quality remained within acceptable limits for fish growth and health (see section 2.2.3). Over the duration of the study, these water quality parameters averaged: 90% saturation for DO, 7.5 for pH, 0.2mg/L and 0.15mg/L for NH_4 and NO_2 respectively. Water temperature was permanently maintained at 24±1°C during the trial.

5.2.4 Analytical methods and histological examination

Fish sampled for whole body analysis (9 fish at the start, and 3 fish per tank at the end of the trial) were sacrificed (see section 2.5.1), freeze dried, homogenized in a blender and stored in sealed polyethylene bags for subsequent ash, protein, lipid and energy analysis. Moisture was determined by weighing the fish before and after the freeze drying process, ash by placing samples in a muffle furnace (550°C for 12h), protein content by macro-Kjeldahl, gross energy by bomb-calorimetry (Parr 1261/1755) and crude fat by the Soxlhet method (see section 2.5.3). Diets and ingredients were also subject to proximate composition analysis according to the same protocols, excepted for moisture which was determined by drying a 10g sample in an oven (105°C) until constant weight has been reached (AOAC, 2003). Fatty acid methyl ester (FAME) profile of FM, PMM75 and dPMM75 diets and carcasses were also established. The analysis was performed by Eclipse Scientific group (Cambridgeshire; UK) using gas chromatography technique.

Standard histological procedures were undertaken (as described in paragraph 5.3.3.1) on livers sampled from three fish per tank at the end of the experiment. Fixation, dehydration clearing and embedding were achieved in the Electron Microscopic Centre of the University of Plymouth while sectioning and mounting were completed in CEFAS Weymouth Laboratory. Prepared slides prepared were examined for any pathological symptoms. Growth performance and feed efficiency were measured in terms of percentage weight gain, survival (%), specific growth rate (SGR, %/day), feed conversion ratio (FCR), protein efficiency ratio (%PER) and protein conversion efficiency (aNPU); these parameters were calculated with the equations given in section 2.6.

5.2.5 Statistical analyses

Statistical interpretations were made using one way analysis of variance at the 5% level of significance. Tukey's *post hoc* analysis was applied to mean values where appropriate (Minitab 13 for windows).

5.3 RESULTS

5.3.1 Biological performances, feed and protein efficiency

Productivity parameters for juvenile gilthead sea bream fed the different dietary treatments are presented in Table 6.3. After five weeks of feeding no differences were found in terms of survival: the low number of dead fish reported resulted in survival rates close to 100% for all treatments (94.67 to 98.67). Significant differences were found in other parameters such as wet weight gain and SGR. Indeed, it was noted that fish fed the fishmeal reference diet had a wet weight gain significantly higher ($P=0.05$) than those fed PMM75 and dPMM75. The same pattern was obviously observed with SGR: fish fed fishmeal had the best growth (3.58%/day) followed by dPMM50 (3.47%/day), SBM/dPMM (3.41%/day), PMM75 (3.24%/day) and dPMM75 (3.19%/day). Feeding the fish to satiation also induced statistical differences as regard total feed intake due to the variable palatability of each diet (the highest inclusion of PMM resulted in the lowest feed intake). This result would partially explain the fact that the performance of the diets regarding FCR were reversed compared to what was described previously for SGR. Feed conversion was significantly improved for diets including the alternative protein sources in comparison with the fishmeal reference. A similar trend was observed for PER: fish fed the blend of SBM/dPMM (diet 5) and dPMM75 (diet 4) were more efficient at converting protein into wet weight gain with PERs of 1.45 and 1.31 respectively (in these fish protein efficiency statistically outperformed fish fed fishmeal).

5.3.2 Carcass composition and nutrient retention parameters

No major differences were observed in gross nutrient composition of fish carcasses presented in Table 6.4. Apart from ash, the percentage of gross nutrients analysed in the whole fish carcass appeared to be higher in the fish sampled at the end of the trial compared with those sampled at the start. Considering those data and the amount of protein consumed, it was established that the highest percentage of protein retained in the carcass was for the fish fed SBM/dPMM according to the aNPU and aNPU(t) parameters.

The fatty acid analysis of diets demonstrated the expected trend with respect to the dietary manipulation in term of lipid sources (Table 6.5). In diet 1 (fishmeal reference) where protein and lipid were all provided by marine ingredients the ratio of n-3/n-6 fatty acid had the highest value with 2.53. In the PMM75 diet, where animal fat was expected to account for 50% of the total lipid content further to the formulation strategy, this ratio decreased to 0.41. This was notably the consequence of a reduction in EPA (from 1.4% to 0.8%) and DHA (from 1.9% to 0.9%) as well as an augmentation of linoleic acid (from 1% to 6.4%). The amount of animal fat present in this last diet was also reflected by an increase in palmitic acid and oleic acid when compared to the reference diet.

The utilization of a defatted source of PMM in the last diet analysed (dPMM75) allowed restoration of the n-3/n-6 fatty acids ratio at 1.14. For this diet, compared to the one where 75% of fishmeal was replaced with full fat PMM, the amount of oleic acid (C18:1n-9) decreased from 32.9% to 24.3% while the level of linoleic acid (18:2n-6) varied from 6.7% to 4.4%. The amount of fish oil being adjusted in the formulation to make up for the extracted animal fat, the quantity of EPA and DHA increased. Amongst the three diets, it was also interesting to note that the level of arachidonic acid (ArA C20:4) varied slightly with the following trend: dPMM75>PMM75>reference.

With reference to the diets, similar profiles were observed in the fatty acid composition of the fish carcasses. In other words carcasses reproduced the variation of fatty acids observed in the diets. The proportion of total PUFA in the carcass was much higher than in the diets and n-3/n-6 ratios were found to be approximately two fold higher in the carcass (indicating that bio-accumulation of n-3 fatty acids is twice as quick as for n-6). Agreeing with what is usually described in wild or farmed gilthead sea bream (Mnari *et al.*, 2007), palmitic (C16:0) and oleic (C18:1n-9) acids were the principal SFA and MUFA regardless the dietary regime. DHA was the dominant PUFA within the carcass of fish fed diet 1 (reference) and 4 (dPMM), whereas linoleic acid (C18:2n-6) appeared to be the first PUFA in carcass of fish fed PMM75. The level of ArA (C20:4) was slightly reduced in the carcass of fish fed the PMM75 in comparison to the two other treatments.

Table 5.2: Carcass composition of gilthead sea bream juveniles used in trial 4. Values are means of 3 replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Carcass composition	Initial fish	Diet 1 FM LT94	Diet 2 PMM75	Diet 3 dPMM50	Diet 4 dPMM75	Diet 5 SBM/dPMM	Anderson-Darling	ANOVA (Kruskal-Wallis)
Moisture (%)	70.60±0.43	69.30±0.46 ^{a}	69.99±0.52 ^{a}	70.81±0.92 ^{a}	69.58±0.13 ^{a}	68.94±0.19 ^{a}	P=0.012	P=0.243
Crude protein *(% dry fish)*	48.58±0.24	52.12±0.74 ^{a}	52.62±0.84 ^{a}	52.09±0.38 ^{a}	51.45±0.10 ^{a}	51.05±0.28 ^{a}	P=0.242	F=1.27 P=0.343
Crude protein *(% wet fish)*	14.28±0.07	16.00±0.23 ^{a}	15.79±0.25 ^{ab}	15.20±0.11 ^{b}	15.65±0.03 ^{ab}	15.85±0.09 ^{ab}	P=0.751	F=3.42 P=0.052
Crude Lipid *(% dry fish)*	21.70±1.34	29.63±1.62 ^{a}	29.34±1.16 ^{a}	31.05±0.21 ^{a}	30.81±0.11 ^{a}	31.00±0.83 ^{a}	P=0.096	F=0.71 P=0.606
Crude Lipid *(% wet fish)*	6.37±0.39	9.09±0.50 ^{a}	8.80±0.35 ^{a}	9.06±0.06 ^{a}	9.37±0.03 ^{a}	9.63±0.25 ^{a}	P=0.738	F=1.14 P=0.392
Gross Energy *(MJ/kg) dry fish*	22.10±0.00	25.36±0.20 ^{a}	24.84±0.25 ^{a}	25.09±0.05 ^{a}	25.05±0.13 ^{a}	25.40±0.13 ^{a}	P=0.231	F=1.95 P=0.179
Gross Energy *(MJ/kg) wet fish*	6.50±0.01	7.79±0.06 ^{ab}	7.45±0.07 ^{cd}	7.32±0.01 ^{d}	7.62±0.04 ^{bc}	7.89±0.04 ^{a}	P=0.430	F=20.71 P=0.000
Ash *(% dry fish)*	13.27±0.09	10.84±0.20 ^{b}	12.23±0.36 ^{a}	11.85±0.11 ^{ab}	12.11±0.29 ^{a}	11.17±0.15 ^{ab}	P=0.932	F=6.45 P=0.008
Ash *(% wet fish)*	3.90±0.03	3.33±0.06 ^{b}	3.67±0.11 ^{a}	3.45±0.03 ^{ab}	3.68±0.08 ^{a}	3.47±0.04 ^{ab}	P=0.234	F=4.44 P=0.026

Table 5.3: Survival, growth performance and feed utilization indices of gilthead sea bream juveniles used in trial 4. Values are means of 3 replicates ± SE. In each row, values with the same superscripts are not significantly different (Tukey's test)

Productivity index	Diet 1 FM LT94	Diet 2 PMM75	Diet 3 dPMM50	Diet 4 dPMM75	Diet 5 SBM/dPMM	Anderson-Darling	ANOVA (Kruskal-Wallis)
Survival (%)	94.67±1.33^{a}	97.33±2.67^{a}	98.67±1.33^{a}	98.67±1.33^{a}	94.67±3.53^{a}	P1=0.003	P=0.521
Initial mean weight (g)	10.07±0.06^{a}	10.08±0.05^{a}	9.92±0.14^{a}	10.14±0.07^{a}	10.19±0.10^{a}	P1=0.039, P2=0.054	F=1.33 P=0.325
Final mean weight (g)	35.31±1.23^{b}	31.32±0.50^{a}	33.35±0.16^{ab}	30.95±0.46^{a}	33.67±0.38^{ab}	P=0.359	F=7.53 P=0.005
Wet Weight gain (%)	250.6±13.2^{b}	210.7±6.6^{a}	236.5±6.2^{ab}	205.4±5.2^{a}	230.3±1.9^{ab}	P=0.285	F=7.24 P=0.005
Feed intake (g)fish^{-1}day^{-1}	1.39±0.01^{a}	0.98±0.02^{b}	1.14±0.03^{ab}	0.98±0.03^{b}	1.01±0.03^{ab}	P1=0.006	P=0.027
SGR (%/day)	3.58±0.10^{b}	3.24±0.06^{a}	3.47±0.05^{ab}	3.19±0.05^{a}	3.41±0.01^{ab}	P=0.307	F=6.49 P=0.008
FCR	1.93±0.07^{b}	1.62±0.01^{a}	1.71±0.06^{ab}	1.65±0.03^{a}	1.50±0.05^{a}	P=0.115	F=9.55, P=0.002
PER	1.12±0.04^{a}	1.28±0.01^{abc}	1.26±0.04^{ab}	1.31±0.03^{bc}	1.45±0.05^{c}	P=0.627	F=9.60 P=0.002
aNPU*	18.74±0.77^{a}	21.12±0.13^{ab}	19.50±0.51^{a}	21.55±0.39^{ab}	24.12±1.07^{b}	P=0.352	F=9.98 P=0.002
aNPU(t)**	21.64±0.88^{a}	25.43±0.16^{bc}	22.71±0.59^{ab}	24.77±0.45^{abc}	27.76±1.24^{c}	P=0.397	F=9.87 P=0.002

*apparent Net Protein Utilization. ** apparent Net Protein Utilization based on true protein intake (Feed intake × % of digestible protein in diet)
Kruskal-wallis multiple comparison test (minitab13)

Table 5.4: Fatty acid Methyl Ester profile of diets and carcasses (expressed as weight percent of total fatty acid). SFA: Saturated Fatty Acid, MUFA: Mono Unsaturated Fatty Acid, PUFA: Poly Unsaturated Fatty Acid.

	Diets			Carcasses		
	FM LT94	PMM75	dPMM75	FM LT94	PMM75	dPMM75
Fat (Bligh & Dyer)	13.5	16.5	7.5	32.43±1.27	30.47±1.91	26.97±2.63
Fat Acid Hydrolysis	14.1	17.2	15.5	38.83±14.20	29.60±2.39	30.03±0.47
SFA						
C6.0 Caproic acid	0.1	0.1	0.1	0.10±0.00	0.10±0.00	0.10±0.00
C8.0 Caprylic acid	0.1	0.1	0.1	0.10±0.00	0.10±0.00	0.10±0.00
C10.0 Capric acid	0.2	0.2	0.2	0.10±0.00	0.10±0.00	0.10±0.00
C12.0 Lauric acid	0.3	0.3	0.3	0.10±0.00	0.10±0.00	0.10±0.00
C14.0 Myristic acid	9.0	4.3	7.6	5.20±0.10	3.43±0.12	4.97±0.06
C15.0 Pentadenoic acid	0.9	0.7	0.8	0.53±0.06	0.43±0.06	0.57±0.06
C16.0 Palmitic acid	23.4	24.6	24.3	18.67±0.25	19.40±0.61	19.00±0.26
C17.0 Heptadecanoic acid	2.2	1.2	2.3	2.20±0.00	1.57±0.06	2.20±0.00
C18.0 Stearic acid	3.7	6.1	5.5	3.60±0.10	4.27±0.23	3.50±0.61
C20.0 Arachidic acid	0.3	0.3	0.3	0.30±0.00	0.27±0.06	0.30±0.00
C22.0 Behenic acid	0.1	0.1	0.2	0.10±0.00	0.10±0.00	0.20±0.00
C24.0 Lignoceric acid	0.1	0.1	0.1	0.10±0.00	0.10±0.00	0.10±0.00
Total SFA	40.4	38.1	41.8	31.10	29.97	31.23
MUFA						
C14.1 Myristoleic acid (n-5)	0.5	0.3	0.5	0.30±0.00	0.30±0.00	0.37±0.06
C16.1 Palmitoleic acid (n-7)	11.4	8.2	10.5	9.40±0.10	8.57±0.15	9.77±0.06
C17.1 Heptadecanoic acid (n-7)	0.4	0.3	0.6	0.90±0.00	0.60±0.00	0.83±0.06
C18.1 Oleic acid (n-9)	18.7	32.9	24.3	20.53±0.45	30.10±0.89	23.13±0.21
C20.1 Gadoleic acid (n-9)	9.2	3.4	3.7	5.30±0.10	2.70±0.10	2.70±0.00
C22.1 Cetoleic acid (n-11)	9.3	2.4	2.9	4.93±0.15	2.10±0.10	2.33±0.06
Total MUFA	49.5	47.5	42.5	41.37	44.37	39.15
PUFA						
C18.2 Linoleic acid (n-6)	1.0	6.7	4.4	2.37±0.21	8.77±0.15	5.20±0.10
C18.3 Linolenic acid (n-3)	0.4	0.7	0.8	0.80±0.10	1.47±0.06	1.17±0.06
C18.3 Linolenic acid (n-6)	0.3	0.3	0.4	0.43±0.06	0.50±0.00	0.53±0.06
C18.4 Octadecatetraenoic acid (n-3)	0.3	0.2	0.6	1.53±0.06	1.00±0.00	1.37±0.06
C20.2 Eicosadienoic acid (n-6)	0.1	0.2	0.3	0.40±0.00	0.33±0.12	0.37±0.06
C20.3 Eicosatrienoic acid (n-3)	0.1	0.1	0.1	0.10±0.00	0.10±0.00	0.10±0.00
C20.3 Eicosatrienoic acid (n-6)	0.1	0.1	0.1	0.17±0.06	0.23±0.06	0.20±0.00
C20.4 Arachidonic acid (n-3)	0.1	0.1	0.2	0.73±0.06	0.47±0.06	0.60±0.00
C20.4 Arachidonic acid (n-6)	0.1	0.2	0.3	0.60±0.00	0.60±0.00	0.70±0.00
C20.5 Eicosapentaenoic acid (n-3)	1.4	0.8	2.3	7.53±0.21	4.03±0.06	6.00±0.17
C22.4 Docosatetranoic acid (n-6)	0.1	0.1	0.1	0.83±0.06	0.57±0.12	0.70±0.00
C22.5 Clupanodonic acid (n-3)	0.1	0.3	0.5	2.10±0.10	1.27±0.06	1.63±0.06
C22.6 Docosahexaenoic acid (n-3)	1.9	0.9	1.9	10.47±0.40	5.37±0.21	6.83±0.15
Total PUFA	6.0	10.7	12.0	28.07	24.70	25.40
Total n-3	4.3	3.1	6.4	23.26	13.71	17.7
Total n-6	1.7	7.6	5.6	4.8	11	7.7
Ratio n-3 n-6	2.53	0.41	1.14	4.84	1.24	2.30
SFA (in sample)	5.3	6.22	3.1	11.87±4.30	8.73±0.52	9.37±0.23
MUFA (in sample)	6.7	7.85	3.19	16.08±5.96	13.12±0.93	11.74±0.24
PUFA acids (in sample)	0.78	1.7	0.88	10.88±3.94	7.30±0.44	7.60±0.02

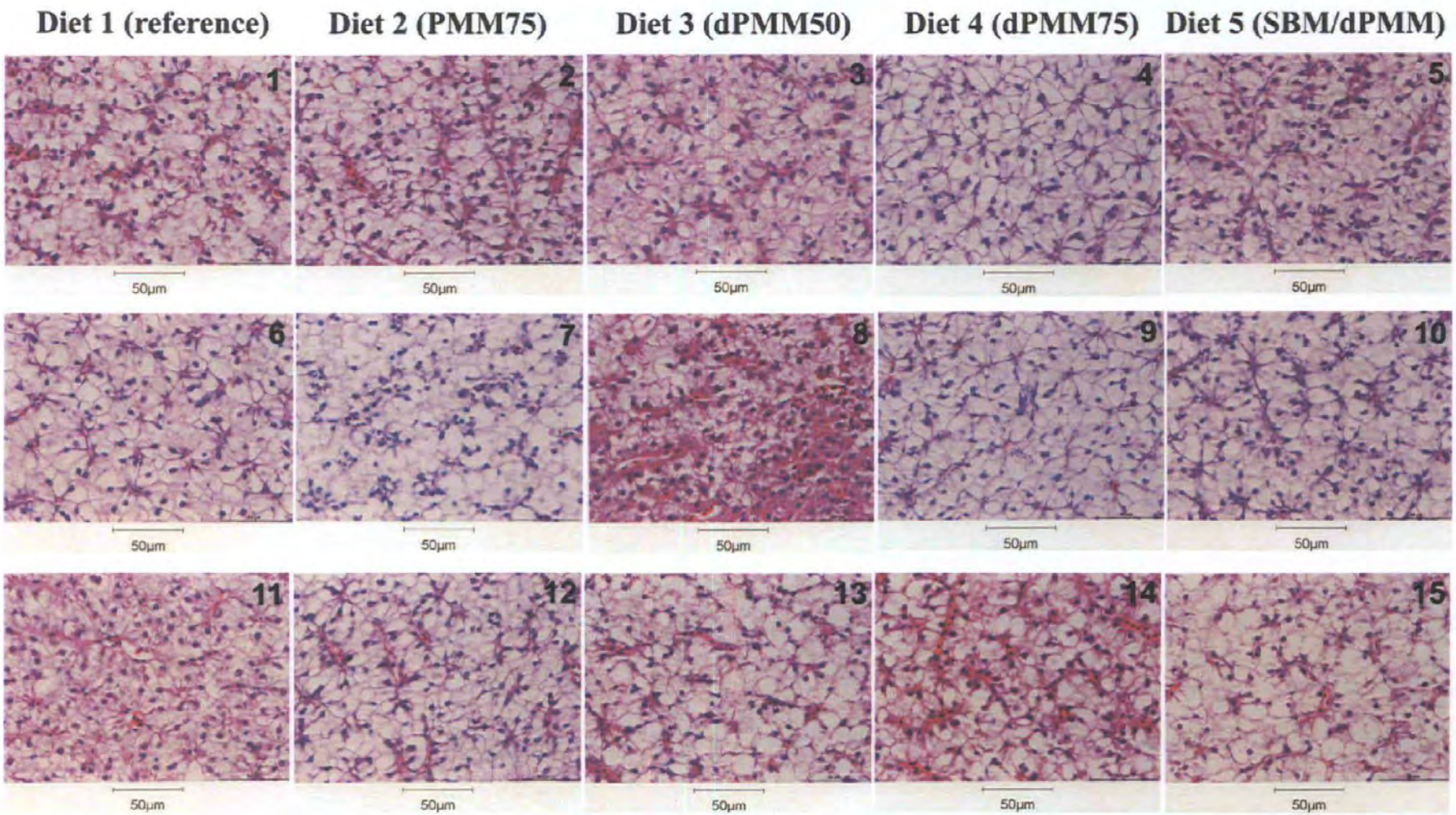


Plate 5.1: Mallory's stained section of livers from one of the three fish sampled in each tank (magnification × 40). Treatments and tanks number are indicated on the figure.

5.3.3 Histological observation on livers

Further to the visual comparison of the few sections obtained, across the different treatments (Plate 5.1), similar histological characteristics were found in the livers of the gilthead sea bream sampled. Qualitative assessment did not show any pathological features such as cloudy swelling, atrophy or necrosis of the hepatocytes. In addition, in terms of fatty degeneration (steatosis) and hepato-cellular vacuolation, light micro-photographs revealed comparable status. Although a certain individual variation was noted, most of the sections obtained were generally indicative of moderate to high level of lipid inclusion.

5.4 DISCUSSION

After five weeks of feeding, results obtained in the present trial tended to confirm the conclusion of chapter 4A (which indicated an optimal substitution rate of fishmeal with PMM below 50%) since the diets with the highest inclusion of PMM were again found to be significantly less efficient compared to the reference for most of the factors measured.

Moreover, with productivity parameters for both PMM75 and dPMM75 showing close agreement, this trial demonstrated that the qualitative variation of lipid induced by a high inclusion rate of PMM in the diet of juvenile gilthead sea bream to replace fishmeal did not influence the performance of the diet in terms of survival, growth, protein efficiency and utilization. Extracting the fat from poultry meat meal to test a "pure" protein source (at a high inclusion level) did not yield any improvement in comparison with the same inclusion level of full fat PMM. Also, fat of poultry origin does not seem to lower palatability of the diet since exactly the same feed intake was observed for both PMM75 and dPMM75 fed fish. Palatability and EAA profile of PMM are presumed to be the main factors limiting growth of sea bream when the full fat grade of PMM is included at a fishmeal replacement rate of 75%.

The present trial also brought evidence that PMM (defatted) have a good potential when associated with SBM. The inclusion of SBM in diet 5 did not lead to a significant reduction of feed intake ($p=0.05$) in comparison with diet 3 (dPMM50). This resulted in good growth performance for the fish fed SBM/dPMM (with SGR as good as the SGR of fish fed fishmeal), while the same group of fish had the best productivity values in terms of FCR, PER and aNPU.

Although both protein sources are said to be deficient in methionine (Nengas *et al.*, 1999; Hertrampf and Piedad-Pascual, 2000; Rawles *et al.*, 2006b), combining SBM to PMM might result in a partial improvement as far as lysine is concerned. Moreover, if this association may enable a partial correction of nutrient imbalance in PMM, it may be beneficial to SBM in terms of nutrient digestibility, level of ANFs and palatability. Indeed, these nutritional factors are known to depress fish performance when SBM is used as a sole protein (Forster, 2001).

The efficacy of SBM to replace fishmeal in diets for gilthead sea bream was examined by several researchers: Robaina *et al.* (1995) suggested a fishmeal replacement rate of 30%, whereas conclusions of Nengas *et al.* (1996) indicated rates of 20% to 35 % depending on the ingredient processing. Very limited information is available on the use of SBM and animal protein mixtures in the diets of sea bream. In a nutrition trial carried out by Fasakin *et al.* (2005) it was observed that tilapia fed either HFM or EFM in a mix with SBM had better performance compared to the fish fed feather meal without SBM. Similarly, Nengas *et al.* (1999) found that a combination of PMM and feather meal improved growth of sea bream. In rainbow trout, blends of feather meal and MBM (Bureau *et al.*, 2000) as well as blends of PMM and SDH (Serwata, 2007) were successfully tested.

The utilization of alternative protein sources with high levels of fat (see chapter 4A), as well as the partial replacement of fish oil with fat of different origin, has proved to be successful in many trials when growth and feed efficiency responses were considered.

Based on their results Martínez-Llorens *et al.* (2007) stated that it is possible to feed gilthead sea bream until they reach commercial weight with a 48% dietary substitution of fish oil for soybean oil. Using diets containing 25% lipid, Izquierdo *et al.* (2003) showed that 60% of dietary FO can be replaced by linseed, rapeseed or soybean oil taken individually or blended, without affecting gilthead sea bream juvenile growth. These results are globally in accordance with what was previously found by El-Kerdawy and Salama (1997) who successfully fed fingerling gilthead sea bream with a diet where 50% of FO (fixed at 9% in the control) was replaced by SO. Other authors have prioritised and recommended the utilization of oil mixtures in order to reduce the possibility of getting unbalanced fatty acid profiles (Montero *et al.*, 2003; Wassef *et al.*, 2004).

In most cases, results would indicate that significant fishmeal and/or fish oil replacements do not affect the minimum requirement level of n-3 HUFA or that fish have certain adaptation capabilities (although marine fish are not known to have the ability to elongate and desaturate C18 PUFA). Feeding sea bass an n-3 fatty acid deficient diet, Skalli *et al.* (2006) observed that most tissues had a certain capacity to respond and regulate DHA content in polar lipids. Within the context of a total FO replacement, the lack of a well balanced fatty acid profile (Sargent *et al.*, 2002) and a lower palatability (Regost *et al.*, 2003) or digestibility (Caballero *et al.*, 2002) are likely to limit the success of marine fish production. In this study, fish requirements for FA were not expected to be impaired since diet manipulation did not result in a reduction of fish oil <5%.

In common with other vertebrates, fish cannot synthesize either 18:2(n-6) or 18:3(n-3) *de novo* (NRC, 1993; Guillaume *et al.*, 1998). Hence one or both of these fatty acids must be supplied preformed in the diet, depending on the EFA requirements. In addition, fish vary considerably in their ability to convert 18-carbon unsaturated fatty acids to longer chain, more highly unsaturated fatty acids of the same series. The EFA requirement of fish is then related, to some extent, to their ability to modify these fatty acids metabolically. A major

difference appears to exist between freshwater and stenohaline marine fish as a result of an apparent deficiency in the enzymes required for elongation and desaturation of C18 precursor in marine fish. In general freshwater fish require either dietary linoleic acid, 18:2(n-6) or linolenic acid, 18:3(n-3) or both, whereas stenohaline marine fish require a combination of dietary eicosapentaenoic acid (EPA), 20:5(n-3), docosahexaenoic acid (DHA), 22:6(n-3) and/or arachidonic acid (ArA), 20:4(n-6) (NRC, 1993).

In addition to assisting in the absorption of fat soluble vitamins, fatty acids function as components of phospholipids in bio-membranes and as precursors for eicosanoids that fulfil a variety of metabolic functions. The fluidity membranes require to function properly depends on a specific balance between saturated and unsaturated fatty acids that changes with temperature (Guillaume *et al.*, 1998). With a blood temperature usually lower than homeotherms, the highly unsaturated fatty acids (HUFA) appear as essential nutrients for fish to ensure a good fluid state in all their cellular membranes (Guillaume *et al.*, 1998). With respect to these important functions it appears imperative to validate these dietary modification with more criteria based on physiological responses, immune functions and disease resistance particularly (Hasan, 2001).

Montero *et al.* (2003) demonstrated that when a single vegetable oil (such as rapeseed, linseed or soybean oil) was used to replace 60% of fish oil in the diet of gilthead sea bream, fish health could be affected in terms of immuno-suppression or stress resistance while the utilization of a blend of those different vegetable oils at the same replacement rate had no negative effects. Similarly the study completed by Mourente *et al.* (2005) revealed that the partial replacement of FO with rapeseed, linseed or olive oil may alter some immune parameters of sea bass. However, classical haematological parameters do not appear to be good indicators of oil source variation in the diet. In sea bream, haematological parameters remained unaffected by dietary treatment when a mixture of vegetable oils was used to replace 60% of dietary fish oil content (Wassef *et al.*, 2007). In

largemouth bass, like in sea bream, values obtained for Hct, Hb and MCHC did not show any significant difference in relation to dietary oil source variation (Subhadra *et al.*, 2006).

As well as satisfying EFA requirements, lipids provide the major source of non-protein dietary energy in the fish diets (NRC, 1993). As mentioned earlier, oil sources may serve an important protein sparing role in modern commercial feed formulations and energy dense diets are produced for both temperate fresh water and marine species. This has become an accepted practice where rapid growth, optimum feed conversion and minimum environmental impact is desirable. The use of high amounts of lipid in the diet (which generally requires the utilization of appropriate antioxidant to avoid peroxidation and rancidity) is known to increase level of lipid deposition in tissues (Caballero *et al.*, 1999). Kaushik *et al.* (1989) reported a decrease in trout body lipid content when digestible energy of the diet was reduced.

Distribution, quantitative importance and composition of fat are aspects that strongly influence fishery products and need to be further investigated. In European sea bass and gilthead sea bream, currently available data indicate that the liver is one of the major sites of lipogenesis and that dietary factors do regulate lipogenic enzyme activities. Following their trial Dias *et al.* (1998) found that key regulatory enzymes in the lipogenic pathway were depressed by elevated levels of dietary lipid. However, in another experiment, the partial replacement of fish oil by a vegetable oil mixture did not modify the activity of the same enzymes (Richard *et al.*, 2006).

Tissue degeneration or steatosis patterns in fish liver were also associated with the modification of lipid source (the high level of plant oil in diets for instance) and imbalance in n-3/n-6 fatty acid ratio (Alexis, 1997; Robaina *et al.*, 1998; Caballero *et al.*, 2004).

In the present study with gilthead sea bream (in which diets were designed to be iso-lipidic), it was not evident that total fat content of the carcass was related to the analysed dietary lipid, energy or n-3/n-6 ratio content of the diet (depending on the method of fat analysis the amount of lipid in the carcass was found to be more or less equal). Besides qualitative assessment of liver sections did not show any evidence of a variation in lipid content when treatments were compared to one another. These observations are nonetheless coherent with the digestibility results presented in chapter 3 indicating that lipids of PMM had high digestibility coefficients.

However, although the tissue fatty acid profiles of sea bream conformed to the expected changes in theoretical dietary fatty acid patterns expected for fish oil replacement with poultry fat, there was a pronounced discrepancy in the measured total PUFA fatty acid content in all diets. Although these values are at the minimum possible range typical for marine oils, the values reported may have been compromised by the methodology used for the extraction of lipid from feed. Various solvent based methods are known to be highly influenced by the conditions employed. Incomplete extraction of fishmeal bound phospholipids would greatly under-estimate the highly unsaturated fatty acids such as C20:5 and C22:6 omega 3 series. It is also possible that oxidation may occur which would also result in low levels in the analytical report. These possibilities are described by Stansby (1990) in a comprehensive text on fish oils in nutrition.

Nonetheless important differences observed for sea bream could be ascribed to the fatty acid composition of the respective diets tested. As far as the these qualitative changes are concerned, it is well established from the literature that the nature of dietary oil influences carcass quality and fatty acid pattern in tissues and organs (Farndale *et al.*, 1999; Izquierdo *et al.*, 2005; Caballero *et al.*, 2006; Piedecausa *et al.*, 2006). In terms of human consumption and consumer acceptance high levels of HUFA in fish muscle that can be obtained with proper diet manipulation would be a desirable benefit (Kaushik, 1997).

Contradictory results exist on the effect of replacing fishmeal with different protein sources. Carcass/muscle total lipid content and composition can be modulated by varying dietary ingredients. However, some studies have reported that the inclusion of increasing levels of plant ingredients does not affect the whole body lipid content of marine fish such as sea bass (Gouveia and Davies, 2000) and sea bream (Pereira and Oliva-Teles, 2002). In the same way, Aoki *et al.* (1996) did not find any difference in the flesh quality between adult red sea bream fed with or without fishmeal as a dietary protein source. On the other hand, other studies completed with marine fish resulted in the modification of muscle total lipid or whole body lipid content (Robaina *et al.*, 1995; Kissil *et al.*, 2000; Kaushik *et al.*, 2004; de Francesco *et al.*, 2007). Nowadays defatted grades of soybean meal are available and more widely utilized in scientific studies (Martínez Llorens *et al*, 2007).

The utilization of a defatted grade of PMM may not prove to be rational economically considering the processing costs and the current issues on fish oil utilization, however, it could constitute a realistic solution in specific conditions. This would allow a reduction in fishmeal utilization while maintaining an adequate fatty acid profile (rich in omega 3) in a "finishing" diet (used during the last month of the growing stage) necessary for the production of high quality filets.

Clearly there is much scope in developing a more secure understanding of the contribution of fat and oils in complete feeds for sea bream and other related marine fish species from natural high energy ingredients. The range of plant and animal by products is extensive and the fatty acid profiles inherent in these lipids may either augment or constrain their use. If the goal is to lower costs by providing lipid for energetic purposes for achieving high productive growth rates, then poultry meat meal would be a useful commodity. However if it is the strategy to market sea bream as a omega 3 rich food, then its inclusion should be limited. The consumer demand for quality products and economic considerations will inevitably dictate the best compromise for the optimum use of these materials.

CHAPTER 6

General discussion

6.1 CONCLUDING REMARKS

6.1.1 Introduction

The primary purpose of this work was to assess the feasibility of partially replacing fishmeal with selected animal by-products in order to reduce the fishmeal demand of the marine fish aquaculture industry. Therefore, using gilthead sea bream as a marine fish model through a sequential series of experiments, this program was developed considering a typical research strategy for testing novel protein sources in fish diets as described by Glencross *et al.* (2007) and reported in Figure 1.15. During the present project, digestibility, palatability, growth performance and feed utilization were the key points investigated in order to advance our understanding and efficacy of using animal by-products in diets formulated for the intensive production of gilthead sea bream. However, the present research program was also designed to emphasise more specifically the health effects of such substitution and explore in more detail the implications of fishmeal replacement on tissue integrity.

The accumulated data and insights gained during this study may allow formulation of cost-effective feeds and optimisation of dietary regimes. In this respect, using some of the data obtained during the course of this project, more advanced feed formulations were attempted with the linear least-cost approach and proposed in a final section of this work (section 6.3). Integrating our data to a certain extent, this work provided a final assessment in terms of practical diet formulation for sea bream with a basic strategy.

6.1.2 Conclusions and recommendations

Based on the findings of this thesis a list of conclusions and recommendations regarding different aspects considered as being involved in the nutritional value of the selected animal by-products may be summarised as follows:

▶ ***Digestibility (trial 1, chapter 3):*** i) PMM has an excellent potential to significantly replace fishmeal in gilthead sea bream diet with respect to digestibility and nutrient profile. ii) Lower digestibility and/or EAA deficiencies should limit the inclusion of feather meal and spray dried haemoglobin in balanced diet for marine carnivorous fish. iii) The processing method applied to feather meal was found not to greatly influence the digestibility of nutrients in this feedstuff. iv) Results obtained with the blends tested questioned the additive properties of digestibility coefficients as well as the synergetic combination of specific animal by-products. However, since a mathematical anomaly may be involved in the low coefficient determined for the feather meal based diet this statement would be subject to further testing. v) Methionine and isoleucine digestibitilty in SDH present much reduced coefficient that does not reflect the high overall protein digestibility of this ingredient.

▶ ***Feed utilization and growth performance (trial 2 and 4, chapter 4A and 5):*** Provided that gilthead sea bream are fed for a period of 5 to 9 weeks with balanced diets designed on a protein digestibility basis: i) Substituting fishmeal with PMM, SDH and EFM at the rates of 25%, 10% and 5% respectively results in biological performance better or comparable to the fishmeal reference. ii) Fish fed high dietary inclusion of PMM (50% of fishmeal replacement and higher) grow lower and are less efficient to convert food into body weight in comparison to the other test diets. iii) The type of animal by-product and inclusion level tested does not affect the gross carcass composition. iv) The qualitative variation of lipid induced by a 75% replacement rate of fishmeal with PMM does not influence performance of the diet in terms of survival, growth and protein efficiency. v) Following the 5 weeks feeding trial, productivity values of fish fed a combination of PMM and SBM were statistically similar to the productivity values of fish fed fishmeal.

► ***Palatability features (trial 2, 3 and 4, chapter 4A and 5):*** i) Incorporating increasing levels of PMM in diets designed to be isonitrogenous (40% digestible protein) and isoenergetic results in lower feed intakes. ii) The comparison of daily feed intake per unit of time for diet containing high and equal amounts of animal by-products suggests that PMM and EFM are significantly less palatable than fishmeal whereas SDH performance proves to be similar the reference. iii) Fat content of PMM does not seem to lower the palatability of the diet in the case of a 57% dietary inclusion.

► ***Health status (trial 2, chapter 4B):*** When gilthead sea bream are fed for a period of 9 weeks using balanced diets formulated with adequate inclusion of animal by-products (i.e. ~7% of SDH, ~11% of EFM and 19% to 57% of PMM) and 40% digestible protein: i) Haematological status does not indicate any significant reduction of the carrying oxygen capacity (anaemia condition). ii) Gut integrity (evaluated in terms of goblet cell density, microvilli length and perimeter ratio) remains unaffected by the dietary treatments (although a trend toward increasing absorptive areas was observed for the higher inclusion of PMM). iii) Liver reveals normal histological structure and no signs of specific pathology apart from a steatosis pattern found in the livers of fish fed PMM50, PMM75 and EFM5.

► ***Nutrient assimilation and product quality (trial 2 and 4, chapter 4A and 5):*** i) At the term of a 9 week feeding trial, the Diet designed with a moderate amount of SDH (7%) provides highest protein efficiency and utilization values, while the diet formulated with high inclusion level of PMM (but the same amount of digestible protein and crude lipid) shows the worst level of protein efficiency and assimilation. ii) The variation in the dietary fatty acid profile induced by a high inclusion of PMM is reflected in the carcass composition of the gilthead sea bream after 5 weeks of feeding.

6.1.2 Experimental methodology issues

It is noteworthy that the results obtained within the framework of the current study must be discussed within a specific context regarding the materials and methodology employed. Methodology used to evaluate digestibility coefficients has, for instance, generated much debate and controversy. Additionally, water temperature, particle size of raw materials and fish size are some other factors that may greatly influence digestibility results as discussed by Nengas (1991). In the same way, findings deduced from the growth trial must be related to specific conditions and protocols such as salinity, stocking density, diet type or feeding regime. For practical reasons, the trials performed within the course of this study were all undertaken in recirculating rearing units. These systems may differ from commercial production units in many ways but allow the maintenance of the environmental conditions at desirable levels. Protocols for diet analysis, diet manufacturing, fish handling and sampling, as well as settings regarding experimental conditions and designs were chosen to be as optimal as possible on the basis of the literature data, as far as the time, facilities and economical situation did permit. It should finally be noted here that the conclusions made on the ability of a specific ingredient to replace fishmeal are obviously dependent on the quality of the fishmeal reference to which they are compared.

6.1.4 Discussion, final assessment on the nutritional potential of animal by-products in gilthead sea bream diets

Clearly, the new generation of animal by-products such as poultry meat meal, spray dried haemoglobin and enzyme treated feather meal appeared valuable protein sources for the partial replacement of fishmeal in diet for gilthead sea bream. Moderate (20%) to more restrained (7-10%) incorporations of the high quality material tested was shown to be feasible without impairing the productive performance of the fish. Although the recent outbreak of avian flu (H5N1 strain) has made the issue of using poultry derived products even more difficult, the new regulation and practices put in place following the BSE crisis

(such as the categorisation of raw material, the interdiction of intra-species recycling as well as effective heat treatments) ensure from now on that no pathogens enter the food chain. Thereby, amongst the products tested, PMM may be seen as the most suitable animal-derived alternative to fishmeal from both the nutritional standpoint and bioethical considerations. While the biological value of this ingredient is excellent, palatability and lipid content of PMM represent two arguments leading to a limitation in its dietary inclusion at around 20% (chapter 3, 4A/B and 5). Amino acid deficiencies (histidine, lysine, methionine) combined with reduced digestibility performance constrain the utilization of feather meal in fish diet. The inherent deficit in isoleucine and methionine as well as the inferior digestibility performance of these EAA represent the major factors affecting the biological value of SDH in sea bream. However, iron and zinc content may constitute another constraint when inclusion of SDH is intended in fish diet. The utilization of EFM and SDH provides excellent results when formulations are based upon digestible protein/EAA and low amounts of ingredients are utilised in the diet. It is our belief that fish diets would benefit, in terms of both economics and nutrition, from moderate inclusion of animal processed proteins used as a single alternative source or blends. Recommendations for optimum replacement rates of fishmeal are indicated in Table 6.1.

Table 6.1: Optimum replacement rate of fishmeal with processed animal protein provided by PDM deduced from different digestibility trial (a), feeding trial (b) or digestibility and growth trials (c) conducted either at the University of Plymouth or at the University of Porto. Estimations are based on Fasakin *et al.* (2005) for red tilapia and Serwata (2007) for rainbow trout. In certain cases inclusions tested and results obtained do not give enough indication to determine an optimum rate accurately.

	sea bream	sea bass	turbot	red tilapia	rainbow trout
PMM	25%[c]	25%[a]	10%[a]	66%[b]	15%[c]
SDH	10%[c]	10%[a]	5%[a]	<66%[b]	10%[c]
EFM	5%[c]	≤5%[a]	≤5%[a]	<66%[b]	<20%[c]

6.2 SUGGESTIONS FOR FUTURE WORK

6.2.1 Extending the research to a wider range of fish species

Extending the research to other marine fish of commercial importance as well as new candidates for aquaculture appears another key challenge. The development of formulated feeds for trout and salmon has effectively been at the forefront of the work in fish nutrition, and diets provided to a variety of fish are still widely based on the knowledge gained on these reference species. Since one of the major trends now observed is toward the diversification of culture fish species, a proper recognition of the metabolic response of each species is required. Work achieved alongside this project on two other Mediterranean marine fish revealed some differences with respect to their ability to digest the animal by-products tested (chapter 3). Additional information and growth trials with sea bass and turbot are required to define more precisely optimum inclusion rates of animal by-products in these marine fish of commercial importance (using the preliminary data obtained with these specific fish).

6.2.2 Developing investigations directed toward the assessment of blended protein sources including animal by-products

Combining protein sources together may help correcting imbalance and deficiencies. The technique of protein complementation consists of combining protein sources to achieve a better amino acid balance than either would have alone. Because of differences in amino acid make-up, when protein sources are combined, the strengths of one make up for the deficiencies in another. With accurate and reliable information on nutrient specification and nutrient availability, the objective is to match an "ideal" amino acid pattern based on the known EAA requirement of the targeted fish. Fractionation has been shown to be a good strategy in order to reduce the level of anti-nutritional factors and optimise nutrient profile when various plant protein sources were included in fish diet. Commercial diets currently manufactured for carnivorous fish such as gilthead sea bream commonly contain

a wide range of protein sources (of plant origin) to limit the inclusion of fishmeal. Blood meal and feather meal are often reported to combine synergistically. However, beyond complementation theory, mixing feedstuffs may also bring the risk of creating antagonistic effects between constituents resulting in inferior digestibility performance as indicated in this project. This justifies/requires more testing to elucidate the causes of such results.

6.2.3 Developing alternative methodology for digestibility study

6.2.3.1 Benefit (interest) of *in vitro* assays and background

Due to the special features of the aquatic environment, fish nutritionists are well aware of the difficulties and errors obtained when determinations of digestibility are carried out *in vivo*. Moreover, if this type of investigation is affected by environment and developmental status of the animal, the *in-vivo* techniques also present the disadvantage of being time consuming, costly and inapplicable to certain fish species. One of the lessons that could be drawn from this project is certainly on the complexity of *in vivo* digestibility studies and the necessity to standardise the procedure.

Over recent years, some research efforts have then been focused on development of more or less simple *in vitro* digestion techniques for the rapid estimation of nutrient digestibility (Bassompierre *et al*, 1997; Gomes *et al*, 1998; Carter *et al*, 1999; Chong *et al*, 2002; Lemos, 2003). However while several *in vitro* digestibility tests have been developed, few have been adopted by industry due to their relative complexity or problems surrounding reliability and/or inconsistencies in predictive ability. As far as *in vitro* techniques are concerned, one major issue relates to the sensitivity of the assays (do they have the potential to separate different feed ingredients?) and how accurately they predict *in vivo* digestibility value.

6.2.3.2 Proposals for an *in vitro* digestibility assay

In vitro evaluation of protein digestibility requires that appropriate enzymes be employed, an applicable digestion-reactor developed (with a technique to separate *digesta* from *ingesta*), and suitable assay conditions are formulated. In other words the *in vitro* digestion method must simulate the conditions in the digestive tract of a marine fish model as closely as possible. Following the pattern of differential pH digestion, the technique could be designed with a two steps sequence, where several parameters (physiological temperature optimum, gastric and intestinal pH, digestion time...) would be established precisely to reproduce the gut conditions of the investigated fish. In order to develop an easy applicable assay (easy to standardize), the utilization of commercial enzymes rather than crude extracts would be preferable (as long as the optimal temperature of these enzyme do not represent a limitation in their applicability to the fish model), but type and amount of enzyme should be determined accurately. With the data published in the literature (Deguara *et al*, 2003; Munilla-Moran and Saborido-Rey, 1996a, 1996b; Hidalgo *et al.*, 1998) a preliminary calibration would then be necessary to determine the quantity of each commercial enzyme that should be use in the incubator to reproduce the enzyme activities measured in the digestive tract of the target species. As far a technique to separate the soluble digestion products from the rest of the ingesta is concerned, an "incubating bag" like the one developed by Ankom technology (sealed polyester bags, 25 micrometers porosity) could be employed.

Regarding the issues mentioned previously, the aim of such work would be first to demonstrate I) that the *in vitro* digestion method based on a fish-specific physiological background can predict the *in vivo* performance II) that *in vitro* digestion method can predict the quality of any ingredients or diets through the digestibility value of its proteins.

6.2.4 Assessing new feed formulae at the production scale

Most growth trials with fish are performed using the juvenile stages of the species where growth is rapid. Additionally, results reported often relate to relatively short term experiments. Longer experimental periods or even growing fish to marketable size would give a more complete picture of the nutritional value of the test ingredients. Then, with the nutritional data obtained on the selected ingredients further to laboratory testing, evaluating dietary inclusion of animal by-products at the production scale would permit to get a final validation and further arguments to restore public confidence and convince retailers. This ultimate step would include formulating diets with more practical ingredients (diet formulated in this project may be considered as semi-purified) and feeding them to marine fish intensively farmed over a longer period (covering nursing and growing stage) in production scale facilities and conditions (in cages at sea for instance). Trials conducted in this project were intended to be as realistic as possible of the intensive rearing conditions applied in commercial operations; however many experimental factors remained representative of research scale rearing facilities and practice. It is, for example, of great importance to mention here that all experimental diets were cold pelleted in the laboratory, with temperatures never exceeding 40°C during drying. Pellets processed by the feed manufacturing industry may have different physical and nutritional properties since much higher temperature are used in steam pelleting and extrusion techniques (these two most common processing methods for the preparation of dry pelleted diets involve the use of varying degrees of heat, moisture and pressure). Thereby, data obtained from the present study must be interpreted in this context. Rearing system, environmental condition, stocking density, husbandry, are factors that may influence fish performance.

6.2.5 Expanding the database on physiological response, nutritional pathology and health related consequences when animal by-products are included in the diet

Although the haematological and histological assessment performed in this study did not indicate any significant dietary effect, it is imperative longer term trials be conducted to confirm these results and ensure more subtle changes, which may impact animal welfare as opposed to gross pathology, do not occur. In terms of histology, future evaluations could take into consideration more tissues such as muscle and bone. Using histological and biochemical (Tartrate Resistant Acid Phosphatase TRAP, and Alkaline Phosphatase ALP assays) approaches, analysis were initiated at the University of Plymouth to assess the potential effect of different diets on the structure and mineral composition (characterisation of vertebral structure, determination of osteoblast and osteoclast activity...) of bony tissues such as vertebra, scales and gills. This potentially represents a new area of investigation where further work and preliminary evaluation will be necessary.

Regarding the issue of gut integrity, data collected through histological and digestibility studies could be completed with other specific measurements: the impact of the experimental diets on the activity of specific gastro-intestine enzyme could, for instance, be examined following the completion of the feeding phase of the trial. It has been well established that diets can modulate lipases, amylases and proteolytic enzymes (Alarcon *et al.*, 1999).

Considering the proteinaceous origin of mitogenic and immunoglobulin function, future work should also focus upon the modulation of immune response by dietary components more specifically. In gilthead sea bream the following immune indicators were for instance used to determine the immune status of the fish through different techniques, assays and/or protocols: Lymphocyte number, phagocytosis, lysozyme, complement, agglutination and immunoglobulins (Esteban *et al.*, 1998; Tort *et al.*, 1996; 1998; Montero *et al.*, 1999; Ortuño *et al.*, 2001; Sitjà-Bobadilla *et al.*, 2005; Contessi *et al.*, 2006). Challenging fish

with pathological agents could constitute another approach developed to evaluate the immune competence of fish that would have previously been subjected to different dietary treatments.

6.2.6 Flesh quality issues: fat deposition and fat quality

The flesh quality of farmed fish is also an issue of prime importance that needs to be addressed in more detail in relation to the accepted practice of fishmeal and fish oil replacement and the resulting situation as regard lipid nutrition. In this field of research, typical flesh quality analysis usually focuses on parameters such as colour, texture and flavour of fillets. Apart from further analysis on the biochemical composition (fatty acids) of white muscle, ensuring final product quality could be achieved through sensory evaluation (which would involve taste panels). This type of test would permit to establish the consumers' preferences and might constitute an important step toward the eradication of the negative image carried by animal by-products. With the view of restoring retailers' confidence, this method (establishing the gustative perception of sea food products by consumer representatives) could certainly provide weighty arguments. With respect to this work, similar project could in the future benefit from the new facilities and expertise of feed technology unit of the University of Plymouth which has shown abilities in conducting organoleptic and texture assays.

6.3 FORMULATION OF PRACTICAL TYPE DIETS FOR GILTHEAD SEA BREAM: DIFFERENT SCENARIOS OBTAINED FROM A LEAST-COST FORMULATION PROGRAMME

6.3.1 Introduction to least cost formulation and brief description of the program utilised (FeedSoft™)

Modern approaches to feed formulation depend increasingly on the use of Linear Least-Cost systems based on appropriate software. Least-cost formulation is combining many feed ingredients in a certain proportion to provide the target animal with a balanced

nutritional feed at the least possible cost. Though least-cost formulation is a mathematical solution based on linear programming, it requires the professional knowledge of animal nutritionists who take into consideration the nutrient requirements of a species and its capability to digest and assimilate nutrients from various available ingredients. For instance, the quality and accuracy of a formula designed for gilthead sea bream will depend on the quantity and quality of details entered as regard the nutritional value of all potential ingredients (e.g. protein digestibility coefficients established in gilthead sea bream...), the prices of these ingredients, and the nutritional needs of gilthead sea bream (digestible protein, amino acids requirements etc...). The final feed formula will only be as accurate as the initial information that was input by the user.

FeedSoft™ (Feedsoft Corporation) is a program that allows the management of a list of ingredients that are available and potentially useful for diet formulation (each ingredient should have corresponding nutrient composition and assimilation data) and offers several options as far as concerned formula specification (nutrient levels, ingredient limits...) (Rossi, 2004).

6.2.2 Principals and settings employed for the least-cost formulations performed

On the basis of the research finding in this thesis relating to the evaluation of the major animal by-products employed, various restrictions and potential nutritional value data were obtained. This allowed several linear least cost formulations to be attempted using the latest raw material prices and full nutritional parameters for the main nutrients.

Animal by-products entered into the program were notably characterised by their digestible protein value (determined in chapter 3) as well as their latest market prices. The raw amino acid composition provided by the manufacturer was employed rather than digestible EAA values. Diets were finally designed to contain 40% digestible protein and 15% lipid, as visible in the nutrient restrictions table presented.

Each scenario was based on diet formulation for juvenile sea bream at the grower stage of development. Feed formulations for advanced production stages towards marketable weights were not attempted due to a lack of information regarding the nutrition constraints and raw material utilization. However, it should be possible to extrapolate the following scenarios to larger fish as it is likely that they would be less sensitive to nutritional constraints compared to faster growing juveniles.

6.3.3 Presentation and discussion around the different scenarios obtained

The strategy presented firstly illustrates a fishmeal high value type diet, without inclusion of any alternative protein concentrates (scenario 1). Scenario 2 depicts the maximum potential for poultry meat meal inclusion replacing fishmeal without prior restriction on EAA requirements. This demonstrated that 40 % inclusion of PMM was feasible. In scenario 3, PMM was fixed at 25% inclusion on the basis of the fish feeding trials reported in chapter 4A which provided evidence that this level was an acceptable inclusion for optimum growth and development of sea bream. Scenario 4 was designed to include a fixed level of haem protein concentrate (7%) that was previously tested for digestibility and growth response (up to 10% fishmeal replacement) with successful results. For digestibility, it was found that PMM and SDH provided a high digestibility coefficient as a blended material. Again PMM was assigned at 25% inclusion in this formulation with fishmeal providing the major balanced. In Scenario 5, feather meal was introduced into the formulation for the first time. Interestingly this ingredient with a high protein concentration but proven low digestibility for protein was accepted at 0.86% into the formulation since it is probably a contributing material to meet the total protein target. In a further formulation (scenario 6) it was necessary to formulate a more complicated feed matrix representing the typical strategy of considering plant protein ingredient (namely soybean meal and maize gluten meal). These are very good alternative proteins that were not the main emphasis of this current project but are routinely used in commercial aqua-feeds including those for

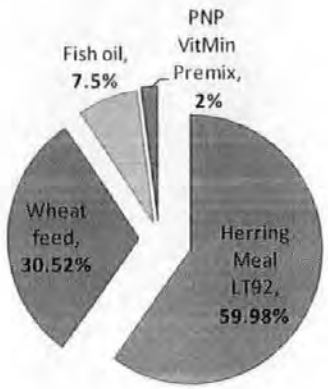
marine species. On this basis fixed levels of PMM (25%) and SDH (7%) were maintained. Additionally a minimum of 12% (maize gluten) and 15% (soybean meal) was set. The formulation resulted in a practical diet which included significant levels of these latter ingredients and a balanced diet was obtained. Finally, a more refined approach was considered in which minimum essential amino acid requirements were set based on the scientific literature (Table 1.1) for methionine, lysine, arginine and tryptophan. Unfortunately there are no current values for the remaining EAA and obviously these would be important for a detailed appraisal of any potential protein rich ingredient. In scenario 7, the primary four limiting amino acids described were incorporated into the nutrient requirement data for sea bream and open formulation made presenting each of the main ingredients with a maximum of 30% for PMM and 12% for maize gluten due to concerns with respect to methionine and lysine level respectively for these sources. However, in this formulation, a methionine analogue was also included to meet this amino acid requirement (methionine is potentially low in PMM). Scenario 7 demonstrated the feasibility of maximising the use of a PMM by-product with support of a crystalline amino acid supplement. Clearly, refinement of feed formulations will depend greatly on the requirement for each of the 10 EAA and in particularly their individual digestibility in respective ingredients and also the digestible amino acid requirement for the species. There is obviously much more work to be undertaken in addressing this issue.

The various scenarios described show a possibility of reducing cost considerably by the progressive substitution of very expensive raw material such as fishmeal. Ultimately the optimum scenario would depend on local availability of raw materials, their nutritional composition and costs. In the scenarios described here it has been shown that the cost per tonne of complete feed was reduced from 570€ (fishmeal based diet) to 470€ (a practical diet employing animal by-products and plant protein ingredients under restricted use). This amounts to a 17.5% reduction in the cost per tonne, based on current raw material prices.

This can obviously be refined further on the basis of more information on the nutritional requirements for sea bream and ingredient nutritional value as reported previously. It should also be stated that these scenarios only offer a model indicative of the findings from this project. They do not reflect the wide potential for many more ingredients available for fish feed formulations, and obviously the cost of feed production could be reduced further.

An additional caution is the fact that the diet formulated in this chapter was based on digestible crude protein (40%) and the gross protein of the diet was allowed to rise to 48%. An increasing discrepancy between DCP and crude protein (CP) would result in elevated faecal nitrogen losses (as seen in scenarios 2, 5, 6 and 7). Consequently this amounts to a further limitation that should be imposed on the upper CP levels in feeds to minimise environmental impact. Finally, the cost benefit analysis is a multi-factorial concept that incorporates dietary nutrition constraints, physiological aspects related to the species as well as the environmental considerations with respect to nutrient losses.

Scenario 1:



Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Herring Meal LT92	0.71			59.98
Wheat feed	0.22			30.52
Fish oil	1.00			7.50
PNP VitMin Premix	0.30	2.00	2.00	2.00

Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.99	0.01
02. Crude Protein	%	40.00	48.00	45.65	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	0.00
06. Crude Fiber	%			2.71	0.00
07. Phosphorous	%	1.00		1.24	
08. Calcium	%	1.00		1.65	0.00
09. Magnesium	%			0.19	
10. Avail. Phos.	%			1.09	
11. Ash	%			1.57	
12. Lysine	%			3.63	0.00
13. Methionine	%			1.32	
14. Met + Cys	%			1.85	
15. Arginine	%			3.25	-0.00
16. Histidine	%			1.13	
17. Threonine	%			1.95	
18. Tryptophan	%			0.55	
19. Leucine	%			3.53	-0.00
20. Isoleucine	%			2.11	
21. Phenylalanine	%			1.87	
22. Valine	%			2.84	
24. Vitamin E	mg/kg			14.61	-0.00
26. Choline	mg/g			3.08	0.00

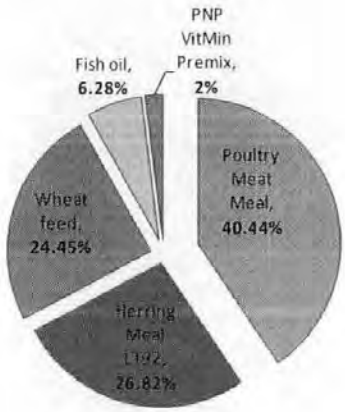
Cost of diet

0.57 €/kg; 570 €/Tonnes

Scenario 2:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Poultry Meat Meal	0.38		60.00	40.44
Herring Meal LT92	0.71			26.82
Wheat feed	0.22			24.45
Fish oil	1.00			6.28
PNP VitMin Premix	0.30	2.00	2.00	2.00



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.42	0.06
02. Crude Protein	%	40.00	48.00	48.00	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	
06. Crude Fiber	%			2.83	-0.00
07. Phosphorous	%	1.00		1.33	
08. Calcium	%	1.00		2.10	0.00
09. Magnesium	%			0.19	
10. Avail. Phos.	%			1.20	
11. Ash	%			1.57	
12. Lysine	%			3.23	-0.00
13. Methionine	%			1.02	
14. Met + Cys	%			1.57	
15. Arginine	%			3.20	-0.00
16. Histidine	%			1.03	
17. Threonine	%			1.81	
18. Tryptophan	%			0.48	
19. Leucine	%			3.19	0.00
20. Isoleucine	%			1.81	
21. Phenylalanine	%			1.75	
22. Valine	%			2.40	
24. Vitamin E	mg/kg			11.66	0.00
26. Choline	mg/g			3.94	0.00

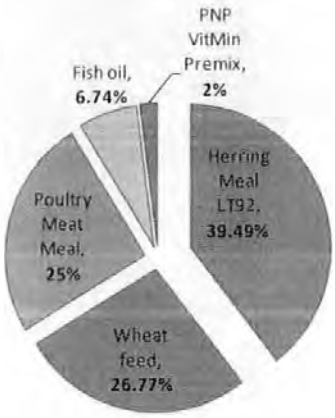
Cost of diet

0.47 €/kg; 470 €/Tonnes

Scenario 3:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Herring Meal LT92	0.71			39.49
Wheat feed	0.22			26.77
Poultry Meat Meal	0.38		25.00	25.00
Fish oil	1.00			6.74
PNP VitMin Premix	0.30	2.00	2.00	2.00



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.64	0.01
02. Crude Protein	%	40.00	48.00	47.10	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	0.00
06. Crude Fiber	%			2.78	0.00
07. Phosphorous	%	1.00		1.30	
08. Calcium	%	1.00		1.93	0.00
09. Magnesium	%			0.19	
10. Avail. Phos.	%			1.16	
11. Ash	%			1.57	
12. Lysine	%			3.39	0.00
13. Methionine	%			1.14	
14. Met + Cys	%			1.68	
15. Arginine	%			3.22	-0.00
16. Histidine	%			1.07	
17. Threonine	%			1.87	
18. Tryptophan	%			0.51	
19. Leucine	%			3.32	-0.00
20. Isoleucine	%			1.93	
21. Phenylalanine	%			1.80	
22. Valine	%			2.57	
24. Vitamin E	mg/kg			12.79	-0.00
26. Choline	mg/g			3.61	0.00

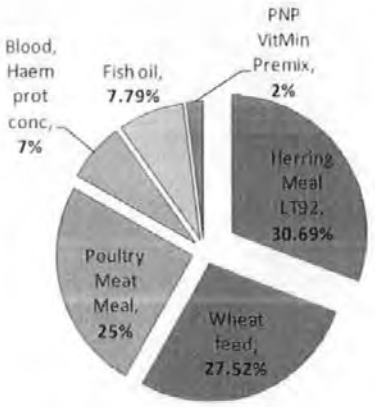
Cost of diet

0.51 €/kg; 510 €/Tonnes

Scenario 4:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Herring Meal LT92	0.71			30.69
Wheat feed	0.22			27.52
Poultry Meat Meal	0.38		25.00	25.00
Blood, Haem prot conc	0.70	7.00	7.00	7.00
Fish oil	1.00			7.79
PNP VitMin Premix	0.30	2.00	2.00	2.00



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.62	0.01
02. Crude Protein	%	40.00	48.00	47.47	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	0.00
06. Crude Fiber	%			2.78	0.00
07. Phosphorous	%	1.00		1.16	
08. Calcium	%	1.00		1.73	0.00
09. Magnesium	%			0.17	
10. Avail. Phos.	%			1.01	
11. Ash	%			1.57	
12. Lysine	%			3.46	0.00
13. Methionine	%			1.00	
14. Met + Cys	%			1.43	
15. Arginine	%			3.06	-0.00
16. Histidine	%			1.40	
17. Threonine	%			1.83	
18. Tryptophan	%			0.51	
19. Leucine	%			3.71	-0.00
20. Isoleucine	%			1.68	
21. Phenylalanine	%			2.01	
22. Valine	%			2.77	
24. Vitamin E	mg/kg			12.74	-0.00
26. Choline	mg/g			3.24	0.00

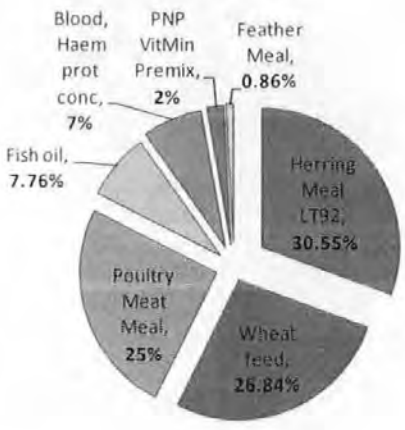
Cost of diet

0.51 €/kg; 510 €/Tonnes

Scenario 5:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Herring Meal LT92	0.71			30.55
Wheat feed	0.22			26.84
Poultry Meat Meal	0.38		25.00	25.00
Fish oil	1.00			7.76
Blood, Haem prot conc	0.70	7.00	7.00	7.00
PNP VitMin Premix	0.30	2.00	2.00	2.00
Feather Meal	0.25			0.86



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.57	0.01
02. Crude Protein	%	40.00	48.00	48.00	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	
06. Crude Fiber	%			2.74	-0.00
07. Phosphorous	%	1.00		1.16	
08. Calcium	%	1.00		1.73	-0.00
09. Magnesium	%			0.17	
10. Avail. Phos.	%			1.01	
11. Ash	%			1.59	
12. Lysine	%			3.47	0.00
13. Methionine	%			1.00	
14. Met + Cys	%			1.46	
15. Arginine	%			3.09	-0.00
16. Histidine	%			1.40	
17. Threonine	%			1.86	
18. Tryptophan	%			0.51	
19. Leucine	%			3.75	0.00
20. Isoleucine	%			1.70	
21. Phenylalanine	%			2.03	
22. Valine	%			2.80	
24. Vitamin E	mg/kg			12.46	-0.00
26. Choline	mg/g			3.23	0.00

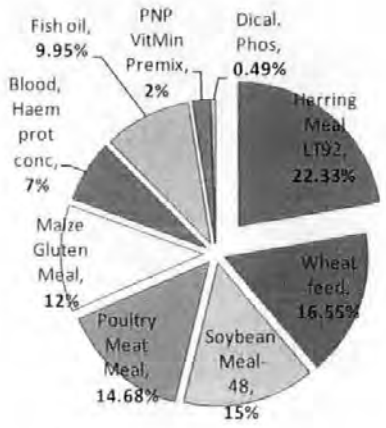
Cost of diet

0.51 €/kg; 510 €/Tonnes

Scenario 6:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Herring Meal LT92	0.71			22.33
Wheat feed	0.22			16.55
Soybean Meal-48	0.28		15.00	15.00
Poultry Meat Meal	0.38		25.00	14.68
Maize Gluten Meal	0.20		12.00	12.00
Blood, Haem prot conc	0.70	7.00	7.00	7.00
Fish oil	1.00			9.95
PNP VitMin Premix	0.30	2.00	2.00	2.00
Dical. Phos	0.20			0.49



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	95.85	0.06
02. Crude Protein	%	40.00	48.00	48.00	
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	-0.00
06. Crude Fiber	%			2.43	-0.00
07. Phosphorous	%	1.00		1.00	
08. Calcium	%	1.00		1.37	-0.00
09. Magnesium	%			0.13	
10. Avail. Phos.	%			0.73	0.00
11. Ash	%			2.55	
12. Lysine	%			3.08	
13. Methionine	%			1.03	
14. Met + Cys	%			1.54	
15. Arginine	%			2.87	0.00
16. Histidine	%			1.43	
17. Threonine	%			1.82	
18. Tryptophan	%			0.51	
19. Leucine	%			4.54	-0.00
20. Isoleucine	%			1.74	
21. Phenylalanine	%			2.29	
22. Valine	%			2.72	
24. Vitamin E	mg/kg			11.14	-0.00
26. Choline	mg/g			2.55	0.00

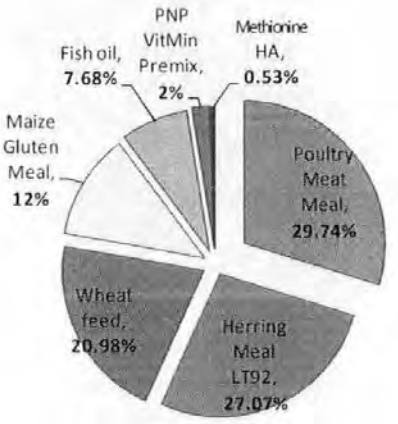
Cost of diet

0.47 €/kg; 470 €/Tonnes

Scenario 7:

Ingredient Restrictions

Ingredient	Price (€)	Min(%)	Max(%)	Usage(%)
Poultry Meat Meal	0.38		30.00	29.74
Herring Meal LT92	0.71			27.07
Wheat feed	0.22			20.98
Maize Gluten Meal	0.20		12.00	12.00
Fish oil	1.00			7.68
PNP VitMin Premix	0.30	2.00	2.00	2.00
Methionine HA	1.50			0.53



Nutrient Restrictions

Nutrient	Units	Min Limit	Max Limit	Actual	Shadow
01. Dry Matter (DM)	%		100.00	96.41	0.06
02. Crude Protein	%	40.00	48.00	48.00	0.00
03. DCP	%	40.00		40.00	
04. Crude lipid	%	15.00		15.00	
06. Crude Fiber	%			2.51	-0.00
07. Phosphorous	%	1.00		1.17	
08. Calcium	%	1.00		1.78	-0.00
09. Magnesium	%			0.18	
10. Avail. Phos.	%			1.04	
11. Ash	%			1.57	
12. Lysine	%	2.00		2.94	-0.00
13. Methionine	%	1.60		1.60	0.02
14. Met + Cys	%			1.74	
15. Arginine	%	2.00		2.97	-0.00
16. Histidine	%			1.04	
17. Threonine	%			1.81	
18. Tryptophan	%	0.24		0.45	
19. Leucine	%			3.98	0.00
20. Isoleucine	%			1.86	
21. Phenylalanine	%			1.97	
22. Valine	%			2.44	
24. Vitamin E	mg/kg			12.95	0.00
26. Choline	mg/g			3.31	0.00

Cost of diet

0.47 €/kg; 470 €/Tonnes

6.4 FINAL WORDS:

This thesis has embraced the conceptual strategy of using a selection of animal by-products as partial substitutes for fishmeal in balanced diet formulations for a major farmed marine species, namely gilthead sea bream. This represents a major fish group which can be found throughout the Mediterranean region and has wider implications for related species globally. This research has provided evidence that it is feasible to meet the needs of this species using high quality animal protein concentrates quite apart from the current trend towards only using plant based raw materials in aqua-feeds. The potential for the use of category three high grade animal derived materials is of course subject to much controversy due to the prevailing adverse public opinion surrounding the perceived risks such as TSE and prion related pathogenic agents associated with these commodities.

The socio-economic implications were not a central aspect for discussion in the remit of this thesis but must of course be considered in the wider feed industry. If progress is to be made in the goal of reducing feed costs, meeting sustainability of resources, traceability of the food chain whilst also meeting animal welfare criteria for fish, then a more pragmatic approach must prevail in future. The scientific objectives to provide a sound framework for using animal by-products in feeds for marine species such as the gilthead sea bream were realised in this work. As aquaculture continues to grow in importance in the world, commercial and industrial applications will become increasingly dependent on the evaluation of novel ingredients, including animal by- products. Such opportunities will have to comply with the social agenda as well as the scientific merits.

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