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DIGESTIBILITY, FEED UTILISATION, GROWTH PERFORMANCE AND HEALTH CRITERIA ASSESSMENT OF MEDITERRANEAN SEA BREAM *(SPARUS AURATA)* FED SELECTED ANIMAL BY-PRODUCTS

Fish Species to be investigated: temperate: European gilthead seabream

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Introduction

This report describes the conclusion of trials conducted for FPRF as agreed in the protocol forwarded initially in 2004 and describes investigations commenced in 2005 and completed in 2006. The main part of the study reports the testing of selected animal by-products for use in the Mediterranean fish species Gilthead sea bream, *Sparus aurata* which is one of the most popular farmed fish in Europe and is typical of most marine *sparids* found in many parts of the world. The application of a comprehensive digestibility trial is the perquisite for subsequent long term fish nutrition trials using balanced diets where various ingredients can be included within the diet to substitute more expensive ingredients in particular fish meal and fish oil. The trials were undertaken to provide a sound foundation for obtaining reliable digestibility coefficient data with respect to protein, lipid, energy and essential amino acids for sea bream. The work is a continuation of the previously successful digestibility trials performed with European sea bass and turbot. Together this embraced the first comparative work of its type for three important marine fish species.

Aquaculture production of high value species has the potential to increase further especially in marine locations where water is not as limiting compared to established freshwater sites employed for carp, *Cyprinus carpio* and rainbow trout, *Oncorhynchus mykiss*. These latter species have been the traditional major freshwater fish farmed for decades in Europe, the USA and India & China. The aquaculture sector is destined to become the main focus for future development with numerous candidate species being reared in different parts of the world (Ferlin & La Croix, 2000). This is especially true for Mediterranean marine fish such as sea bass and sea bream.

For example, there is considerable output of Gilthead sea bream, *Sparus aurata*, sea bass, *Dicentrarchus labrax* and turbot, *Scophthalmus maximus* in southern European regions close to the Mediterranean and North African States such as Tunisia, Algeria, Egypt and Libya. These have now become established aquaculture species making a valuable contribution for these nations.

It is generally accepted that fish such as gilthead seabream do not have a definitive protein requirement as such, but require instead a balanced complement of the 10- essential amino acids (EEAs) to meet known requirements.

There is a broad range of alternative protein sources of potential value for inclusion in farmed fish diets and these are mainly derived from plant and animal by-products as well as yeast, bacteria, algae and

single cell proteins. Tacon, 2005 has extensively reviewed the range of raw materials available for different fish species and regional practice. These are also applicable to the Mediterranean area.

In fish, an adequate protein level must supply the nitrogen source (N) for the synthesis and redevelopment of tissue and body protein during growth and different stages of development and especially reproductive demand (Wilson & Halver, 1986).

The concept of an 'ideal' dietary protein that is perfectly balanced to meet the exact needs of the animal is now accepted in farm animal nutrition and has become the basis for swine and poultry production (Cole & Van Lunen, 1994). This has also been confirmed for fish in a number of studies and is advocated for the formulation of advanced salmonid diets and for marine fish in general. It is now recommended that the minimum protein requirement where all the amino acids are equally limiting and commensurate with the maximum inclusion of non-protein energy forms the basis for the main specifications in most fish diets.

Animal derived proteins are stated to possess a fairly good amino acid balance and relatively high protein content. They may however vary in terms of their digestibility; amino acid profile and ash level but none the less provide a reasonable partial substitute for fishmeal in diets for farmed fish. Animal by-products such as poultry meat meal, steam hydrolysed / enzyme treated feathermeal and blood meals derived from abattoirs have considerable potential in fish and shrimp feeds. Williams et al (1997) reviewed the applications of rendered protein meals for use in aquaculture. For most species it was reported that even above 30% inclusion, there were no detrimental effects on fish and prawns or adverse taste of the products for the consumer. Although these materials have proven to be effective substitutes and secondary protein sources to fishmeal in temperate, tropical and marine fish species, their role must be addressed in the light of new information and public confidence in commercial animal based feeds.

One of the more promising ingredients available is Poultry Meat Meal (PMM), the rendered product of poultry processing by-products, manufactured from inedible portions of poultry, excluding feathers.

PMM has also been tested in diets for Chinook salmon *Oncorhynchus tshawytscha*, (Brannon et al., 1976; Roley et al. 1977; Fowler 1981a,b; 1990, 1991), Coho salmon *Oncorhynchus kisutch* (Markert et al. 1977; Higgs *et al*. 1979) and Atlantic salmon *Salmo salar* (Bergström 1973). Poultry meat meal has been studied as a partial fishmeal replacement in the diets of channel catfish *Ictalurus punctatus* (Brown *et al*. 1985) rainbow trout *Oncorhynchus mykiss* (Alexis, *et al*.1985; Bureau 1999, 2000). More recently Tibbetts et al. (2006); Subhadra et al. (2006); Rawles et al. (2006); and Zhou et al. (2004) have been able

to evaluate the potential of various animal by- products in diets for a number of marine species in accordance with fish nutrition protocols. These studies have shown that it is feasible to partially or include high levels to substitute and possibly replace fishmeal with suitable materials from either avian or porcine sources. However despite these optimistic results, there has been little information regarding the digestibility and full nutrition trial assessment in a major European marine species such as the gilthead seabream. This has been mainly due to concerns with respect to efficacy and safety resulting from previous perceived problems with prion related pathogens within the food chain. The present research was conducted objectively with category three defined materials or high standard to obtain reliable information to characterize the suitability of animal by products for sea bream.

Trial 1; Digestibility

Materials and methods

Diets

These were designed to primarily assess the digestibility coefficients of the assigned animal by-products within balanced diets for Gilthead sea bream. All diets were produced from the test materials provided by Prosper de Mulder Ltd (UK) and were namely, standard heat treated feather meal, enzyme treated feather meal, poultry meat meal, Spray Dried Haem, SDH (American Protein Corporation, USA) and blends of each feathermeal with SDH and Poultry Meat Meal (PMM). The reference diet was based on a prime quality low temperature fishmeal LT-94 Icelandic (Skretting UK).

The technical characteristic of the materials are based on specifications provided by the relative manufacturers. Poultry by-products were provided by Prosper De Mulder Group, Market Harborough, England.

Steam Hydrolysed Feathermeal was a mixed poultry feather source hydrolysed at 5.5 bars pressure for approximately 30 minutes. This was dried by an indirect steam drier (Rotadisc) to ~5% moisture. Enzyme Feathermeal was heated to 50C in presence of an enzyme and mixed for 30 minutes. Hydrolysis was followed by processing at 2 bars pressure for 15 minutes and steam heated to ~5% moisture. Poultry Meat Meal was form mixed poultry sources deemed fit for human consumption. The material was minced to <3mm introduced into a continuous process (Rotadisc) to evaporate water, sterilize in presence of natural fats. The residence time is about 90 minutes with a maximum temperature of 125 deg C. The resulting

material is concentrated by an expeller press to remove fat. The protein rich fraction is cooled and milled. The haemoglobin (Haem Protein Concentrate) was manufactured by American Protein Corporation (APC) Des Moines, Iowa, USA. The AP301 product is whole porcine blood, from animals slaughtered fit for human consumption. The blood is chilled and separated into plasma and red blood cell fraction (Haemoglobin). The latter is spray dried to produce a dry (<5% moisture) Haemoglobin powder.

Diets were prepared using a California Pellet Mill (CPM) in which all dry ingredients, vitamins and mineral premixes were uniformly mixed together before the addition of marine fish oil and de-ionised water. The resulting mixture was extruded through a 4mm aperture die and the resulting pellets air dried by convection until moisture content was <10%. The diets were all stored in plastic sealed containers and frozen prior to there use in the trials.

Table 1, Gilthead sea bream diet formulation (g/kg) (40/30% inclusion)

	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Fishmeal LT94	600	200	200	200	250	250	200	200
Marine Fish oil	100	120	120	100	150	137	100	100
Corn starch	212	201	201	212	186	195	214	201
Dextrin	68	59	59	68	94	98	66	59
Vitamins	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Minerals	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
HFM	0	400	0	0	0	225	0	0
EFM	0	0	400	0	0	0	0	300
PMM	0	0	0	400	0	0	300	0
SDH	0	0	0	0	300	75	100	100
Total	1000	1000	1000	1000	1000	1000	1000	1000

Table 2, Gilthead sea bream diet (nutrient analysis in %)

	Fishmeal	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	41.4	45.6	42.1	40.9	43.3	41.8	42.0	44.8
Lipid	20.91	8.46	10.84	23.02	12.94	8.56	17.79	15.18
Ash	9.2	4.8	5.9	8.1	5.8	5.5	7.5	5.4
Energy (MJ/kg)	22.1	23.1	23.1	21.9	22.5	21.7	22.0	22.9
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

Table 2 displays the proximate compositional analysis of the diets for protein, lipid, ash and energy as determined from standard AOAC methods. Amino acid profiles are also included for all 10 essential amino acids (EAA's).

Fish and experimental protocol

Juvenile Gilthead sea bream (36 g mean weight) were obtained from Aguarela-Sociedade de Piscicultura, Lda, Aveiro, Portugal and transferred to the experimental facility. The fish were acclimated for 3-weeks with medicated feed and there-after fed with a commercial sea bass diet. Fish were then transferred to specially designed digestibility trial tanks base on the Guelph system. The tanks dimensions were 40cm length, 17.5 cm width, and 27-38cm depth with a volume of 60L. These tanks had sloping floors and faecal material could be voided and recovered in external conical transparent separation chambers fitted with a valve.

25 fish were stocked per tank and the experimental diets allocated in triplicate to comprise 24 tanks in total within a semi-closed re-circulation system. The water temperature was held at 25C+/- 1C and salinity between 33-34ppt. The photoperiod was 12h light; 12 h dark throughout the study.

Sea bream were fed to satiation twice daily and after a period of 3-weeks fed each test diet, the faecal collection process was commenced. Uneaten feed was removed from the faecal collection traps at the base of each tank and this procedure was repeated until sufficient material could be recovered.

Faeces was oven dried at 105 deg C until constant dry matter was attained and ground into a fine powder and stored in air-tight containers until subsequent analysis.

Chemical analysis

All diets and faecal samples obtained from fish were analysed according to the protocols defined in AOAC and these were Kjeldahl Nitrogen for protein, and standard oven drying at 105 C for moisture until material attained constant weight. Ash was determined after ignition of samples at 550 C and subsequent calculation of residual material. Lipid was however extracted by a modified Folch method as described by Davies and Serwata previously**).** Energy values were determined by bomb calorimetry (Parr Instruments) using a standard Adiabatic bomb calorimeter with reference to benzoic acid as the standard for calibration.

Prior to amino acid quantification samples were subjected to 6N HCL hydrolysis for 24h in sealed glass ampoules, for tryptophan analysis the samples were subjected to 4N Methane Sulfonic acid hydrolysis for 16 hours. Amino acid analysis was undertaken using a Dionex Electrochemical Detector following chromatographic separation.

Chromic oxide (inert dietary marker) was analysed by a modification of the method of Furukawa & Tsukahara (1966) as reported by Gouveia and Davies (2000).

Digestibility calculations

The calculation of digestibility was undertaken according the following equation and for each nutrient in turn as determined in diets and corresponding faecal samples.

Digestibility of the nutrient components in diets were calculated according to equation (1) and the respective ingredient by the ratio of test ingredient contribution and reference diet as stated in equation (2). These are described by Lupatsch et al (1997) as applied to sea bream and established for many other fish species in aquaculture.

Formula 1:

ADC (%) = 100-[100× ($Cr_2O_{3\ food}$ / $Cr_2O_{3\ faeces}$) × (Nutrient $_{faeces}$/Nutrient $_{food}$)].

(Cr_2O_3 and nutrient in g kg^{-1})

Formula (2)

Partial digestibility coefficients were calculated using:

**$DC_T=[DC_D-(DC_r \times r)/t$

**Where DC_D is the digestibility coefficient of the nutrient in the diet (%); DC_R is the digestibility coefficient of the nutrient in the reference ingredient (%); DC_T is the digestibility coefficient of the nutrient in the test (%); r is the contribution of the nutrient

Results & Discussion

Faecal composition

The data shown in Table 3 summarizes the analysis of the faecal material obtained from each of the diets fed to 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 concentrations of marker and specific nutrients in diets.

Gross nutrient digestibility

Table 4A, shows the calculated apparent digestibility coefficient (ADC) profiles of each experimental diet mixtures and the coefficients for the specific ingredients tested. These results were obtained for juvenile sea bream conditioned to the experimental diets for a defined period and it can be viewed that they are representative of typical conditions for this species with respect to the feeding and temperature conditions.

The data shows the combined digestibility coefficients for a range of test diet mixtures fed to sea bream for all the major nutrient components important for diet formulations.
Extrapolation of the data using a specific calculation provided ADC values on an ingredient specific basis. 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 is very low for some ingredients.

The data presented in the table 4A provides values based on the standard Guelph (Cho) approach that incorporates the ratios of the test ingredient to the reference diet (40:60 in this case) as well as a calculation derived from the actual ratios of nutrients from each component of the mixed diets.
The latter is deemed to be more accurate and is the technique advocated by Forster (1999) for obtaining coefficients of digestibility of nutrients in feeds for fish.
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 of 82%- 88% for DM, energy and crude protein. High highest coefficient values for an alternate ingredient was obtained for poultry meat meal PMM with 80.61% (78.86%) for crude protein based on the Cho or Forster calculation respectively that showed close agreement. Lipid digestibility was 90% (91.53%) again showing very close values using the two calculation approaches. The overall energy digestibility value of 71.98% was consistent with the material and its performance in the nutritional trial was comparable with other fish species evaluated previously.
Disappointingly, both feathermeal sources (HFM & EFM) did not perform well in this evaluation for the Gilthead sea bream and protein digestibility was very poor without any indication that enzyme treatment had any benefit n improving digestibility of protein for this species. Coefficients were no greater than 23.6% and calculations based on the Cho ratio method yielded much lower values of 4-6%. Similar

discrepancies were obtained for the lipid digestibility coefficients with the higher values being for the Cho ratio at 59.29% and 66.03% for HFM and EFM respectively.

A spray dried haem protein concentrate produced reasonably good protein digestibility coefficients for sea bream with values of 78.86% & 80.61% for the two methods. Lipid values showed a large discrepancy with a DC of 65.83 using Guelph and 21.1 for Forster methods.

Digestibility values obtained for the blended mixture of either HFM, EFM with SDH appeared to be very poorly digested based on each method employed. This was especially noted for the protein content of these blended ingredients and it would appear that significant ingredient interaction has occurred within the composite diet mixtures. It is speculated that a complex may have occurred between the spray dried haem protein and the feathermeal resulting in a very poorly available protein within the blend that is quite unavailable to the digestive enzymes present in the sea bream intestinal tract.

Table 3, Nutrient composition of the faeces in % (±SEM, N=3) for the Gilthead sea bream trial and inert marker level

	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Crude protein	18.26±1.85	43.2±0.36	44.17±1.5	19.0±0.89	20.8±3.06	39.9±1.16	25.0±1.08	35.5±2.14
Crude Lipid	10.1±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	17.2±0.97
Energy (MJ/kg)	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
Chromic Oxide	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
Tryptophane	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

Table 4 A, Dry matter and nutrients apparent digestibility coefficients in Gilthead sea bream (Mean ± SEM, N=3)

ADC of diets	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Dry matter	71.82±1.45	48.75±6.41	52.57±2.63	65.81±2.89	68.33±3.63	42.5±10.9	59.57±2.07	40.24±1.82
Protein	87.49±1.71	51.55±5.78	50.09±4.40	84.04±2.00	84.26±3.85	44.7±11.6	76.01±0.77	52.51±4.31
Lipid	88.91±0.55	77.06±3.21	79.76±0.29	89.69±0.89	81.98±2.45	72.54±5.51	77.89±1.55	64.16±1.01
Energy	82.76±0.99	60.41±4.86	63.11±2.05	78.45±1.76	77.96±2.90	54.89±8.10	69.14±1.75	50.09±1.72
ADC of ingredients	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Protein (Forster)	87.49±1.71	23.56±9.04	22.37±8.56	80.61±5.32	80.77±9.80	12.58±6.83	65.74±1.84	27.01±6.96
Protein (Cho)	87.49±1.71	6.22±4.28	4.28±4.28	78.86±7.06	74.5±14.7	0<±n/a	58.80±2.81	6.52±3.50
Lipid (Foster)	88.91±0.55	33.5±13.3	24.23±3.06	91.53±2.75	21.1±21.1	4.56±4.56	45.65±7.29	0<±n/a
Lipid (Cho)	88.91±0.55	59.29±7.32	66.03±0.83	90.88±2.07	65.83±9.31	34.6±17.7	61.37±4.48	27.02±3.29
Energy (Cho)	82.76±0.99	26.9±10.9	33.64±6.53	71.98±5.16	66.7±11.9	10.73±5.54	48.69±5.67	3.34±1.87

Table 4B, Apparent availability (%) of essential amino acids in diets (left) and test ingredients (right) consumed by Gilthead sea bream

	Fishmeal	HFM	EFM	PMM	SDH	HFM/SDH	PMM/SDH	EFM/SDH
Arginine	91.72/91.72	54.51/32.29	58.51/28.14	84.73/79.01	83.15/74.70	40.78/-1.27	89.89/88.37	53.74/19.49
Histidine	85.27/85.27	36.27/-20.5	54.65/8.72	88.51/92.00	89.74/92.34	56.70/33.08	92.42/96.78	64.09/32.32
Isoleucine	87.13/87.13	51.04/24.78	56.38/10.24	82.41/76.38	72.02/29.11	33.88/-18.8	89.18/92.31	54.07/4.47
Leucine	89.22/89.22	48.31/18.90	48.87/11.6	83.42/78.10	87.71/86.68	43.40/4.81	90.88/92.14	56.87/8.34
Lysine	92.69/92.69	69.79/32.65	70.19/38.49	90.69/89.07	90.28/88.35	74.33/49.81	93.60/94.14	73.83/57.92
Threonine	84.76/84.76	37.98/5.30	44.51/14.79	77.02/70.44	79.52/75.00	25.54/-27.6	84.97/85.14	40.99/8.71
Tryptophan	87.99/87.99	36.67/-29.3	44.29/-9.79	82.76/76.91	87.73/87.43	42.52/-21.6	89.74/91.25	55.97/22.36
Valine	85.32/85.32	40.42/16.92	42.40/-21.9	80.65/76.47	84.17/83.53	32.21/-1.18	88.65/91.02	51.75/1.41
Methionine	88.38/88.38	56.03/-0.25	77.12/57.98	88.78/89.29	75.50/57.45	55.37/-4.93	90.01/91.65	64.75/28.76
Phenylalanine	83.55/83.55	43.13/12.22	44.50/14.08	75.90/67.84	82.53/81.77	29.26/-22.1	86.30/88.64	47.55/-6.45

Essential Amino Acid (EAA) availability

Table 4B displays the digestibility coefficients of the essential amino acids of the test ingredients and these show considerable differences for each of the animal by-products tested. It is clearly evident that in the main, trends occur that follow those seen for DC values for crude protein. From data it is seen that all EAA's within fishmeal are very highly available with coefficients ranging between 83.55- 92.69% for Phenylalanine and lysine respectively.

Lysine availability was very much reduced in both feathermeals at 32.65 and 38.49% respectively with a slight improvement for the EFM treatment. Lysine availability was >89% for PMM and 88.35% for spray

dried haem and 94% for the SDH/PMM blend. Lysine availability is generally regarded as fairly good indication of protein quality in terms of overall digestibility and degree of protein damage during processing.

Likewise leucine availability also showed very similar trends and was especially inferior for the blended composite ingredients SDH/HFM and SDH/EFM. Interestingly, methionine was very poorly digested within the standard feathermeal but was appreciably better in the enzyme treated meal (EFM). This sulphur containing amino acid is especially associated with feathermeal protein and is of importance in feed formulation since it is essential to fish.

Tryptophan was also well digested from fishmeal (87.99%) compared to the feathermeal sources but was again marginally better for the EFM. This essential amino acid was available by 77% for poultry meat meal fed to sea bream and higher at 87.43% for spray dried haem.

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.

Conclusions

The data presented in this report has provided a means to confidently screen the by-products for their efficacy as potential feed ingredients for Mediterranean Gilthead sea bream, *Sparus aurata*.

The values for protein and energy for individual animal by-products enabled balanced diet formulations to be designed and this was then implemented in a subsequent experiment to assess the best performing ingredients in a longer term nutritional trial with sea bream. These were the PMM at varying inclusion level up to 75% replacement of fishmeal protein, and low inclusions of enzyme treated feathermeal and spray dried haem.

The diet protein levels were assigned to provide 40% digestible protein using the values obtained from the previous digestibility trials for the protein sources. The formulation of the diets is shown in Table 5 and these diets are therefore isonitrogenous with respect to digestible protein level and essentially isocaloric in terms of lipid and carbohydrate content.

The trial was initiated in April 2006 and completed in August 2006. Full nutritional performance indicators such as SGR, FCR, ANPU and body compositional analysis were undertaken as well as

haematological measurements of health and relevant histological appraisal. The investigation was conducted in the Fish Nutrition Facility of the University of Plymouth in accordance with the institutional codes of practice of the Ethical Committee and the UK 1986 Animal Scientific Procedures Act.

Trial 2: Growth trial

Experimental protocol

Source and characteristics of ingredients:

Rendered animal protein tested (Poultry Meat Meal, Enzyme Feather Meal and Spray Dried Haemoglobin) were provided by UK's rendering plants (Prosper de Mulder, Doncaster UK). The high quality grade, low temperature Norwegian fish meal (LT-94) was obtained from Skretting Aquaculture (a Nutreco company, Preston UK). The proximate composition of all the main ingredients is given in table 5. A brief description of those raw materials is also provided within the notes of this table.

Table 5, proximate composition of major ingredients used as protein sources in the experimental diets.

	FM (LT94)	PMM	EFM	SDH
Moisture (%)	7.44±0.01	5.95±0.03	10.13±0.20	9.20±0.01
Crude Protein (%)	73.53±0.18	65.24±1.11	92.17±0.10	96.13±0.11
Crude Lipid (%)	10.04±0.30	12.40±0.05	2.44±0.04	0.01±0.00
Gross energy (MJ/Kg)	21.25±0.09	20.95±0.12	22.95±0.13	22.25±0.09
Ash (%)	13.27±0.29	17.00±0.21	2.61±0.06	2.63±0.39

PMM: ***Poultry Meat Meal (Natural Fat / Atmospheric)***: Mixed species poultry material (from animals slaughtered fit for human consumption) are reduced in size by mincing to less than 30mm, and then introduced into a continuous process (Rotadisc) that evaporates the water in the presence of natural fat levels and sterilises the components. The residence time is approximately 90 min 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 an antioxidant.

PMM: ***Poultry Meat meal (Added Fat / Atmospheric)***: Mixed species poultry material (from animals slaughtered fit for human consumption) are reduced in size by mincing to less than 30mm, and then introduced into a continuous process which contains high levels of poultry fat (1 part Raw Material / 5 parts Fat). The water is evaporated and the components are sterilised during the process. The residence time is approximately 30 minutes and the maximum temperature reached is 135-140°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 an antioxidant.

SDH: ***Spray Dried Haemoglobin***: The raw material used is whole porcine blood, from animals slaughtered fit for human consumption. 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 dry (<5% moisture) haemoglobin powder. The product is finaly cooled and bagged prior to despatch.

EFM: ***Enzyme hydrolysed Feather Meal***: Mixed species poultry feathers are heated to 50°C in the presence of an enzyme and co-factor mixture, and continually mixed for 30 minutes. Following this enzyme hydrolysis, the feathers are pressure

processed at 2 bars for 15 minutes. The enzyme hydrolysed feather meal is then dried in a Rotadisc drier to ~5% moisture, cooled, milled and stored.

Diet preparation:

Six diets were formulated and manufactured with animal by-products in order evaluate their potential as fish meal substitutes in a long term feeding trial. Those were including a control diet (where high quality Fish Meal were the only protein source) and 5 experimental diets (where Spray Dried Haemoglobin (SDH), Enzyme Feather Meal (EFM) and Poultry Meat Meal (PMM) were partially replacing Fish Meal (FM) with the different rates indicated in table 6). Using the digestibility coefficients pre-established, all diets were designed to contain 40% digestible protein and 15% lipids.

Diets were prepared using the California Pellet Mill of the University of Stirling (Scotland) in which all ingredients, vitamins and mineral premixes were uniformly mixed together before the addition of marine fish oil and de-ionised water. The resulting mixture were extruded through a 3 mm aperture die and the resulting pellets air dried by convection in a warm air cabinet (37°C) until moisture content was inferior to 10%. The diets were all stored and conserved in plastic sealed containers prior and during their use in the trial.

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 in 4 of the 16 tanks that compose the rearing system, then redistributed in 8 tanks a few weeks later and finally randomly assigned to the 16 tanks so that the stocking density equals 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 triplicates.

Facilities and experimental conditions:

Trial was conducted in the experimental facilities of the University of Plymouth (nutrition aquarium) in a closed marine system made up with sixteen 104L square fiberglass tanks. Within this closed rearing system, the natural sea water used was recirculated through mechanical and biological filters (located below the culture tanks) to ensure its purity. The water treatment system was actually consisting in sediment traps (horizontal screen filters and sedimentation chamber), a 1000L bio-filter compartment (submerged filter) and a D-Deltec protein skimmer. Partial water changes (amounting to approximately 20% of the system's volume) were nevertheless achieved every week while filters were cleaned daily to avoid any accumulation of waste products. Each tank (covered with grid to prevent fish from escaping) was supplied with the filtered sea water at a rate of $10L.min^{-1}$ (resulting in an important water exchange rate 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 33-34ppm, 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 ($CaOH_2$).

All groups of fish were fed by hand twice a day (two successive rounds for each meal). Fish were fed to satiation (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 (in order to get FCR values reflecting diets quality). The fish were fasted prior to the weekly weighing. The feeding occurred 6 days a week, except for the day of weighing. Also, the quantities of feed were adjusted accordingly based on new weekly fish biomass.

Analytical methods:

- *Feed efficiency, growth and survival indices:*

In order to follow growth and feed utilization, each group of fish were then weekly batch weighted. With the raw data collected the following growth and feed efficiency related parameters were established: Specific Growth Rate (SGR) = $[(\text{Ln } FBW) - (\text{Ln } IBW)/T] \times 100$ where *Ln* is natural log, *FBW* is Final Body Weight (g), *IBW* is Initial Body Weight (g) and *T* is time in days; Food Conversion Rate (FCR) = FI / WWG, where *FI* is feed intake (g) and *WWG* is wet weight gain (g).

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 content were then determined), ground into a homogeneous mass and stored in air tight plastic containers. Results obtained from those analyses allowed us to calculate nutrient retention indices such as: Protein Efficiency Ratio (%PER) = [(increase in wet biomass (g))/(amount of protein consumed (g))]×100; and Protein Conversion Efficiency (%PCE) = [(increase in carcass protein (g))/(amount of protein consumed (g))] ×100. Livers weight were also determined from another group of fifteen fish withdrawn at the start of the trial (during the final sampling, liver weighted were those dissected for histological examination). The information collected was used to calculate the Hepatosomatic 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 K= (Weight (g) × 100) / Length (cm) ^{3}. All mortalities were recorded and took into consideration to calculate the daily feed ration.

• *Chemical analysis of diets and fish carcasses:*

Diets, major ingredients and fish carcasses (sampled before and after the feeding trial) were subject to proximate composition analyses. 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.

Haematological analyses:

• *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 one unique aliquot) to realize erythrocyte, determine haematocrit values as well as total haemoglobin concentration.

- *Haematocrit determination:*

Two haematocrit values were obtained for each of the five fish sampled. Heparinised capillary tubes were filled three quarter 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.

- *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).

- *Erythrocyte counts:*

Erythrocyte counts were performed on diluted blood samples (1:100 dilution in Dacie's fluid) with a Neubauer haemocytometer. 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), 5 small squares in the centre of the grid were counted under a light microscope.

Table 6: Formulation (%) and Nutrient composition (±SEM) of experimental diets for Gilthead sea bream fed selected animal protein by-products for growth and feed performance trial

	FM LT94	PMM 25	PMM 50	PMM 75	EFM 5	SDH 10
Fishmeal	64	48	32	16	60.8	57.6
Poultry Meat	0	19	38	57	0	0
Enz. Feather	0	0	0	0	10.8	0
S. Dried Haem	0	0	0	0	0	6.8
Marine Fish Oil	7.4	6.77	6.22	5.67	7	7.95
Starch	11.33	11.33	11.33	11.33	11.33	11.33
Dextrin	5.67	5.67	5.67	5.67	5.67	5.67
Vitamin	0.5	0.5	0.5	0.5	0.5	0.5
Mineral	0.5	0.5	0.5	0.5	0.5	0.5
αcellulose	10.6	8.23	5.78	3.33	3.4	9.65
Total	100	100	100	100	100	100
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

Results

Growth performance

The results of the nutritional trial are displayed in Tables 6 and 7 which depict the growth and feed utilization performance of sea bream fed each diet and serological profiles respectively at the end of the 9 week investigation.

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 and feed conversions varying between 1.3 and 1.43. These are typical of juvenile sea bream and were in accordance with conventional data for this species under intensive fish farm situations.

The substitution of the fish meal component of the reference diet with 25%, 50 and 75% PMM resulted in a gradual trend for reduced final mean weight of sea bream which was not however significant. A longer feeding trial may have resulted in a more significant depression in growth performance. The SGR (Specific Growth Rate) data also indicated a progressive depression in relative growth rate which was again not significant. Reference to protein utilization (PER and NPU) provide additional data that support a trend for reduced protein utilization. A significant reduction was found for PER for PMM substitution at

75% of the protein but not for NPU. It would be reasonable to state that PMM could be included at up to 25% of the protein without detriment to growth performance.

Enzyme treated feather meal at 5% substitution of protein and Spray Dried Haem at 10% protein substitution resulted in the highest final mean weight for sea bream but was still not significant compared to the control. This was also reflected in the values for net protein utilization but was still not significant.

The carcass composition data of sea bream analysed at the end of the nutrition trial for the experimental diets were consistent with the profile of sea bream in the scientific literature. There were no obvious differences in moisture, lipid, protein and ash component in the final fish carcasses and the levels of these nutrients showed typical values for sea bream with slightly elevated levels of protein and lipid compared to the initial carcass profile of sea bream at the end of the nutrition trial.

Health assessment

The condition factor (K) and Hepato Somatic index (HSI) are displayed in table 7 with each parameters showing no significant changes following administration of the experimental diets containing animal by-products. These values were all supportive of good conformation of the sea bream after 9 weeks and a liver weight indicating optimum weight relative to body weight.

The haematology included measurement of haematocrit haemoglobin and total red blood cell count for sea bream at the end of the experimental period. The haematocrit value and supporting haemoglobin concentration indicated good erythrocyte levels in accordance with known values for teleost fish species. The red blood cell counts (RBCC) were also consistent and typical of healthy fish. It was concluded that diets did not affect the physiological function of the major blood components measured in this study.

Discussion & Conclusion

The integrated preliminary digestibility trial was a successful validation of selected animal by-products for use in formulated diets for the Gilthead sea bream *Sparus auratus* an important fish cultivated in the Mediterranean region of Europe and with related *Sparidi* species in other parts of the world.

The investigations using a preliminary digestibility appraisal followed by a nutrition trial with gilthead sea bream was compliant with the strategic approach presented by Glencross et al (2007) for the general evaluation of fish feed ingredients in aquaculture practice.

The results were used to evaluate the best performing ingredients for the subsequent nutrition trial with juvenile sea bream with experimental diets based on the known nutritional constraints for this species

obtained from the current literature. The experiment was conducted under conditions that were deemed to be representative of the optimum environmental conditions appropriate to aquaculture practice for bream in the Mediterranean and this accounted for the rapid growth response of the fish providing reassurance that the data reported was relevant and reliable. These experiments were performed under controlled conditions of temperature and photoperiod for this species.

At all stages, the fish within both trials exhibited high appetite rates and were healthy and disease free. The control fishmeal, fed fish group was the reference for each test treatment and all diets were designed to be iso-Nitrogenous and iso-Caloric with respect to digestible protein (based on the first trial) and in terms of lipid and energy. It was apparent that no palatability problems limited overall feeding response, but some constraints of feed intake are apparent and these probably were the cause of the reduction in SGR of the fish receiving proportionally higher levels of poultry meat meals. Although not deemed to be significant, elevated inclusion of poultry meat meals above 50% substitution of fish meal should be cautioned, however Feed Conversion Ration (FCR) was actually improved due to the reduced feed intake. In the growth study, enzyme treated feathermeal was tested at a low level (5%) and spray dried haem at (10%) and good performances were obtained. This complies to the work of Fowler (1991) who examined the potential of poultry by-product meals for Chinook salmon diets with good success. Haematocrit and haemoglobin levels were well within normal levels for this fish species. The haematocrit ranged from 42-37% whilst haemoglobin levels ranged from 7.81 to 7.25 g/dl, no significant differences were observed within any of these parameters. It is apparent from digestibility studies that lower coefficients of digestibility is encountered in bream for feathermeal and this limited its use in diets for growth studies. Similar findings have been reported for other fish species but Bureau et al (1999) obtained reasonably high values for feathermeal protein in test diets for rainbow trout. Spray dried haem is also a potential problem due to the very high iron and copper levels in this concentrate and the tendency to cause rancidity of the oil in feeds with inherent instability and off-flavours greatly reducing palatability. However very small inclusions would be of value as a natural organic source of iron and copper in fish diets especially those formulated with high inclusions of plant proteins. The benefits of various blood based by-product meals have been advocated by Tacon (2006) for use in aquafeeds with some concern to the use of haem concentrates containing very high levels of iron and copper.

In these experiments, the animal by-products were evaluated in isolation in relatively simple formulations whereas in practice, multiple ingredients would be used and their interactions either producing synergistic or even antagonistic effects to fish performance. These area areas for future consideration, as digestibility

of proteins and amino acids are not always additive in feeds and may contribute varying levels resulting in imbalances or complementary associations. The importance of maintaining an optimum amino acid balance in complete feeds for marine fish is essential and this must be addressed when high levels of animal by-products are used to replace fish meal in diets for Gilthead sea bream and other fish species as stated by Rawles et al. (2006).

The production of ingredients is a continuing process of development and refinement with major industrial advances in biotechnology leading to improvements that have elevated the nutritional value of animal by products such as poultry meat meal, feathermeal and blood meals for use in aquafeeds. This project has demonstrated that production of juvenile sea bream is feasible by moderate inclusions of specific animal by products to reduce the fishmeal burden. Evidently more work is needed to test these materials on fish approaching market size and there remains the problem of consumer acceptance which can be partially addressed by means of taste studies to assess fish eating quality and overall texture. Future investigations of this kind would increase awareness of the contribution animal proteins and fats can make in generating sustainable solutions to modern marine aquaculture of fin fish.

Future work would focus primarily on reducing the level of fat in poultry meat meal using various de-fatting technologies to remove the contribution of hydrogenated poultry fat which constrains the supplementation of fish oil in complete diets for fish. These limitations may result in essential fatty acid deficiencies at high poultry meat meal inclusion levels. De-fatting of material would produce a more protein rich ingredient and would enable nutrient dense feed formulations for aquaculture, the resulting animal fat could be exploited for use as a bio-fuel given the current world demand for alternative energy resources.

In conclusion, this work has highlighted the opportunities for further exploitation in aquafeeds for an important Mediterranean farmed species namely, gilthead seabream. It is likely that related fish species would result in similar growth rates and feed utilisation such as red seabream (*Pagrus major*), potentially sea bass (*Dicentrachus labrax*) and more recently barramundi (*L. calcarifer)*. All these species are currently being reared in many regions of the world and specialised feed formulations are required to meet their nutritional requirements for optimum growth and performance. From the current research investigation it is evident that poultry meat meal, enzyme treated feathermeal, and spray dried haem could offer a valuable new source of protein in balanced diets for these marine species. In this respect, further work would validate this concept.

Table 6, Growth performance, feed utilization and carcass composition of Gilthead sea-bream juveniles

Values are means of 3 or 2 (treatment 1 and 2) replicates ± SEM. In each row, values with same superscripts are not significantly different (Tukey's test)

Productivity index	Diet 1 Control	Diet 2 PMM25	Diet 3 PMM50	Diet 4 PMM75	Diet 5 EFM5	Diet 6 SDH10	Anderson-Darling	ANOVA
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
PER	1.51±0.02^{bc}	1.60±0.01^{cd}	1.48±0.02^{b}	1.37±0.01^{a}	1.54±0.02^{bcd}	1.62±0.02^{d}	P=0.492	F=23.02 P=0.000
aNPU	27.29±0.90^{ab}	28.12±0.18^{ab}	26.32±0.76^{ab}	25.56±0.20^{a}	28.21±0.65^{ab}	29.06±0.59^{b}	P=0.526	F=4.58 P=0.020
aNPU(t)	31.44±1.04^{a}	32.88±0.21^{a}	32.00±0.92^{a}	33.9±0.26^{a}	34.54±0.80^{a}	34.47±0.71^{a}	P=0.696	F=2.59 P=0.094

Carcass composition	Initial fish							
Moisture (%)	68.60±0.24	67.39±0.05 ^{a}	65.91±4.05 ^{a}	66.77±2.08 ^{a}	65.97±2.09 ^{a}	67.24±2.12 ^{a}	66.58±2.43 ^{a}	P=0.227	F=0.37 P=0.860
Crude protein *(% wet fish)*	16.19±0.32	17.23±0.31 ^{a}	17.05±0.05 ^{a}	17.08±0.21 ^{a}	17.57±0.05 ^{a}	17.36±0.24 ^{a}	17.16±0.14 ^{a}	P=0.091	F=1.11 P=0.415
Crude Lipid *(% wet fish)*	10.55±0.30	11.73±0.42 ^{a}	13.09±0.57 ^{a}	12.28±0.92 ^{a}	12.42±0.63 ^{a}	11.48±0.31 ^{a}	12.29±0.23 ^{a}	P=0.769	F=0.79 P=0.578
Gross Energy (MJ/kg) *wet fish*	7.91±0.04	8.22±0.39 ^{a}	8.98±0.42 ^{a}	8.54±0.24 ^{a}	8.52±0.34 ^{a}	8.36±0.22 ^{a}	8.59±0.17 ^{a}	P=0.430	F=0.64 P=0.673
Ash *(% wet fish)*	3.35±0.05	3.43±0.02 ^{a}	3.55±0.02 ^{a}	3.51±0.02 ^{a}	3.68±0.08 ^{a}	3.51±0.14 ^{a}	3.54±0.10 ^{a}	P=0.195	F=0.76 P=0.599

Table 7, Health related parameters established at the end of the trial (week9): General parameters and haematology

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

Morphometry	Diet 1 Control	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 Control	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

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