EFFECT OF EXTRUSION ON THE NUTRITIONAL VALUE OF PEAS (*Pisum sativum*), CHICKPEAS (*Cicer arietinum*) AND FABA BEANS (*Vicia faba*) AND INCLUSION IN FEEDS FOR EUROPEAN SEABASS (*Dicentrarchus labrax*) AND GILTHEAD SEABREAM (*Sparus aurata*)

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by

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To my parents

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged the work described in this thesis is the result of my own investigations.

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Date: _____

Abstract

The current general economic and food crises have generated an unsettled future for food and feed production and prices in general. Increasing demand, prices and fluctuations in supply in world markets for fishmeal, fish oil, soybean meal, maize and wheat meal emphasize the need to reduce the dependence of the fish feed industry on these ingredients by increasing choices among a wider range of raw materials. Legume seed such as peas, chickpeas and faba beans are promising ingredients for aquafeeds due to their high protein compared to cereals but also for their energy content. The objective of the present study was to investigate the use of these legumes in both high and low inclusion level in diets for the two main species farmed in the Mediterranean countries namely, European seabass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.).

In Chapter 3 the effects of different processing conditions were examined on whole seed flours of tested ingredients with respect to both nutritional and antinutritional factors and physical characteristics of the experimental diet pellets including high and low levels of each legume. Extrusion of raw material resulted in a clear reduction in trypsin inhibitors (TI) with chickpeas showing a decrease of up to 90% and complete inactivation for wheat flour, while for peas and faba beans reduction was less than 50% in most cases. Extrusion processing was less effective in the reduction of phytic acid and total tannins, occasionally reaching 22% and 18% respectively. Oligosaccharides and total NSP were not drastically affected by processing, however faba bean NSP showed greater reduction. A redistribution of soluble and insoluble NSP fractions was noted for chickpea and pea flours after extrusion. Physical characteristics of the pellets were not negatively affected for any of the tested diets.

In Experiment I the effects of total or partial wheat substitution by legumes were investigated on nutrient digestibility, gastrointestinal evacuation rate and serum glucose response of European seabass. Use of legumes in seabass and seabream diets resulted in an overall increase in gastrointestinal evacuation time and a delay in glucose load. Specifically, gastric evacuation time was greatly delayed when seabass was fed a diet with high levels (30%) of chickpeas, while foregut evacuation time was mostly delayed by the diet including a high level (30%) of faba beans. In addition, glucose levels in seabass serum were also affected by the type of carbohydrates ingested with wheat

starch showing more rapid increase and decrease of glucose compared to fish fed pea and chickpea diets, while faba bean starch resulted in a lower glucose peak.

In Experiment II growth, digestibility, hematological parameters, histological effects and fillet organoleptic characteristics and the interaction between starch inclusion level (8% and 16% or 17% and 35% of legumes respectively) and legume type were estimated when tested legumes replaced wheat in European seabass diets. Digestibility coefficients were satisfactory for all nutrients (>93%) while legume diets at a low level had beneficial effects on growth parameters when compared to the control diet, with chickpeas showing a significant improvement in SGR (P<0.05). High level legume diets did not result in any negative effect on growth. HSI was increased with increasing starch/legume inclusion in the feed and serum glucose also increased for fish fed high levels of faba beans and chickpeas. Carcass proximate composition was not affected by replacement of wheat in the diets, excluding the increase of fat content in fish fed chickpeas. Sensory analysis showed no differences between fish fed the control and high legume inclusion diets.

Lastly in Experiment III growth, hematological parameters, histological effects and the interaction between starch inclusion level (low and high) and legume type were evaluated when tested legumes were included in gilthead seabream diets. Decreased, but not significantly so, growth was observed for all diets including legumes compared to the control. Poorer SGR were observed for pea and faba bean diets when these legumes were included at high levels. Liver glycogen increased with increasing starch level, but HSI did not differ significantly for any of the diet treatments.

Histological examination of hindgut did not show pathological effects, such as enteritis, for in either species or for any of the diets. Increased absorptive vacuoles were found for control and pea diets (high level) only for seabass.

The findings of this thesis showed that the two important species cultivated in Mediterranean countries responded differently to the same raw materials used at high levels in the diets. Overall legumes had a strong effect on gastrointestinal evacuation reducing the rate of feed or digesta passage. Peas, chickpeas and faba beans successfully replaced wheat in seabass diets resulting in improved growth coefficients. However, when the same legumes included in seabream diets growth performance was not improved compared to the wheat based diet.

Acknowledgements

Abbreviations and Acronyms

ADC(s)   Apparent Digestibility Coefficient(s)

ANF   Antinutritional Factor(s)

ANOVA   Analysis of Variance

BW   Body Weight

CP   Chickpeas

DE   Digestible Energy

DF   Dietary Fibre

DP   Digestible Protein

EFA   Essential Fatty Acid(s)

FCR   Feed Conversion Ratio

FI   Feed Intake

B   Faba Beans

P   Field Peas

GET   Gastric Evacuation Time

GER   Gastric Evacuation Rate

HSI   Hepatosomatic Index

HUFA   Highly Unsaturated Fatty Acids

IAA   Indispensable Amino Acid(s)

I-NSP   Insoluble Non Starch Polysaccharides

NI   Nitrogen Intake

NSP   Non Starch Polysaccharides

PER   Protein Efficiency Ratio

PPV   Protein Productive Value

RSO   Raffinose Series Oligosaccharides

SGR   Specific Growth Rate

S-NSP   Soluble Non Starch Polysaccharides

TI   Trypsin Inhibitors

VSI   Viscerosomatic Index

Table of Contents

List of Figures

List of Tables

Chapter 1. General Introduction

1.1   Carbohydrates in fish nutrition

1.1.1   General

Numerous investigations have detailed the role of carbohydrates in the nutrition of farmed fish species (Wilson, 1994; Jauncey, 1998; Dabrowski and Guderley, 2002; Hemre et al., 2002; Krogdahl et al., 2005). Carbohydrates are non-essential in fish diet as the energy they supply can be replaced by protein or lipids and glucose requirements can be met by gluconeogenesis (Hemre et al., 2002) however, carbohydrates and especially starches can be generally utilized well (Wilson, 1994) and they can also enhance protein sparing effect for growth (Jauncey, 1998). Their digestion and utilization is species dependant with carnivorous fish being less able to utilise them than omnivorous and herbivorous (Krogdhal et al., 2005). Carbohydrates are intrinsic components of feedstuffs, most especially those of plant origin and in that context some dietary content is unavoidable. In addition their low cost per kJ and the beneficial effect on physical properties of the diets make carbohydrate important constituents of fish diets (Jauncey, 1998; Krogdhal et al., 2005).

1.1.2   Carbohydrate fractions

Carbohydrates can be classified into a number of categories according to their chemical structure and properties and these vary considerably amongst different carbohydrate types (Knudsen, 1997). The main carbohydrate classes of interest in animal feeds are starch, dietary fibre (DF) and low molecular weight sugars (LMW-sugars) or oligosaccharides (Knudsen, 2001).

Starch consists of two glucose crystalline polymer structures: amylose and amylopectin, with amylopectin being dispersed in amylose (Gallant et al., 1992). Amylose is a linear molecule in which the glucose units are connected with $\alpha(1\rightarrow4)$ glycosidic bonds and the degree of polymerization ranges between 600-6000 glucose units. Amylopectin is a branched helical crystalline molecule in which the glucose units are connected with $\alpha(1\rightarrow6)$ bonds for the branch point and $\alpha(1\rightarrow4)$ bonds for the linear parts, and polymerization for a single molecule may exceed 106 single glucose units (Bornet, 1993).

Starch can also be classified according to the digestive response, as readily digestible starch, slowly digestible starch and resistant starch, and resistant starch in turn can be divided into physically inaccessible, resistant starch granules and retrograded starch (Cairns et al., 1996). Starch digestibility in fish is variable ranging from 25% to 95% (Wilson, 1994; Shiau, 1997) depending on the fish species, the botanical origin and complexity of the starch, inclusion level in the diet, feeding strategy and processing methods such as cooking or extrusion (Wilson, 1994). In general processed starch is better digested than raw starch (Peres and Oliva-Teles, 2002; Venou et al., 2003).

DF can be categorized as lignin and non starch polysaccharides (NSP) (Knudsen, 1997) and these are commonly found in plant cell walls (Knudsen, 2001). Lignin basic units are phenylopropanes built in a branched network and NSP are divided into water soluble NSP (S-NSP) and water insoluble (I-NSP) (Knudsen, 1997; Knudsen, 2001). S-NSPs include pectins, hydrocolloids and some hemicelluloses, while I-NSP include cellulose and some hemicelluloses. The most common single molecules included in both NSP fractions to build the polysaccharide polymers are arabinose, xylose

(pentoses), mannose, glucose, galactose (hexoses), rhamnose, fucose (6-deoxyhexoses) and uronic acids (glucuronic and galacturonic acids) (Knudsen, 1997).

Animals including fish typically lack the enzymes needed to degrade NSP in the digestive tract that can be only fermented by intestinal flora (Dabrowski and Guderley, 2002). The presence of the S-NSP reduces the evacuation rate and increases the evacuation time of the digesta in the intestine (van der Klis and van Voorst, 1993; quoted in Choct, 1997), while the insoluble polysaccharides cause the opposite effects (Kirwan et al., 1974). Pectins and gums tend to increase the viscosity of the digesta in chicken resulting in lower digestibility and absorption (Choct et al., 1995), probably by obstructing the ability of enzymes to act in the bulk of digesta. NSP (guar galactomannans and alginates) inclusion in salmon (*Salmo salar*) diets resulted in reduced availability of diet nutrients (Storebakken, 1985; Storebakken and Austreng, 1987). Some NSP bind with bile acids, lipids and cholesterol causing increases in bile acid production from the liver with possible effects on the absorption of fats and cholesterol from the gut in rats (Levrat et al., 1996; Favier et al., 1997).

Finally, oligosaccharides are composed of raffinose, verbascose and stachyose as basic units. The oligosaccharides are often present in legume seeds causing flatulence or diarrhoea in monogastric animals due to a lack of the enzyme α-galacosidase which is necessary for breaking the sugar bonds (Siddhuraju and Becker, 2001; Vinjamoori et al., 2004). Oligosaccharides can be metabolised in the intestine of carp (*Cyprinus carpio*) by microbes, liberating short chain fatty acids, carbon dioxide and methane gas (Kihara and Sakata, 2002), a procedure that is known to exist in the hind gut of monogastric animals (Smiricky-Tjardes et al., 2003), however, in fish these studies are very scarce. Hung et al. (1990) found increased water and osmolality values in the distal

intestine of white sturgeon (*Acipenser transmontanus*) when fed high levels (27%) of sucrose, lactose or fructose compared to glucose, dextrin and raw corn starch.

1.1.3 Carbohydrate metabolism in fish

The ability of fish to utilize dietary carbohydrate varies among fish species, due to anatomical and functional differences of the gastrointestinal tract and the digestive associated organs (Krogdahl et al., 2005). In general fresh- and warm-water fish have much higher intestinal amylase activity than marine and cold-water fish, and herbivorous and omnivorous fish utilize carbohydrates better than carnivorous fish species (Dabrowski and Guderley, 2002).

Teleost fish are considered to have a high glucose tolerance compared to mammals, as wide fluctuations of blood glucose levels are normally found (Dabrowski and Guderley, 2002), with carnivorous species showing more persistent hyperglycaemia (Peres and Oliva-Teles, 2002) than omnivorous fish (Furuichi and Yone, 1981). Fish have been characterised as resembling higher animals with insulin dependent diabetes mellitus (Kelley, 1993) because they exhibit similar symptoms (Shimeno, 1991), however the persistent hyperglycaemia generally coincides with transient hyperinsulinemia (Moon, 2001).

There are digestive enzymes that function to break down nutrients in foods into compounds that can be absorbed across the brush border membrane of the enterocyte. These enzymes are mainly excreted into the lumen. The location in which these enzymes act characterizes this as extracellular digestion, membrane linked digestion or intracellular digestion (Rust, 2002). Carbohydrate digestion is an extracellular procedure that involves hydrolysis of complex carbohydrates in the stomach, intestine

and caecae as well as in the brush-border section of intestines where enzymes such as maltase and sucrase are present (Rust, 2002; Harpaz et al., 2005).

The major enzymes required for carbohydrate digestion are apparently present in fish (Rust, 2002) and also the enzymes for the major carbohydrate metabolic pathways, such as glycolysis, tricarboxylic acid cycle, pentose phosphate shunt, gluconeogenesis and glycogen synthesis, have also been demonstrated to be present (Hemre et al., 2002). Even though the various enzymes and metabolic pathways for glucose metabolism have been detected, the overall role and contribution of dietary carbohydrates to the total energy requirements of fish remains unclear (Krogdahl et al., 2005).

Enzyme distribution and concentration in the gut varies with the different intestinal morphologies and feeding habits of fish (Lunstedt et al., 2004). It is reported that carnivorous fish are characterized by higher trypsin and chymotrypsin activity (Eshel et al., 1993), while herbivorous and omnivorous fish are characterized by higher amylase activity (Hofer et al., 1982; Munilla-Moran and Saborido-Rey, 1996; Hidalgo et al., 1999). Changes in the type of diet, the source or the quantities of nutrients present, may change the enzyme concentrations, which in turn can affect the digestion and absorption of nutrients (Deguara et al., 2003).

Hormones related to glucose metabolism, apart from insulin, such as glucagon, glucagon-like peptides, insulin-like growth factors, growth hormone, somatostatins, cortisol and catecholamines are also present in fish (Moon, 2001). The hormonal control mechanisms of teleosts sometimes resemble those of mammals, such as the targets and mechanisms of actions of glucagon, but generally there are many differences between them (Hemre et al., 2002).

1.1.4 Effects of dietary carbohydrates in fish

Excessive amounts of carbohydrate in fish diets have been associated with increased glycaemia (Wilson, 1994; McGoogan and Reigh, 1996) and feed intake in rainbow trout (*Oncorhynchus mykiss)* and European seabass (*Dicentrarchus labrax*) (Hilton and Slinger, 1983; Perez et al., 1997). Increased hepatosomatic index (HSI) has also been associated with dietary carbohydrates in trout (Kim and Kaushik, 1992) and European seabass (Lanari et al., 1999; Perez et al., 1997), but no such effect was found for salmon (Arnesen et al., 1995). Excessive glycogen deposition in liver is also related to the presence of carbohydrates in diets for both salmon (Arnesen et al., 1995) and trout (Kim and Kaushik, 1992), while fat deposition in liver is associated with high carbohydrate content in diets for European seabass (Lanari et al., 1999). In addition, different morphological changes in liver tissue have been found in European seabass fed different starch sources (Russell et al., 2001).

Excessive fibre in aquaculture diets may also lead to a decrease in feed utilization by obstructing the action of digestive enzymes and diluting nutrient density (Booth et al., 2001). Non-starch polysaccharides may reduce utilization of other nutrients as they can bind water and minerals and absorb compounds such as sterols and acids (Krogdahl et al., 2005). There are cases that these properties can be beneficial to the animals, but most commonly they have negative impacts on nutrient utilization (Krogdahl et al., 2005).

1.2 World and Mediterranean aquaculture

1.2.1 General

Aquaculture production has been growing at an average annual rate of 10.5% per year worldwide for marine fish species and 8.8% for all aquaculture since 1970,

compared with 2.8% for terrestrial livestock and 1.2% for capture fisheries (SOFIA, 2006). China is the biggest world producer, contributing 69.6% of aquaculture production by quantity and 51.2% of world aquaculture value (SOFIA, 2006). Aquaculture quantities by category and their respective values for 2004 are shown in Figure 1.1.

Aquaculture Quantities and Values
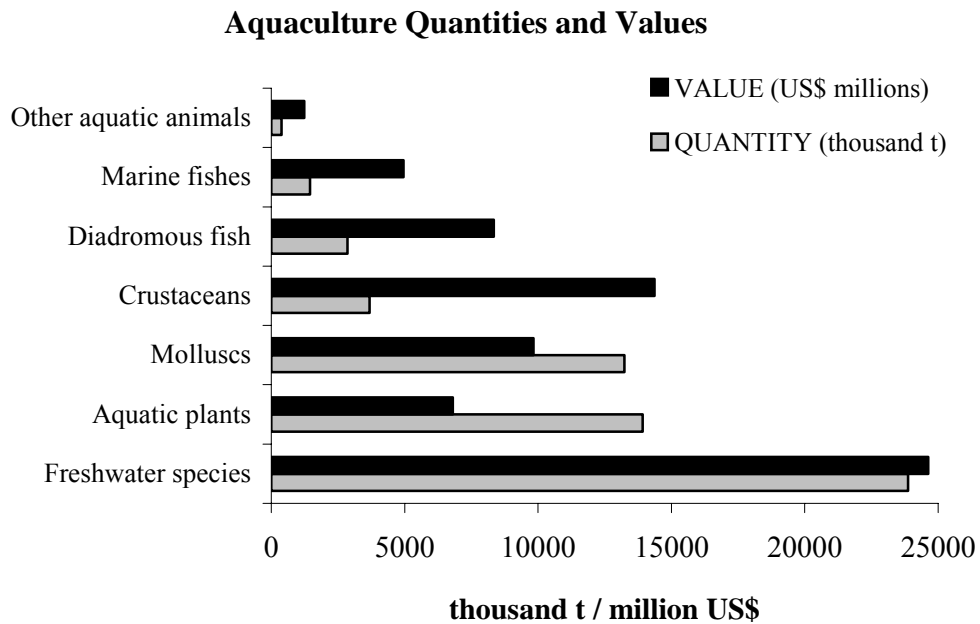
Figure 1.1. World aquaculture production in quantities and values in 2004. (Source: SOFIA, 2006)

1.2.2 Mediterranean Aquaculture

In 1985, Mediterranean fish farming was estimated to produce 374t of European seabass (*Dicentrarchus labrax* Linnaeus, 1758) and gilthead seabream (*Sparus aurata* Linnaeus, 1758), where Spain, Greece and France provided the initial impetus for production (FAO, 2008a). By 1990, production had increased to 3,876t where Greece, Spain and Italy had emerged to dominate with nearly 80% of all production. Five years

later production had increased twelve-fold to over 47,000t while, in 2000, combined Mediterranean production exceeded 130,000t, and in 2006 it was more than 190,000t (FEAP, 2008).

Currently, the production leaders are Greece with nearly 41% (79,000t) of total production, while the other important producing countries are Turkey (28% - 53,000t), Spain (15% - 28,000t) and Italy (9.3% - 17,800t). More than 90% of this production sector is thus focused on 4 Mediterranean countries (Aquamedia, 2008).

1.2.3 Seabass

France and Italy were the pioneers of reliable mass-production techniques for juvenile seabass in the late 1960s and by the late 1970s these techniques were dispersed and developed in most Mediterranean countries (FAO, 2008a). European seabass was one of the first marine non-salmonid species to be commercially cultured in Europe and at present is a very important commercial fish widely cultured in Mediterranean areas, with the most significant producers being Greece, Turkey, Italy and Spain (FAO, 2008a) as demonstrated in Figure 1.2.
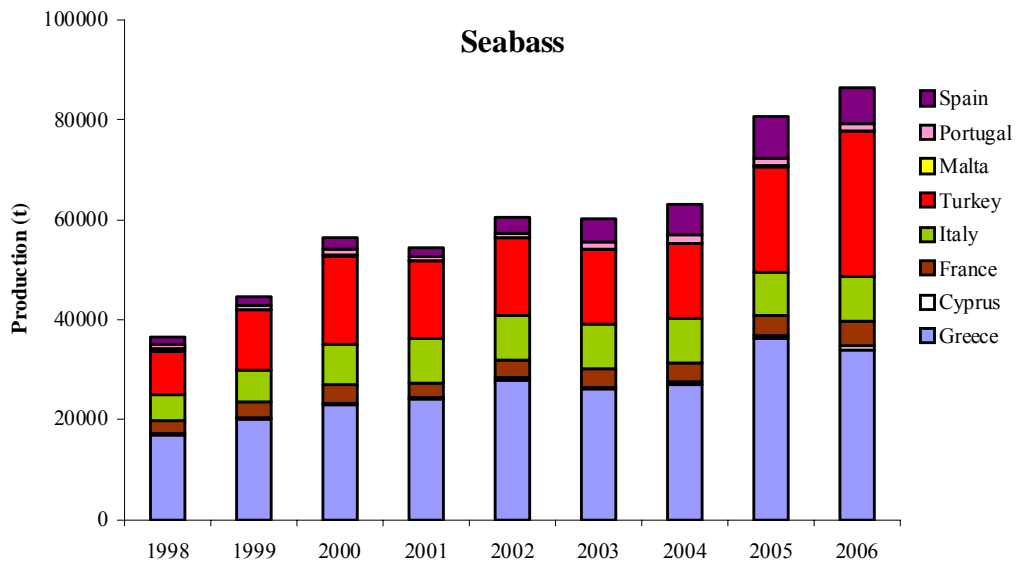
Figure 1.2. Aquaculture production of seabass in Mediterranean countries from 1998 to 2006. (Source: Aquamedia, 2008)

European seabass belongs to the family Moronidae (Superorder Teleostea, Order Perciformes, Class Actinopterigii), it is a euryhaline fish which is found in subtropical climates (66°-13°N) in the eastern Atlantic (from Norway to Morocco, the Canary Islands and Senegal) and also in the Mediterranean and Black Seas (Fishbase, 2008a). Seabass inhabits the littoral zone on various kinds of bottoms in estuaries, lagoons and occasionally rivers, and enters coastal waters and river mouths in summer, but migrates offshore in colder weather and occurs in deep water during winter in the northern range (Fishbase, 2008a). Seabass is a gonochoristic species with spawning taking place from December to March in the Mediterranean Sea and up to June in the Atlantic Ocean (Haffray et al., 2006). The eggs and larvae are greatly dispersed in the first 3 months of their life and adults migrate over several hundreds of kilometres (Haffray et al., 2006).

Seabass are predators and their natural diet consists mainly of shrimps, molluscs and fishes (Smith, 1990). Other common species detected in seabass stomachs are nekton including zoobenthos (amphipods), benthic copepods, crabs, insects, fish eggs and larvae, other planktonic invertebrates, mysids, cladocerans, planktonic crustaceans and copepods (Fishbase, 2008b)

Despite the commercial importance of seabass, there are no official tables for the nutritional requirements of this species, but there are numerous studies in this area as indicated in Table 1.1. The optimum dietary protein level for juvenile or fingerling seabass has been reported by Hidalgo and Alliot (1988) and Peres and Oliva-Teles (1999a) to be around 50%. Perez et al. (1997) observed optimum growth with 45% protein for fingerlings and Dias et al. (1998) with 43% compared to 52% protein inclusion level for 6g seabass.

Table 1.1. Seabass protein/fat ratio requirements

Reference	Composition of the diets % Protein / % Fat	Results
Alliot et al. (1974)	32,44,52,62 / 8,10, 12, 14	Best results 52/12
Hidalgo and Alliot (1988)	30,40,50,60 / 12	Best results 50%
Ballestrazzi et al. (1994)	44,49,54 / 12	49-54 equal results
Perez et al. (1997)	40-55 / 18-6	45/14 & 45/12 equal
Dias et al. (1998)	43,52 / 9,18	The final growth and PER were best for 43/18
Lanari et al. (1999)	48 / 11, 15, 19	19% fat improved growth but did not improve FCR
Peres and Oliva-Teles, (1999a)	48 / 12, 18, 24, 30	12-24% did not affect feed efficiency, but 30% adversely affect the results

Data for the quantitative requirements for the indispensable amino acids (IAA) for seabass are available from Kaushik (1998) and presented in Table 1.2. These results are based on determination of whole body IAA composition as this is considered to be representative of the IAA requirement profile (Mambrini and Kaushik, 1995). Results from other authors agree with these values concerning arginine (Alexis, 1997), lysine (Abd El-Hady and Habiba, 2003), methionine (Thebault et al., 1985) and threonine (Tibaldi and Tulli, 1999).

Table 1.2. A/E ratios and an estimation of indispensable amino acid (IAA) requirements as g/16 g N for seabass

IAA	A/E*	Requirements
Arginine	146.6	4.6
Lysine	153.6	4.8
Histidine	49.8	1.6
Isoleucine	84	2.6
Leucine	138.8	4.3
Valine	91.2	2.9
Methionine+Cystine	72.5	2.3
Phenylalanine+Tyrosine	83.4	2.6
Threonine	86.3	2.7
Tryptophan	19.4	0.6

*A/E ratio = IAA/total IAA x 1000. Lysine requirements are available and theoretical requirements were calculated for all other IAA (Source: Kaushik, 1998)

Optimal dietary fat level for European seabass fingerlings was estimated by Alliot et al. (1974) to be 12.5%. More recent studies (Peres and Oliva-Teles, 1999b) found no differences in growth performance of seabass fed diets ranging from 12 to 24% lipids, while with 30% lipids growth was depressed. On the contrary Lanari et al. (1999)

obtained better growth performances when the dietary fat was 19% compared to 11 or 15% in ongrowing seabass diets and similar results were observed by Dias et al. (1998) with 18% lipid (compared to 9%) in fingerling diets.

Starch rich grains are commonly included in feed formulations for substituting part of the protein and fat as an energy source and to improve the mechanical properties of the pelleted feed (Lanari et al., 1999). Pregelatinised starch is sufficiently digested by seabass (Spyridakis et al., 1986; quoted in Lanari et al., 1999) while Perez et al. (1997) found no significant differences in growth of seabass fed diets with 16 to 28% gelatinised starch, but depression occurred with 33% gelatinised starch.

Available data on vitamin and mineral requirements of marine species are very scarce. For seabass a requirement for vitamin C was demonstrated but not quantified (Alexis, 1997; Henrique et al., 1998). Fournier et al. (2000) showed that seabass require 120mg of ascorbic acid per kg diet for maximum growth to maintain normal skin collagen concentration and hepatic ascorbic acid saturation.

The digestible protein (DP) to digestible energy (DE) ratio of the diets also affects growth rate and feed utilization efficiency (Oliva-Teles, 2000). According to Dias et al. (1998) optimum protein to energy ratio of diets for seabass should be 19mgkJ^{-1} in diets with at least 20kJ DEg^{-1}. Lupatsch et al. (2001) determined the efficiencies of utilization of DE and DP for maintenance and growth in *D. labrax*, by feeding fish of various sizes at increasing feeding levels, from zero to maximum voluntary feed intake. The relationship between DE intake and energy gain was found to be linear and was independent of feed intake and body weight (BW). The requirement for DE for maintenance was calculated to be 43.6kJ BW (kg)$^{-0.79}$ day^{-1} and for DP 0.66g BW (kg)$^{-0.69}$ day^{-1}. The partial efficiency utilization for growth was 0.68 and 0.52 for DE and DP, respectively.

1.2.4   Seabream

Traditionally, seabream was extensively cultured in Italy and Egypt in coastal lagoons and salt water ponds taking advantage of the natural migration of juveniles from the sea into the coastal lagoons (FAO, 2008a). Intensive rearing systems were developed in early 1980s in Italy, and large-scale juvenile production was achieved in the late 1980s in Spain, Italy and Greece (FAO, 2008a). This species shows high adaptability to intensive culture conditions and nowadays Greece, Turkey, Spain and Italy are the main producers as demonstrated in Figure 1.3.
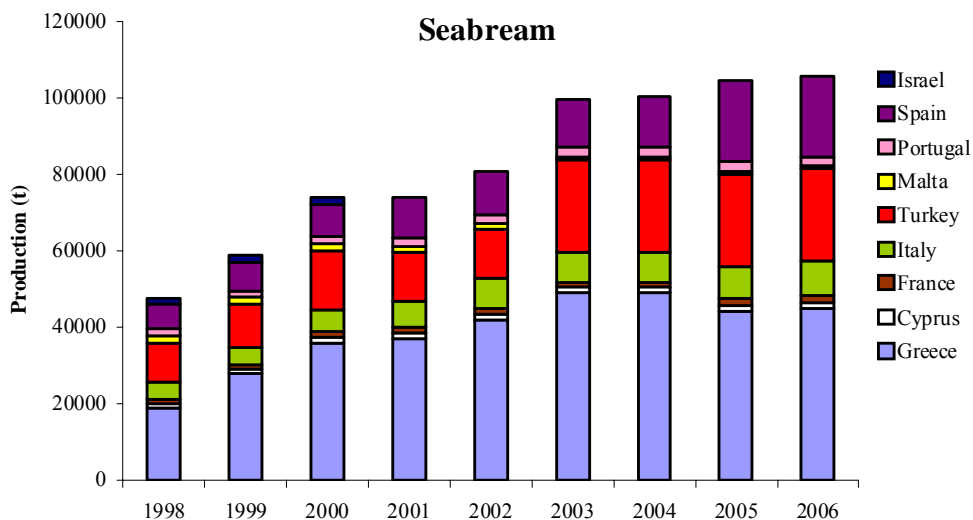
Figure 1.3. Aquaculture production of seabream in the Mediterranean countries from 1998 to 2006 (Source: Aquamedia, 2008)

Gilthead seabream, belongs to the family of Sparidae, (Superorder Teleostea, Order Perciformes, class Actinopterigii). *S. aurata* is a euryhaline fish, which is found along the eastern Atlantic coasts from Great Britain to Senegal and also in the Mediterranean Sea. The species inhabits seagrass beds and sandy bottoms as well as the surf zone, commonly to depths of about 30m but adults may occur to 150m depth. Due

to its euryhaline and eurythermal habits it is found both in sea and brackish water including lagoons and estuaries especially during the early stages of its life cycle (FAO, 2008a). Gilthead seabream is a protandrous hermaphrodite and the females can lay 20,000-80,000 eggs per day for a period of up to 4 months (FAO, 2008a).

Gilthead seabream is mainly a carnivorous fish and accessorily herbivorous (Bauchot and Hureau, 1990). The most common feed items reported in seabream stomach are phytoplankton, zoobenthos items including amphipods, benthic copepods and crustaceans, ostracods, molluscs, annelids, polychaetes, fish eggs and larvae, other planktonic invertebrates, cladocerans, planktonic crustaceans and copepods (Fishbase, 2008b).

Despite the commercial importance of seabream, there are no official tables for the nutritional requirements of this species, but there are a number of studies in this area listed in Table 1.3.

Sabaut and Luquet (1973) estimated the optimum protein requirement for maximum growth of juvenile seabream using semi-purified diets to be 40%, but more recent research reevaluates this percentage with practical diets to be 45-46% (Vergara et al., 1996a; Nengas et al., 1997). Similar results were observed when growing fish were fed 47 and 51% protein level when the fat level was also high (21%) (Santinha et al., 1999).

Table 1.3. Seabream protein/fat ratio requirements

References	Composition of the diets % Protein / % Fat	Results
Sabaut and Luquet (1973)	10-60 / 8	Minimum 40% protein
Santinha et al. (1996)	40,45,50,55 / 12	Best results 55%
Marti-Palanca et al. (1996)	45,52,60 / 8,12,17	Best results 60/12
Vergara et al. (1996a)	42,46,52,58 / 9,15	Best results 52/15
Santinha et al. (1999)	47,51 / 15,21	47/21 & 51/21 Equal
Fountoulaki et al. (1997)	40,46,51 / 11,16,21	46/16 & 51/21 Equal
Nengas et al. (1997)	38,45,51 / 10,15,20	Best results 45/15

In table 1.4 the requirements for the IAA for seabream are shown with the values representing the whole body IAA of seabream (Kaushik, 1998) as it is considered to be representative of the IAA requirements profile (Mambrini and Kaushik, 1995). Similar results from other authors were observed for arginine (Tibaldi et al., 1994) and lysine (Tibaldi and Lanari, 1991).

Table 1.4. A/E ratios and an estimation of indispensable amino acid (IAA) requirements as g/16 g N for seabream.

IAA	A/E	Requirements
Arginine	162.4	5.4
Lysine	149.6	5.0
Histidine	49.9	1.7
Isoleucine	78.9	2.6
Leucine	134.3	4.5
Valine	88.5	3.0
Methionine+Cystine	73.1	2.4
Phenylalanine+Tyrosine	86.4	2.9
Threonine	84.8	2.8
Tryptophan	19.4	0.6

*A/E ratio = IAA/total IAA x 1000. Lysine requirements are available and theoretical requirements were calculated for all other IAA (Source: Kaushik, 1998).

Gilthead seabream have been reported to require a minimum of 6.5% cod liver oil in the diet in order to maintain best growth and feed utilization (Kalogeropoulos et al., 1992). According to the same author, seabream juveniles require 0.9% highly unsaturated fatty acids (HUFA) in the diets and best growth performance was obtained with an eicosapentaenoic/docosahexaenoic fatty acid ratio (EPA/DHA) of 2/1 (Ibeas et al., 1997). Marais and Kissil (1979) observed best growth with a 9% lipid diet, the lowest value tested (9-16%) but according to Vergara et al. (1996b) best growth for 42g seabream was observed with a diet including 15-16% fish oil and no improvement in growth was found by raising the lipid percentage to 21% (Santinha et al., 1999).

As mentioned above, fish do not use carbohydrates efficiently. Seabream seems to have a ratio of amylolytic to proteolytic enzymes higher than rainbow trout and this may influence the carbohydrate digestion capability of these species (Hidalgo et al., 1999).

Available data on vitamin and mineral requirements of marine species are very scarce. For seabream a requirement for vitamin C was demonstrated but not quantified (Alexis, 1997; Henrique et al., 1998) and some limited data for pyridoxine are available (Kissil et al., 1981). Preliminary results showed a dietary phosphorus requirement around 0.75% of the diet (Oliva-Teles and Pimentel-Rodrigues, 2004).

1.3   Feed formulation

1.3.1   General

Successful fish culture depends on supply of diets containing optimal levels of energy and nutrients for growth. Diet preparation is in fact a compromise between the ideal situation and practical considerations (Hardy and Barrows, 2002). The main objective when formulating a fish diet is to provide a nutritionally balanced mixture of

ingredients to support the maintenance, growth, reproduction and health of the animal at an affordable cost (NRC, 1993). As feed is one of the principle costs in feed production (Lupatsch et al., 2001), formulations must be based on our knowledge of nutritional requirements for them to be economically viable.

In intensive aquaculture, fish feeds are formulated to be dense in nutrients and energy, based mostly on ingredients of marine origin, particularly fish meal and fish oil (Kaushik, 2000). When the requirements of certain fish species are known, it is not difficult to formulate a diet that contains all nutrients at an appropriate level. However, there are practical matters to consider that make this task complicated. The most important of these are the price and the availability of the ingredients, diet acceptability to the farmed fishes, pelletability of the formulation, pellet storage and handling requirements (NRC, 1993). What is also crucial with regard to the aquatic environment, is that the pellets should remain intact in water until fish consume them, while the nutrients should be highly available to the fish (NRC, 1993) to minimize excretions and therefore avoid settlement of organic matter on the bottom with possible impacts on the sediment under and around sea cages (Mente et al., 2006)

1.3.2   Commonly used ingredients and the limitations

The ingredients that are used in fish feed are either by-products of human food technology or products produced directly for fish consumption. Ingredients used in commercial fish diets can be classified as sources of protein (amino acids), fats (EFA), carbohydrates, vitamins and minerals (NRC, 1993). It is also very important to cover the energy requirements of fish, although energy is not considered to be a nutrient in an independent sense.

The main protein source that is used in fish feeds is fishmeal. When prepared from good quality, whole fish it is one of the highest-quality protein sources. Depending on the fishmeal quality, protein level ranges from 56% to 76%, it is also a rich source of energy, EFA and minerals and is highly digestible and palatable to most fishes (NRC, 1993). Fishmeal availability is limited due to somewhat stable production and increased demand (SOFIA, 2006) and thus it is an expensive ingredient (Josupeit, 2008) that contributes greatly to the final cost of fish feeds.

Soybean meal is an available ingredient with high protein content and one of the best amino acid profiles among protein-rich plant feedstuffs for meeting most of the essential amino acid requirements of fish (Mohsen, 1989; quoted in NRC, 1993). It has long been used as a substitute for animal protein in aquaculture feeds. The antinutritional factors (ANF) that are present in soybean seeds and the considerable rise in soybean price over the last year (Josupeit, 2008) are restricting factors for their use in fishfeeds. In addition, the strict rules of EU countries adopted in 2003 relating to the release of genetically modified seeds into the environment, their traceability, labelling and their use in animal feeds (Euractiv, 2006) also proved to be a limiting factor in their use in fishfeeds.

Many other plant protein sources are used, proteins that are extracted from oilseeds such as rapeseed and sunflower, or proteins from cereals like wheat and maize gluten (Aslaksen et al., 2007). Their availability is high, but their use is limited either due to ANF (Aslaksen et al., 2007) or due to imbalanced amino acid profile; for example the lysine deficiency of glutens (Davies et al., 1997; Pereira and Oliva-Teles, 2003).

Fats and oils are the main sources of energy but they also provide the EFA, with marine fish oils containing 10-25% HUFA (NRC, 1993). Marine oils are obtained from

processing of marine animals and are classified as fish oils, fish liver oils and marine mammal oils (Hertrampf and Piedad-Pascal, 2000). Their use in fish feeds is restricted by the availability, the high prices (SOFIA, 2006) and the possible contaminants present like dioxins or dioxin-like polychlorinated biphenyls that can be transferred to, and accumulated in, the edible parts of farmed fish (Bell et al., 2005). Plant oils such as soybean, canola (Glencross et al., 2003), linseed (Bell et al, 2004), rapeseed and palm oil (Karalazos, 2007) can be used as substitutes for fish oil, although their fatty acid compositions differ significantly, with vegetable oils containing lower levels of n-3 fatty acids (particularly HUFA) than fish oil. The use of vegetable oils in fish diets can differentiate the fatty acid composition of the produced fish (Bell et al., 2001; 2003), but this effect can be largely overcome after a finishing fish oil diet before harvest (Bell et al, 2004).

Carbohydrate sources such as cereals are present in fish diets as a binder as well as an inexpensive energy source (Davis and Arnold, 1995). Cereal whole grains contain 62-72% starch and this is an important binding agent in steam–pelleted and extruded feeds (NRC, 1993). Many by-products of grain industry as for example wheat, oat, corn, rice, milo or rye by-products are valuable ingredients for animal feeds and subsequently for fish feeds (Hardy and Barrows, 2002). Legumes such as peas, beans and chickpeas contain also considerable amounts of starch that could be utilised by fish as energy source (Booth et al., 2001). The use of these plant ingredients could be limited when their fibre content is high (Nengas et al., 1995) or when they are not processed, as raw starch is considered a poor energy source (Peres and Oliva-Teles, 2002). However, digestibility of raw starch can be enhanced considerably by gelatinization through heat treatment (Peres and Oliva-Teles, 2002) which is a common practice in commercial feeds.

Other materials that are used in fish feeds in lower quantities are vitamin and mineral premixes, feed binders, carotenoid supplements, drugs and antibiotics, probiotics, enzyme supplements, antifungal agents, antioxidants, fibre, colourings, flavourings and water (Hardy and Barrows, 2002).

1.3.3   The need to identify new/novel feed ingredients

The current general economic and food crisis brought about by increasing oil prices as well as the expansion of the bio-ethanol and bio-diesel industry have generated an unsettled future for food and feed production and prices in general. Increasing demand, prices and fluctuations in supply in the world markets for fishmeal, fish oil, soybean meal, maize and wheat meal, emphasize the need to reduce their incorporation in feeds and at the same time increase the range of raw material sources.

Fishmeal and fish oil production have been more or less stable over the last few decades (SOFIA, 2006), while events such as the El Ninõ in 1997-1998 have shown that fish oil and meal can become unpredictable raw materials for aquaculture production in specific years.

Furthermore, since European production of soybean is extremely limited, due mainly to climatic and geographical constraints (P.E.A., ÉCLAIR, Programme, 1993; quoted in Gouveia and Davies, 1998), it is considered worth exploring the possibilities of using other plant sources which are cultivated in European and Mediterranean countries, thus limiting the expense of importation. Peas, chickpeas and faba beans with their protein and starch inclusion levels could successfully replace wheat and partially replace other plant or animal proteins.

1.4 Legume seeds

1.4.1 General

Grain legumes are the harvested seed of leguminous crops, which includes beans and peas and other closely related species within the family Fabaceae. Legumes play an important role in the traditional diets of many regions around the world because of their high nutritional value for humans. They are low in fat content and they are an excellent source of protein, dietary fibre and a variety of micronutrients and phytochemicals (Messina, 1999). Legumes are also traditional sources of plant proteins for ruminants and monogastric animals and can provide a range of benefits both for farms and feed manufacturers (FAO, 2002). Soybeans are the most extensively evaluated and most commonly used plant protein source in fish diets. The nutritional value of legumes such as lupins, field peas (Burel et al., 2000) have been evaluated in rainbow trout and turbot (*Psetta maxima*), faba beans in salmon (Aslaksen et al., 2007), while cow peas, chickpeas and vetch (Allan et al., 2000) in silver perch (*Bidyanus bidyanus*) as potential protein or/and energy sources. Legumes usually contain more lysine than cereals but they have lower digestibility of sulphur amino acids (Allan et al., 2000).

The EU is the major producer of peas (2.8mmt) grown essentially for animal feed, while Canada has significantly increased production (2.1mmt) since 2000 (Grain legumes, 2007). The major producer of faba bean is China (1.8mmt), but important harvests are also produced in Australia, the United Kingdom and France. Chickpeas are mainly produced in India (5.3mmt) (Grain Legumes, 2007).

Field pea is the main cultivated arable protein crop in the EU with spring type, white-flowered, low-tannin varieties being the most popular (Grain legumes, 2006). Faba beans have been used for decades for human consumption in the south and for

cattle or pigeon feed in the northern part of Europe, while currently areas of cultivation are expanding ranking this crop as the second most cultivated legume in Europe (Grain legumes, 2006). Chickpea is cultivated exclusively in the south of Europe and is mainly destined for direct human consumption. The 'kabuli type' (large white seeds) is the most popular type for both cultivation and consumption in Europe (Grain legumes, 2006). The areas cultivated with each legume in E.U. are represented in Figure 1.4.

EU grain legume crops (areas in 2004)
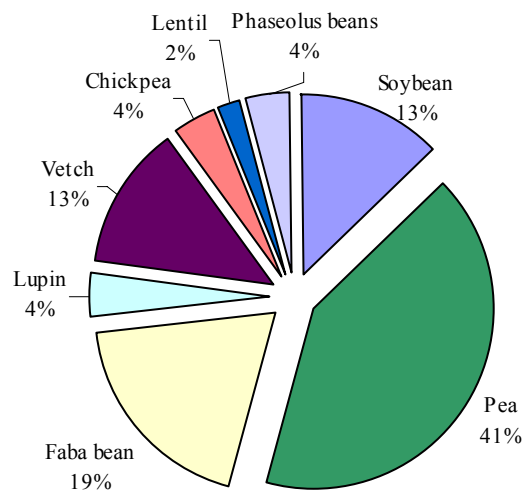
Figure 1.4. Areas as a percentage of total in the EU cultivated with legume seeds (Source: Grain legumes, 2006)

1.4.2 Field peas in aquaculture feeds

Field or feed or green pea (*Pisum sativum* L.) is a legume with potential due to the fact that it has been used in livestock feeds for a long time as a source of energy and protein, but has only recently been evaluated in feed for aquatic species (Davis et al., 2002). The average protein content of whole peas is around 21% which is low compared to soybean and lupins, but it is high compared to cereals, and it is rich in starch (around 45%) and has an energy content of 15.8kJg^{-1} (Sauvant et al., 2004). Field

peas have been evaluated as potential feed ingredients, whole or dehulled, raw or processed, for several aquatic species including European seabass (Gouveia and Davies, 1998; 2000), Australian silver perch (Allan et al., 2000), Atlantic salmon (Carter and Hauler, 2000), rainbow trout (Gomes et al., 1995a), turbot (Burel et al., 2000) and blue shrimp, *Litopenaeus stylirostris* (Cruz-Suarez et al., 2001) and other species as presented in Table 1.5.

In general most studies indicate that dehulled peas and extruded pea seed meal have higher Apparent Digestibility Coefficients (ADCs) for energy and crude protein than whole or raw peas respectively (Booth et al., 2001; Booth et al., 2002; Davis et al., 2002; Thiessen et al., 2003; Allan and Booth, 2004).

Table 1.5. Results concerning protein and energy digestibility and growth in one case of field pea and diets including field pea for different aquaculture species.

Fish Species	Inclusion rate	Field Pea	Protein digestibility %	Energy digestibility %	Reference
Silver perch *Bidyanus bidyanus*	50%	Whole-extruded Dehulled-extruded Whole-raw Dehulled-raw	85.3 90.4 84.3 87.8	71.3 74.9 63.7 70.1	Allan and Booth (2004)
Silver perch	15%, 30% 45%, 60% 75%	Dehulled	The weight gain reduced as the inclusion rate of peas increased		Booth and Allan (2003)
Rainbow trout *Oncorhynchus mykiss*	20%	Whole-raw Dehulled-raw Dehulled-extruded Air-classified Protein	90.9* 91.4* 93.5* 94.6*	54.6* 56.8* 78.4* 87.0*	Thiessen et al. (2003)
White shrimp *Litopenaeus vannamei*	25%	Whole-raw Whole-extruded Dehulled-raw Dehulled-extruded Whole-micronized	77.4 81.6 78.1 79 83.3	72.6 76.7 77.3 79.1 78.9	Davis et al. (2002)
Seabream *Sparus aurata*	17.5% (1) 35% (1) 19% (2) 37% (2)	1.Dehulled-defibred-extruded-microground 2.Infrared radiation	92.8 81.4 89.7 90	92.2 77.7 79.7 68.7	Pereira and Oliva-Teles (2002)
Silver perch	15%	Cold pelleted Steam pelleted Extruded	78.9 83.7 80.7	58.6 63.5 70.0	Booth et al. (2002)
Silver perch	30%	Whole peas Dehulled Protein concentrate	87.6 89.2 92.4	71.6 75.3 82.4	Booth et al. (2001)
Seabass *Dicentrarchus labrax*	20% 40%	Pea seed meal	88.4 89.1	73.6 72.1	Russell et al. (2001)
Blue shrimp *Litopenaeus stylirostris*	30%	Whole - raw Whole - extruded Dehulled - raw Dehulled - extruded Whole - micronized	87.3 89.3 89.1 88.2 87.7	-	Cruz-Suarez et al. (2001)
Silver perch	29.7%	Pea seed meal	81*	51*	Allan et al. (2000)
Seabass	10% 20% 30%	Pea seed meal	94.2 94.3 94	90.8 89.6 88.4	Gouveia and Davies (2000)
Atlantic salmon *Salmo salar*	20.5 27.5	Extruded Protein concentrate	95.2 95.5	88.8 89.2	Carter and Hauler (2000)
Nile tilapia *Oreochromis niloticus*	30%	Extruded pea seed meal	92.6*	89.2*	Fontainhas-Fernandes et al. (1999)
Seabass	20% 40%	Pea seed meal	88.4 89.1	64.8 56.7	Gouveia and Davies (1998)
Rainbow trout	30%	Pea seed meal	80.4*	59.2*	Gomes et al. (1995a)

*Numbers with an asterisk represent apparent digestibility of the ingredient included in the test diets.

1.4.3 Chickpeas in aquaculture feeds

Chickpea (*Cicer arietinum* L.) is grown in tropical sub-tropical and temperate regions as an annual plant and it is considered drought tolerant (Bhardwaj et al., 1999). Based on seed colour and site of origin, chickpeas are classified into two types, the kabuli and the desi type that differ in their nutrient composition, with kabuli types having a lower fibre, higher starch and fat content than desi types (Gill et al., 1996). The average protein content of the Mediterranean kabuli type seed is 20%, starch content 45% and energy 17.5kJg^{-1} (Sauvant et al., 2004).

Research on this plant material as an ingredient in aquaculture feeds is very scarce. Chickpeas have been evaluated as a potential fish feed ingredient by Allan et al. (2000) and Booth et al. (2001) for silver perch (Table 1.6).

Table 1.6. Results concerning protein and energy digestibility of chickpea and diets including chickpeas.

Fish Species	Inclusion rate	Chickpeas	Protein digestibility %	Energy digestibility %	Reference
Silver perch *Bidyanus bidyanus*	29.7%	Whole chickpeas (desi type)	82.2*	54.8*	Allan et al. (2000)
Silver perch	29.7%	Whole chickpeas Dehulled	88.1 87.5	72.1 74.2	Booth et al. (2001)
Silver perch	29.7%	Whole chickpeas Dehulled	79.2* 79.8*	54.8* 61.3*	Booth et al. (2001)

*Numbers with an asterisk represent apparent digestibility of the ingredient included in the test diets.

1.4.4 Faba beans in aquaculture feeds

Faba beans (*Vicia faba* L.) are also known as field beans, broad beans and horse beans and the varieties can be divided into summer and winter (Hertrampf and Piedad-Pascal, 2000) or white and coloured flower varieties with low and high tannin contents respectively (Sauvant et al., 2004). The average protein content of faba beans is 26%,

the average starch content is 38% and energy content is estimated to 16.2kJg^{-1}. Faba bean is a common human food in developing countries and it is also used as animal feed, mainly for pigs, horses, poultry and pigeons in industrialized countries. The nutritional value of faba beans is high and it is considered in some regions to be a good substitute for meat or skimmed milk (Duke, 1981).

Research on this plant material as an ingredient in aquaculture feeds is scarce. Both digestibility and nutritional value of faba bean meal have proved favourable for rainbow trout and common carp (Grabner and Hofer, 1985) and they have also been tested in more recent studies in silver perch (Allan et al., 2000; Booth et al., 2001), in Nile tilapia, *Oreochromis niloticus* (Fontainhas-Fernandes et al., 1999) and in rainbow trout diets (Gomes et al., 1995b) as listed in Table 1.7.

Table 1.7. Results concerning protein and energy digestibility of faba beans and diets including faba beans for different aquaculture species.

Fish Species	Inclusion rate	Faba beans	Protein digestibility %	Energy digestibility %	Reference
Silver perch *Bidyanus bidyanus*	29.7%	Faba beans	90.5*	59.2*	Allan et al. (2000)
Silver perch	29.7%	Whole beans Dehulled Protein concentrates	90.5* 96.4* 94.3*	59.2* 59.6* 73*	Booth et al. (2001)
Silver perch	29.7%	Whole beans Dehulled Protein concentrates	89.7 91.3 91.7	74.0 74.0 77.1	Booth et al. (2001)
Nile tilapia *Oreochromis niloticus*	30%	Faba bean meal	87.5*	68.0*	Fontainhas-Fernandez et al. (1999)
Rainbow trout *Oncorhynchus mykiss*	30%	Faba bean meal (fijord)	80.2*	60.2*	Gomes et al. (1995b)

*Numbers with an asterisk represent apparent digestibility of the ingredient included in the test diets.

1.4.5 Challenges and limitations

1.4.5.1 Potential of peas, chickpeas and faba beans

As mentioned before peas, chickpeas and faba beans are all legumes and they are good sources of both protein and starch. In this respect these three legumes offer flexibility to the feed manufacturer as they can replace partially both energy (cereals like wheat that is also used as binding agent) and protein (such as soybean or other plant and animal protein) (Fraser, 2005). These replacements could be very important if they do not have a negative impact on fish growth, fish health and final product quality.

'Green' issues, environmental impacts and sustainability are all of considerable current concern and these legumes may have advantages in this respect. Their cultivation is suitable for climate conditions in southern Europe. This coincides with regions where European seabass and gilthead seabream are also farmed, potentially reducing transport costs and also soybean imports. In addition, cultivations of legumes may decrease nitrogen pollution through their ability to naturally fertilize poor fields that for years have been cultivated with cereals and they can also increase total yields through rotation (Grain legumes, 2006). After harvesting legumes a substantial amount of nitrogen is left in the field through incorporation of the plant residues adding at the same time the needed organic matter to maintain and improve soil health, long term fertility and sustainability of the ecosystem (Gaur et al., 2008). Chickpeas have an additional advantage over peas and faba beans that they can be cultivated in a wide range of different soil qualities (Davies et al., 1985) and they have low water demand (Loss and Siddique, 1997), as in their normal growing season they do not depend on rainfall water, considering that they cultivated on non-irrigative fields (Gaur et al., 2008).

1.4.5.2 Antinutritional factors

Plant materials are commonly used in fish feeds but their inclusion level is often limited due to lower levels of available protein and palatability issues compared to animal products as well as due to the presence of ANF (Tacon, 1993). As already mentioned before, some important ANF of relevance include protease inhibitors, phytates, tannins, lectins, oligosaccharides and non-starch polysaccharides (Francis et al., 2001) and their inactivation includes a variety of methods such as dehulling, germination, soaking and enzyme addition or heat treatment such autoclave, roasting and extrusion (Francis et al., 2001). Indicative contents of ANF in peas, chickpeas and faba beans are presented in Table 1.8.

Table 1.8. Indicative content of antinutritional factors in legume seeds

Antinutritional factors	**Field pea**	**Faba bean**	**Chickpea**
Oligosaccharides (%)	3.69	2.93	1.99
Phytate (%)	0.48	0.66	0.63
Tannins total (%)	0.25	0.75	0.49
Tannin activity hulls (mg g^{-1})	11.06	21.73	5.43
Tannin activity cotyledons (mg g^{-1})	0.16	0.22	0.11
Trypsin inhibitor activity (mg g^{-1})	1.01	0.39	4.79
Chymotrypsin inhibitor activity (mg g^{-1})	1.60	0.40	7.72

Data extracted from Peterson and Mackintosh (1994) and Saini (1995) (quoted in Booth et al. (2001).

1.5   Feed processing and extrusion

1.5.1   General

Most commercial fish feeds are processed by compression pelleting or extrusion and other manufactured forms include moist and semi-moist, microencapsulated and micropulverized feeds (NRC, 1993). Processing techniques such as heat treatment, solvent extraction, flaking and grinding can improve the nutritional value of the diet ingredients and this in turn could be very beneficial for several fish species (Gouveia and Davies, 2000)

1.5.2   Processing Methods

Processing of the feed mixture through grinding, steam conditioning and extrusion is currently a big issue for fish feed companies, as these processes inevitably affect both the physical and chemical characteristics of a feed (Hilton et al., 1981). These characteristics include water stability and durability, pellet hardness, nutrient availability and digestibility (Hardy, 1989). Other factors influenced by processing such as the palatability and organoleptic properties of a diet may affect the amount of feed consumed by a target species (Mackie and Mitchell, 1985).

Since the method of pelletisation has a major influence on the nutritional characteristics of feeds it is important to ensure that ingredients identified as having potential in aquaculture feeds are incorporated into experimental diets that reflect current commercial processing (Oliva-Teles et al., 1994). There are a lot of different processing methods to produce pellets, but there are just few general techniques (Hardy and Barrows, 2002) described below .

1.5.2.1 Pelleting

Cold pellet extrusion involves no thermal activity and the wet mixture is forced through a plate with holes drilled into it. The resulting noodles are cut into appropriate lengths by an external cutting blade as they leave the die (Hardy and Barrows, 2002).

Compressed pelleting is a process which forces a feed mixture through holes in a metal die by the action of a roller located inside the die. Before this, the mixture can be exposed to dry steam for some seconds to increase the temperature to about 85ºC and the moisture to about 16% (Hardy and Barrows, 2002), but compressed pellets can also be produced without any steam as well (Thomas et al., 1997).

Extruded pellets are made in the same general way as compressed pellets, but the use of different dies and physical conditions results in a very different product (Hardy and Barrows, 2002) as described below.

1.5.3 Extrusion

Extrusion requires more elaborate equipment and higher inputs of moisture, heat and pressure than simple pelleting. Usually, mixed ingredients are finely ground before entering the pre-conditioner where steam treatment takes place until the mixture is converted into a mash that may or may not be pre-cooked (pre-conditioning) before entering the extruder. The mash, which contains around 25% moisture, is compacted and heated to 104º - 148ºC under pressure in the barrel of the extruder. As the material is squeezed through die holes at the end of the barrel, and external pressure decreases, part of the water in the superheated dough immediately vaporizes and causes expansion of the feed particles (NRC, 1993) leaving a large number of air-filled interstices. The extruded particles have high moisture content and require external heat for drying. Thus, after extrusion the particles must pass through a drying process to reduce

moisture to a safe level for storage (NRC, 1993). The air trapped inside the particles enables, when needed, the coating under vacuum with oil and other heat-sensitive materials giving an important advantage to this method.

Extrusion processing can impart a wide range of different physical characteristics to the final product due to the different settings in pre-conditioning, the types and number of screws (single or twin-screw) in the extruder, the screw speed, the temperature, the mechanical shear and the duration of every application (Thomas et al., 1997). This manufacturing method has the advantage of short cooking time and high productivity, but high operating costs.

1.5.3.1 Effects of extrusion on feed nutrients

Manufacturing processes can influence the utilization of nutrient ingredients and feed intake in fish (Booth et al., 2000). The effects of processing can differ for different species and it is important to apply the appropriate processing technique to maximize production efficiency at the lowest possible cost (Tacon, 1990). The use of extrusion increases nutrient availability of plant meals especially in relation to the amount of DE available through starch gelatinization. Dias et al. (1998) reported that inclusion of extruded wheat in the diet had an advantage over raw wheat improving energy digestibility of the diet for European seabass. More specifically extrusion processing gelatinizes starch and improves the digestion of starch in salmonids (Thodesen and Storebbaken, 1998).

Mild extrusion processing usually enhances the digestibility of plant proteins (Håkansson et al., 1987), while Booth et al. (2002) found increased feed efficiency and DP of extruded diets compared with cold and steam pelleted diets for silver perch.

Extrusion seems not only to improve protein and carbohydrate digestibility, but also lipid digestibility. The influence on lipid digestibility could be related to a decrease in indigestible carbohydrates (Storebakken et al., 1998).

Extrusion effects have been investigated in some ingredients, and resulted in increased nutritional value of rapeseed and peas for rainbow trout (Gomes et al., 1993), improvement of digestibility of canola meal for chinook salmon *Oncorhynchus tshawytscha* (Satoh et al., 1998), and increased nutritional value of dehulled pea seed meal for European seabass (Gouveia and Davies, 2000). However, Oliva-Teles et al. (1994) reported that extrusion had no effect on the nutritional value of full fat soybean in rainbow trout diets and, according to Sorensen et al. (2002), differences in extrusion temperatures (100, 125 and 150°C) caused no significant differences in ADC of crude protein, individual amino acids or energy in fish meal and wheat meal based diets for rainbow trout.

While processing techniques can improve the nutritional and physical qualities of diet ingredients, the heating process can also have detrimental effects. Damage to proteins and losses in nutritional value with destruction of IAA and reduction in amino acid availability, with lysine being the most sensitive, may be observed during processing (Papadopoulos, 1989). Allan et al. (2000) also reported that excessive heat during the rendering process could reduce the digestibility of proteins and amino acids, damage lysine and contribute to low nitrogen digestibility in animal meals. Generally, severe heating in combination with low moisture content have resulted in reduction of digestibility of most amino acids in fishmeal (Ljokjel et al., 2000). In addition, heat labile vitamins can also be lost at elevated temperatures, for example ascorbic acid had been shown to be unstable during heat treatments such as steam conditioning and extrusion (Slinger et al., 1979).

1.5.3.2 Effects of extrusion on feed ANF

Various processing procedures can decrease levels of a number of ANFs and, concurrently, increase the protein and starch availability of the plant seeds as has been shown by several authors (Gomes et al., 1993; Oliva-Teles et al., 1994; Pfeffer et al., 1995; Abd El-Hady and Habiba, 2003; Wang et al., 2004). Specific protease inhibitors, lectins, antivitamins and α-amylase inhibitor are heat labile factors (Abd El-Hady and Habiba, 2003; Wang et al*.,* 2004), which means that extrusion processing can reduce or minimize their activity. According to the same authors, saponins, non-starch polysaccharides, antigenic proteins, phytoestrogens and some phenolic compounds that are classified as ANFs are heat resistant.

Legume extrusion cooking may allow reduction of ANF and therefore improve the nutritional quality at a cost lower than other heating systems (baking, autoclaving, etc.) due to more efficient use of energy and better process control with greater production capacities (Alonso et al., 2000a). The digestibility coefficients of legumes increased after extrusion, compared to raw legumes both *in vitro* and *in vivo,* namely in rats (Alonso et al., 2000b), but also in rainbow trout (Cheng and Hardy, 2003a; Cheng and Hardy, 2003b) and silver perch (Allan and Booth, 2004).

1.6   General objectives

The objective of the present study was to investigate peas, chickpeas and faba beans as potential feed ingredients for European seabass and gilthead seabream in extruded commercial-type diets. Each one of these legumes were used up to an inclusion level of 35% as an energy source replacing wheat and as a protein source replacing partially different protein sources in each experiment. Specifically the objectives were:

1) To elucidate the effect of different processing conditions on the whole seed flour of the tested ingredients with respect to both nutritional and antinutritional factors and to examine the physical characteristics of experimental diet pellets including high and low levels of each legume.

2) To investigate the effects of wheat substitution in high fish meal diets on both digestibility, gastrointestinal evacuation rate and glucose load of seabass.

3) To estimate growth, digestibility, haematological parameters, histological effects and fillet organoleptic characteristics when the tested legumes replaced wheat and also the interactions between inclusion level of starch (low and high) and legume type in European seabass diets.

4) To estimate growth, haematological parameters and histological effects when the tested legumes replaced wheat and also the interactions between inclusion level of starch (low and high) and legume type in gilthead seabream diets.

Chapter 2. General Materials and Methods

2.1 Experimental diets

2.1.1 Processing system

Extrusion processing of raw legumes and diets took place at the BioMar TechCentre (Brande, DK). A CLEXTRAL BC 45 twin-screw extruder was used, with screw diameter of 55mm and an overall active length of 800mm. All legumes and wheat were ground to fine flour (1.5mm) just before heat processing. Legumes and diets were treated in a 'home made' single-screw preconditioner before entering the extruder. The temperature at the first part of the extruder, at the middle part and at the outer die of the extruder was constant for each treatment. The extrudates were conveyed into a 6 level GEELEN dryer. After drying the pellets entered into a GEELEN cooler to cool quickly to room temperature.

2.1.2 Diet production conditions

When feeds were manufactured the extruder was operated at approximately 340rpm and the feeder was set to deliver approximately 130-135gmin^{-1}. Water addition was adjusted in the preconditioner and on the extruder barrel using metering pumps with variable settings (calibrated in kgh^{-1}). Extrusion temperature at the outer die was 70ºC. Experimental diets for experiments I, II and III (described in section 3.2.3) were produced as practical-type extruded pellets under the same conditions.

2.2 Experimental animals and husbandry

2.2.1 Experimental fish

The experimental animals used in this study were European seabass for experiments I and II and gilthead seabream for experiment III. All *in vivo* experiments took place in the facilities of the Institute of Aquaculture of the Hellenic Centre for Marine Research in Athens (HCMR, Helliniko, Athens, Greece).

2.2.2 Facilities

Fish for experiments I and II, with respect to digestibility trials, were held in enclosed tanks at the aquarium facilities of HCMR. Fish for experiments II and III, with respect to growth trials, were held in small cages placed in cement tanks. All trials were carried out in triplicate.

2.2.2.1 Digestibility tanks

Digestibility trials were carried out in 15 cylindroconical fibreglass tanks of 250L in two groups in order to test all 7 diets in triplicate; in the first group 5 diets were tested and in the second group three diets were tested including in both groups the control diet. The conical bottom of each tank ended in a horizontal pipe and this pipe ended in the faecal trap (Figure 2.1). Water inlet was via a pipe placed vertically at the water surface. The pipe had several holes on the same side and the unidirectional water flow set up a gentle circular current. The current moved faeces to the bottom of the tank immediately after they were voided into the water. The inflow rate was approximately 150Lh^{-1} and water was pumped continuously from the sea via a mechanical filter (5µm) before entering the tanks. One airstone diffuser was located

in each tank to provide sufficient oxygen, especially in the case of failure of the water supply.

2.2.2.2 Growth tanks and cages

Growth trials were carried out in 2 cement tanks in the outdoor facilities with dimensions 5m x 6m x 1.50m. Into the 2 cement tanks were placed a total of 21 small cages (Figure 2.2). The cage dimensions were 1m x 1.5m and 1.3m height and they were suspended about 20cm from the tank bottom. $400Lh^{-1}$ were pumped continually from the sea via a mechanical filter (5µm) to each tank. Stone diffusers were placed in the tanks to provide sufficient oxygen as assurance, especially in the case of failure of the water supply.

2.2.2.3 Water quality

Oxygen concentration was approximately $8\pm1mgL^{-1}$, pH ranged from 7.8 to 8.0 and salinity was 38ppt. Temperature of the water is defined for every experiment in the respective chapter.

Figure 2.1. Digestibility tanks and faecal trap

Figure 2.2. Cages for growth trial

2.2.3 Feeding

Fish in experiments I, II and III were carefully hand fed to make sure no feed escaped from the cages. European seabass were fed to satiation for digestibility and growth trials, while one specific meal (1% of the wet bodyweight) was given to the fish before measuring gastrointestinal evacuation time and rate. Restricted feeding was applied to gilthead seabream in experiment III to avoid overfeeding as seabream is known to be voracious especially at high temperatures. The feed consumed was recorded daily.

2.3 Sampling procedures

2.3.1 Feed sampling

At the beginning of each experiment sufficient amounts of the diets were collected to complete the analyses under investigation. Diets were thoroughly milled and kept refrigerated at 4°C until analysed.

2.3.2 Faeces sampling

Faeces were collected in a trap using a modification of the Guelph method (Cho et al., 1982). The faecal trap was surrounded with ice during faecal settlement to minimize bacterial degradation. Faecal samples were removed every morning, prior to feeding, in centrifuge tubes. Faeces were centrifuged and kept at -20°C until they were freeze-dried and subsequently analysed.

2.3.3 Fish sampling

Prior to any experimental procedure (e.g. weighing and blood sampling) all fish were anaesthetized using phenoxyethanol/ethanol (1:1, v/v) in a concentration of $0.4mlL^{-1}$ (Ekmann, personal communication). After the experimental procedure the fish were placed in clean aerated sea water and allowed to recover before being returned to the experimental tanks. Measurements of fish weight were made at the beginning and throughout growth experiments.

In growth experiments (II and III) fish were fasted for 40 hours and blood samples were taken prior to any other sampling procedure. Fish were killed with a sharp blow to the head. Two pools of 5 fish per cage were taken for whole body proximate composition analysis, 2 pools of 4 fish for fillet analysis, 8 fish for blood sampling, liver and viscera weight, 2 pools of 3 livers for glycogen and fat analysis and the internal organs of 3 fish per cage for histological analysis.

Where fish were sacrificed for tissue sampling they were first anaesthetized and then killed with a sharp blow to the head such that death was instantaneous. Whole fish, tissue, intestine contents and blood samples were immediately stored as described in the methodology of the appropriate chapter for each experiment. All samples were placed in appropriate containers i.e. plastic tubes, centrifuge tubes, beakers, plastic bags etc and stored as required until further analyses.

2.4 Proximate analysis of diets, tissues and faeces

The chemical compositions of the experimental diets, fish tissues and faecal samples were determined by proximate analyses based on methods described in AOAC (1990).

2.4.1 Lyophilization or freeze-drying

During freeze-drying samples are quickly frozen onto the walls of a vessel that is then attached to an ice trap that is kept at -40°C and a vacuum is applied. The water in the frozen sample sublimes and is deposited in the trap. Samples are left undisturbed until all the ice has sublimed and the sample container is no longer cold. Then air is slowly admitted to the system, and the vessels are removed.

2.4.2 Moisture

Approximately 1.5g of ground sample was placed in a pre-weighed porcelain cup. Moisture content was determined as the loss in weight on drying at 105°C for 24 hours.

2.4.3 Ash

After moisture determination samples were placed in a furnace and ash content was determined as the residue after ignition at 550°C for 12 hours.

2.4.4 Crude protein

Crude protein content was determined by Kjeldahl analysis (nitrogen x 6.25) using a Kjeltec Autoanalyser (Tecator). Crude protein is determined by estimating the total nitrogen content of a material, assuming that all nitrogen is proteinaceous in origin. Approximately 200mg of sample was added to the digestion tubes. A tablet containing 7.5mg selenium as a catalyst was added to each sample together with 3 glass balls to aid boiling. Samples were then digested for 1 hour at 410°C with 6ml concentrated H_2SO_4 and 1ml of H_2O_2 was added to assist clarifying the sample. After cooling, 20ml of distilled water was added to each tube and the samples were distilled in Kjeldahl apparatus and titrated.

2.4.5 Acid hydrolysis

Extruded samples were hydrolysed using a Soxtec System 1047 hydrolysis unit (Tecator Application note 92/87). Approximately 1g of sample, 1g of celite and 100ml of HCl (3M) were added to each tube. All tubes were placed in the hydrolysis unit in a fume cupboard and boiled gently for 1 hour. The solutions in the tubes were then filtered through glass thimbles, which were then dried in an oven at 60°C overnight. The following day the samples were extracted with petroleum ether according to Soxhlet method as described in 2.4.6.

2.4.6 Crude fat

Crude fat was determined by exhaustive Soxhlet extraction using a Soxtec System HT6 (Tecator application note 67/83). Approximately 3g of sample were added to a thimble and placed in the extraction unit. Samples were boiled for 30min. in petroleum ether and rinsed for 2 hours and the extracted lipid was completely collected in the extraction cups. After extraction the solvent was evaporated and the extracted material weighed. Total lipid content was estimated by the weight difference of the cups before and after the extraction.

2.5 Lipid determination according to the phosphovanillin method

The phosphovanillin method of Nengas et al. (1995) is a micro method appropriate for faeces analysis where total sample quantities are very low (approximately 70mg). Lipids were extracted from the faeces using chloroform/methanol 2/1 solution. The solvent was then evaporated and the remaining lipids oxidised with hot concentrated sulphuric acid. The resulting pink colour produced by reaction with the phosphovanillin reagent was measured in a spectrophotometer at 530nm.

2.6 Lipid determination according to Folch method

Folch is a gravimetric method appropriate for analysis of samples with high moisture contents (Folch et al., 1957), such as livers. The lipids are extracted in chloroform/methanol (2:1, v/v).

250-300mg of homogenized liver sample stored at –20ºC, was weighed and added to test tubes. 5ml of chloroform/methanol (2:1, v/v) were added to each tube and homogenized carefully in a bath of iced water. Dilutions were filtered under vacuum in a new pre-weighed tube. To the filtered solution 1ml of NaCl 0.9% was added, the contents vortex mixed and centrifuged at 2000rpm for 5min. The upper phase was discarded and approximately 1ml of Folch reagent (chloroform/methanol/NaCl 0.9%, 3:48:47) was added. The upper phase was discarded again and the remaining solution was evaporated with nitrogen and put in the oven for 1h at 60°C. Samples were left to cool and weighed. The fat content of the samples was calculated by the weight difference of the tubes before and after the extraction.

2.7 Glycogen determination in liver tissue

Glycogen was extracted from liver tissue using citrate buffer and quantitavely hydrolysed to glucose by amyloglucosidase and then glucose was measured colorimetrically with the Human kit (cat. No. 10260, HUMAN). The method is described by Murat and Serfaty (1974).

100-120mg of frozen homogenized liver was weighed into test tubes and placed in ice. 5ml of citric acid buffer (0.1M, pH 4.3) were added to each tube. Samples were homogenized carefully and 100µl of the amyloglucosidase enzyme solution were added to each tube followed by vortex mixing. Samples were incubated for 24h at room temperature and then centrifuged at 3000rpm for 10min. Supernatant was diluted (1/10)

in citric buffer and 100μl of the last dilution were pipetted to new tubes and 2.5ml of the HUMAN reagent were added. Tubes were incubated for 10min at room temperature and absorbance measured at 500nm. Glycogen content was calculated as:

Glycogen, %=Glucose Standard (mg)$\dfrac{Abs_{std} \times 5 \times 0.5}{Abs_s \times 0.05 \times 0.1} \times 100$

Where, mg of glucose standard=0.02mgml^{-1}, Abs$_{std}$ is the absorbance of standard, Abs$_s$ is the absorbance of sample, 5/0.05 the 1st dilution and 0.5/0.1=the 2nd dilution.

2.8 Determination of Yttrium oxide (Y$_2$O$_3$)

Yttrium oxide was included in the tested diets as a marker and was determined in diets and freeze-dried faeces according to Refstie et al. (1997). Approximately 150–200mg of sample was weighed and combusted at 550ºC overnight in glass scintillation vials. When cooled 5ml of HCl/HNO$_3$ (2:1 v/v) was added and the samples were boiled until colourless. When cooled a few drops of water were added, the sample was dissolved in 1.25ml HNO$_3$ (concentrated) and diluted to 25ml with distilled water. The concentration of Yttrium was measured using an ICAP spectrometer. Analysis took place in the labs of Plymouth University and at Stirling University with the support of technical staff.

2.9 Carbohydrate determination

2.9.1 Determination of Starch

This method is used to determine starch according to the directions of the commercial kit; Megazyme Total Starch Assay Kit (Megazyme International, Ireland) (McCleary et al., 1992). Starch hydrolysis proceeds in two phases; in phase I, starch is partially hydrolysed and totally solubilised and in phase II, the starch dextrins are

quantitatively hydrolysed to glucose by amyloglucosidase. For diet samples, complete solubilisation of starch was achieved by cooking the sample in the presence of thermostable α-amylase. However, for legume samples that contained high levels of resistant starch, complete solubilisation and dextrinisation required pre-treatment with dimethyl sulphoxide (DMSO) at 100ºC.

2.9.2 Determination of free glucose, sucrose and raffinose-series oligosaccharides

This method is used to determine three different oligosaccharides; glucose, sucrose and the sum of raffinose series oligosaccharides (RSO) (raffinose, verbascose and stachyose), followed the directions of the commercial kit; Megazyme Raffinose/D-Glucose Assay Kit (Megazyme International, Ireland). This method is applied to raw and extruded legumes after very fine grinding (ball milling).

RSO are hydrolysed to galactose, glucose and fructose using α-galactosidase and invertase. The glucose is then determined using glucose oxidase/peroxidase reagent spectrophotometrically. Raffinose, verbascose and stachyose are determined as a group and not each one separately. The results are expressed on a molar basis, since RSO contain one mole of glucose.

2.9.3 Determination of total non-starch polysaccharides

Determination of DF as NSP with spectrophotometric measurement of constituent sugars was carried out according to Englyst et al. (1994) with a modification. NSP content was calculated using the equation of a standard curve derived from a standard sugar solution. At the first stage starch present in the sample was completely removed enzymically, and the NSPs were broken into simple sugars under acidic hydrolysis and

measured as a single value for total sugars by spectrophotometry. Values were obtained for total, insoluble and soluble NSP.

The samples were treated with DMSO and consecutively with different enzymes; heat stable amylase (Termamyl 120L Type L, Novozyme), pancreatin (Sigma, Cat.No.P-1750), pullulanase (Promozyme 400L, Novozyme) and pectinase (Sigma, Cat. No. P4716-10KU). After NSP isolation by enzymatic hydrolysis, they were precipitated and washed repeatedly. Different precipitating and washing procedures were followed for the measurement of total and for insoluble NSP. The colour of the samples was obtained with 3,5- dinitrosalicylic acid (DNS) and the absorbance measured at 530nm. The amount of S-NSP was calculated as the difference between total and insoluble NSP.

2.10 Antinutritional factors determination

2.10.1 Determination of total tannins

Tannins were extracted from samples according to Budini et al. (1980) and determination was based on a formation of the Prussian Blue method (Graham, 1992).

Tannins were extracted by gently refluxing the samples for 35min in 2N HCl. The contents were filtered while still hot into a 250ml volumetric flask and made up to volume with distilled water. The extract was incubated with $K_3Fe(CN)_6$ (0.016M) and $FeCl_3$ (0.02M in 0.1N HCl) and then treated with 6M H_3PO_4 and 1% gum acacia. The colour was measured spectrophotometrically at 700nm and the measurement of tannins derived from the equation of a catechin standard curve.

2.10.2 Determination of phytic acid

Determination of phytate was carried out according to Latta and Eskin (1980) using anion-exchange column (AEC) with resin (AGI-X4, 100-200 mesh chloride form, Bio-Rad Laboratories). Determination of phytate is based on its precipitation as insoluble ferric phytate in acid solution. Phytate was extracted from the samples with dilute HCl and then the extracts were eluted through the AEC to separate inorganic phosphorus and avoid overestimation of phytate in the sample. Phytate was eluted with 0.7M NaCl solution and determination of phytate was based on its reaction with Wade reagent (0.03% $FeCl_3$ $6H_2O$ and 0.3% sulfosalisylic acid) and a phytic acid standard curve.

2.10.3 Determination of trypsin inhibitors

Trypsin inhibitors (TI) were determined according to the method of Smith et al. (1980) with some modifications. The method involves extraction of the inhibitors from the sample at pH 9.5 and mixing unfiltered suspensions with bovine trypsin. The activity of the remaining trypsin was then measured on BAPNA substrate (N-benzoyl-DL-arginine-p-nitroanilide hydrochloride) under standard conditions. The p-nitroaniline released was measured spectrophotometrically at 410nm. This provided a linear measure of the residual trypsin activity (within the limits of the method), so that the amount of pure trypsin inhibited per unit weight of sample can be calculated.

2.11 Serum analysis

2.11.1 Determination of chemiluminescence

Chemiluminescence was determined according to Marnila et al. (1995). The procedure took place in the Pathology & Immunology lab of HCMR under the

supervision of Dr. Morgane Henry. Blood serum is separated from erythrocytes by centrifugation for 10min at 17000rpm. In quadruplate wells of a white flat-bottomed 96 wells microplate (Nunc), 10μl of 10mM luminol were added to 265μl of diluted blood and kept for 10min at room temperature to allow the background chemiluminscence to stabilize. Then, 25μl of non-opsonised zymosan at 5mgml^{-1} were added to each well and chemiluminescence was read every 3min for 2 hours in a GeniosPro luminometer (Tecan, Austria). Results were expressed as the peak chemiluminescence in relative luminescent units (rlu).

2.11.2 Determination of glucose in blood serum

The glucose oxidase method, GOD-PAP, was used for glucose measurement, without extracting the protein (cat. No. 10260, HUMAN). 10μl of the serum was added to a 10ml tube and 1ml of the monoreagent added. Samples and the standards were left for 10min at 20-25ºC and the absorbance was measured spectrophotometrically at 500nm. Serum glucose was calculated as:

$$C, mgdL^{-1} = 100 \times \frac{Abs_s}{Abs_{std}} \quad \text{or} \quad C, mmolL^{-1} = 5.55 \times \frac{Abs_s}{Abs_{std}}$$

Where Abs_s is the absorbance of sample and Abs_{std} the mean absorbance of standards.

2.11.3 Determination of total protein in blood serum

Serum protein was measured with the photometric method of Biuret, (cat. No. 10570, HUMAN). 20μl of serum was added to a 10ml tube and 1ml of monoreagent added. Samples and the standards were left for 10min at 20-25ºC and absorbance measured spectrophotometrically at 546nm. Serum protein was calculated as:

$$C, \text{gdL}^{-1} = 8 \times \frac{Abs_s}{Abs_{std}} \quad \text{or} \quad C, \text{gL}^{-1} = 80 \times \frac{Abs_s}{Abs_{std}}$$

Where Abs_s is the absorbance of sample and Abs_{std} the mean absorbance of standards.

2.11.4 Determination of triacylglycerols in blood serum

Serum triacylglycerols were measured with the enzymatic method L.C.F., Photometric method GPO-PAP (cat. No. 10724, HUMAN). 10μl of serum was added to a 10ml tube and 1ml of monoreagent added. Samples and standards were left for 10min at 20-25ºC and absorbance measured spectrophotometrically at 546nm. Serum triacylglycerols was calculated as:

$$C, \text{mgdL}^{-1} = 200 \times \frac{Abs_s}{Abs_{std}} \quad \text{or} \quad C, \text{mmolL}^{-1} = 2.28 \times \frac{Abs_s}{Abs_{std}}$$

Where Abs_s is the absorbance of sample and Abs_{std} the mean absorbance of standards.

2.11.5 Determination of cholesterol in blood serum

Serum cholesterol was measured with the enzymatic method L.C.F., Photometric method GHOD-PAP (cat. No. 10028, HUMAN). 10μl of serum was added to a 10 ml tube and 1ml of buffer solution added. For standards, 10μl of standard solution was added to a 10ml tube and 1ml of monoreagent added. Samples and standards were left for 20min at 20-25ºC and absorbance measured spectrophotometrically at 546nm. Serum cholesterol was calculated as:

$$C, \text{mgdL}^{-1} = 200 \times \frac{Abs_s}{Abs_{std}} \quad \text{or} \quad C, \text{mmolL}^{-1} = 5.17 \times \frac{Abs_s}{Abs_{std}},$$

Where Abs_s is the absorbance of sample and Abs_{std} the mean absorbance of standards.

2.12 Histology of internal organs

Tissues from organs related to digestion were dissected from European seabass and gilthead seabream in experiments II and III. Liver, anterior and posterior intestine, spleen and kidney were collected. The samples were fixed in formalin until processing. All formalin fixed tissues were routinely dehydrated in ethanol, methanol and embedded in paraffin according to standard histological techniques. Sections of 4μm were cut and stained with haematoxylin and eosin. Histological examination was performed by light microscopy. Histology procedures took place in the histology lab of HCMR and Dr. George Rigos assisted with examination of the sections.

2.13 Determination of physical characteristics of the pellets

2.13.1 Texture analysis

For texture determination of the pellets the Stable Micro Systems TA-XT2 Texture Analyser was used and calibrated every several samples with a 5kg weight. For sample examination a blade (knife edge) was placed on the texture analyzer and cut through the sample at a speed of 1mms^{-1} from a distance of 5mm. The blade approach was applied by pressing the blade through the pellet vertical to the flat surface of the 'pellet cylinder'. The shear force (Newton, N) was measured as the maximum force required cutting through the samples that was equal to the pick height of the given curve on a force (N)–time (s) diagram.

2.13.2 Water activity

For the determination of water activity a Rotronic Hygrometer A3 was used. Extruded feed samples were examined using this device in a simple appliance. This parameter is very important for extruded products their possible storage time. This device can measure the relative humidity of the space around the sample. The principle of the method is based on the change of conductivity of the ingredients when moisture is present. The sample (15-20g) is placed in a small plastic container and when temperature and relative humidity of the sample space are stabilized the device gives the final value. Each measurement lasted for approximately 5min and all measurements took place at 23°C with no replicates.

2.13.3 Pellet density

Pellet density d was measured by the formula d=m/V, where m is the mass determined in an analytical balance and V the cylinder volume of the pellet. Dimensions of 12 pellets, chosen to have the shape of a well formulated cylinder, were determined with a calliper.

2.13.4 Water absorption

Water absorption of the pellet was measured by immersing 15 pellets in sea water in triplicate groups for 5s and then determining moisture as described in section 2.4.2. The final value was calculated by subtracting the initial moisture content of the diet from the measured value.

2.13.5 Pellet settling velocity

In a volumetric cylinder of 1L and 34cm height, sea water, of salinity 38, and temperature at 20°C, was added and pellets left to fall through the water and reach the bottom of the cylinder. The time (t) needed for each pellet to pass through 34cm of the cylinder was measured and the velocity (v) of the pellets of each diet determined according to the formula $v=34/t$ cms^{-1}.

2.14 Calculations

Calculation of digestibility coefficients

ADCs for control and test diets were calculated according to the formula:

ADC % = 100 x [1- (F x Dy)/(D x Fy)]

Where F=nutrient or energy concentration in faeces, D=nutrient or energy concentration in diet, Dy=yttrium concentration in diet and Fy=yttrium concentration in faeces.

Energy in diets and faeces was determined according to the formula (Blaxter, 1989):

Energy (kJg^{-1}) = 23.6xP+17.3xS+39.5xF

Where P=% protein in faeces or in diet, S=% starch in faeces or in diet, F=% fat in faeces or in diet.

The following formulae were applied to the data:

Feed Conversion Ratio:

$$FCR = \frac{daily\ FI\ (g)}{daily\ wet\ weight\ gain\ (g)}$$

Specific Growth Rate:

$$\text{SGR, \%/day} = \frac{[\ln(W_1) - \ln(W_0)]}{total\ days} \times 100$$

Hepatosomatic Index:

$$\text{HSI, \%} = \frac{W_{Liver}}{BW} \times 100$$

Viscerosomatic Index:

$$\text{VSI, \%} = \frac{W_{Viscera}}{BW} \times 100$$

$$\text{Nitrogen Intake, NI, (g)} = \frac{PI}{6.25}$$

Protein productive value (g protein gain x g protein ingested^{-1}):

$$\text{PPV} = \frac{P_1 W_1 - P_0 W_0}{P_F \times cumulative\ F.I.}$$

In the above formulae FI is feed intake, PI is protein intake W is the weight of the sampled fish in grams; W_0 and W_1 are the initial and the final fish mean weights in grams; W_{liver} and $W_{viscera}$ are the weights in grams of the liver and viscera, respectively, of the sampled fish; P_0 and P_1 are the initial and final protein concentrations of the fish; P_F is the protein concentration of the feed on a dry matter basis; cumulative feed intake was determined in grams on a dry matter basis.

2.15 Statistics

All the data are presented as means±SD (n=3). The statistical analyses for all experiments were performed using SPSS 13.0 (SPSS Inc) and Statgraphics Plus 2.1.

Gastrointestinal evacuation models in experiment I were plotted in Excel (Office Microsoft) and regressions were performed using the statistical program Statgraphics. The linear fits and the comparison of slopes were tested at a significance level P=0.05.

Significant differences between dietary treatments were determined by one-way or two-way Analysis of Variance (ANOVA, General Linear Model) depending on the case. Post-hoc tests were used to rank the groups and the main effects respectively at a significant level (P=0.05), while Tukey post-hoc test applied for one-way ANOVA and Bonferroni for two-way ANOVA.

The experimental designs for the experiments I, II and III were factorial with 2 factors (2 inclusion levels x 3 different legume types). A control diet that was absolutely comparable to 3 out of 6 experimental diets was also tested. Hence, the effects of the one factor were analyzed with one-way ANOVA, but also one-way ANOVA applied for all seven diets. The effects of two factors and their interactions were analysed by two-way ANOVA. When significant interactions of the two factors were observed, multiple comparison testing was performed to look at the simple main effects (instead of the main effects), that is the main effect of one factor at a given level of the other (Zar, 1999).

Data were tested for their normality by One-Sample Kolmogorov-Smirnov Test and Homogeneity of Variances by Levene test in experiments I, II and III. Data that were identified as non-homogeneous (Levene's test) were subjected to square root, exponential or log transformation before analysis.

Possible differences among the different dietary groups for their organoleptic characteristics were checked by non Parametric Kruskal-Wallis test (SPSS, 13.0) in experiment II.

Pearson correlation in SPSS 13.0 used to evaluate any significant ($P<0.01$ and $P<0.05$) positive or negative correlation among data (normally distributed), fat

metabolism parameters in experiment II and III and fillet characteristics in experiment II.

Chapter 3. Effects of extrusion on peas, chickpeas and faba beans and evaluation of pellet physical properties

3.1   Introduction

Legumes like faba beans, chickpeas and field peas are valuable potential sources of both protein and energy for European seabass and gilthead seabream feeds. All these crops are cultivated in Mediterranean countries as well as in the rest Europe. This could be an advantage with a positive effect on the final price of the product due to minimization of transport costs, and also in development of agricultural production with vertical integration between seed and fish feed production. It is, however, well known that the use of legumes, especially for carnivorous species, is limited due to the presence of various ANF.

To overcome this problem, a variety of techniques have been already examined in plant materials like soaking (Frias et al., 2000), boiling (Marquez and Alonso, 1999), autoclaving (Mansour et al., 1993), microwaving (Marconi et al., 2000), roasting, dehulling, germination, fermentation (Chitra et al., 1996), supplementation with enzymes (Riche and Garling, 2004) and extrusion cooking (Abd El-Hady and Habiba, 2003). To destroy or eliminate the effects of ANF and to improve the physical (texture and palatability) and chemical (starch gelatinization) characteristics of feeds, modern fish feed technology applies extrusion processing (Sorensen et al., in press). Extrusion processing, as described in section 1.5.3, includes a series of different pieces of apparatus such as grinder, preconditioner, extruder, drier and cooler that have a wide range of abilities and which can differentiate the final product depending on steam and water quantity, pressure, mechanical shear, temperatures, duration of application and die dimensions (Thomas and van der Poel, 1996; Thomas et al., 1997).

Regarding ANFs, TI is a crystalline globular protein that has been shown to depress the growth rate of mammals, chickens and fish and also to cause pancreatic hypertrophy (Hendricks, 2002). Phytic acid is the hexaphosphate of myo-inositol and is a constituent of all cereals and oilseed meals and has the capacity to tightly bind divalent cations present in seeds, such as calcium, magnesium and zinc rendering them unavailable to animals when ingested (Hendricks, 2002). Tannins are secondary compounds of various chemical structures widely occurring in the plant kingdom and are generally divided into hydrolysable and condensed tannins. Their antinutritional effects include interference with digestive processes by binding enzymes or by binding to feed components such as proteins or minerals (Liener, 1989). Tannins also reduce the absorption of vitamin B_{12}, while they are known to interact with other antinutrients. For instance interaction between tannins and lectins seems to reduce the inhibitory action of tannins on amylase (De Boer and Bickel, 1988) and their interaction with cyanogenic glycosides reduces the deleterious effects of the latter (Bromley, 1994).

Oligosaccharides are low molecular weight carbohydrates containing α-galactosidic and β-fructosidic linkages. Sucrose, and RSO (raffinose, stachyose and verbascose) have been indicated as the causative factors of osmotic effects in the intestine and anaerobic fermentation of these sugars results in increasing gas production (Van Barnevelt, 1999). NSP are important constituents of grain legumes and cereals (Grabner and Hofer, 1985), and their negative effects in fish may be due either to binding to bile acids or obstructing the action of digestive enzymes and movement of substrates in the intestine (Storebakken et al., 1998). The NSP, and particularly the S-NSP, are more detrimental to growth of fish than the oligosaccharides (Refstie et al., 1999) because they have the ability to trap water and

form gum-like masses in the intestine which increase the viscosity of intestinal contents and obstruct digestive enzyme activity as explained in section 1.1.2.

Extrusion is effective in the inactivation of TI (Aslaksen et al., 2007), while temperature does not seem to improve bioavailability of minerals bound by phytic acid (Francis et al., 2001). The positive effect of extrusion on digestibility of all nutrients in plant feedstuffs could be attributed to a partial degradation of NSP (Francis et al., 2001). However, this degradation is not sufficient to consider NSPs as heat-labile ANF (Alonso et al., 2001).

Plant materials can affect the pelleting properties of feed mash when included in fish diets (Thomas et al., 1998). Hardness, durability, water absorption, settling velocity and density are the main physical characteristics and some of these could be affected when new feedstuffs are included in diets, whilst different type of starch (e.g. amylose/amylopectin ratio, starch granules) and fibres (soluble or insoluble) in combination with processing conditions can also differentiate the final product (Thomas et al., 1998). The legumes tested in the present study have high starch contents and NSP (Englyst and Hudson, 1996; Knudsen, 1997) and were included up to $36g100g^{-1}$ in diets. It was thus essential to investigate the effects of their incorporation on the physical properties of the pellets.

The aim of the present research was to investigate the effect of two different preconditioning temperatures and three different drying temperatures in combination on the chemical composition and some key ANF of peas, chickpeas and faba beans. Proximate composition, TI, total tannins, phytic acid, oligosaccharides and NSP were determined, before including whole seed flours in seabass and seabream diets using commercial pellet manufacturing conditions. The same batches of legume seeds were

contained in the experimental diets, for seabass and seabream in the *in vivo* trials (Experiments I, II and III).

3.2   Materials and Methods

3.2.1   Tested ingredients

Three different legumes were used for the present experiment: 1) middle size, kabuli–type chickpeas (CP) and 2) an early-middle season and low tannin variety and light beige seed colour peas (P), both cultivated, in the area of Thessaly in central Greece and 3) a common faba bean (horse beans) variety (B), cultivated in Denmark.

3.2.2   Processing of tested ingredients

Extrusion processing of raw materials took place at the BioMar TechCentre (Brande, DK) using the system described in detail in section 2.1.1. Pea, chickpea and faba bean whole seeds were ground to fine flour (1.5mm) and processed separately. Wheat (W) was only tested for one processing treatment and for two different grinding sizes (1.5mm and 0.2mm).

Flours were first treated in the preconditioner at temperatures below, above and 'standard' for commercial processing procedures before entering the extruder. The temperature was approximately 70ºC for the low (Lp), about 90ºC for the standard (Sp) and 100ºC for the high (Hp) temperature preconditioner treatment. One sample was taken of each material during the standard preconditioner treatment (Sp/-), at the extruder exit, without being dried. These samples contained more than 20% moisture and they were freeze dried before the analysis (P6, CP6, B6).

The extruder was operated at 380rpm and the feeder was set to deliver 107-116gmin^{-1}. Moisture contents were consistent in both barrels with a water flow of

12.5kgh^{-1} in the extruder barrel. The extrudates were conveyed into a 6 level dryer and the temperature lowered from 120-90ºC for the standard (Sd) and from 150-120ºC for high (Hd) drier treatment. Processing conditions are presented in summary in Table 3.1.

Table 3.1. Processing conditions for peas, chickpeas, faba beans and wheat in preconditioner (p), extruder and drier (d) at low (L), standard (S) or high (H) temperature.

	Processing conditions					
	1	2	3	4	5	6
	Lp/Sd	Sp/Sd	Sp/Hd	Hp/Sd	Hp/Hd	Sp/-
Preconditioner (ºC)	70	90	90	100	100	90
Extruder						
Extruder rpm	380	380	380	380	380	380
Ampere	19	18	20	19	18	19
Feeder (gmin^{-1})	113	113	113	113	113	113
Water (Kgh^{-1})	12.5	12.5	12.5	12.5	12.5	12.5
Outlet die (ºC)	110	110	110	110	110	110
Front (ºC)	95	95	95	95	95	95
Middle (ºC)	70	70	70	70	70	70
Drier						
Level 1 (ºC)	120	120	150	120	150	-
Level 6 (ºC)	90	90	120	90	120	-
Cooling	YES	YES	YES	YES	YES	YES

3.2.3 Diet formulation

All diets were manufactured at the BioMar TechCentre (Brande, DK) using a twin-screw extruder as described in section 2.1. Legumes were ground to pass a 1.5mm screen. All diets contained 0.1% yttrium oxide (Y_2O_3) as an indigestible marker. Processing of the tested diets was decided to be the common commercial procedure. The extruder was operated at approximately 340rpm and the feeder was set

to deliver 135gmin^{-1}. Mass moisture content was maintained in both barrels with a water flow of 6kgh^{-1} in the extruder barrel while the temperature in the outlet die was approximately 70ºC. The dryer temperatures on the different levels ranged from 110-90ºC and the cooler was set at 11 ºC.

3.2.3.1 Diets for Experiment I

Chickpeas, field peas, and faba beans were included in diets that were formulated to be isoenergetic (20kJg^{-1}) and isonitrogenous (42g100g^{-1} protein) containing 15g100g^{-1} (CP15, P15 and B15) or 30g100g^{-1} (CP30, P30 and B30) of each legume plus a wheat based (23%) control diet. In diets containing 15g100g^{-1} of legumes, half of the wheat was replaced compared to the control, whilst in diets containing 30g100g^{-1} of legumes wheat was completely replaced (excluding 1g100g^{-1} of wheat in CP30 diet). The pellet diameter was intended to be 4.5mm (Table 3.2).

3.2.3.2 Diets for Experiment II

The same legumes were included in the experimental diets of Experiment II. Six practical type extruded diets were formulated containing approximately 17g100g^{-1} (L diets) or 35g100g^{-1} (H diets) of each legume plus the control diet (Table 3.3). All diets were intended to be isoenergetic (20kJg^{-1}) and isonitrogenous (40g100g^{-1} protein) and were formulated to include similar levels of fishmeal (26g100g^{-1}) and high levels of plant protein sources (soybean meal, high protein sunflower and corn gluten). In L diets, legumes completely substituted wheat compared to the control diet. However, to formulate H diets it was necessary to reduce plant proteins (soybean and high protein sunflower) by half, to maintain the protein and energy content. The diameter of the pellets was intended to be 4.5mm.

3.2.3.3   Diets for Experiment III

Six practical type extruded diets were formulated containing approximately 17g100g^{-1} (L diets) or 35g100g^{-1} (H diets) of each legume (P, CP, B) plus the control diet (Table 3.4). All diets were intended to be isoenergetic (19kJg^{-1}) and isonitrogenous (42g100g^{-1} protein) and were formulated to include equal levels of fishmeal and plant protein sources (soybean meal, high protein sunflower, corn gluten and the tested legumes). In L diets, legumes completely substituted wheat compared to the control diet. However, H diets were formulated as practical, low cost and sustainable diets and thus it was necessary to reduce plant proteins differently (soybean and high protein sunflower and gluten), to maintain the protein, energy and IAA content. The pellet diameter was intended to be 4mm.

3.2.4   Chemical analysis

Proximate analysis was based on methods of AOAC (1990) as described in section 2.4. ANF (total tannins, phytic acid and TI) were determined by the methods described in section 2.10. Starch, total, soluble and insoluble NSP were determined according to the chemical methods described in section 2.9. All ingredients were ball-milled prior to analysis for the materials to be more consistent, especially for the enzymatic methods.

3.2.5   Physical characteristics of the diet pellets

Texture analysis to determine the pellet hardness, water activity, pellet density, water absorption and settling velocity were determined as described in section 2.13.

Table 3.2. Seabass diet composition used in experiment I.

Raw material (g 100g^{-1})	Control	B15	CP15	P15	B30	CP30	P30
Fishmeal 70%, Norway	61.4	57.1	57.9	57.7	54.1	55.0	55.3
WHEAT	**24.2**	**13.1**	**12.9**	**12.5**		**1.3**	
FABA BEANS		**15.0**			**30.0**		
CHICKPEAS			**15.0**			**30.0**	
FIELD PEAS				**15.0**			**30.0**
SA Fish oil, Peru	14.1	14.5	13.9	14.5	15.6	13.4	14.4
Premix[1]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Proximate composition (%)							
Ash	9.5	10.1	9.6	10.0	10.0	9.8	9.9
Crude fat	17.3	18.1	17.6	20.1	20.8	19.4	18.9
Crude protein	42.3	43.8	42.9	42.9	44.3	44.0	43.1
Total Starch	15.8	14.8	14.2	14.6	11.7	12.4	13.3
Wheat starch[2]	15.8	8.9	8.8	7.8	0.0	0.9	0.0
Legume starch[2]	0.0	5.9	5.3	6.8	11.7	11.5	13.3
Total NSP[3]	5.0	5.4	4.8	5.2	6.9	5.1	6.1
S-NSP[4]	1.6	2.5	1.5	1.8	2.4	1.5	2.3
I-NSP[5]	3.4	2.6	3.4	3.6	3.7	3.6	4.6
Total carbohydrates	20.8	20.2	19.0	19.7	18.7	17.5	19.4
Water content	6.7	6.3	7.4	6.6	8.3	6.8	7.3
Energy (kJg^{-1})	20.1	19.5	20.6	20.7	20.2	19.9	20.1

[1]Vitamins (per kg/premix): Vitamin A (UI) 1,250,000, Vitamin D3 (UI) 250,000, Vitamin E (ppm) 43,750, Vitamin B1 (ppm) 2,500, Vitamin B2 (ppm) 5,000, Vitamin B6 (ppm) 2,500, Vitamin B12 (ppm) 7.5, Vitamin K3 (ppm) 2,500.
Minerals (per kg/premix): Zinc (ppm) 25,000, Iodine (ppm) 300, Copper (ppm) 1,250, Manganese (ppm) 7,500, Cobalt (ppm) 250, Selenium mineral (ppm) 62.5. Mineral premix also includes Y_2O_3 to give final concentration of 0.1% in the diet
[2]Calculated from total starch values and the concentration of each ingredient in the diet.
[3,4,5]Total, soluble and insoluble non starch polysaccharides

Table 3.3. Seabass diet compositions used in Experiment II.

Raw materials (g100g^{-1})	Control	BL	CPL	PL	BH	CPH	PH
Fishmeal 70%, Norway	24.1	25.0	24.9	25.6	24.2	23.4	25.3
Hi Pro Soya 48%, Brazil	16.0	16.0	16.0	16.0	8.0	8.0	8.0
Sunflower	16.0	16.0	16.0	16.0	8.0	8.0	8.0
Corn Gluten	10.0	7.5	8.2	7.4	10.0	10.0	10.0
WHEAT	**17.0**						
FABA BEANS		**16.5**			**34.0**		
CHICKPEAS			**16.5**			**35.0**	
FIELD PEAS				**16.5**			**33.5**
SA Fish oil, Peru	15.4	18.0	17.4	17.6	15.7	14.6	14.2
Premix[1]	0.4	0.4	0.4	0.4	0.4	0.4	0.4
MCP[2]	0.3	0.6	0.6	0.5	0.7	0.6	0.6
Proximate Composition (%)							
Ash	7.0	7.3	7.4	7.5	7.1	7.1	7.2
Crude fat	19.3	19.4	20.0	19.2	18.3	18.3	16.8
Crude protein	40.2	39.6	39.9	40.6	39.9	39.1	38.5
Total Starch	11.1	7.4	7.6	9.0	14.4	15.8	17.8
Total NSP[3]	8.8	9.3	8.1	8.9	9.4	7.6	8.9
S-NSP[4]	2.0	1.7	2.5	2.1	2.1	2.1	2.6
I-NSP[5]	6.8	7.6	5.6	6.8	7.4	5.5	6.3
Total carbohydrates	19.9	16.8	15.8	17.9	23.9	23.4	26.7
Water content	8.3	8.2	8.5	6.7	6.0	5.5	4.4
Energy (kJg^{-1})	20.8	19.9	20.4	20.0	20.4	20.3	19.7

[1]Vitamins (per kg/premix): A (UI) 1,250,000, D3 (UI) 250,000, E (ppm) 43,750, B1 (ppm) 2,500, B2 (ppm) 5,000, B6 (ppm) 2,500, B12 (ppm) 7.5, K3 (ppm) 2,500, Niacin (ppm) 12,500, B3 (ppm) 10,000, Biotin (ppm) 75, C (ppm) 25,000 (stable to extrusion), Folic acid (ppm) 2,500, Ethoxyquin (ppm) 10,000.
Minerals (per kg/premix): Zinc (ppm) 25,000, Iodine (ppm) 300, Copper (ppm) 1,250, Manganese (ppm) 7,500, Cobalt (ppm) 250, Selenium mineral (ppm) 62.5. Mineral premix also includes Y_2O_3 to give final concentration of 0.1g100g^{-1} in the diet
[2]Mono calcium phosphate (as a phosphate source)
[3,4,5]Total, soluble and insoluble non starch polysaccharides

Table 3.4. Seabream diet compositions used in Experiment III.

Raw materials (g100g[-1])	Control	BL	CPL	PL	BH	CPH	PH
Fishmeal 70%, Norway	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Hi Pro Soya 48%, Brazil	20.0	20.0	20.0	18.5	7.5	6.1	3.3
Sunflower cake 37	11.1	11.1	9.4	9.7	3.0	3.0	1.8
Gluten[1]	13.9	8.9	8.9	12.2	12.0	13.4	17.4
WHEAT	**12.0**		.				
FABA BEANS		**17.5**			**35.0**		
CHICKPEAS			**17.5**			**35.0**	
FIELD PEAS				**17.5**			**35.0**
SA Fish oil, Peru	14.7	15.2	14.8	14.8	12.2	11.2	11.2
Premix[2]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
MCP[3]	3.0	2.0	3.0	3.0	5.0	6.0	6.0
Proximate composition (%)							
Ash	7.6	7.1	7.1	7.1	7.7	7.8	7.7
Crude fat	18.5	17.8	17.5	16.5	14.5	15.7	13.3
Crude protein	43.6	44.2	42.3	42.2	42.2	42.0	41.2
Starch	9.3	8.0	8.2	9.4	15.7	15.9	18.3
Total NSP[4]	8.5	8.8	9.6	8.9	9.8	7.1	7.0
S-NSP[5]	2.1	0.6	2.2	1.9	2.4	1.7	1.2
I-NSP[6]	6.3	8.2	7.4	7.0	7.4	5.4	5.8
Total carbohydrates	17.8	16.8	17.9	18.3	25.4	23.0	25.3
Water content	8.0	8.3	8.2	8.4	7.4	7.1	6.7
Energy (kJg[-1])	19.2	18.8	18.3	18.1	18.4	18.9	18.1

[1]Gluten: mixture of corn gluten/wheat gluten 1/1,
[2]Vitamins (per kg/premix): A (UI) 1,250,000, D3 (UI) 250,000, E (ppm) 43,750, B1 (ppm) 2,500, B2 (ppm) 5,000, B6 (ppm) 2,500, B12 (ppm) 7.5, K3 (ppm) 2,500, Niacin (ppm) 12,500, B3 (ppm) 10,000, Biotin (ppm) 200, C (ppm) 25,000 (stable to extrusion), Folic acid (ppm) 1,500, Ethoxyquin (ppm) 10,000,
Minerals (per kg/premix): Zinc (ppm) 25,000, Iodine (ppm) 300, Copper (ppm) 1,250, Manganese (ppm) 7,500, Cobalt (ppm) 250, Selenium mineral (ppm) 62,5, Calcium (ppm) 262,500, Magnesium (ppm) 2750, Sodium (ppm) 340. Mineral premix also includes Y_2O_3 to give final concentration of 0.1g100g[-1] in the diet
[3]Mono calcium phosphate (as a phosphate source).
[4,5,6]Total, soluble and insoluble non starch polysaccharides

3.3 Results

3.3.1 Proximate composition and ANFs of tested ingredients

Statistical analysis was not performed between the treatments because each set of processing conditions was applied only once. Mean values of replicates of both chemical and physical analysis would only support ANOVA with respect to analytical precision, while tests comparing values without replicates (e.g. Friedman test) were not appropriate for the present data (Dytham, 1999). Additionally, the identity of the carbohydrates, variances among seed varieties (Saini and Knights, 1984), differences in ANF's chemical methods and in processing equipment and temperatures (e.g. single or twin-screw extruders) make quantitative comparisons to similar studies difficult. Nevertheless, some useful conclusions can be drawn.

Proximate composition, S-NSP, I-NSO and total NSP, F-glucose (free glucose), sucrose and RSO and ANFs of raw and extruded faba beans are presented in Tables 3.5 and 3.6, of chickpeas in Tables 3.7 and 3.8, peas in Tables 3.9 and 3.10 and wheat results are presented in Tables 3.11 and 3.12.

Higher protein content was found for the raw faba beans with $31.4g100g^{-1}$, followed by chickpeas ($27g100g^{-1}$), peas ($24.4g100g^{-1}$) and wheat ($17.7g100g^{-1}$), fat content was very low and similar for faba beans, peas (ca. $1.6g100g^{-1}$) and wheat ($1.8g100g^{-1}$) and higher for chickpeas ($5.5g100g^{-1}$). Faba beans had higher ash content ($4.3g100g^{-1}$) compared to chickpeas, peas and wheat (3.5, 3.3, and $2.3g100g^{-1}$)

Starch content was highest for raw wheat $66.2 \text{ g}100g^{-1}$ followed by peas and chickpeas (ca. $42g100g^{-1}$) and faba beans ($36.9g100g^{-1}$) and extrusion generally increased starch values of the processed materials.

Faba beans included highest levels of total NSP (18.3-23.7g100g^{-1}), followed by peas (14.0-16.4g100g^{-1}) and chickpeas (12.9-13.8g100g^{-1}), while the ratio I-NSP/S-NSP (I/S) in raw materials was similar for peas and chickpeas (3.7) and higher than faba beans (2.1). Faba beans contained the highest levels of phytate (3.2g100g^{-1}) and tannins (1.3g100g^{-1}) and raw chickpeas contained the highest levels of TI (14.7mgg^{-1}), but after heat treatment non linear changes were observed for these parameters.

Chickpea and field pea NSP values do not seem to be much affected by processing, with only a small trend of reduction in total and I-NSP, whilst similar results for faba beans were found only for Sp/- processing. For all the other processing methods in faba beans total NSP, S-NSP and I-NSP seem to be slightly reduced.

Total oligosaccharide values were higher for raw peas (6.6g100g^{-1}) followed by chickpeas (5.1g100g^{-1}), faba beans (3.9g100g^{-1}) and wheat (1.7g100g^{-1}), free glucose amounts were negligible. However, higher values were obtained for faba beans (0.2g100g^{-1}). Sucrose values were similar for raw chickpeas, peas and wheat (ca. 1.1-1.4g100g^{-1}) and lower for faba beans (0.9g100g^{-1}), RSO were higher in raw peas (5.3g100g^{-1}), 3.7g100^{-1} in chickpeas, 2.8g100g^{-1} for faba beans and only 0.5g100g^{-1} for wheat. Heat processing slightly reduced total oligosaccharides in most of the cases in peas and increased them in faba bean and chickpea.

TI were reduced for all processed products with the highest effect for the legumes to be in chickpeas (86-92%) while TI in wheat were not detected after heat treatment. Overall, phytate and total tannins were affected to a lower extent or they were unaffected by processing. Phytate values were reduced up to 22% for B, 16% for CP and 18% for P, while total tannins reduced by up to 11% for B, 18% for CP and 12% for P.

Table 3.5. Proximate composition on dry matter basis of raw and processed faba beans

FABA BEANS	Processing*	Proximate composition								Oligosaccharides			
		ash	protein	Fat	starch	S-NSP[1]	I-NSP[2]	I/S[3]	Total NSP[4]	F-glucose[5]	sucrose	RSO[6]	Total
raw	-	4.3	31.4	1.6	36.9	7.6	16.1	2.1	23.7	0.20	0.9	2.8	3.9
B1	Lp/Sd	4.5	32.1	2.0	38.1	4.9	15.6	3.2	20.5	0.14	0.9	3.4	4.4
B2	Sp/Sd	4.3	31.2	2.0	39.6	5.4	14.4	2.6	19.8	0.14	0.9	2.9	3.9
B3	Sp/Hd	4.2	30.8	1.7	35.0	3.9	14.4	3.7	18.3	0.11	1.2	2.9	4.2
B4	Hp/Sd	4.2	30.8	2.0	37.0	3.6	15.6	4.4	19.2	0.11	1.4	2.6	4.1
B5	Hp/Hd	4.2	31.7	2.0	39.1	3.6	14.7	4.1	18.3	0.13	1.4	2.4	3.9
B6	Sp/-	4.4	31.2	2.1	34.0	8.6	9.8	1.2	18.4	0.17	1.2	2.7	4.1

[1]S-NSP: soluble non starch polysaccharides, [2]I-NSP: insoluble non starch polysaccharides, [3]I/S: Insoluble/Soluble NSP fraction, [4]Total NSP: total non starch polysaccharides, [5]F-glucose: free glucose, [6]RSO: raffinose-series oligosaccharides.
*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.6. Antinutritional factors on dry matter basis of raw and processed faba beans and reduction percentages

FABA BEANS	Processing*	Phytate		Total tannins		Trypsin inhibitors	
		g100g^{-1}	Reduction,%	g100g^{-1}	Reduction,%	mgg^{-1}	Reduction,%
raw	-	3.20		1.28		2.20	-
B1	Lp/Sd	2.87	10.3	1.14	10.9	1.24	43.6
B2	Sp/Sd	2.80	12.5	1.21	5.5	1.33	39.3
B3	Sp/Hd	3.21	-	1.25	2.3	1.02	53.7
B4	Hp/Sd	3.32	-3.7	1.31	-2.3	1.38	37.1
B5	Hp/Hd	3.17	1	1.25	2.3	1.55	29.7
B6	Sp/-	2.48	22.5	1.28	-	2.20	35.7

*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.7. Proximate composition on dry matter basis of raw and processed chickpeas

CHICKPEAS	Processing*	*Proximate composition*								*Oligosaccharides*			
		ash	protein	Fat	starch	S-NSP[1]	I-NSP[2]	I/S[3]	Total NSP[4]	F-glucose[5]	sucrose	RSO[6]	Total
raw	-	3.5	27.0	5.5	41.9	2.9	10.9	3.7	13.8	0.04	1.4	3.7	5.1
CP1	Lp/Sd	3.6	27.5	5.1	47.4	3.8	9.8	2.6	13.6	0.05	1.4	3.5	5.0
CP2	Sp/Sd	3.6	26.7	5.3	45.0	4.1	9.4	2.3	13.5	0.02	2.0	3.5	5.5
CP3	Sp/Hd	3.5	27.5	5.6	41.2	3.5	9.4	2.7	12.9	0.01	2.1	3.4	5.5
CP4	Hp/Sd	3.5	27.3	5.5	45.5	3.8	9.4	2.5	13.2	0.03	2.0	3.5	5.5
CP5	Hp/Hd	3.5	27.8	5.6	45.6	3.2	10.2	3.2	13.4	0.02	2.1	3.4	5.5
CP6	Sp/-	3.5	26.5	5.0	44.2	3.9	9.7	2.5	13.6	0.01	1.6	4.0	5.6

[1]S-NSP: soluble non starch polysaccharides, [2]I-NSP: insoluble non starch polysaccharides, [3]I/S: Insoluble/Soluble NSP fraction, [4]Total NSP: total non starch polysaccharides, [5]F-glucose: free glucose, [6]RSO: raffinose-series oligosaccharides.
*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.8. Antinutritional factors on dry matter basis of raw and processed chickpeas and reduction percentages

CHICKPEAS	Processing*	Phytate		Total tannins		Trypsin inhibitors	
		g100g^{-1}	Reduction,%	g100g^{-1}	Reduction,%,	mgg^{-1}	Reduction,%
raw	-	2.12		0.49		14.66	-
CP1	Lp/Sd	1.78	16.0	0.49	-	1.63	88.9
CP2	Sp/Sd	2.02	4.7	0.43	12.2	1.66	88.7
CP3	Sp/Hd	1.75	17.5	0.43	12.2	1.81	87.7
CP4	Hp/Sd	2.14	-0.9	0.40	18.4	1.62	89.0
CP5	Hp/Hd	1.83	13.7	0.41	16.3	2.09	85.8
CP6	Sp/-	1.86	12.3	0.46	6.1	1.20	91.8

*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.9. Proximate composition on dry matter basis of raw and processed field peas

FIELD PEAS	Processing*	Proximate composition								Oligosaccharides			
		ash	protein	Fat	starch	S-NSP[1]	I-NSP[2]	I/S[3]	Total NSP[4]	F-glucose[5]	sucrose	RSO[6]	Total
raw	-	3.4	24.4	1.6	42.2	3.5	12.9	3.7	16.4	0.03	1.3	5.3	6.6
P1	Lp/Sd	3.7	25.6	1.9	43.2	4.6	10.9	2.4	15.5	0.05	1.3	4.2	5.6
P2	Sp/Sd	3.5	25.6	2.0	44.2	4.8	9.8	2.1	14.6	0.05	1.3	4.5	5.9
P3	Sp/Hd	3.4	25.3	1.8	44.9	5.7	8.9	1.6	14.6	0.05	1.4	4.5	6.0
P4	Hp/Sd	3.5	25.6	1.9	44.2	4.8	10.5	2.2	15.3	0.06	1.2	5.3	6.6
P5	Hp/Hd	3.3	24.4	1.8	45.3	3.7	11.2	3.0	14.9	0.05	1.2	4.9	6.2
P6	Sp/-	3.8	26.8	2.7	44.0	4.3	9.7	2.3	14.0	0.07	1.2	5.0	6.3

[1]S-NSP: soluble non starch polysaccharides, [2]I-NSP: insoluble non starch polysaccharides, [3]I/S: Insoluble/Soluble NSP fraction, [4]Total NSP: total non starch polysaccharides, [5]F-glucose: free glucose, [6]RSO: raffinose-series oligosaccharides.
*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.10. Antinutritional factors on dry matter basis of raw and processed field peas and reduction percentages

FIELD PEAS	Processing*	Phytate		Total tannins		Trypsin inhibitors	
		g100g^{-1}	Reduction,%	g100g^{-1}	Reduction,%,	mgg^{-1}	Reduction,%
raw	-	1.20		0.78		2.74	-
P1	Lp/Sd	1.16	3.3	0.67	11.5	1.26	54
P2	Sp/Sd	1.22	-1.7	0.69	9.0	1.17	57.5
P3	Sp/Hd	1.11	7.5	0.71	6.4	1.41	48.5
P4	Hp/Sd	0.99	17.5	0.78	1.3	1.12	58.9
P5	Hp/Hd	1.13	5.8	0.73	-	1.32	51.8
P6	Sp/-	1.87	2.5	0.77	11.5	1.27	53.7

*Processing treatments with low (L), standard (S) and high (H) preconditioner (p) temperature and standard (S) or high (H) drier (d) temperature.

Table 3.11. Proximate composition on dry matter basis of raw and processed wheat

WHEAT*	Processing**	Proximate composition								Oligosaccharides			
		ash	protein	Fat	starch	S-NSP[1]	I-NSP[2]	I/S[3]	Total NSP[4]	F-glucose[5]	sucrose	RSO[6]	Total
raw	-	2.3	17.7	1.8	66.2	2.8	7.7	2.8	10.4	0.05	1.1	0.5	1.7
W1 (1.5mm)	Sp/Sd	1.9	14.8	2.0	70.6	3.0	7.3	2.4	10.3	0.02	1.1	0.3	1.4
W2 (0.2mm)	Sp/Sd	2.4	19.0	2.0	65.8	2.9	7.3	2.6	10.1	0.03	1.2	0.5	1.8

[1]S-NSP: soluble non starch polysaccharides, [2]I-NSP: insoluble non starch polysaccharides, [3]I/S: Insoluble/Soluble NSP fraction, [4]Total NSP: total non starch polysaccharides,
[5]F-glucose: free glucose, [6]RSO: raffinose-series oligosaccharides.
*Grinding size of 1.5mm or 0.2mm.
**Processing treatments with standard (S) preconditioner (p) temperature and standard (S) drier (d) temperature.

Table 3.12. Antinutritional factors on dry matter basis of raw and processed wheat and reduction percentages

WHEAT		Phytate		Total tannins		Trypsin inhibitors	
	Processing*	g100g^{-1}	Reduction,%	g100g^{-1}	Reduction,%,	mgg^{-1}	Reduction,%
raw	-	1.68		0.45		1.00	
W1 (1.5mm)	Sp/Sd	1.59	5.4	0.39	14.3	-	100
W2 (0.2mm)	Sp/Sd	1.73	-3.0	0.46	-1.5	-	100

*Processing treatments with standard (S) preconditioner (p) temperature and standard (S) drier (d) temperature.

3.3.2 Physical characteristics of the diets used in experiments I, II and III

Physical properties of diet pellets for Experiment I were examined (Table 3.13) and it was found that among the seven diets CP30 pellets were the hardest (13.8N), while B30 pellets were less hard (11.1N). Seawater absorption in 5s was lower for B30 (2.5% total moisture) and higher for B15 (6.8%). Settling velocity was slower for CP15 (6.7cms^{-1}) and faster for control (11.2cms^{-1}). Density was significantly higher for the control and lower for the diets including chickpeas. Pellet diameters varied from 4.8-5.3mm.

Differences were found for diet pellets produced for experiment II (Table 3.14) for hardness after texture analysis. BH (16.4N) and CPH (16.0N) gave the highest values and CPL (11.1N) the lowest. Seawater absorption in 5s was lower for CPH (3.7% of total moisture) and higher for control (5.7%). Settling velocity was slower for BH (4.8cms^{-1}) and faster for CPL (9.1cms^{-1}). Pellet density was lower for diets CPH and BH (0.94gcm^{-3}) and higher for diet CPL (1.10gcm^{-3}). Measurements of pellet diameters showed that the pellet sizes among the diets varied from 4.5-4.9mm.

Hardness and values were very similar among the pellets of diets tested for experiment III (Table 3.15). Water absorption was lower for diet BL (2.8%) and higher for PH (5.1%). Settling velocity was slower for the control (7.3cms^{-1}) and faster for CPH (9.5cms^{-1}) and PH (9.3cms^{-1}). Pellet density ranged from 0.99gcm^{-3} for the control to 1.16gcm^{-3} for diet PH. Measurements of pellet diameters showed that the values for the diets varied from 4.1-4.3mm.

Water activity (a_w) values of the diets used in all three Experiments ranged from 0.44-0.61.

Table 3.13. Physical characteristics of the diets used in Experiment I.

	Hardness (N)	Water absorption (%)	Velocity (cms^{-1})	Density (gcm^{-3})	Pellet size (mm)	a_w at 23˚C
Control	12.4±1.3	5.2±0.6	11.2±1.8	1.28±0.12	4.8±0.2	0.61
B15	12.4±1.8	6.8±0.3	10.2±3.6	1.11±0.11	5.3±0.3	0.59
CP15	11.9±1.4	4.0±0.5	6.7±3.2	1.04±0.09	5.2±0.1	0.53
P15	12.3±1.8	5.4±0.1	10.0±2.1	1.16±0.06	5.2±0.1	0.53
B30	13.8±2.2	2.5±0.6	8.4±2.2	1.13±0.08	5.1±0.2	0.52
CP30	11.1±1.4	5.7±0.5	7.4±2.2	0.99±0.06	5.3±0.2	0.57
P30	12.3±1.9	3.7±0.2	10.9±1.4	1.13±0.05	4.9±0.2	0.54

Table 3.14. Physical characteristics of the diets used in Experiment II.

	Hardness (N)	Water absorption (%)	Velocity (cms^{-1})	Density (gcm^{-3})	Pellet size (mm)	a_w at 23˚C
Control	13.2±0.6	5.7±0.2	7.3±1.4	1.00±0.04	4.9±0.1	0.57
BL	12.6±0.9	5.3±0.2	8.0±0.2	1.01±0.03	4.7±0.1	0.59
CPL	11.1±0.8	5.2±0.7	9.1±0.7	1.10±0.07	4.5±0.1	0.51
PL	14.2±1.2	4.1±1.0	8.1±1.0	1.02±0.05	4.8±0.1	0.59
BH	16.4±1.1	4.1±0.4	4.8±0.8	0.94±0.06	4.9±0.2	0.51
CPH	16.0±0.6	3.7±0.3	6.0±0.1	0.94±0.04	4.8±0.1	0.55
PH	15.6±1.5	4.2±0.3	8.6±0.6	1.04±0.06	4.8±0.2	0.48

Table 3.15. Physical characteristics of the diets used in Experiment III.

	Hardness (N)	Water absorption (%)	Velocity (cms^{-1})	Density (gcm^{-3})	Pellet size (mm)	a_w at 23˚C
Control	14.3±0.8	3.6±0.5	7.3±1.1	0.99±0.07	4.3±0.2	0.50
BL	13.2±0.6	2.8±0.5	8.5±0.9	1.02±0.04	4.2±0.1	0.44
CPL	12.7±2.1	3.8±0.6	8.7±1.0	1.05±0.05	4.1±0.2	0.53
PL	14.3±1.2	2.5±0.4	8.4±1.1	1.11±0.09	4.1±0.1	0.49
BH	14.8±0.5	4.5±0.5	8.1±2.1	1.03±0.07	4.2±0.2	0.54
CPH	14.4±0.8	5.0±0.4	9.5±1.1	1.08±0.07	4.2±0.2	0.49
PH	13.9±1.3	5.1±0.4	9.3±0.9	1.16±0.05	4.1±0.1	0.53

3.4 Discussion

3.4.1   Raw materials and processing

Small differences in the ash, protein and fat composition of legumes and wheat among the processing treatments could be attributed to normal variations in the analytical methods. Extrusion did not affect either pea or faba bean protein values in other studies (Alonso et al., 2000a; Alonso et al., 2000b) or ash and ether extract of extruded peas (Alonso et al., 2000b). Protein and ash were also measured in raw and extruded peas, chickpeas and faba beans by Abd El-Hady and Habiba (2003) and no differences were found again for any of the legumes before and after processing, while the absolute concentration of protein and ash was slightly higher in the present study only for faba beans.

Starch analysis showed an overall increase in the processed materials that could be related to composition changes in the resistant starch. Resistant legume starch corresponds to physically inaccessible starch that is entrapped in the cellular matrix (Englyst et al., 1992) that in most cases is destroyed by processing (Gonzalez-Soto et al., 2006). Thermal processing such as cooking has been proven to cause a significant reduction of resistant and poorly digestible starch and an increase in rapidly digestible starch in peas (Periago et al., 1996), chickpeas and common beans (Marconi et al., 2000). However, in some cases (potato and banana starch) amylose retrogradation may occur resulting in resistant starch when raw materials are either freeze dried or autoclaved (Gonzalez-Soto et al., 2006). In this respect, highly digestible starch may have increased and this is possibly reflected in the final starch values, while resistant starch formation is less likely to occur in legume starches. The increased values

observed for total starch in processed legumes could be possibly attributed to the easier breakage of extruded starch granules by amylase during analysis.

Total NSP were reduced in all extruded faba beans and peas compared to respective raw product values. Extruded chickpeas also had lower values but the reduction was smaller and these results are in agreement with the results of Alonso et al. (2000b) for extruded peas. A redistribution of S-NSP and I-NSP has been found in rice after press cooking (Sagum and Arcot, 2000) or in cooked chickpeas and common beans (Marconi et al., 2000) with increase of S-NSP and decrease of I-NSP (reduction of ratio I/S-NSP) after treatment. This may be comparable to the results for the extruded chickpeas and peas in the present study although total NSP were not affected in the case of pressure cooking (Marconi et al., 2000). Anguita et al. (2006) also noted that extrusion can increase solubilization of pea NSP, however, solubilisation is less and absolute numbers are higher in the present study and this may be attributed to differences in field pea variety. The redistribution of S-NSP and I-NSP fractions could be attributed to the partial solubilization or depolymerization of hemicellulose and insoluble pectic substances (Vidal-Valverde et al., 1992). Faba bean and wheat flour that underwent drying processing in the present study showed decreased values of S-NSP as opposed to peas, chickpeas and the results of the aforementioned authors but, when faba bean flour was not dried, S-NSP increased. Different behaviours amongst legumes could be attributed to differences in the synthesis of the NSP fraction; for example pea uronic acids and mannose seem to increase, while xylose does not change under extrusion and rhamnose and arabinose are stable (Alonso et al., 2000b). Different results were reported by Periago et al. (1996) between raw and cooked peas NSP fractions, indicating that differences could be expected in the results.

Alonso et al. (2000b) found that pea sucrose, raffinose and verbascose were increased after extrusion processing, but significant differences were only observed for verbascose values, while stachyose values significantly decreased. In a later work the same authors (Alonso et al., 2001) found that pea and kidney bean sucrose, stachyose and verbascose did not differ before and after extrusion, while raffinose significantly decreased in kidney beans. In addition, autoclaving rapeseed for 1.5 hours resulted in decreased levels of sucrose and stachyose (Mansour et al., 1993). In this respect the result of extrusion on total oligosaccharides could also be dependent on the fraction composition that could be variable among different pulses or seed parts such as hulls, dehulled seeds or protein concentrates (Knudsen, 1997).

Phytic acid values in processed ingredients vary in the literature. For instance, Alonso et al. (1998; 2000a; 2000b; 2001) found significant reduction in peas (5.9%), in faba beans (29%) and kidney beans (4%) after extrusion at 150°C (in outer die). However, according to Abd El-Hady and Habiba (2003) findings much lower reduction of phytate was observed between raw and extruded legumes at 140°C or 180°C (barrel temperature). Gualberto et al. (1997) showed that extrusion did not affect cereal bran phytate content. In the present study extruded peas, chickpeas and faba bean phytate values either remained the same or decreased up to 17.5, 17.5 and 22.5% respectively compared to the raw legumes. The reduction of phytate though was not consistent for the different processing methods and thus further investigation is imperative.

Francis et al. (2001) in their review of antinutrients mentioned that the effect of thermal treatment on substances like tannins is still not clear. Total tannins in the current study did not seem to be affected drastically, with most of the values in processed legumes being slightly lower than the values in raw materials. In some cases though that was not the case. In this respect the results are partially in accordance with

those of Abd El-Hady and Habiba (2003) who found a slight decrease in tannins in peas, chickpeas and faba beans after different extrusion temperatures. Nonetheless Alonso et al. (1998, 2000a, 2000b) presented significant reduction in both tannins and polyphenols in peas and faba beans when extruded at 150°C and this reached 92% when compared to the raw legumes. Reduction of condensed tannins was also observed for peas and kidney beans by Alonso et al. (2001) under the same extrusion conditions. Differences in tannin reduction could be attributed to higher temperatures or to the duration of processing methods and these are not always clear especially for the extrusion appliance. It has also been suggested that heat treatment possibly reduces extractability by increasing polymerisation of tannins and thus showing lower values after analysis (van der Poel et al., 1991).

TI are known to be heat sensitive (Francis et al., 2001) and they can be reduced or completely destroyed in different plant materials and under different processing methods (Marquez and Alonso, 1999; Frias et al., 2000; Romarheim et al., 2005). High values of TI in raw chickpeas, similar to the present study, have also been reported from other authors (Abd El-Hady and Habiba, 2003). The highest reduction of TI among legumes was noted in chickpeas reaching 92%, while for faba beans it was up to 44%, for peas up to 59% and in wheat TI was not detected in the extrudates. Reduction of 95% in peas was found by Alonso et al. (2000a; 2000b) and 99% in faba beans (Alonso et al., 1998), while in another study (Abd El-Hady and Habiba, 2003) no TI was detected in any of the tested legumes after extrusion processing no matter the initial quantity of the ANF in the raw seeds or the temperature applied. Complete inactivation of TI in the present study was only observed for wheat flour, even though no high temperatures were applied to this material.

3.4.2 Pellet physical characteristics

Experimental diet pellets were not affected negatively by the addition of low or high levels of legumes with respect to hardness, settling velocity, water activity and pellet density. The pellets did not crumble in the sacks, while transferring or stored, however, a disadvantage of BH diets and to a lesser extent of CPH diets for both Experiments II and III was a small percentage of floating pellets that was not quantified. Pellet physical properties are dependent on the ingredients and the nutrient composition (Briggs et al., 1999; Thomas et al., 1998), on the pellet size (Chen et al., 1999; Vassalo et al., 2006) on the processing techniques (Schwertner et al., 2007) as well as on carbohydrate binders such as cellulose (Hansen and Storebakken, 2007) and chitosan (Santos et al., 2002).

Water activity values were within the safety limits (Schwertner et al., 2003) for all diets indicating that long storage even at room temperature is possible without microbial development and spoilage of the pellets. Although Schwertner et al. (2003) found a good correlation between water content and water activity comparing different pelleting methods, it is not necessary for this correlation to exist (Water activity, 2007). In the present research this fact is justified as no correlation between the two parameters was found.

Settling velocity was obviously positively related to the density of the pellets. The pellets used in the Experiment I had higher average velocity (9.26cms^{-1}) and density (1.12gcm^{3}) than the values observed for diets of experiments II (7.41cms^{-1} and 1.01gcm^{3}) and III (8.55cms^{-1} and 1.06gcm^{3}). Although bigger pellets are related to higher sinking rates (Chen et al., 1999; Vassallo et al. 2006), the small difference in pellet size was possibly less important than the density difference. Higher density levels

of pellets manufactured for Experiment I could be related most possibly to the high level of fishmeal included in this diet ($51\text{-}59\text{g}100\text{g}^{-1}$) in comparison to the rest of the diets ($25\text{-}28\text{g}100\text{g}^{-1}$) (Sorensen et al., in press).

Concerning hardness, diets for Experiment I showed lower average values (12.30N) than diets for Experiment II (14.17N) and III (13.95N). Higher values were observed for H diets compared to L diets in both Experiments II and III. This could be related to the high inclusion levels of legumes and their carbohydrate properties as Sorensen et al., (in press) found increasing hardness when fishmeal was replaced by soybean meal. This result can be considered a positive effect on pellet quality as harder pellets are possibly also more durable (Sorensen et al., in press) and could be used in automatic feeders with less losses.

3.5 Conclusions

From this study it could be concluded that peas, chickpeas and faba beans are raw materials that could potentially be used in seabass and seabream diets without causing negative effects on the pellet physical characteristics. Levels up to $35\text{g}100\text{g}^{-1}$ of each legume are acceptable for producing pellets that could be manufactured, transported and stored. ANF in these materials are not prohibitive for their use in feeds, as total tannin levels are comparatively low compared to those known to affect fish growth performance (Francis et al., 2001), phytate problems could be possibly overcome by mineral supplementation (Porres et al., 2004) and trypsin inhibitors are significantly inactivated under extrusion. In vivo studies in the following chapters will provide more information about the use of peas, chickpeas and faba beans in seabass and seabream diets.

Chapter 4. Experiment I - Effects of peas, chickpeas and faba beans on nutrient digestibility and gastrointestinal evacuation in European seabass (*Dicentrarchus labrax*)

4.1 Introduction

Wheat is an important source of carbohydrate in aquafeeds for Mediterranean species included at levels up to 200gkg^{-1} (Campbell, personal communication). Wheat starch is a good binder and is also considered a good source of energy (Hertrampf and Piedad-Pascal, 2000). However, recent expansion of global biofuel industries has led to increased demands for starch and sugar-rich feedstocks with consequent price increases for such crops (Runge and Senauer, 2007). Indeed, wheat is expected to undergo further price increases in the future (Runge and Senauer, 2007). Furthermore, limited European production of soybean (Grain legumes, 2006) and the general position of EU countries with respect to genetically modified soybeans reinforce the importance of plant sources other than soybean in aquafeeds (Euractiv, 2006).

Legumes represent a significant constituent of global grain production (Grain legumes, 2007) and are highly valued feed components for both ruminants and monogastric livestock due to their relatively high protein and energy values compared to cereals (Peterson and Mackintosh, 1994). Several legumes, including field peas, chickpeas and faba beans, may be able to replace wheat carbohydrates since they are characterized by high carbohydrate content (Knudsen, 1997). Moreover, the protein content of legumes may enable partial replacement of fishmeal (Gouveia and Davies, 1998), which has been consistently high in price over the last few years (Josupeit, 2008).

Many nutritional deficiencies in legumes (e.g. limiting levels of sulphur amino acids and tryptophan) can be overcome by the complementary use of cereal grains or the addition of low cost synthetic amino acids (Booth et al., 2001). Allan et al. (2000) have reported that cystine and glycine availability was relatively low in field peas and

faba beans respectively, while limited availability of several amino acids was found in chickpeas in the same study. A further drawback to the use of legumes in fish diets is the presence of a variety of endogenous ANF including protein inhibitors, amylase inhibitor, phytic acid, tannins and lectins that adversely affect enzyme action or the absorption of minerals and other nutrients (Tacon, 1993). The indigestible carbohydrate content of plant ingredients has often been considered an ANF (Hilton et al., 1983). ANF in legume seeds can be partially or totally inactivated by heat processing methods such as roasting, autoclaving, extruding or cooking or by other processing methods such as soaking, germination or enzyme addition, prior to inclusion in fish feeds (Francis et al., 2001).

Gelatinized wheat is known to be highly digestible by seabass (Peres and Oliva-Teles, 2002), while legume starches are known to be digested at a slower rate in humans (Tharanathan and Mahadevamma, 2003). The rapidness of starch digestion can be reflected in the glucose loading in blood after each feeding (Hemre and Hansen, 1998). The period needed for the fish to recover from glucose loading depends on the starch level and the water temperature (Hemre et al., 1995). However, the glucose curve of European seabass has only been investigated after glucose injection (Peres et al., 1999), but not after feeding different starch sources.

Gastrointestinal evacuation indicates the rate profile of the time needed for an animal to digest a meal and is affected by the physical and chemical composition of the diet (Jobling, 1987). Gastrointestinal evacuation rate has proven to be a very important parameter for modelling daily feed intake (Jobling, 1981) as it is partly responsible for control of appetite in fish (Riche et al., 2004). However, pelleted fish feeds have not been thoroughly investigated in farmed fish in this respect, since most of the pertinent literature deals with 'prey' type feeds, either experimental or natural (Bromley, 1994;

Andrade et al., 1996), feeding frequency (Ruggerone 1989; Zarate et al., 1999), restoration of appetite (Riche et al., 2004) and gastric modelling for prey feed (Salvanes et al., 1995; Andersen and Beyer, 2005).

The objectives of the present study were to evaluate the effect of partial or total replacement of wheat by whole grain flours of field peas, chickpeas and faba beans, on diet digestibility and to determine gastrointestinal evacuation time for extruded diets containing these raw materials at 30% level fed to European seabass.

4.2   Materials and methods

4.2.1   Feed ingredients and feed formulation

Chickpeas, field peas and faba beans were included in the experimental diets containing $15g100g^{-1}$ (CP15, P15 and B15) and $30g100g^{-1}$ (CP30, P30 and B30) of each legume plus a control diet as described in detail in section 3.2.3.1.

4.2.2   Fish and experimental design

Three hundred fish of initial weight 152.3±12.1g were transferred from a commercial farm (located in Epidavros, Greece) to the facilities of the laboratory of Fish Nutrition and Pathology, Institute of Aquaculture, Hellenic Centre for Marine Research in Athens. A total of 20 fish were stocked randomly into each one of the 15 digestibility tanks (section 2.2.2.1) and acclimatized for two months being fed the experimental diets. Faeces were collected and processed as described in section 2.3.2. Seabass were fed daily to appetite, twice a day (9.00am and 16.00pm). Faecal samples were removed every morning prior to feeding for 8 days.

4.2.3 Water quality

Sea water was supplied continuously as described in section 2.2.2.3 Water temperature was controlled at 18±1ºC during the experimental period.

4.2.4 Gastrointestinal evacuation

Following the digestibility trials, high level diets were selected to be compared for gastrointestinal evacuation time to control diet as these diets expected to show stronger effects (control, CP30, B30 and P30). Fish were adapted to the diets for 2 weeks and tested in triplicate groups. Fish were fasted for 72h before being fed the experimental meal to make sure that the gastrointestinal tract was empty. Six fish per treatment were sacrificed at 0, 8, 16, 24, 32 and 48 h after feeding a single meal equal to 1% (diet wet weight) biomass, following phenoxyethanol/ethanol (1/1, 0.4mlL^{-1}) induced anaesthesia (Ekmann, personal communication). The time intervals were selected based on test sampling and taking into account the low temperature as proposed by Finstad (2005).

The method used was the serial slaughter technique. This method involves sacrificing fish at regular time intervals after they have been fed. Fishes were anaesthetized, blood samples were taken and then killed with a blow to the head and the digestive tract was carefully removed and separated into three parts: stomach, foregut and hindgut. Foregut was defined as the section from the pyloric sphincter to the ileocaecal valve and hindgut from the ileocaecal valve to the anus. The gut contents were removed and placed in pre-weighed tubes. Samples were freeze-dried and dry weights were used to estimate the gastric evacuation time (GET) in comparison to the BW of each fish.

Geometric means of stomach and intestinal contents were regressed against time in order to examine possible fit to a model for calculating time and rate of gastric and intestinal evacuation.

4.2.4.1 Stomach

Data from all treatments best fitted an exponential model, in the case of stomach and gastric evacuation rates (GER) calculated using the formula described by Elliott (1972):

$$W_t = A^{-rt}$$

Where W_t is the geometric mean weight of stomach dry digesta at time t, A is a parameter calculated from the formula of the regression and r is the rate of gastric evacuation. Data were plotted in a logarithmic form as

$$LnW_t = lnA – rt,$$

The slope of r corresponding to GER was calculated. GET was calculated from the above equation as the time (t) necessary to empty 50%, 75% and 90% of the stomach content.

4.2.4.2 Foregut

Data for the foregut fitted a linear model. The time needed for the digesta to completely pass the foregut was estimated from the intercept on the x axis and the rate of evacuation by the slope of each equation.

4.2.4.3 Hind gut

Hind gut data best fitted a quadratic model. Points from each curve were estimated to determine the time of maximum intestinal content and evacuation time.

4.2.5 Chemical analysis

Proximate compositions of feeds, freeze-dried faeces, carcass and fillet were determined according to AOAC (1990). Faecal and feed samples were analyzed for starch, and faeces fat determined according to the phosphovanillin method (Nengas et al., 1995). Feeds were also analyzed for total and insoluble NSP (Englyst et al., 1994) and yttrium oxide (Refstie et al., 1997). Blood samples were centrifuged and plasma was analyzed for glucose (section 2.11.2). All chemical methods are described in detail in Chapter 2, General Materials and Methods.

4.2.6 Calculation of digestibility coefficients

ADCs for control and test diets were calculated according to the formulae described in section 2.14. Energy in diets and faeces was determined according to the formula of Blaxter (1989), also presented in section 2.14.

4.2.7 Statistical analysis

ADCs were analysed by one-way ANOVA using SPSS 13.0. Significant differences were evaluated using Tukey post-hoc test at a significance level of $P \leq 0.05$. Regressions were performed using the statistical program Statgraphics Plus 2.1. The linear fits and the comparison of slopes were tested at a significance level of $P = 0.05$.

4.3 Results

4.3.1 Digestibility

ADCs for all diet nutrients tested are summarized in Table 4.1. Digestibility coefficients were high for all diets, but as a consequence of low standard deviations ANOVA revealed significant differences between diets. Protein ADC was significantly

lower for Control and higher for diet B15. Starch digestibility was significantly lower for diet P30 (92.3%) and Control and significantly improved for all three 15% diets. Finally, energy ADCs showed similar trends to starch ADCs demonstrating best values for B15 (96%).

Table 4.1. Apparent Digestibility Coefficients for Protein, Fat, Starch and Energy

	% ADC protein	% ADC Fat	% ADC Starch	% ADC Energy
Control	92.9±0.3[a]	96.7±0.6[ab]	94.0±1.9[ab]	94.3±0.5[a]
Diet CP15	93.2±0.4[ab]	97.2±0.4[bc]	96.4±0.6[cd]	95.0±0.3[b]
Diet P15	93.9±0.2[bc]	97.7±0.2[c]	96.4±0.5[cd]	95.6±0.2[bc]
Diet B15	94.8±0.2[d]	97.7±0.1[c]	97.0±0.7[d]	96.0±0.0[c]
Diet CP30	93.0±0.7[a]	96.2±0.5[a]	94.5±0.2[bc]	94.2±0.4[a]
Diet P30	92.9±0.5[a]	97.0±0.2[bc]	92.3±1.1[a]	94.2±0.3[a]
Diet B30	94.2±0.2[cd]	97.7±0.1[c]	95.7±0.2[bcd]	95.6±0.1[bc]

Different superscripts in each column show significant differences among the values according to Tukey's test (P≤0.05).

4.3.2 Gastric evacuation

GET was evaluated as the geometric mean of dry matter of feed per g of BW of each fish. The values observed immediately after feeding (0h) were lower than 1%BW partially because of the calculation of dry matter and partially because some sampled fish had eaten less than 1%. From the curves generated for each part of the gastrointestinal tract (Figure 4.1), the following exponential equations were determined:

Control: $y = 0.827\ e^{-0.13x}$, $R^2 = 0.98$

B30: $y = 0.804\ e^{-0.08x}$, $R^2 = 0.97$

CP30: $y = 0.655\ e^{-0.07x}$, $R^2 = 0.96$

P30: $y = 0.938\ e^{-0.12x}$, $R^2 = 0.95$
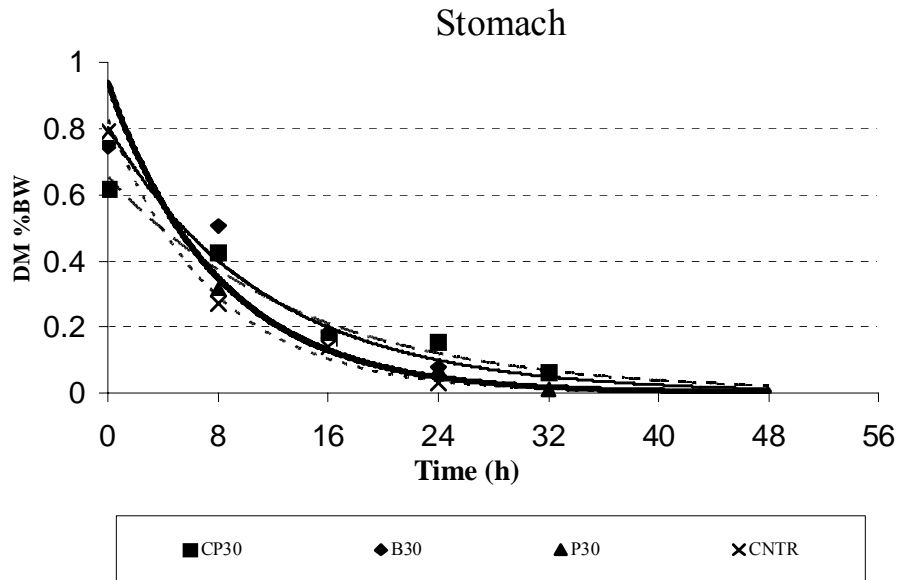
Figure 4.1. Stomach exponential curves show gastric evacuation time (GET) as dry matter % of BW over the 32 hour sampling period in fish fed the four experimental diets.

Table 4.2 describes the GET in relation to the percentage of feed remaining in the stomach. The Control diet required 5.33h to evacuate half of the initial feed content, followed by P30, 5.59h, B30 with 7.95h and longer for CP30, 9.80h. Comparison of the slopes for GET (Figure 4.2) indicated significantly longer GET for CP30 compared to Control and P30 diets, while B30 did not differ from all other diet emptying times (Table 4.3).
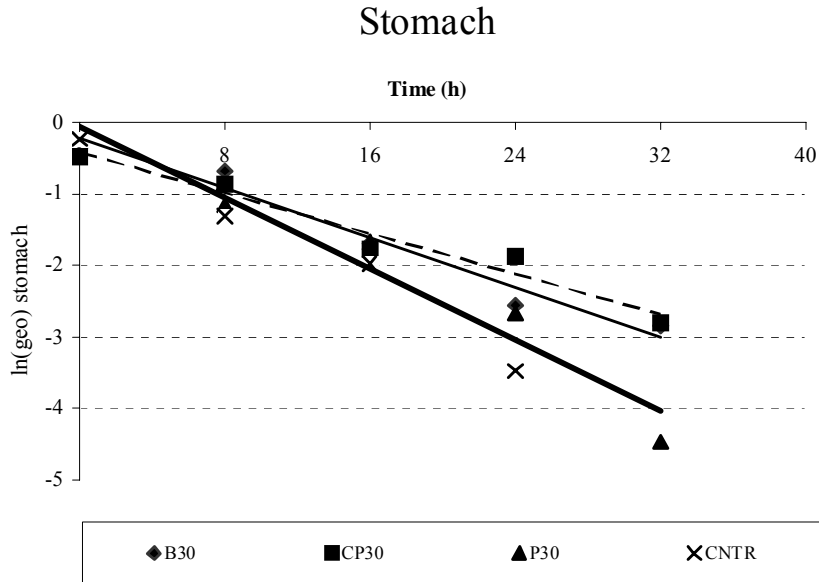
Stomach

Figure 4.2. Stomach curves plotted linearly in a logarithmic form show the gastric evacuation time (GET) for dry matter % of BW over the 32 hour sampling period in fish fed the four experimental diets.

Table 4.2. The gastric evacuation time (GET, h) for European seabass fed the diets and correlation coefficient (r) of the regression lines based on an exponential model of gastric evacuation.

Diet	GET (h) 50%	GET(h) 75%	GET (h) 90%	R	R^2
Control	5.3	10.7	17.7	-0.9892	97.85
P30	5.6	11.2	18.6	-0.9696	94.02
B30	8.0	15.9	26.4	-0.9849	97.00
CP30	9.8	19.6	32.6	-0.9753	95.11

Table 4.3. Coefficients of the stomach linear (y=αx+β) curves and gastric evacuation rate (GER). Slope, intercept and R^2 of the regression lines following the logarithmic transformation and GER (gmin^{-1})

Diet	Slope (a)	GER (*10^{-3})	Intercept (β)	R^2
Control	-0.1300^b	2.17	0.1896	0.98
P30	-0.1241^b	2.07	0.064	0.95
B30	-0.0872^{ab}	1.45	0.2173	0.97
CP30	-0.0707^a	1.18	0.4228	0.96

Different superscripts in each column show significant differences among the values (P≤0.05).

4.3.3 Foregut evacuation

Foregut was evacuated in 31.8h for fish fed the control diet, 38.3h for CP30, 39.5h for P30 and 46.5h for B30 (Table 4.4). The rate of the evacuation is also shown in Table 4.4. The linear relationships are shown in Figure 4.3.

Table 4.4. Slope and intercept of the linear equations (shown in figure 4.3) of each diet for gut evacuation and the time needed (FGET 100%) for the foregut to evacuate completely.

Diet	Slope (a)	Intercept (β)	FGET 100% (h)	FGER (*10^{-3}) (g min^{-1})
Control	-0.0049^a	0.1557	31.8	0.082
P30	-0.0043^b	0.1699	39.5	0.072
B30	-0.0031^c	0.1441	46.5	0.052
CP30	-0.0035^b	0.1339	38.3	0.058

Different superscripts in each column show significant differences among the values (P≤0.05).
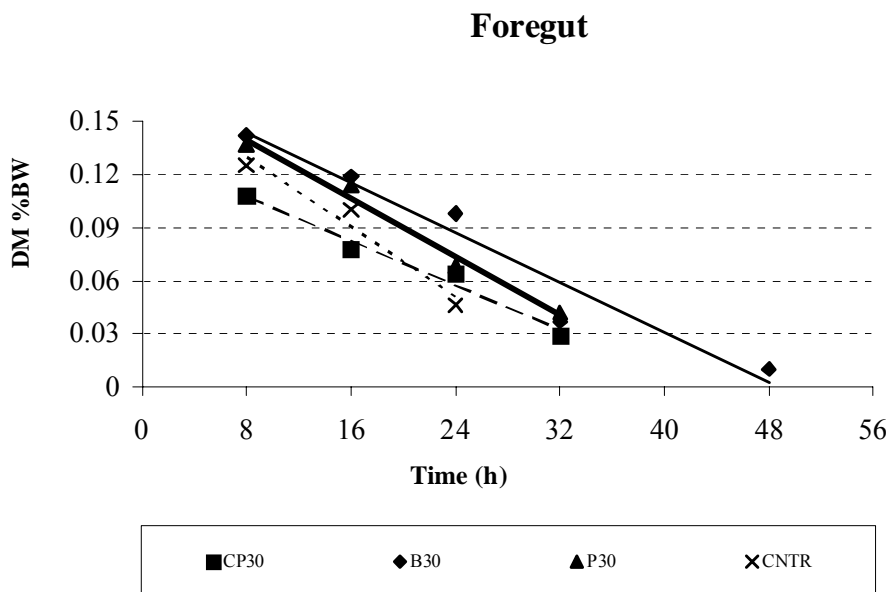
Foregut

Figure 4.3. Foregut linear relationship curves for dry matter % of BW over the 48hour-sampling period in fish fed the four experimental diets

4.3.4 Hindgut evacuation

Hindgut evacuation was 34.2h in the control diet, 41.4h in P30, 51.9h for CP30 and 51.7 for B30. All data for hindgut are presented in Table 4.5 and the quadratic curves in Figure 4.4.

Table 4.5. α, β and γ are the coefficients of the quadratic equations ($y=\alpha x^2+\beta x+\gamma$). The time at which the curve reaches its maximum and the time required for the hind gut to evacuate completely (HGET 100%).

Diet	α ($*10^{-5}$)	β ($*10^{-3}$)	γ ($*10^{-3}$)	Max curve (h)	HGET 100% (h)
Control	-7.785	3.12	-16	20.1	34.2
P30	-2.141	0.78	-4.37	18.2	41.4
B30	-3.339	1.77	-1.06	26.1	51.7
CP30	-2.222	1.20	-2.5	27.0	51.9
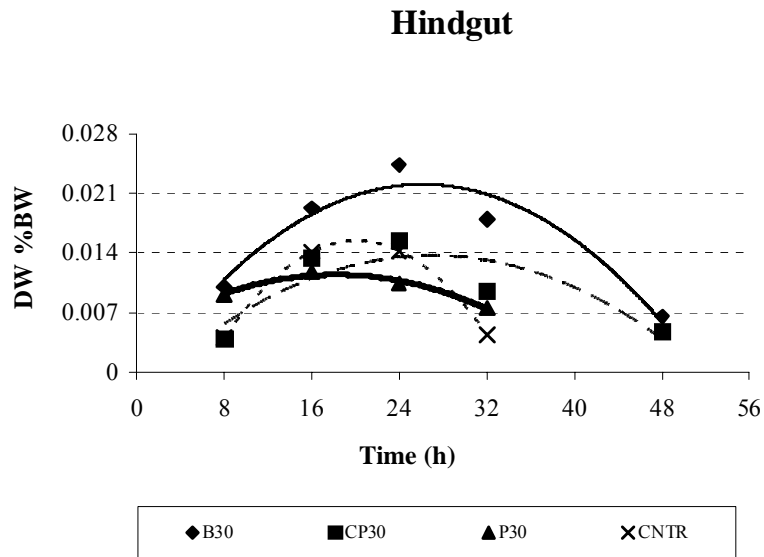
Hindgut

Figure 4.4. Hindgut quadratic curves describe the evacuation of dry matter % of BW over the 48 hour sampling period in fish fed the four experimental diets.

4.3.5 Serum glucose

In the fist sampling (0 hours after feeding) serum glucose values ranged from 4.56 to 5.30mmoldl^{-1} (Table 4.6). After 8 hours fish fed the wheat based control diet showed the most rapid increase that became most obvious by 16h, the curve was similar for CP30 and P30, while B30 showed a delay with glucose only start increasing after 8 hours. After 24 hours, control, CP30 and P30 had a peak, while diet B30 had a peak value after 32 hours. Fish fed the control diet showed a rapid decrease of serum glucose at 32 hours in contrast to the rest of the fish fed the legume diets that had a 'smoother' glucose decline (Figure 4.5). Standard deviations were, not unexpectedly, high and values were normally distributed but they were not homogenous. Transformations did not lead to homogeneity and thus no statistical analysis was applied.
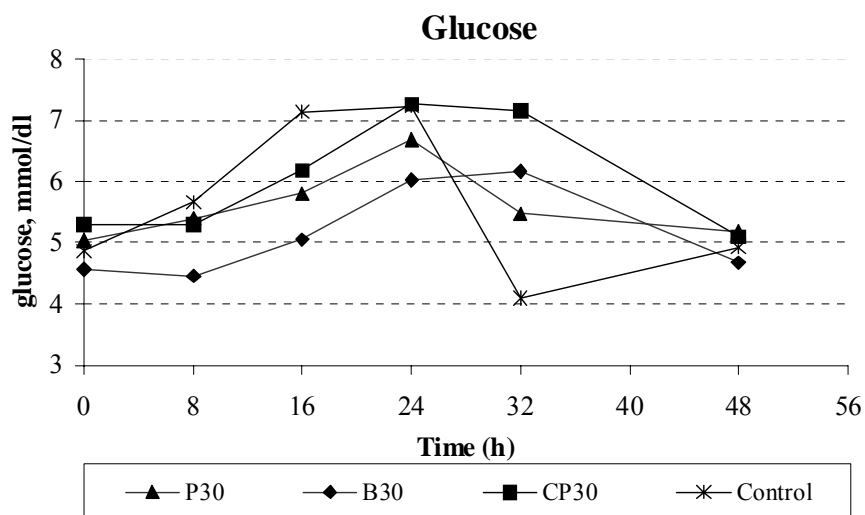
Figure 4.5. Curves describe plasma glucose load (mmoldl^{-1}) over the 48 hour sampling period in fish fed the four experimental diets.

Table 4.6. Plasma glucose values (mmol/dl) measured over 48 hours, in 8 hours intervals for diets control, P30, B30 and CP30.

Hours*	Plasma glucose (mmol/dl)			
	Control	P30	CP30	B30
0	4.87±0.53	5.04±1.83	5.30±1.13	4.56±0.97
8	5.67±1.62	5.39±1.31	5.30±0.95	4.45±0.72
16	7.14±1.49	5.80±0.02	6.18±1.40	5.05±0.51
24	7.24±1.45	6.69±1.18	7.27±1.31	6.02±0.87
32	4.10±0.54	5.47±1.52	7.15±1.01	6.17±1.23
48	4.94±0.53	5.18±1.31	5.10±1.94	4.67±0.31

*Hours of sampling after feeding.
Numbers are given as means±SD (n=6)

4.4 Discussion

In the present study all three legumes tested proved valuable as carbohydrate replacers at both inclusion levels tested in seabass as indicated by their ADCs. However, best values were observed for faba bean diets. Similar studies evaluating legumes for carbohydrate replacement in fish diets are very limited in the literature. Gouveia and Davies (1998) tested diets containing 20 and 40% field pea in European seabass and showed no differences for protein ADCs, but carbohydrate digestibility was significantly lower for the 40% substitution level. The differences in energy and carbohydrate digestibility of field pea diets between this and the present study can be attributed to different processing of the feeds used. Extrusion, as opposed to compressed pellet, was used in the present study, possibly resulting in enhanced carbohydrate digestibility and thus improved energy utilization. The same authors in a more recent study (Gouveia and Davies, 2000) tested extruded pea seed meal in diets for European seabass up to 30% and all nutrient ADCs did not differ for the different inclusion levels, while both protein and carbohydrate ADCs were higher than in their previous research (Gouveia and Davies, 1998). When pea seed meal was included at 18% and substituted for wheat in Atlantic salmon extruded diets (Aslaksen et al., 2007) all nutrient digestibilities were lower compared to those found in the present study, while starch ADC decreased as opposed to the present findings. The same authors also evaluated 22% faba bean substitution for wheat and found similar values to the control ADC, contrary to decreased starch ADC, which disagrees with the current results. No similar studies appear to be available for chickpeas, with the exception of Booth et al. (2001) who investigated digestibility in Australian silver perch and found values of 88.1 and 72.1% for protein and energy ADCs, respectively.

There is little published information on how different starch sources influence utilization of other nutrients in the diet. It seems as though more easily digestible starch (gelatinized) cannot influence protein digestibility but can improve energy and starch digestibility (Peres and Oliva-Teles, 2002).

According to Knudsen (2001), differences in starch digestibility among different diets are more likely to be due to the structure of the cell walls that enclose the starch granules, different amylase/amylopectin ratios, and the presence of α-amylase inhibitors rather than to differences in digesta viscosity and intestinal fermentation activity. Taking into account the fact that amylase/amylopectin ratios vary widely among different plant species and among different varieties of the same species of cereals and legumes, it is not possible to relate this parameter to starch digestibility, since this ratio was not determined in the present study. In general wheat starch includes 20-30% amylose while legumes include 30-60% amylose and higher values of amylose can adversely affect starch digestibility (Hoover and Zhou, 2003). However, in the present study, higher starch ADC was observed for diets combining wheat and legume starch rather than the diets including only one type of starch.

NSP in monogastric animals can delay intestinal absorption of glucose, possibly through a reduced rate of gastric emptying, leading to delayed absorption (Knudsen, 2001). Furthermore, soluble dietary NSP can increase the viscosity of the stomach contents (Storebakken, 1985). In the current study, total, soluble and insoluble NSP did not play any significant role in starch digestibility, however, the diet with the higher inclusion level of total NSP (B30) showed the longest evacuation time for the gut and one of the highest values for starch digestibility.

In most GER studies to date, the aim has been investigation of the nutritional habits of wild fish by creating specific models (Andersen and Beyer, 2005), comparing

pelleted and prey feed (Andrade et al., 1996) or to investigate the effects of prey size (Santos and Jobling, 1991) and temperature (Finstad, 2005). The majority of these studies showed that the relationship between food remaining in the gastrointestinal tract and time can be described by a linear, square root or exponential equation (Bromley, 1994). In the present study with seabass, the mathematical models giving best fit were found to be different for each part of the tract for the high level diets being exponential for the stomach, linear for the foregut and quadratic for the hindgut. Stomach curves followed the formula described by Elliott (1972) and supported by Persson (1986) as a model, fitting better for voluntary feeding. However, no comparable data are available for fore and hind gut models.

High standard deviations were observed for the hindgut dry weight values in the present study, due to the small length of the hind gut, the low quantity of the sample obtained and increased mucus content present in this part of the intestine. Hindgut quadratic curves show that this part of the intestine accumulates the indigestible contents of foregut, reaches a maximum point and defecation starts or continues at a faster rate. Along with this observation no correlation was found between gastrointestinal evacuation time and digestibility of diet nutrient which is in agreement with the findings of Sveier et al. (1999).

In evacuation rate studies, it is preferable to measure the meal size of each individual fish (Bromley, 1994). During communal feeding it is difficult to control the amount of feed eaten by individual fish, but the results can be less biased than when fish fed individually or forced fed (Bromley, 1994). For this reason the variance in initial stomach content is expected to be high (Jensen and Berg, 1993; Bromley, 1994). These initial variations in meal size are also reflected in the variations measured in the amount of feed retrieved from the different parts of the digestive tract. Such substantial

variations were also determined in this study, but to avoid extreme values when a fish was overfed or under-fed it was rejected and immediately replaced by another during the sampling procedure.

A single meal experimental protocol was followed in the present trial, as in most similar published GET experiments. Multiple meal experiments usually show that the second meal reduces the time of first meal evacuation and also increases the emptying time of the second meal (Elliott, 1975). In aquaculture the most common practice is feeding more than one meal per day. Hence, whilst the results presented here give a comparative evaluation among the different diets, absolute values would be different in multiple meal production processes.

In practical terms, inclusion of legumes in diets for seabass clearly increased the GET of the feed with faba bean having the strongest effect. NSP inclusion level may have affected this delay (Knudsen, 2001), as indigestible particles of the feed are the last to remain in the stomach after the digestible part of the diet has been expelled and their evacuation is slow (Bromley, 1987). NSP could also have affected hindgut evacuation time. Different extruded starch sources of wheat and corn result in different GER for gilthead seabream (Venou et al., 2003). It is possible that the structure of the extruded starch molecules of the tested legumes could result in this delay.

Legume starches promote slow and moderate postprandial glucose and insulin responses and result in lower glycaemic indexes in humans (Tharanathan and Mahadevamma, 2003), while fish seem to have a longer postprandial hyperglycaemia after carbohydrate intake (Brauge et al., 1994). Peres et al. (1999) in a glucose tolerance test of seabass at 22ºC found higher range between the lower and the higher value compared to the present study and a peak 3-6 hours after a glucose injection, while in a similar test with salmon at 2-3ºC (Hemre and Hansen, 1998) the peak was found 3

hours after the injection. The present study was performed to evaluate the effect of different starch sources on the glycaemic curve and thus the results are not comparable to the pre-mentioned studies. The increase of evacuation time in the foregut could have also affected the glucose absorption rate. Diets control, CP30 and P30 evacuated 90% of the digesta after 32, 38 and 39 hours respectively and the glucose was rapidly reduced for the wheat-starch diet, while a delay in glucose reduction found for pea and chickpea starch diets. Fish fed diet B30 evacuated the digesta after 46 hours, showing at the same time a better glucose regulation with a lower peak in glucose curve and a strong possibility of correlation of these two facts.

4.5 Conclusions

In conclusion, all three legumes tested proved to have potential as feed ingredients for European seabass, mainly as carbohydrate replacers for wheat and to a lesser extent for protein substitution. Digestibility coefficients showed satisfactory values for all diets either including low or high levels of legumes. In addition starch digestibility was slightly improved for the diets that combined wheat and legume starch source. GET was significantly delayed by the inclusion of chickpeas, while foregut evacuation rate was reduced for all legume diets with faba beans showing the strongest effect. Glucose levels in seabass serum was also affected by the type of carbohydrates ingested with wheat starch showing more rapid increase and decrease of glucose compared to fish fed on pea and chickpea diets, while faba bean starch had a delay in glucose peak and a lower value as well.

Chapter 5. Experiment II - Effects of peas, chickpeas and faba beans included at two levels in European seabass (*Dicentrarchus labrax*) diets

5.1 Introduction

Legumes are cultivated extensively representing a significant part of global grain production (Grain legumes, 2007) and are highly valued feed components for both ruminants and monogastric livestock due to their relatively high protein and energy values compared to cereals (Peterson and Mackintosh, 1994). Several legumes, including field peas, chickpeas and faba beans, are characterized by high carbohydrate content (Knudsen, 1997) and thus may potentially replace wheat carbohydrates in fish diets. The relatively high crude protein content of legumes may also enable partial replacement of protein rich ingredients such as fishmeal (Gouveia and Davies, 1998) or commonly used plant protein sources that could be very significant economically.

Carbohydrates in feeds for farmed fish represent 10-40 percent of the total energy (Hemre et al., 2002) with wheat being one of the important carbohydrate sources. Wheat starch is a good binder and is also considered a good source of energy (Hertrampf and Piedad-Pascal, 2000). However, present high demand for starch and sugar-rich materials (fermentable carbohydrate) due to expansion of the biofuel industry has led to the price rises for these crops (Runge and Senauer, 2007) and for this reason wheat may suffer further price increases in future years (Runge and Senauer, 2007). Limited European production of soybean and the reaction of EU countries to genetically modified soybeans (Euractive, 2006), reinforces the importance of using plant sources, other than soybean, in aquafeeds.

ANF such as protein inhibitors, amylase inhibitor, phytic acid, tannins and lectins adversely affect enzyme action or mineral and other nutrient absorption (Tacon, 1993), but they can partially or totally inactivated by heat processing (Francis et al., 2001). Furthermore, the indigestible carbohydrate content of plant ingredients has often been

considered to be an ANF (Hilton et al., 1983) that cannot be reduced or inactivated by heat processing. However, some I-NSP can be converted into the soluble form (Anguita et al., 2006) increasing digesta viscosity and changing the interaction between enzymes and digesta (Edwards et al., 1988), as well as the absorption rate (Johnson and Gee, 1981). Legumes are deficient in sulphur amino acids (methionine and cystine) and tryptophan, but these deficiencies can be overcome by the complimentary use of cereal grains or the addition of low-cost synthetic amino acids (Booth et al., 2001). Cystine and glycine availability has been found to be low in field peas and faba beans, respectively, while limited availability of several amino acids was found in chickpeas (Allan et al., 2000).

The influence of legume seeds as a carbohydrate source on fish blood parameters has received little attention. Glucose tolerance tests tend to show the relatively slow removal of carbohydrate from the plasma compartment (Shikata et al., 1994), however, glucose may be of primary importance as an oxidative substrate to some cells and tissues in fishes, with expected large inter-species variability (Hemre et al., 2002). Interesting differences exist in the effectiveness of different carbohydrates with respect to lipogenesis and protein sparing. In sturgeon, for instance, glucose and maltose are more lipogenic and can result in increased plasma triacylglycerols and cholesterol than carbohydrates such as fructose, sucrose, lactose, dextrin and starch or cellulose (Hung et al., 1989)

There is some evidence that legumes, like soybean, can cause antigenic activity in mammals (Lalles, 1993) and in salmonids (Burrells et al., 1999), suggesting that there are mechanisms involving the immune system and substances present in soybean or peas (Esparza et al., 1996), however, these processes are complex and not yet clear. The hindgut of fish is sensitive to antigen stimulation and strong immune responses are

caused when antigens are delivered to this part of the digestive tract (Ellis, 1995). Thus, enteritis and immunological parameters seem to be related in rainbow trout (Rumsey et al., 1994). Histological changes have been found in this part of intestine in Atlantic salmon fed soybean meal (Baeverfjord and Krogdahl, 1996).

Carbohydrate level in fish diets has been investigated in several species such as rainbow trout (Austreng et al., 1977; Bergot, 1979; Brauge et al., 1994; Krogdahl et al., 2004), salmon (Arnesen et al., 1995; Krogdahl et al., 2004), tilapia (Wang et al., 2005) and European seabass (Perez et al., 1997; Lanari et al., 1999). Most of these studies link carbohydrate level to liver size (HSI), liver fat and liver glycogen, while Hemre and Hansen (1998) and some of the afore mentioned authors (Bergot, 1979; Arnesen et al., 1995) also correlate these characteristics to the carbohydrate source.

The objectives of the present study were to evaluate the effect of total replacement of wheat by whole grain flours of field peas, chickpeas and faba beans and the interactions of starch/legume level and legume type on diet digestibility, growth performance and organoleptic characteristics of European seabass and to evaluate blood serum indices and immunological factors as well as the histology of internal organs for European seabass.

5.2 Materials and methods

5.2.1 Feed ingredients and experimental diets

Three different legumes including field peas, chickpeas and faba beans represented by P, CP and B respectively, were included in six practical type extruded diets containing approximately $17g100g^{-1}$ (L diets) or $35g100g^{-1}$ (H diets) of each legume plus the control diet as described in detail in section 3.2.3.2.

5.2.2 Fish and experimental design

5.2.2.1 Digestibility

Four hundred fish of initial weight 176±11g were transferred from a commercial farm (located in Epidavros, Greece) and a total of 20 fish were stocked randomly in each digestibility tank (section 2.2.2) and acclimatized for two months, being fed the experimental diets. Digestibility trials were carried out in triplicate, seabass were fed to satiation, twice a day (9.00am and 16.00pm) and faeces were collected as described in section 2.2.2. Faecal samples were removed every morning for 8 days, immediately prior to feeding, and processed as described in section 2.3.2.

5.2.2.2 Growth

Seven hundred seabass of initial weight 97.9±4.8g were transferred from the same farm and randomly distributed into 21 cages of 1.5m^3 as described in section 2.2.2. Fish were weighed at the start of the trial, mortalities were recorded and dead fish were removed, while fish were hand fed twice a day (9:00am and 16:00pm) to satiation. Growth experiment took place from August to November and lasted for 14 weeks.

5.2.4 Sampling procedure

At the beginning of the experiment fish were weighed and a sample of 6 fish were taken as representative of the initial population to determine whole body proximate composition. At the end of the 14-week trial fish were fasted for 40h and 8 fish per tank anaesthetized and blood samples taken prior to any other sampling procedure. Sampling procedure is described in section 2.3.3.

5.2.5 Water quality

The average temperature of the water was at 25±1ºC during the digestibility experimental period (June and July) and 17.3-28ºC during the growth period with an average temperature of 23.4ºC, as temperatures below 20ºC were recorded only in the last week of the experiment. Water quality is described in section 2.2.2.3

5.2.6 Chemical analysis

Proximate analysis, faecal fat, starch, yttrium oxide, liver fat, liver glycogen, serum glucose, total protein, triacylglycerols, cholesterol and chemiluminescence were also determined. All chemical methods are described in detail in General Materials and Methods.

5.2.7 Calculation of nutritional parameters

Growth parameters, ADCs, FCR, SGR, PPV and HSI formulae are presented in section 2.14.

5.2.8 Histology

Liver, spleen, kidneys and intestine (foregut and hindgut) were dissected from three fish per cage. Tissue samples were treated as described in section 2.12.

5.2.9 Sensory analysis

A total of 3 fish per cage (18 fillets) being fed control and H diets were harvested, placed into ice and, within 2-6 hours, they were gutted, filleted and whole fillets were wrapped in aluminium foil and steam cooked for 20min Sixteen trained assessors evaluated flesh taste intensity, flavour intensity, colour, oiliness, elasticity, hardness,

stickiness and general acceptance from 0 to 5 in a ranking test. The training of assessors and the design of the test were performed according to the general quantitative descriptive analysis from Murray et al. (2001).

5.2.10 Statistical analysis

All data are presented as means±SD (n=3) and were analysed by one-way or two-way ANOVA as described in section 2.15. Chemiluminescence results were evaluated by One-Sample Kolmogorov-Smirnov Test and Homogeneity of Variances tested with the Levene test. Pearson correlation in SPSS 13.0 was used to evaluate any significant ($P<0.01$ and $P<0.05$) positive or negative correlation among fat metabolism parameters and among fillet characteristics

5.3 Results

5.3.1   Digestibility and blood serum

5.3.1.1 Digestibility one-way ANOVA

High digestibility values were recorded for all diets (Table 5.1). However, significant differences were detected after one-way ANOVA between control and L diets, due to low standard deviations. ADCs for protein ranged from 93.3-95.5%, for fat from 96.1–97.9%, for starch from 97.6-99.1% and for energy from 94.7-96.8%. The wheat based control diet showed the highest and peas the lowest ADC values. Significant decreases were noted (compared to the control) for protein digestibility in PL and CPL, for fat in diet PL, for starch in diets BL and PL and for energy in diets CPL and PL. ANOVA was also applied for comparison of all seven diets indicating increase of protein and energy ADCs for all H diets compared to CPL and PL, but not

108

to control diet, while fat ADC was significantly lower for PL diet. Starch digestibility was significantly increased for H when compared to diet PL.

5.3.1.2 Digestibility two-way ANOVA

Two-way ANOVA applied between L and H diets revealed significant interaction for all ADCs and therefore main effects were not examined and the simple main effects (the effect of one parameter at a given level of the other) were analysed.

Protein ADC ranged from 93.3–95.3%. A significant interaction of inclusion level and legume type was observed (Figure 5.1a) showing that inclusion level significantly affected P and CP diets with H diets giving increased protein ADC compared to the respective L diets. Regarding the effect of inclusion level in L diets, significant increase was noted for diet BL compared to CPL and PL.

Fat ADC ranged from 96.1–97.9% and a significant interaction of inclusion level and legume type was observed (Figure 5.1b). Specifically, for H diets BH increased fat ADC compared to CPH and for L diets PL showed decreased values compared to BL and CPL. Regarding legume type, diet PL resulted in lower fat ADC compared to PH.

Starch ADC ranged from 97.4-98.8% and a significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.1c) showing that inclusion level significantly affected CP diets with CPL giving increased values. Regarding the inclusion level effect in L diets, CPL significantly increased starch ADC compared to BL and PL, while in H diets PH decreased compared to BH.

Energy ADC ranged from 94.7–96.6% and a significant interaction of inclusion level and legume type was observed (Figure 5.2d). In L diets, diet P showed

significantly lower energy ADC compared to B and CP. Comparing the legume level PH showed significantly higher energy ADC than PL.

5.3.1.3 Serum one-way ANOVA

One-way ANOVA showed that fish fed the wheat-based control diet did not differ in any of the tested serum parameters compared to fish fed L diets.

Specifically, blood serum glucose showed significantly elevated values in seabass fed diet CPH, followed by diet BH, while all other treatments resulted in lower values compared to both of these (Table 5.2). Cholesterol levels were significantly higher for diet CPH only when compared to diet BH, while total protein and triacylglycerol levels did not show any significant difference among experimental groups.

5.3.1.4 Serum two-way ANOVA

Two-way ANOVA among L and H diets revealed significant overall effects and interactions for serum glucose and cholesterol and simple main effects were examined.

Serum glucose ranged from $3.63–10.79 \text{mmoldl}^{-1}$ 40 hours after feeding. A significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.2a), showing that inclusion level significantly affected CP diets with CPH giving significantly increased glucose values. Regarding the effect of inclusion level in H diets, PH exhibited significantly reduced glucose compared to BH and BH compared to CPH.

Serum protein ranged from $5.95–7.28 \text{gdl}^{-1}$ and significant effects were observed only for inclusion level (Figure 5.2b). Specifically, fish fed H diets had higher serum protein levels than fish fed L diets.

Serum cholesterol ranged from 10.20–13.32mmoldl^{-1} and significant effects were observed only for legume type, showing that B diets significantly decreased cholesterol levels compared to CP and P diets (Figure 5.2d).

Serum triacylglycerols ranged from 2.79–3.90mmoldl^{-1} and did not show any significant differences among the examined parameters (Figure 5.2c).

Table 5.1. Apparent Digestibility Coefficients (ADCs, %) of the diet nutrients in seabass

ADCs	ONE-WAY ANOVA							TWO-WAY ANOVA P		
	Control	BL	CPL	PL	BH	CPH	PH	level	legume	level x leg.
Protein	95.5±0.6^{C,c}	94.9±0.7^{BC,bc}	93.8±0.5^{AB,ab}	93.3±0.4^{A,a}	95.3±0.4^{C}	95.3±0.2^{C}	95.2±0.3^{C}	0.000	0.015	0.041
Fat	97.9±0.2^{B,b}	97.7±0.5^{B,b}	97.3±0.3^{AB,b}	96.1±0.5^{A,a}	97.9±0.2^{B}	97.0±0.5^{AB}	97.6±0.2^{B}	0.022	0.003	0.005
Starch	99.1±0.4^{D,b}	98.0±0.2^{AB,a}	98.8±0.2^{CD,b}	97.6±0.3^{AB,a}	98.2±0.4^{BC}	97.8±0.2^{AB}	97.4±0.3^{A}	0.033	0.001	0.010
Energy	96.8±0.2^{C,c}	96.2±0.6^{BC,bc}	95.6±0.4^{AB,ab}	94.7±0.4^{A,a}	96.6±0.3^{C}	96.4±0.1^{BC}	96.4±0.3^{C}	0.000	0.008	0.023

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.
P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.
Values are expressed as mean±SD (n=3)

Table 5.2. Blood serum glucose, triacylglycerols, cholesterol (mmoldl^{-1}) and protein (gdl^{-1}) levels of seabass.

Blood serum	ONE-WAY ANOVA							TWO-WAY ANOVA		
	Control	BL	CPL	PL	BH	CPH	PH	level	legume	level x leg.
Glucose (mmoldl^{-1})	4.95±0.20^{BC}	4.28±0.13^{AB}	4.40±0.18^{AB}	4.30±0.51^{A}	7.66±0.86^{CD}	10.79±1.35^{D}	3.63±0.83^{AB}	0.000	0.000	0.000
Protein (gdl^{-1})	6.86±0.83	6.16±0.65	6.51±0.39	5.95±0.44	7.14±0.98	7.28±1.04	7.00±0.52	0.017	0.606	0.939
Triacylglycerols (mmoldl^{-1})	3.87±0.24	2.79±0.49	3.90±0.48	2.97±0.43	2.89±0.71	3.07±0.38	2.86±0.86	0.326	0.157	0.384
Cholesterol (mmoldl^{-1})	12.49±1.88^{AB}	11.25±0.51^{AB}	12.34±0.62^{AB}	12.63±1.03^{AB}	10.20±0.93^{A}	13.32±0.54^{B}	12.13±0.69^{AB}	0.599	0.001	0.088

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.
P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.
Values are expressed as mean±SD (n=3)
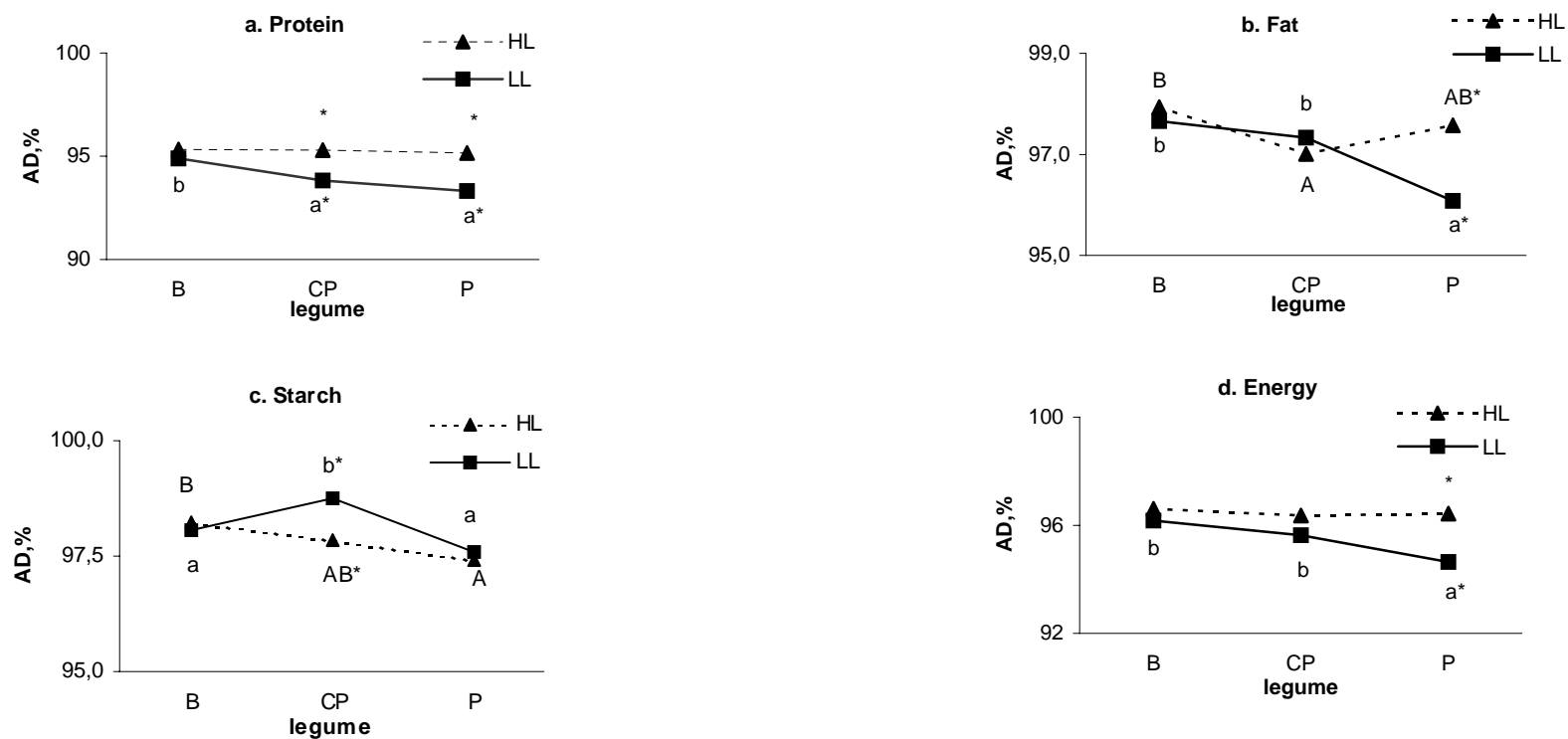
Figure 5.1. Means of seabass ADC of protein (a), fat (b), starch (c) and energy (d) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction.

For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL are marked with an asterisk.
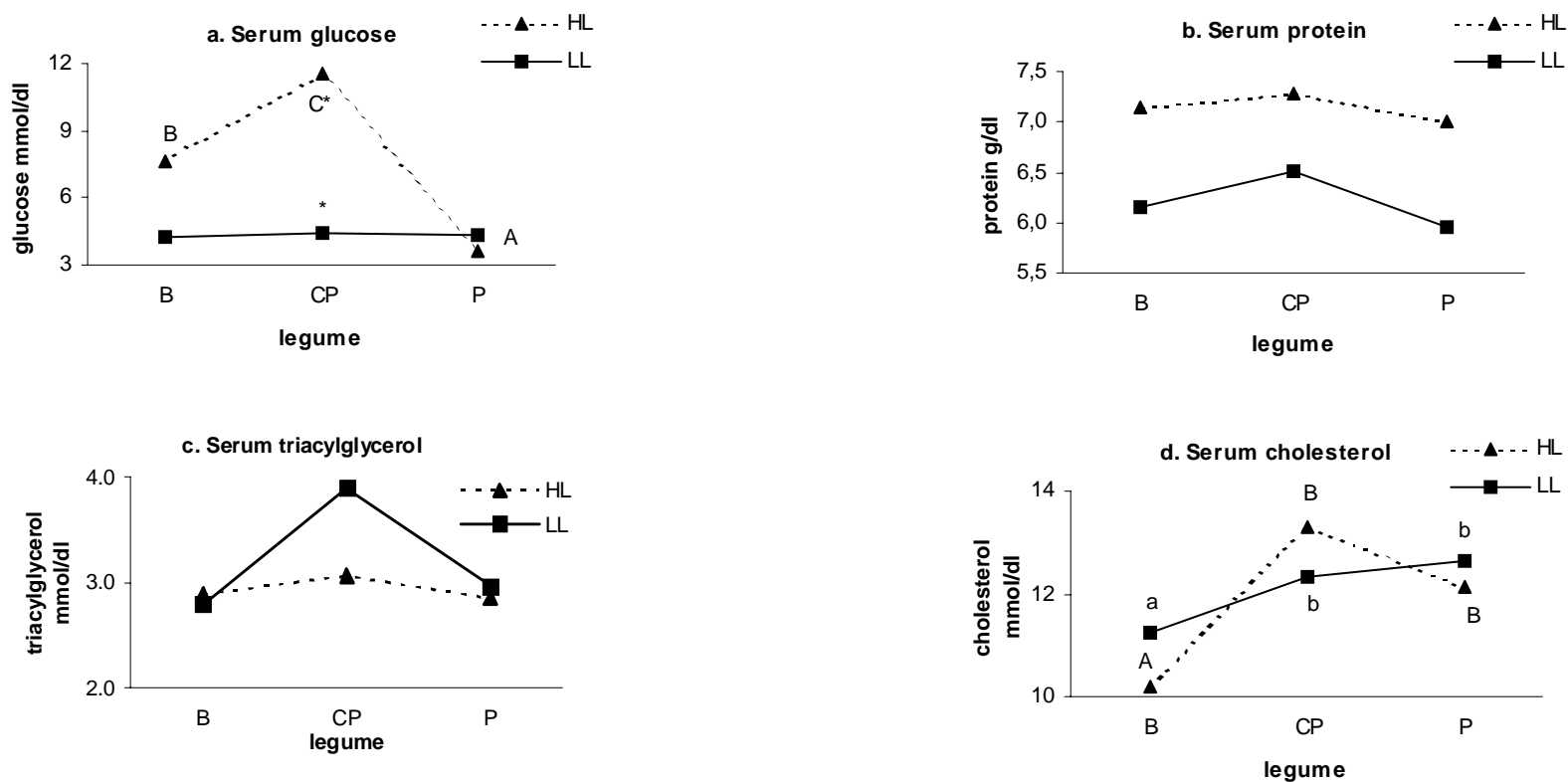
Figure 5.2. Means of seabass serum glucose (a), total protein (b), triacylglycerols (c) and cholesterol (d) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction. For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk.

5.3.2   Growth and liver characteristics

5.3.2.1 Growth one-way ANOVA

Satisfactory growth was observed for all diets (Table 5.3) and significant differences for only SGR were detected by one-way ANOVA between the control and L diets. FCR ranged from 1.24–1.34, SGR from 0.86–1.00 and PPV from 0.27–0.30. Significant improvement was noted in SGR only for fish fed diet CPL. Regarding liver characteristics, glycogen values ranged from 9.3–10.3%, total lipids from 28.4–32.7% and HSI from 2.3–2.7%BW. Significantly decreased HSI was observed in fish fed L diets compared to the control. ANOVA was also applied among all seven diets indicating that fish fed diet CPL had significantly improved FCR than CPH and improved SGR when compared to CPH and control. Total liver fat was significantly lower for fish fed diet PH compared to BL, while diets CPH and PH resulted in higher HSI than the rest of the diets.

5.3.2.2 Growth two-way ANOVA

Two-way ANOVA among L and H diets revealed significant overall effects and interactions for some of the growth parameters and simple main effects were examined.

For FCR a significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.3a), showing that inclusion level significantly affected fish fed the P and CP diets with H diets giving increased FCRs. Regarding the effect of inclusion level in L diets, significant improvement (in this case reduction) was noted for fish fed diets CPL and PL compared to diet BL.

SGR ranged from 0.87–1.00 and significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.3b), showing that inclusion level significantly affected fish fed CP diets with a high level adversely affecting growth rate. With respect to the effect of inclusion level, no significant effect was noted among H or L diets

PPV ranged from 0.27–0.30 and a significant effect of legume type was shown from two-way ANOVA (5.3c), but no significant effect of inclusion level or interaction. Specifically, both diets containing CP significantly improved PPV compared to diets containing B.

Liver glycogen ranged from 9.4–11.5% and significant effects were observed only for inclusion level with H diets resulting in higher glycogen levels than L diets (Figure 5.4a).

Liver fat ranged from 25.2–32.7% and significant effects were observed again only for inclusion level (Figure 5.4b). Particularly, BL and PL resulted in lower liver fat values than BH and PH diets respectively.

HSI ranged from 2.3–2.9%BW and significant effects of inclusion level and legume type were shown from two-way ANOVA (Figure 5.4c), but no interaction was noted. Specifically, when H diets were fed fish showed increased HSI and among the three legume types lower values were observed for fish fed the B diets compared to CP and P.

A strong positive correlation (P<0.01) was found between glycogen and HSI, while a negative correlation was observed for glycogen and liver fat. A significant (P<0.01) negative correlation was also noted for serum glucose with HSI and carcass fat

($P<0.05$). No other correlations were found among HSI, liver fat, blood serum cholesterol, triacylglycerols, glucose and carcass fat content (Table 5.4).

Table 5.3. Growth indicators of seabass fed diets with high (H) or low (L) level of legumes.

	ONE-WAY ANOVA							TWO-WAY ANOVA		
	Control	**BL**	**CPL**	**PL**	**BH**	**CPH**	**PH**	level	legume	level x legume
Initial BW (g)	102.4±11.0	100.1±5.6	95.0±1.9	98.6±5.3	96.0±9.1	96.3±2.7	96.5±5.9			
Final BW (g)	250.5±16.9	264.6±15.3	268.4±11.4	264.2±8.9	242.0±10.7	238.0±9.5	256.0±18.6			
FCR[1]	1.34±0.07^{AB}	1.31±0.02^{AB}	1.24±0.04^{A}	1.27±0.03^{AB}	1.32±0.05^{AB}	1.36±0.04^{B}	1.35±0.02^{AB}	0.001	0.776	0.043
SGR[2]	0.86±0.06^{A,a}	0.93±0.01^{AB,ab}	1.00±0.02^{B,b}	0.95±0.04^{AB,ab}	0.89±0.06^{AB}	0.87±0.02^{A}	0.94±0.02^{AB}	0.03	0.415	0.033
PPV[3]	0.27±0.02	0.27±0.01	0.30±0.01	0.29±0.00	0.29±0.01	0.29±0.01	0.30±0.00	0.098	0.016	0.288
NI[4] (%BW)	4.29±0.12^{ABC}	4.65±0.33^{BC}	4.68±0.15^{C}	4.49±0.11^{ABC}	4.15±0.16^{ABC}	3.93±0.30^{A}	3.95±0.34^{AB}	0.001	0.505	0.692
FI[5] (%BW)	1.08±0.07	1.13±0.00	1.14±0.00	1.11±0.02	1.10±0.03	1.11±0.02	1.17±0.04			
Liver characteristics										
Glycogen (%)	10.3±1.4^{ab}	9.4±1.1^{a}	9.8±1.0^{ab}	9.5±1.2^{a}	10.8±0.8	10.5±0.8	11.5±0.8	0.003	0.597	0.379
Total lipid (%)	28.4±3.0^{AB,ab}	32.7±5.2^{B,b}	30.2±5.4^{AB,ab}	28.4±3.0^{AB,ab}	28.1±4.6^{AB}	31.9±3.6^{AB}	25.2±2.9^{A,a}	0.028	0.191	0.052
HSI[6] (%BW)	2.67±0.02^{B,b}	2.34±0.03^{A,a}	2.43±0.05^{A,a}	2.48±0.05^{A,a}	2.73±0.1^{B}	2.93±0.06^{C}	2.92±0.05^{C}	0.000	0.001	0.294

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.

P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.

Values are expressed as mean±SD (n=3)

[1]Feed Conversion Ratio, [2]Significant Growth Rate, [3]Protein Productive Value, [4,5]Nitrogen and Feed Intake measure as a percentage of Body Weight, [6]Hepatosomatic index

Figure 5.3. Means of seabass growth characteristics; FCR (a), SGR (b) and PPV (c) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction.

For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk.
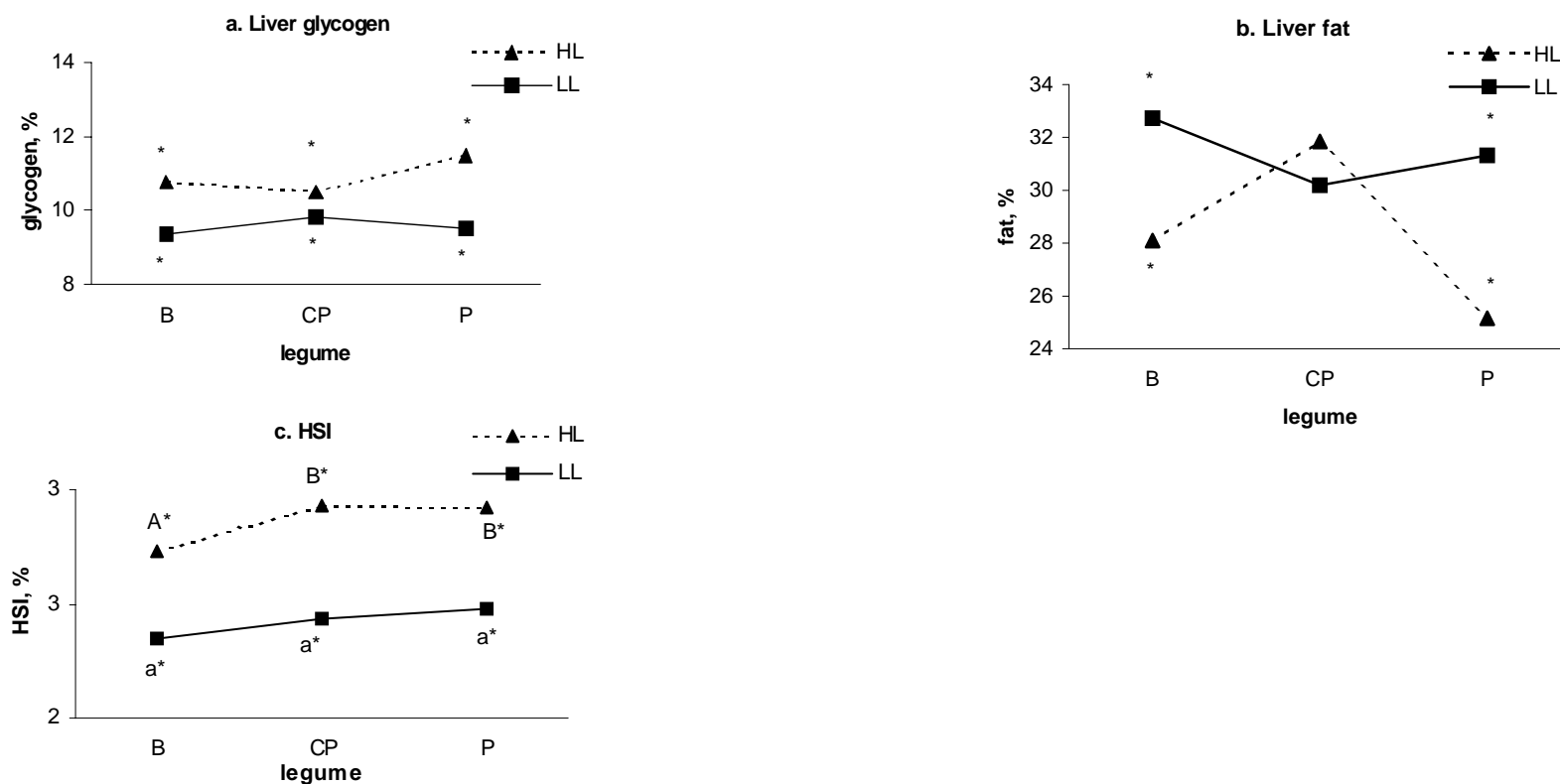
Figure 5.4. Means of seabass liver characteristics; glycogen (a), fat (b) and hepatosomatic index (HSI) (c) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction. For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk.

Table 5.4. Correlation coefficients for serum, liver and carcass characteristics of seabass

	Glucose	Triacylglycerols	Cholesterol	HSI	Liver Fat	Glycogen
			Serum			
Triacylglycerols	-0.006					
Cholesterol	0.074	0.383				
HSI	0.562**	-0.065	0.149			
Liver Fat	-0.004	-0.077	0.131	-0.382		
Glycogen	0.256	-0.031	-0.090	0.661**	-0.675**	
Carcass Fat	-0.547*	0.324	0.351	-0.482*	0.274	-0.386

* Correlation is significant for $P<0.05$ (2-tailed).
** Correlation is significant for $P<0.01$ (2-tailed).

5.3.3   Carcass and fillet

5.3.3.1 Carcass

One-way ANOVA was applied among control and L diets to examine the effects of wheat substitution by legumes on carcass composition (Table 5.5). In this respect fish fed diet CPL showed significantly reduced moisture and increased lipid content, while carcass protein content was not affected by the carbohydrate source. ANOVA among all seven diets showed significantly elevated moisture and decreasing carcass fat for fish fed diet BH compared to diets PH, control and CPL, while significantly higher protein for fish fed diet CPH found but only against diet BL.

Two-way ANOVA among L and H diets revealed significant overall effects and interactions for carcass composition values and simple main effects were examined. Carcass ash values were not homogenous as revealed from Levene's test and no tested transformation led to homogeneity and therefore this parameter was not examined.

Carcass moisture content ranged from 59.9–62.9%. A significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.5a), showing that inclusion level significantly affected the B diets with H diets giving increased moisture content. Regarding the effect of inclusion level in L diets, significant reduction was noted for CPL compared to BL and PL and for H diets significant reduction was noted for PH compared to BH.

Carcass protein ranged from 15.7-16.5% and significant effects were observed only for inclusion level (Figure 5.5b). Specifically, fish fed H diets had higher protein contents than fish fed L diets.

Carcass fat ranged from 16.9-20.4% and a significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 5.5c), showing that inclusion level significantly affected B and CP diets with L diets giving significantly increased fat content. Regarding the effect of legume type significantly less fat was measured in fish fed PL compared to CPL, and BH compared to PH.

5.3.3.2 Fillet

Seabass fillet characteristics were satisfactory for all measured diets. One-way ANOVA was applied for comparison of fillet characteristics among control and H diets although no further results could be revealed from this analysis (Table 5.6). Fillet ash values were not homogenous as revealed from Levene's test and no tested transformation led to homogeneity and therefore this parameter was not examined.

Fillet moisture ranged from 72.9–73.8% and was significantly higher for fish fed diet BH only compared to the control, while protein ranged from 19.7–20.3% and was significantly higher for control compared to diet PH and finally ash content ranged from 1.3–1.6%. Fillet yield ranged from 41.0–44.2%BW and the percentages of fillet muscle (35.4–38.1%BW) were significantly greater for fish fed diet PH, although the flesh protein content for this fillet was the lowest.

According to Pearson correlation, nitrogen deposition had a strong positive correlation ($P<0.01$), while protein had a strong negative correlation with fillet yield.

Table 5.5. Carcass proximate composition (%) and Gross energy (kJg^{-1}) of seabass

Components	Initial	ONE-WAY ANOVA							TWO-WAY ANOVA		
		Control	BL	CPL	PL	BH	CPH	PH	level	legume	level x leg
Moisture	71.7±0.6	61.2±0.3^{AB,b}	61.8±0.2^{BC,b}	59.9±0.2^{A,a}	61.6±0.6^{BC,b}	62.9±0.1^{C}	61.5±1.3^{BC}	61.2±0.2^{AB}	0.019	0.002	0.038
Crude protein	16.4±0.5	16.1±0.2^{AB,a}	15.7±0.4^{A,a}	16.0±0.2^{AB,a}	15.9±0.2^{AB,a}	16.1±0.4^{AB}	16.5±0.4^{B}	16.2±0.1^{AB}	0.014	0.146	0.830
Crude lipid	4.3±0.3	18.8±0.3^{B,a}	19.2±0.2^{BC,ab}	20.4±0.7^{C,b}	18.8±0.6^{B,a}	16.9±0.5^{A}	18.2±0.7^{AB}	18.7±0.4^{B}	0.000	0.008	0.009
Ash	5.0±0.3	2.9±0.2	2.7±0.0	2.7±0.3	2.9±0.0	2.9±0.1	2.8±0.2	2.7±0.1			
Gross energy	5.54	11.2±0.1	11.27±0.1	11.8±0.3	11.2±0.2	10.5±0.2	11.1±0.4	11.2±0.2			

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.
P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.
Values are expressed as mean±SD (n=3)

Table 5.6. Fillet muscle proximate composition of seabass

Components	Control	BH	CPH	PH
Moisture	72.9±0.2^{a}	73.8±0.5^{b}	73.2±0.3^{ab}	73.3±0.4^{ab}
Crude protein	20.3±0.2^{b}	20.2±0.2^{ab}	20.0±0.3^{ab}	19.7±0.0^{a}
Crude lipid	4.6±0.3	4.1±0.3	4.7±0.3	4.7±0.2
Ash	1.5±0.1	1.6±0.1	1.3±0.0	1.3±0.0
Fillet muscle (%BW)	35.4±1.5^{a}	36.3±2.2^{a}	37.0±2.2^{ab}	38.1±1.1^{b}
Fillet yield (%BW)	41.0±1.6^{a}	41.5±1.8^{a}	42.5±2.1^{a}	44.2±1.6^{b}

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05).
Values are expressed as mean±SD (n=3)

a. Carcass moisture

b. Carcass protein

c. Caracass fat
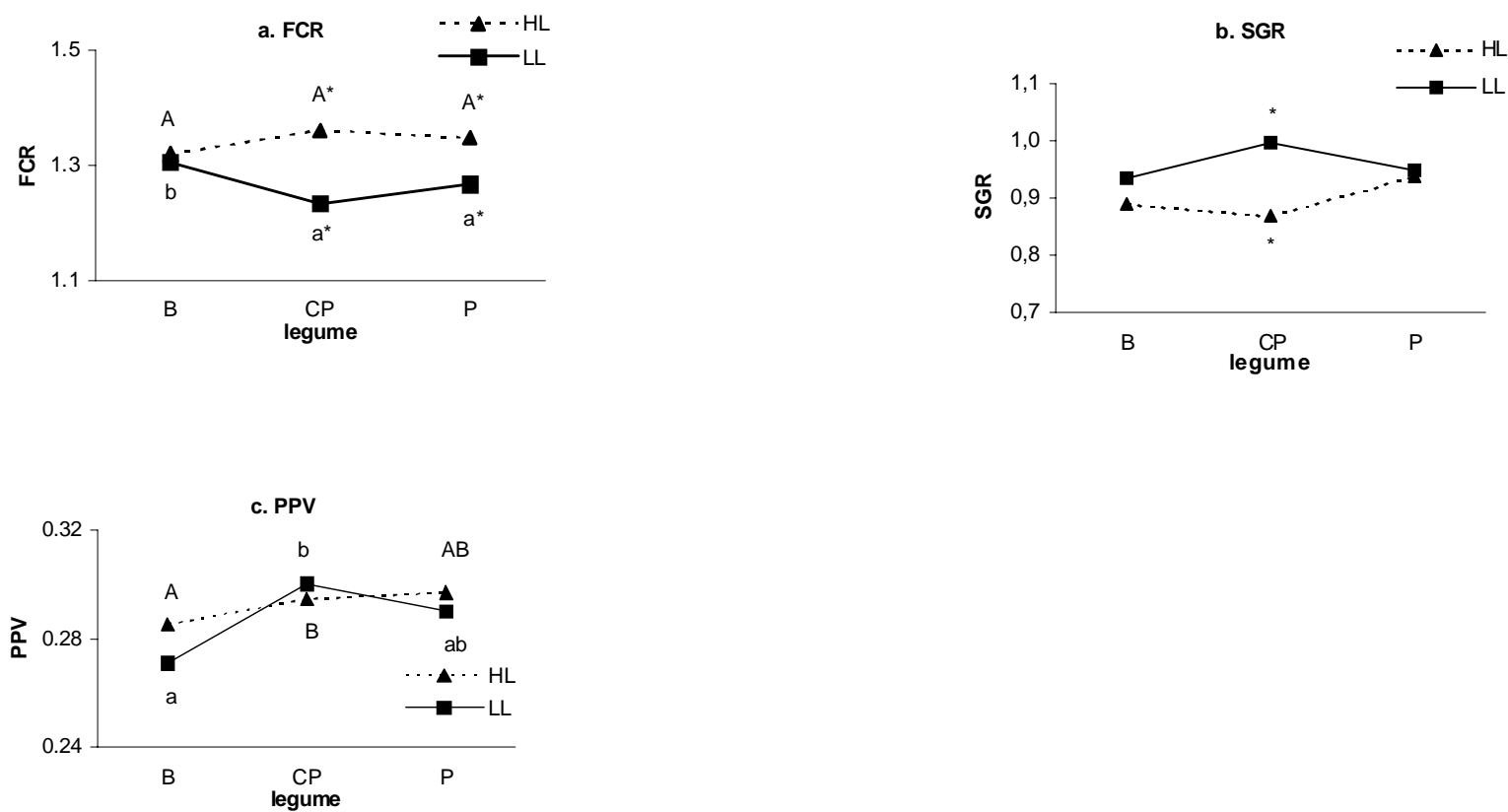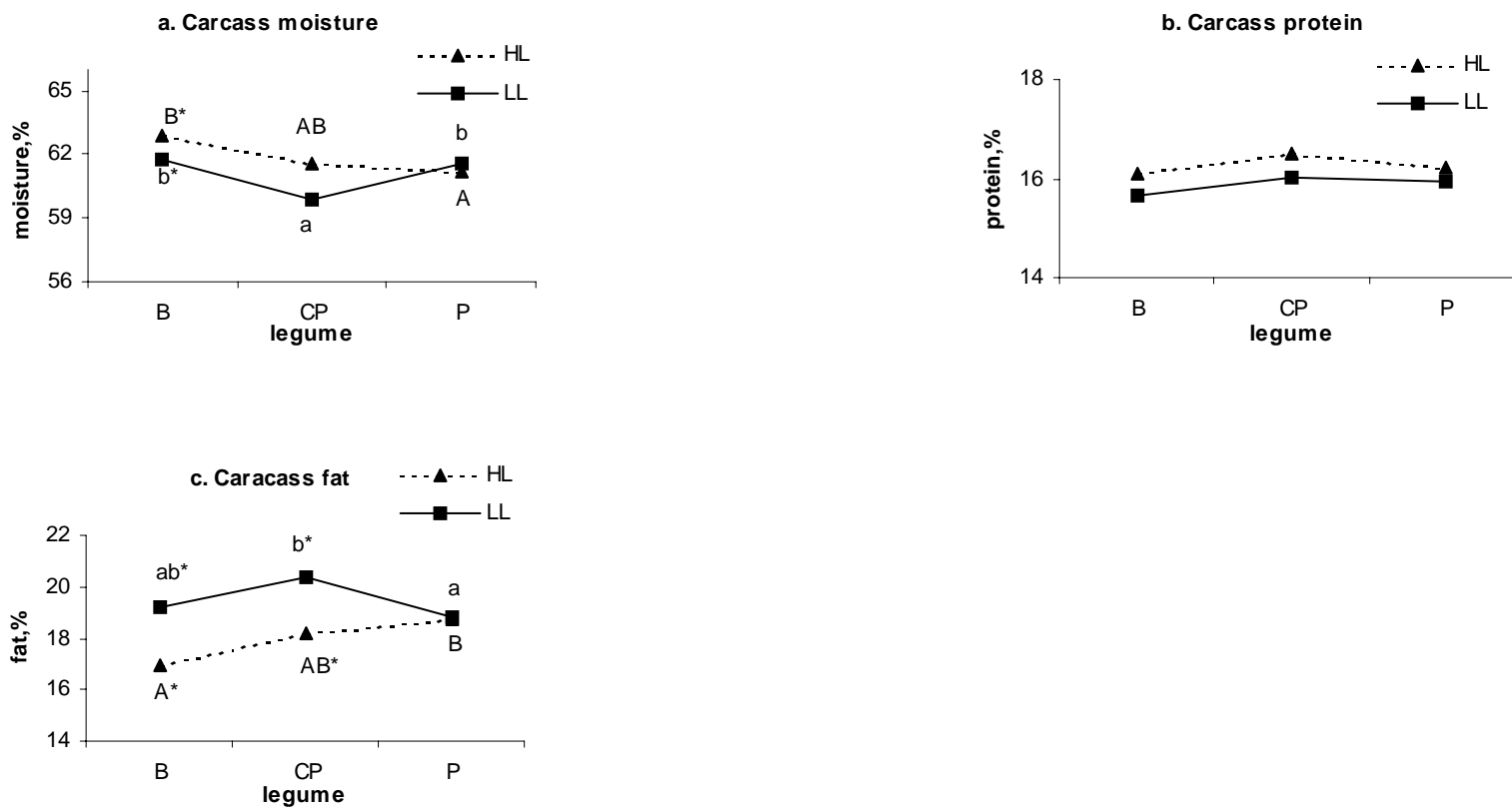
Figure 5.5. Means of seabass carcass moisture (a), protein (b) and fat (c) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction.

For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk.

5.3.3.3 Sensory analysis

No statistically significant differences were found among the organoleptic characteristics of the fish fed the different legume-containing diets as shown in Figure 5.6.
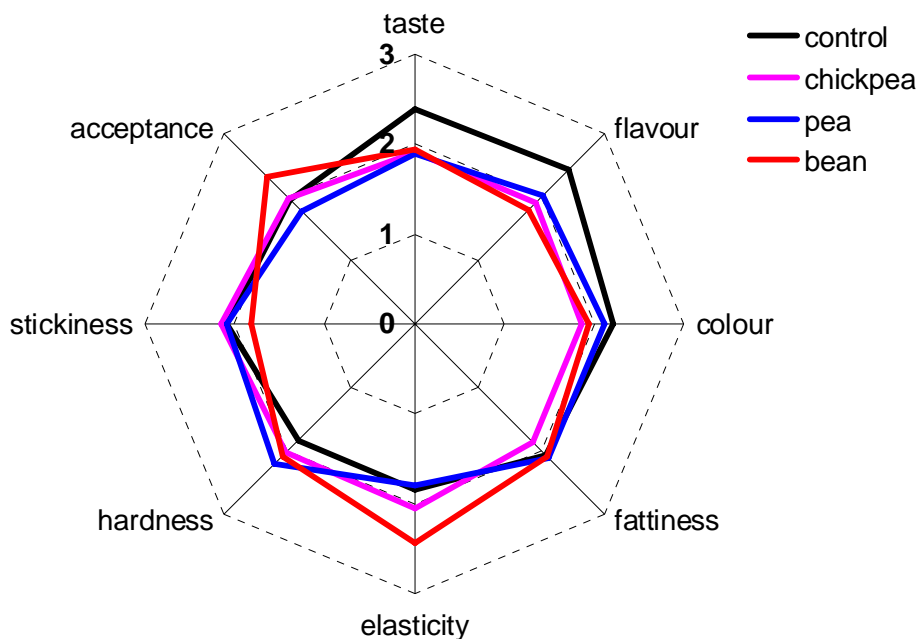
Figure 5.6. Sensory analysis of seabass fillet fed the control or the diets containing high legume levels.

5.3.4 Histology

Examination of histology sections for liver, spleen, kidneys and foregut did not show any pathological abnormalities that could be related to nutritional treatment. Fish fed either diet PH or control induced increased number and abnormal size and arrangement of absorptive vacuoles in hindgut, however the rest of the diets containing legumes did not show any pathological signs.

5.3.5 Immunology

None of the fish groups fed the diets tested showed significant evidence of either immunosuppression or immunostimulation (Figure 5.7) compared to control fish. However, diet BH resulted in significantly lower blood chemiluminescence than diet BL. There was also a tendency for immunosuppression of chemiluminescence by CPH, however this was not significant. Peas have a tendency to immunostimulate as judged by blood chemiluminescence at both inclusion levels even if not statistically significant.
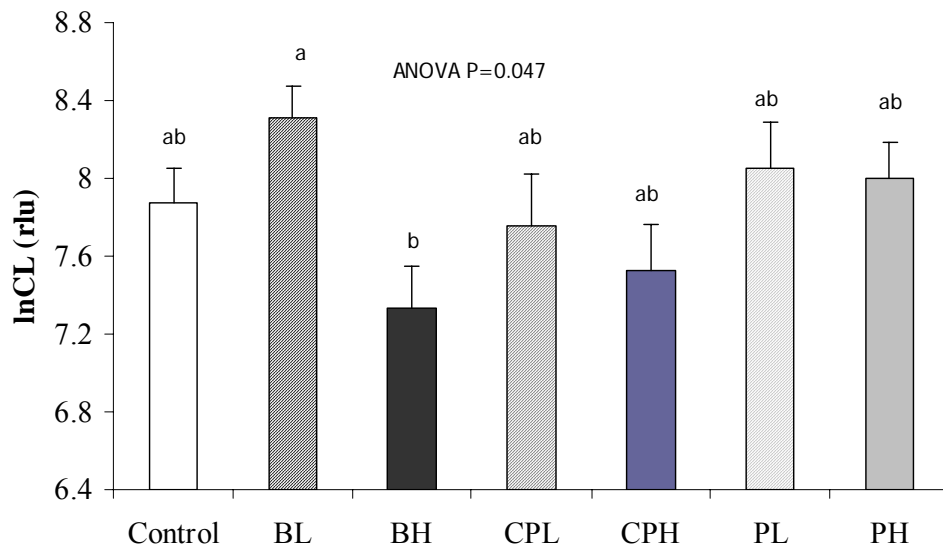
Figure 5.7. Effect of tested dietary legumes on seabass serum chemiluminescence (CL) activity (P<0.05) (rlu, relative luminescent units).

5.4 Discussion

In the present study all three legumes proved valuable carbohydrate replacers at both inclusion levels in seabass as indicated by their ADCs, growth indices, carcass composition, fillet yield and sensory analysis, taking also into account the low fishmeal inclusion level of the diets. Tested diets gave satisfactory digestibility coefficients and with respect to growth performance, PPV showed no differences among treatments, while FCR and SGR showed improved values for diet CPL and reduced for the control.

5.4.1 Digestibility

Objectively, digestibility values obtained in this study were all very high, while when compared to respective values in experiment I (92.9 vs. 94.2% for protein, 96.2 vs. 97.7 for fat, 92.3 vs. 97.0% for starch and 94.2 vs. 96.0% for energy) the values were slightly increased, despite the fact that in Experiment I diets included almost double fishmeal than the diets of Experiment II and the same procedures were followed in both experiments. The increase of digestibility might be attributable to possible leaching resulting from the high plant meal content of the tested diets in the present study that resulted in a more liquid form of faeces (Hung et al., 1990) or to a higher enzyme activity at higher temperatures (18 vs. 25°C) as proved *in vitro* for seabass (Papoutsoglou and Lyndon, 2005). However, Moreira et al. (2008) found an increase only in starch ADC for seabass fed at 25°C when compared to 18°C. Nevertheless, two-way ANOVA among diets L and H revealed significant differences for inclusion level, for legume type and for interaction of the two parameters, but the control diet resulted in higher overall values.

Similar studies evaluating legumes for carbohydrate replacement in fish diets are very limited in the literature. In particular Gouveia and Davies (1998) fed diets

containing 0, 20 and 40g100g^{-1} field pea meal to European seabass and showed no differences for protein (88-89%) and lipid (92-94%) ADCs, but the respective values for carbohydrate (71, 65 and 57%). and energy (78, 73 and 72%) for the high replacement level were significantly lower than the control. The ADC values of pea diets tested in the present study were higher than those reported by Gouveia and Davies (1998), most probably due to different processing procedures of the feeds (extrusion vs pelleted). This view is supported by the fact that the same authors in a more recent study (Gouveia and Davies, 2000) evaluated extruded pea seed meal for the same species at up to 30% and all nutrient ADCs were higher than in their previous work (Gouveia and Davies, 1998) approaching the values of the present study. Gouveia and Davies (2000) showed no differences for all nutrient ADCs at all inclusion levels, in contrast to the present study where diet PH showed improved protein digestibility and both pea diets showed reduced starch ADC compared to the control diet. When pea seed meal was included at 18g100g^{-1} and substituted for wheat in Atlantic salmon extruded diets (Aslaksen et al., 2007) all nutrient ADCs were lower (85, 50, 91 for protein, starch and fat) compared to those found in the present study, but all followed the same trends. In the same work, diets including whole faba bean (22g100g^{-1}) for wheat replacement showed protein ADC (84%) similar to the control (85%), and decreased starch ADC (50 vs 55%), which agrees with the current results. For chickpeas no similar studies are available but some information exists on their digestibility in other farmed species (Booth et al., 2001). Protein and energy ADCs of chickpeas (30% in the diet) have been estimated in Australian silver perch to be 88.1 and 72.1% respectively (Booth et al., 2001).

Increasing starch levels have been noted to decrease starch and to increase protein and energy ADC (Brauge et al., 1994) in rainbow trout diets, while juvenile seabass fed

diets including 10, 20 or 30% of gelatinized starch showed decreased protein, starch and energy ADCs at the highest starch level (Moreira et al., 2008). However, these findings agree only for CP diets in the present study, although lower starch levels were included. It is a paradox that all L level diets resulted in lowered ADCs and yet fish fed these diets showed improved growth parameters. This could be explained by the consistently high ADC values recorded, the low numerical differences, and to the extremely small standard deviations obtained within treatments (that lead to statistically significant differences). It is also important to note that greater differences were found for protein ADCs and although most of the growth parameters showed the opposite trend, protein utilization was higher for the H diets.

5.4.2   Growth

The growth and feed utilization parameters recorded for diets tested in this work were consistent with those reported elsewhere for European seabass. Legumes are commonly used for fishmeal protein substitution in the form of concentrated proteins or in combination with other plant protein sources (Gomes et al., 1995a), but there are very limited studies on wheat substitution. No significant differences were noted in all growth parameters when juvenile European seabass were fed pelleted diets containing 20 and $40g100g^{-1}$ of pea seed meal (Gouveia and Davies, 1998), which agrees with the current results for both high and low pea seed meal diets. Furthermore, protein efficiency ratio (PER) and nitrogen deposition and utilization were improved with increasing levels of pea seed meal in extruded diets, when FCR and SGR values did not differ significantly (Gouveia and Davies, 2000), while in the present research no differences were noted for any of these values. Thiessen et al. (2003) carried out a study replacing wheat meal with dehulled field pea meal ($25g100g^{-1}$) in rainbow trout diets

and demonstrated that, although SGR, FCR and PER were improved, they did not differ significantly when compared to the control diet, this is also in accordance with the present findings for diet PL. For faba beans and chickpeas no relevant growth studies are available, but in the present research SGR and FCR were significantly different only between CP diets with improved values for CPL, while the rest of the diets did not differ significantly. Overall, diet CPL resulted in higher performances, with the most improved SGR and FCR values, while the lowest respective values were noted for diets CPH and the control. L diets showed improved weight gain, FCR, SGR and FI indicating that substitution of wheat by any of the tested legumes at a $17g100g^{-1}$ inclusion level could probably affect growth parameters of seabass, probably due to the different carbohydrates included in legumes compared to cereals.

However, when faba beans and chickpeas were included at a $35g100g^{-1}$ level, FCR and SGR were adversely affected, compared to the respective low legume diet, however only significantly for CP. Difference in plant protein and carbohydrate sources included in the control diet did not lead to significant differences in all afore mentioned parameters, however, values were slightly increased for the legume diets, apart from the FCR value for diet CPH, but this could be expected as different carbohydrate sources have been noted to have various effects. Specifically, different carbohydrate sources affected FCR values but not SGR in salmon (Hemre and Hansen, 1998) and effects on growth performance in rainbow trout were also found by Bergot (1979). This leads to the conclusion that peas, chickpeas and faba beans are very good replacers of wheat and partially of soy or sunflower protein and it is possible that the tested high-starch legumes may offer better starch and protein sources than the commonly used materials (especially wheat and soybean). However, more research with longer period trials should be done in this area to generate definitive conclusions.

5.4.3   Carcass and fillet composition, HSI, liver glycogen, liver fat

In the current study European seabass showed no negative effects on carcass proximate composition of either low (PL) or high (PH) field pea containing diets when compared to the control. Pea seed meal was successfully included in diets of juveniles of the same species with equivalent results (Gouveia and Davies, 1998; Gouveia and Davies, 2000). Pereira and Oliva-Teles (2002) carried out evaluation of growth parameters of gilthead seabream fed two differently processed pea seed meals at similar inclusion levels to those in the present study. The same authors demonstrated that no significant differences occurred in carcass proximate composition and HSI for gilthead seabream. For seabass in this study carcass composition also did not differ between control and pea diets, however, HSI was significantly higher than the control for PH and significantly lower for PL. Higher carcass protein values observed for diet CPH, but this was significantly lower only against diet BL. Results on carcass fat regarding dietary carbohydrate level are controversial as salmon showed decreasing values of fat with increasing carbohydrate levels (Refstie and Austreng, 1981), while Wang et al. (2005) found the opposite in tilapia diets, but these results could be species related, as these two species have very different capacity to utilize carbohydrates. Carcass fat was significantly higher for diets CPL and BL compared to CPH and BH respectively that is in agreement with Refstie and Austreng (1981) in rainbow trout that reduced carcass fat with elevating levels of carbohydrates.

High-inclusion diets were chosen to be compared with the control diet for fillet yield and organoleptic characteristics, as the diets that were more likely to show differences against the control. Fillet yield was higher for diet PH, followed by CPH, BH and lowest for the control diet. This could be attributed to the higher carbohydrate content of H diets compared to the control (Lanari et al., 1999), but this should be

clarified by further research. Pearson correlation showed that protein level of fillet muscle had a strong negative correlation, with fillet yield.

Increased but not significant values were found for liver glycogen in H compared to L diets, most probably due to the increase in digestible starch (Kim and Kaushik, 1992). High glycogen contents associated with increasing carbohydrate levels have been noted by several authors in salmon (Refstie and Austreng, 1981; Arnesen et al., 1995), tilapia (Wang et al., 2005), rainbow trout (Austreng et al., 1977; Bergot 1979) and seabass (Moreira et al., 2008). HSI values showed the same trend as glycogen deposition, but this time the differences were significant and agreed with other studies in trout (Kim and Kaushik, 1992), in silver perch (Stone et al., 2003), tilapia (Wang et al., 2005) and seabass (Moreira et al., 2008), but not in salmon (Arnesen et al., 1995). A strong correlation was also found between HSI and liver glycogen content and this is in agreement with the results published for several other species (Hemre et al., 2002), while increased glycogen deposition seemed to induce enlargement of liver (Stone et al., 2003). Liver fat content has been associated with high carbohydrate contents in diets for seabass by Lanari et al. (1999) but this fact was not confirmed in this research with all H diets resulting in lower liver fat content than L diets, but this difference could be explained by the lower inclusion levels of starch in the present study for the respective values of low and high levels.

5.4.4 Sensory analysis

Different carbohydrate sources in salmon feeds (Young et al., 2006) were noted to cause no impact on flesh quality, however organoleptic characteristics were not evaluated in that study. The similar levels of fishmeal and fish oil included in the tested diets could be the reason that no significant differences were noted for the organoleptic

characteristics of the fillets observed from seabass fed control and H diets as the fishmeal (Kaushik et al., 1995) as the source of fat (Waagbo et al., 1993) in the diet play a determinative role on sensory characteristics.

5.4.5 Haematological parameters

Measurements on serum in seabass, not unexpectedly, resulted in high ranges of the observed values within the same experimental groups. In contrast to mammals, fish have proved to be 'glucose resistant' (Mommsen and Plisetskaya, 1991) and most teleost fishes have wide limits of glucose levels not only between species but also within species at different life stages or under certain feeding regimes (Hemre et al., 2002). Serum analysis has been investigated partially in some species aiming to study effects of certain ingredients in fish diets. For instance Hung et al. (1989) found that different carbohydrate sources could affect total cholesterol and triacylglycerols levels in blood plasma when included in sturgeon (*Acipenser transmontanus*) diets. However, not much information exists about the influence of legume seeds in fish as a wheat replacer in all four blood serum parameters measured in the present research.

Stone et al. (2003) found that raw pea starch at a $30g100g^{-1}$ level did not affect plasma glucose in Australian silver perch 3 hours after feeding, compared to different carbohydrate sources at the same percentage and the same was found for salmon when fed oat and maize meals as carbohydrate sources (Arnesen et al., 1995). In the present study 40 hours after feeding no differences were found for pea diets, although extruded, however, chickpeas at high dietary levels significantly increased glucose compared to the control diet, implying a positive effect of starch level on glucose serum values. Arnesen et al. (1995) also found a significant positive correlation between plasma

glucose and liver glycogen, but in the present study this correlation was positive, although not significant.

Total serum protein did not vary much among diet treatments, probably due to the same fishmeal and total protein inclusion level of the diets and this is in agreement with Hemre and Hansen (1998) who found no effect on this parameter with different carbohydrate sources. Starch level does not seem to play an important role either.

Triacylglycerols did not show any differences among the experimental groups however all diets showed a decreasing trend, excluding diet CPL, that was similar to the control levels. Similarly, levels of triacylglycerols were not affected by the carbohydrate source in salmon (Hemre and Hansen, 1998)

In both mammals and humans, different plant constituents have been reported to lower plasma cholesterol levels (Wester, 2000), but this effect seems to be related to the NSP content of the plant materials (Levrat et al., 1996; Favier et al., 1997). Cholesterol was higher for diet CPH and lower for diet BH, but no other significant differences were found. Both faba bean diets showed a decreasing trend that could be related to the effect of this ingredient. It is also important to note that no correlation was found among serum cholesterol, triacylglycerols and HSI, liver and carcass fat content and none between liver glycogen and the inclusion level of NSP (total NSP, I-NSP or S-NSP).

5.4.6 Immunology

Occurrence of certain legume ANF has been associated with immunological disturbances in mice fed diets including legumes (Macarulla et al., 1992; Martinez et al., 1992; Esparza et al., 1996) and in salmon (Krogdahl et al., 2000) and rainbow trout (Burrells et al., 1999) fed with soybean meal diets. No similar reports are available for the legumes tested in the present study or in European seabass. Experimental diets

included soybean meal at 16 or 8g100g^{-1} in L and H diets respectively and these combinations could act either in a synergistic or antagonistic manner in low level diets. Faba bean diets showed immunosuppressive tendencies at the high inclusion level (BH) and immunostimulatory effects at the low level (BL), however all the other diets did not show any significant differences, but further study is needed to confirm these results.

5.4.7 Histology

It might be expected that diets containing both soy and tested legumes (in L diets) might induce histopathological changes compared to the control diet. However, increased numbers, abnormal size and arrangement of absorptive vacuoles were found only in the distal intestine of fish fed the control diet and to a lesser extent diet PH. Findings in control diet may be due to the 16g100g^{-1} soybean inclusion level while sunflower and corn gluten may not contribute to these effects, as they have been proven not to cause histological abnormalities in Atlantic salmon (Aslaksen et al., 2007). The fact that L diets did not show any changes could be attributed to a possible antagonistic effect of some plant ingredients with problem agents of soy. Diet PH induced increased number of absorptive vacuoles that could be related to whole pea seed meal ANF, however, these hypotheses requires further investigation. For L diets findings are in agreement with (Aslaksen et al., 2007) who did not observe histological abnormalities in Atlantic salmon fed diets including 18 and 22g100g^{-1} of peas and faba beans respectively, while (Sitja-Bobadilla et al., 2005) found histological changes in gilthead seabream only when they were fed diets containing 100% plant proteins.

5.5 Conclusions

The inclusion of any of the three legumes up to 17% in seabass diets proved to be beneficial in terms of growth with chickpea diet giving the best growth values, but also higher carcass fat content. Fish fed on diets including 35% of legumes showed decreasing growth trends compared to control and 17% inclusion level diets. However, the differences should be investigated in longer term experiments. In pathological terms, enteritis was not induced in any of the fish groups and only high inclusion of pea seed meal caused an increased number of absorptive vacuoles in hindgut. No sensory differences were found among steam cooked fillets of seabass fed either the control or the high legume level diets.

Chapter 6. Experiment III - Effects of peas, chickpeas and faba beans included at two levels in gilthead seabream (*Sparus aurata*) diets

6.1 Introduction

Aquaculture production of gilthead seabream in Mediterranean countries is still rising with Greece being the leading producer at almost 50,000t in 2006 (Aquamedia, 2008). As already mentioned in the 'General Introduction' gilthead seabream farming is considered a very important sector for the local economy (FAO, 2008b). Use of locally available feed ingredients from terrestrial agricultural production in the region could directly contribute to the sustainability and cost-effectiveness of fish farming in Greece. In this respect cultivation of legumes, as appropriate for Mediterranean-type climates (Sulas, 2005), could be encouraged if proven to give satisfactory performance in seabream diets. In addition the faba beans used in the present study are legumes produced in high quantities in EU countries (Figure 1.4) and considered of high nutritional value.

The total capacity of fishfeed companies in Greece, presently nine plants, is between 250,000–300,000mt, whilst the national requirements are 200,000mt. The most common ingredients used in aquafeeds in Greece are mostly imported with only approximately 5% being derived from local agricultural production, including wheat and glutens (Alexis, personal communication, EPAN report 2008).

Over the last five years there has been a gradual, or in some cases rapid, rise in the prices of all ingredients used in the livestock feeds with a parallel increase in the cost of feeds and in turn in livestock production (Beek, 2007). Alternative and sustainable ingredients must therefore be evaluated as potential solutions to the sustainable development of the aquaculture industry. The prices of wheat for the last three years greatly varied: the prices (€/t) are presented in ascending order in Table

6.1 with the required respective prices of the tested legumes for a least cost diet included up to 35g100g^{-1} (Karacostas, personal communication).

Table 6.1. Wheat prices (€/t) for years 2005-2008 in EU, in ascending order with the respective maximum cost of faba beans, peas and chickpeas to formulate a least cost diet for seabream.

Seabream product 4.5mm (44/19)			
Wheat (€/t)	Faba beans (€/t)	Peas (€/t)	Chickpeas (€/t)
120	240	205	270
140	255	220	285
160	270	235	300
180	285	250	315
200	305	270	335
220	320	290	350
240	335	305	365
260	345	320	375
280	350	320	385

Calculations are based on BioMar's commercial product (44/19, 4.5mm) for seabream and for maximum inclusion level 35% (Karacostas, personal communication).

Plant materials are the most promising alternative ingredients and indeed locally produced materials are already used in most of the European countries that have developed aquaculture, such as Norway, Denmark, Scotland, France and Spain (FEAP, 2008). The most important exception is soybean that is very commonly used but production in the EU is very limited (Grain legumes, 2006). In addition, legumes such as peas and especially chickpeas can be produced with more environmental friendly cultivation techniques as they do not have high demands for water (Bhardwaj et al., 1999), they can be cultivated in sandy soil, and they do not need nitrogen fertilization if nodulated (Oplinger et al., 1990). Furthermore, such cultivation could help the poor and arid areas of Greece to develop through agricultural production. On the other hand faba beans are commonly produced in most European countries (Schneider, 2002) and could potentially partially or totally replace soybean meal.

Therefore, this study had a dual role; firstly to investigate the effects of different starch sources in seabream diets and secondly to estimate the performance of fish fed diets including high levels of Greek varieties of peas and chickpeas or faba beans produced in the EU in place of imported plant sources like soybean and sunflower meal. Specifically, the effect of total replacement of wheat by whole grain flours of field peas, chickpeas and faba beans and the interactions of starch level and legume type on growth performance were evaluated alongside haematological serum indices and histology of internal organs in gilthead seabream.

The present research was complementary to the project "Utilization of pulses and carob seed germ meal as feed constituents of seabass and seabream", funded by the European Union (Community Support Framework 2000-2006, Operational Programme "Competitiveness") and carried out by The Hellenic Centre for Marine Research, BioMar Hellenic, Selonda and National Agricultural Research Foundation (N.AG.RE.F.).

6.2 Materials and methods

6.2.1 Feed ingredients and experimental diets

Three different legumes including field peas, chickpeas and faba beans represented by P, CP and B respectively, were included in experimental diets. Six, practical type extruded diets were formulated containing approximately $17\text{g}100\text{g}^{-1}$ (L diets) or $35\text{g}100\text{g}^{-1}$ (H diets) of each legume plus the control diet as described in detail in section 3.2.3.3.

6.2.2   Fish and experimental design

Seven hundred seabream of initial weight 92.6±5.0g were transferred from a Selonda farm (in Karistos, Evia) and randomly distributed into 21 cages as described in section 2.2.2. Fish were weighed at the start of the trial, mortalities were recorded and dead fish were removed. Fish were hand fed twice a day (9:00am and 16:00pm) a total of $1.5g100g^{-1}$ BW.

Before the experiment parasites were detected and fish were treated in low salinity water for 6 days. In the 6th week of the experiment a new outbreak of parasites stressed the fish and they required treatment with copper sulphate for 15 days, at a daily dose of 0.8ppm under flowing water. During this period fish were fed normally, but their appetite was obviously reduced, especially for the first 5 days of treatment. Mortalities were recorded and dead fish were weighed.

6.2.3   Sampling procedure

At the beginning of the experiment fish were weighed and a sample of 6 fish were taken as representative of the initial population to determine whole body proximate composition. At the end of the 13-week trial fish were fasted for 40h and 8 fish per tank anaesthetized and blood samples taken prior to any other sampling procedure. Sampling procedure is described in section 2.3.3.

6.2.4   Water quality

Average water temperature ranged from 21-28ºC during the growth period (May-August) with an average temperature of 25ºC. Water quality is described in section 2.2.2.3.

6.2.5 Chemical analysis

Proximate analysis, liver fat, liver glycogen, serum glucose, total protein, triacylglycerols, cholesterol and chemiluminescence were determined. All chemical methods are described in detail in General Materials and Methods.

6.2.6 Calculation of nutritional parameters

Growth parameters, FCR, SGR, PPV, VSI and HIS formulae are presented in section 2.14.

6.2.7 Histology

Liver, spleen, kidneys and intestine (foregut and hindgut) were dissected from three fish per cage. Tissue samples were treated as described in section 2.12.

6.2.8 Statistical analysis

All data are presented as means±SD (n=3) and were analysed by one-way or two-way ANOVA as described in section 2.15. Pearson correlation in SPSS 13.0 was used to evaluate any significant ($P<0.01$ or $P<0.05$) positive or negative correlation among fat metabolism parameters.

6.3 Results

6.3.1 Growth

6.3.1.1 Growth one-way ANOVA

Growth for all diets was lower than expected most probably due to the parasite infection during the experiment and only for FCR significant differences were

detected by one-way ANOVA between control and L diets. FCR ranged from 1.58–1.79, SGR from 0.84–0.92, PPV from 0.18–0.25 and VSI from 6.4–8.1%. Significant improvement was noted in FCR only for fish fed the control diet when compared to fish fed diets PL, PH or BH, while SGR was better for control diet but it did not differ from any of the diets. Regarding liver characteristics, glycogen values ranged from 6.9–10.7%, total lipids from 8.7–13.2% and HSI from 1.0–1.2%. Decreased but not significant values for HSI were observed in fish fed L diets compared to the control, while glycogen decreased significantly for fish fed diet CPL when control and L diets were compared. ANOVA also applied for all seven diets showing that PH diet resulted in high liver glycogen when compared to CPL and BL (Table 6.2).

6.3.1.2 Growth two-way ANOVA

Two-way ANOVA among L and H diets revealed significant interactions between legume type and inclusion level only for liver fat content, while significant overall effects were found for some of the growth parameters and simple main effects or main effects were examined where required.

For FCR (1.68-1.78), SGR (0.84-0.87), VSI (6.7-8.1%) and HIS (1.0-1.3%) no significant differences occurred for inclusion level and legume type or their interaction showing that none of the parameters examined were affected by diet type.

PPV ranged from 0.18–0.25 and a significant effect of legume type and inclusion level was shown from two-way ANOVA. Specifically, fish fed P diets had lower PPV than fish fed CP diets and L diets showed higher PPV values when compared to respective H diet values.

Liver glycogen ranged from 6.9–10.7% and significant effects were observed for inclusion level and legume but not for their interaction, showing that fish fed the H

144

diets had higher liver glycogen levels than fish fed L diets and P diets giving higher values than CP and B diets (Figure 6.1a)

Liver fat ranged from 9.5–13.2% and a significant interaction of inclusion level and legume type was observed and therefore simple main effects were analysed (Figure 6.1b). For H diets diet BH showed significantly lower liver fat than CPH and PH and for L diets, CPL showed significantly higher liver fat. Regarding legume type no differences were found between L and H diets in pairs.

Table 6.2. Growth indicators of seabream fed diets with high (H) or low (L) level of legumes

| | ONE-WAY ANOVA | | | | | | | TWO-WAY ANOVA | | |
	Control	BL	CPL	PL	BH	CPH	PH	level	legume	level x legume
Initial W(g)	95.1±7.7	89.9±1.8	92.0±3.5	96.0±8.5	90.9±6.2	94.8±0.9	89.4±1.0			
Final W(g)	216.2±16.0	188.2±2.8	200.1±3.7	200.9±10.3	188.1±8.0	206.1±4.6	187.9±9.7			
Wgain (g)	127.4±5.1	109.3±2.7	117.5±7.1	109.3±4.8	107.2±5.8	117.9±9.0	110.0±8.9			
Mortalities	5	9	4	8	8	4	6			
FCR[1]	1.58±0.07[A,a]	1.72±0.06[AB,ab]	1.69±0.03[AB,ab]	1.78±0.05[B,b]	1.79±0.07[B]	1.68±0.05[AB]	1.76±0.03[B]	0.543	0.024	0.237
SGR[2]	0.92±0.08	0.85±0.036	0.86±0.02	0.85±0.03	0.84±0.02	0.87±0.05	0.86±0.02	0.939	0.487	0.832
PPV[3]	0.22±0.01[AB]	0.22±0.02[AB]	0.25±0.02[B]	0.23±0.01[AB]	0.22±0.01[AB]	0.23±0.02[B]	0.18±0.02[A]	0.026	0.011	0.139
NI (%BW)[4]	4.57±0.40[B,b]	4.39±0.29[AB,ab]	3.55±0.66[A,a]	4.25±0.22[AB,ab]	4.02±0.36[AB]	3.56±0.05[A]	4.03±0.10[AB]			
FI (%BW)[5]	1.37±0.10	1.40±0.10	1.39±0.03	1.40±0.11	1.44±0.09	1.38±0.07	1.33±0.06			
VSI[6]	7.73±0.14	8.08±0.34	7.67±0.96	7.76±0.23	6.67±1.20	8.08±0.20	6.42±0.91	0.048	0.230	0.100
Liver Characteristics										
Glycogen (%)	9.5±1.0[BC,b]	7.7±0.4[AB,ab]	6.9±0.2[A,a]	9.7±1.2[BC,b]	9.4±0.3[BC]	8.7±1.0[ABC]	10.7±0.4[C]	0.001	0.000	0.576
Total lipid (%)	12.7±0.5[BC,b]	10.7±0.3[AB,a]	13.2±1.0[C,b]	9.4±0.4[A,a]	9.5±0.7[A]	12.0±0.9[BC]	11.7±1.7[ABC]	0.473	0.000	0.004
HSI[7] (%BW)	1.17±0.20	1.02±0.17	1.07±0.19	1.01±0.21	0.99±0.20	1.30±0.31	1.06±0.30	0.477	0.397	0.618

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.

P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.

Values are expressed as mean±SD (n=3)

[1]Feed Conversion Ratio, [2]Significant Growth Rate, [3]Protein Productive Value, [4,5]Nitrogen and Feed Intake measure as a percentage of Body Weight, [6]Viscerasomatic index, [7]Hepatosomatic index

6.3.2   Serum

6.3.2.1 Serum one-way ANOVA

One-way ANOVA showed that fish fed the wheat-based control diet had lower serum glucose than all the L diets, but significantly lower only against diet PL. Triacylglycerol levels were lower for control and CPL and significantly lower than PL and PL compared to BL, while serum cholesterol and protein did not show significant differences between control and L diets groups (Table 6.3).

One-way ANOVA for all diets of blood serum glucose showed that control diet was significantly different only from diet PL that gave the highest glucose values. PL revealed the highest value also after comparing means of all diets, while CPH showed the lowest serum glucose. Triacylglycerol levels were significantly lower for fish fed diet CPL when compared to PL and BL and when all mean values were compared CPL and BH showed significantly lower values than BL, PL and CPH. Cholesterol levels were significantly lower for diets PH and BH compared to the rest of the diets and total protein levels did not show any significant differences among experimental groups.

6.3.2.2 Serum two-way ANOVA

Two-way ANOVA among L and H diets revealed significant overall effects and interactions for serum glucose, triacylglycerols and cholesterol and simple main effects were examined.

Serum glucose ranged from 4.9–7.7mmoldl^{-1}. A significant interaction of inclusion level and legume type was observed and therefore simple main effects were analysed (Figure 6.2a). Inclusion level significantly affected P and CP diets with PH

and CPH giving significantly lower glucose values compared to PL and CPL respectively. Regarding the effect of inclusion level in L diets, PL exhibited significantly increased glucose values compared to BL and CPL, while for H diets PH showed significantly increased values than BH and CPH.

Serum protein ranged from 5.6–6.9gdl^{-1} and significant effects were observed only for legume type (Figure 6.2b). Specifically, fish fed H diets had lower serum protein levels than fish fed L diets, but for P diets this was not the case.

Serum triacylglycerols ranged from 2.8–4.5mmoldl^{-1} and significant interaction of inclusion level and legume type was observed. Inclusion level significantly affected all legume type diets with BL and PL giving significantly increased triacylglycerol values compared to BH and PH respectively and CPL lower values than CPH. Regarding the effect of inclusion level in H diets, BH exhibited significantly reduced triacylglycerols compared to PH and diet PH compared to CPH, while for L diets CPL showed significantly reduced values than PL and PL compared to BL (Figure 6.2c).

Serum cholesterol ranged from 5.8–9.5mmoldl^{-1} and significant interaction of inclusion level and legume type was observed. Inclusion level significantly affected B and P diets with BL and PL giving significantly increased cholesterol values compared to BH and PH respectively. Regarding the effect of inclusion level in H diets CPH exhibited significantly increased values compared to BH and PH, while for L diets PL showed increased values only compared to diets CPL and BL (Figure 6.2d).

6.3.3   Carcass composition

6.3.3.1 Carcass one-way ANOVA

One-way ANOVA was applied among control and L diets to examine the effects of wheat substitution by legumes on carcass composition of seabream (Table 6.4). In this respect fish fed diet BL showed significantly reduced fat lipid content than fish fed diets control and PL, while carcass moisture, protein and ash content were not affected by carbohydrate source. When the same analysis was applied to all values only carcass protein levels revealed differences with BH and PH giving higher values only against diet BL.

6.3.3.2 Carcass two-way ANOVA

Two-way ANOVA among L and H diets revealed significant overall effects and interactions for carcass ash and protein values and simple main effects were examined.

Carcass moisture content ranged from 62.8–64.0% and carcass fat from 14.3-15.9% and no significant effects found for these parameters.

Carcass protein ranged from 15.7–17.7% and a significant interaction of inclusion level and legume type was observed and therefore the simple main effects were analysed (Figure 6.1c). Specifically, regarding legume level for fish fed L diets, BL had lower protein contents than fish fed diet CPL, while for legume type fish fed diet BH had significantly higher protein carcass content than BL.

Carcass ash ranged from 3.1–3.9% and a significant interaction of inclusion level and legume type was observed (Figure 6.1b). Legume type significantly affected B diets with BL diets giving significantly increased ash content than BH.

A significant positive correlation (P<0.01) was found between cholesterol and triacylglycerol levels Table 6.5 and a negative correlation (P<0.01) between liver fat and serum glucose, between I-NSP and liver fat and between S-NSP and serum triacylglycerol levels.

Table 6.3. Blood serum glucose, triacylglycerols, cholesterol (mmoldl^{-1}) and protein (gdl^{-1}) levels of seabream.

Blood serum	ONE-WAY ANOVA							TWO-WAY ANOVA		
	Control	BL	CPL	PL	BH	CPH	PH	level	legume	lev. x leg.
Glucose (mmoldl^{-1})	5.48±0.41^{AB,a}	6.26±0.55^{B,a}	6.19±0.35^{C,a}	7.73±0.62^{C,b}	6.40±0.55^{B}	4.88±0.46^{A}	6.12±0.32^{AB}	0.001	0.001	0.017
Protein (gdl^{-1})	6.00±0.43	5.63±0.80	6.31±0.37	6.25±0.37	5.86±0.69	6.88±0.44	6.00±0.21	0.476	0.047	0.421
Triacylglycerols (mmoldl^{-1})	3.25±0.13^{ABC,a}	4.48±0.12^{D,c}	2.85±0.31^{A,a}	3.91±0.16^{CD,b}	2.76±0.29^{A}	3.87±0.35^{BCD}	3.21±0.21^{AB}	0.002	0.215	0.000
Cholesterol (mmoldl^{-1})	8.55±0.49^{B}	8.75±0.27^{B}	8.48±0.66^{B}	9.50±0.47^{B}	5.80±0.57^{A}	8.69±0.74^{B}	5.92±0.33^{A}	0.000	0.004	0.000

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.
P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.
Values are expressed as mean±SD (n=3)

Table 6.4. Carcass proximate composition (%) and Gross Energy (kJg^{-1}) of seabream

Component	Initial	ONE-WAY ANOVA							TWO-WAY ANOVA		
		Control	BL	CPL	PL	BH	CPH	PH	level	legume	lev. x leg.
Moisture	66.7	64.0±3.4	63.9±1.1	63.2±0.8	63.4±3.3	63.1±0.9	63.7±2.0	62.8±1.0	0.250	0.422	0.323
Crude protein	16.9	16.6±0.4^{AB}	15.7±0.9^{A}	16.9±0.2^{AB}	16.8±0.8^{AB}	17.7±0.5^{B}	16.6±1.3^{AB}	17.1±0.5^{B}	0.013	0.704	0.004
Crude lipid	12.0	15.2±1.7^{b}	14.3±0.8^{a}	14.9±1.0^{ab}	15.9±0.8^{b}	15.3±1.1	14.8±1.6	15.3±0.7	0.808	0.139	0.162
Ash	3.4	3.4±0.5	3.7±0.6	3.2±0.8	3.3±0.7	3.1±0.4	3.9±1.2	3.9±0.6	0.283	0.655	0.018
Gross energy	8.7	9.9	9.4	9.9	10.3	10.2	9.8	10.1			

Different superscripts in each row show significant differences among the values according to Tukey test (P≤0.05). Upper case letters show significant differences among all diets and lower case letters between control and L diets.
P-values revealed from two-way ANOVA for level (Low and High), legumes (B, CP and P) and for interaction of level and legume.
Values are expressed as mean±SD (n=3)

Table 6.5. Correlation coefficients for serum, liver and carcass characteristics of seabream

	Serum						
	Glucose	Triacylglycerols	Cholesterol	HSI	Liver Fat	Glycogen	Carcass Fat
Triacylglycerols	0.048						
Cholesterol	0.085	0.581**					
HSI	0.022	-0.070	0.228				
Liver Fat	-0.564**	-0.195	0.150	0.218			
Glycogen	0.198	-0.126	-0.370	-0.130	-0.343		
Carcass Fat	-0.056	0.111	0.013	-0.385	0.157	0.097	

* Correlation is significant at 0.05 level (2-tailed).
** Correlation is significant at 0.01 level (2-tailed).

a. Glycogen

b. Liver fat

c. Carcass protein
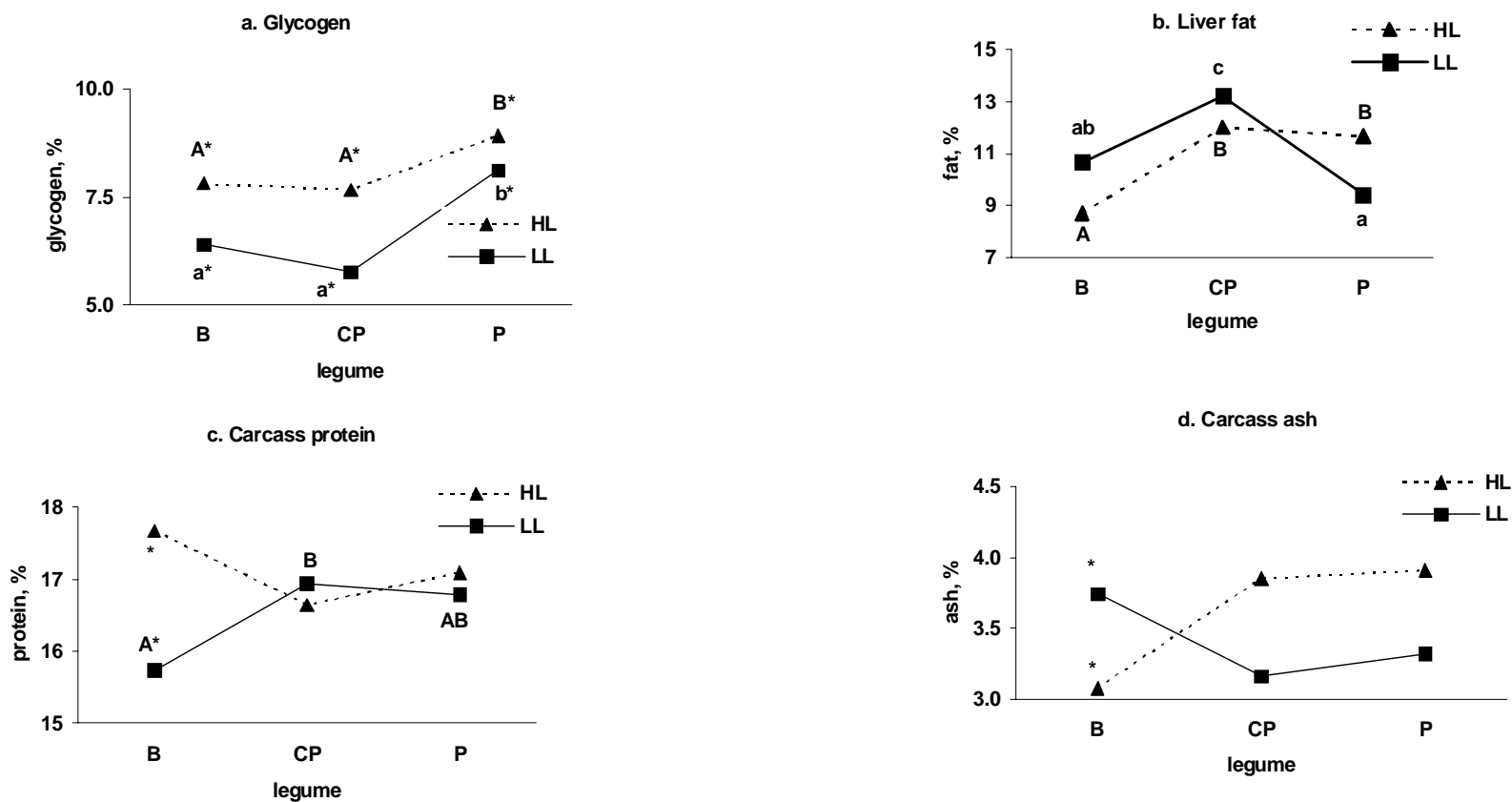
d. Carcass ash

Figure 6.1. Means of seabream; liver glycogen (a), liver fat (b), carcass protein (c) and carcass ash (d) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction. For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk
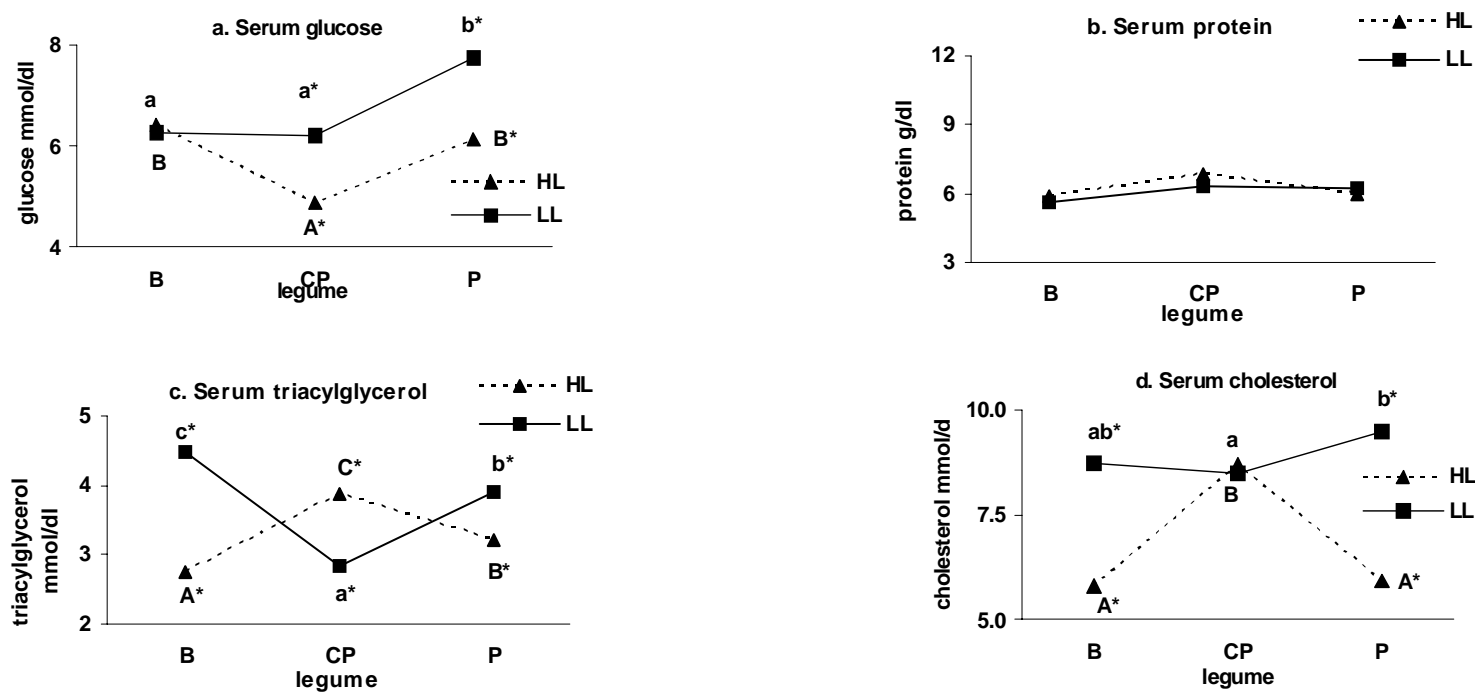
Figure 6.2. Means of seabream serum glucose (a), total protein (b), triacylglycerols (c) and cholesterol (d) for the six experimental diets are presented in figures with two-way ANOVA, showing the effects of the two factors (inclusion level L&H and legume type) and their interaction. For each legume level (L), values denoted with different letters are significantly different; uppercase or lowercase letters correspond to H (high) and L (low) level respectively within each legume. Significant difference between LL and HL marked with an asterisk.

6.3.4 Immunology

One-way ANOVA showed significant differences in chemiluminescence among the fish groups fed diets including different legume types, but not between L and H levels of each legume. In this respect P diets showed significant immunostimulation compared to all diets, followed by B and CP diets, with CPH revealing the lowest values (Figure 6.3).
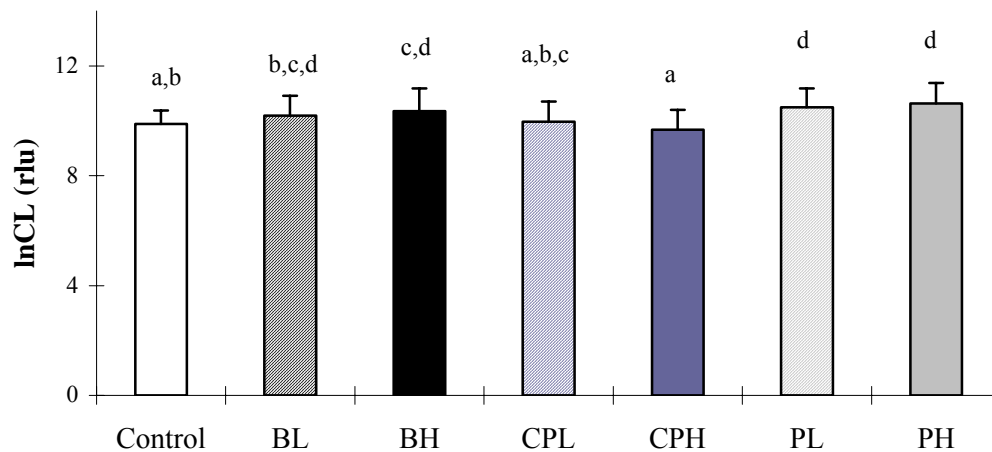
Figure 6.3. Effect of tested dietary legumes and significant differences ($P < 0.05$) on seabream serum chemiluminescence activity (rlu, relative luminescent units).

6.3.5 Histology

Examination of histology sections for internal organs did not show any pathological abnormalities that could be related to nutritional treatment. Liver, foregut, hindgut spleen and kidneys did not show any differences among the treatments, however, in spleen and kidney samples sparse melano-macrophage centres were detected.

6.4 Discussion

6.4.1 Growth

According to the experimental results seabream growth was not negatively affected by wheat substitution by legumes and the high inclusion level of peas, chickpeas or faba beans in the diets. Chickpea diets showed the best performances when fed to the fish with similar values to the control diet and this could be an indication that ANF in chickpeas had lower effects on the fish in relation to growth size.

Growth performance was not improved by wheat substitution by legumes, either by the high inclusion level of legumes. However, none of the P diets significantly affected growth rates which is in agreement with Gouveia and Davies (1998; 2000) who found that in juvenile European seabass SGR values were not affected when fish were fed pelleted diets containing 20 and $40g100g^{-1}$ of raw or $10\text{-}30g100g^{-1}$ extruded pea seed meal compared to a wheat and fishmeal-based control diet. In the latter study Gouveia and Davies (2000) found that PER values, nitrogen deposition and utilization were improved with increasing levels of pea seed meal in extruded diets, while for seabream in the present study no differences were found for this parameter. In rainbow trout, when wheat meal was replaced with dehulled field pea meal ($25g100g^{-1}$) although SGR, FCR and PER values were improved, they did not differ significantly when compared to a control diet (Thiessen et al., 2003). The results of the current trial showed a significant negative effect on seabream FCR values for both P diets that is not in agreement with any of the previously mentioned studies. Differences of fat inclusion level of the diets or the parasitic infection and treatment in the present experiment could possibly have affected these results.

For faba beans and chickpeas no relevant growth studies are available, but in this research FCR was not significantly different for diets CPL, CPH and BL compared to the control, while a high level of faba beans adversely affected feed conversion ratio. Overall, CP diets resulted in higher performances among legume diets, with the most improved SGR and FCR values, while lower values were noted for P diets and BH. Interactions between legume type and level of starch/legume were not found for any of the growth parameters. It is interesting to note that both CP diets showed increased PPV values, however this improvement was significant only against diet PH.

Substitution of wheat as a carbohydrate source in L diets did not affect SGR or PPV values, but it had a negative effect on FCR when fish were fed pea seed meal. Different carbohydrate sources also affected FCR values but not SGR in salmon (Hemre and Hansen, 1998), and growth performance in rainbow trout (Bergot, 1979).

In diets including high levels of legumes, recipes were formulated with the intention of them being of lower cost. At the same time they employed materials commonly used in BioMar's commercial seabream diets and thus there is a more complicated combination of plant proteins. However inclusion of $35g100g^{-1}$ of the tested ingredients as the main starch source in the diet can give very useful information. In this respect the high starch level of any of the legumes did not affect negatively growth performance of seabream, but the high level of faba bean and peas adversely affected FCR.

6.4.2 Carcass composition, HSI, liver glycogen, liver fat

Gilthead seabream in the current study showed no negative effects on carcass proximate composition of either low (PL) or high (PH) field pea containing diets when compared to the control. Processed pea seed meals at similar inclusion levels as in the

present study were tested in gilthead seabream diets (Pereira and Oliva-Teles, 2002) and the authors demonstrated that no significant differences occurred in carcass proximate composition and HSI. This is in agreement with the results of the present study that showed no differences in carcass composition or HSI between control and pea diets. Equivalent results were also found for seabass juveniles fed pea seed meal diets (Gouveia and Davies, 1998; Gouveia and Davies, 2000). Higher carcass protein values observed for diets BH and PH compared to BL and PL respectively, however, significance was found only for faba bean diets. Carcass fat did not differ between high and low starch diets in contrast to the results of Refstie and Austreng (1981) that showed decreasing fat values with increasing carbohydrate levels and Wang et al. (2005) and Moreira et al. (2008) who found the opposite for tilapia and juvenile seabass diets respectively. Wang et al. (2005) did not find any differences in carcass composition between diets including 6 or 14% starch, but only compared to the higher starch levels, while Moreira et al. (2008) found increasing carcass fat with increasing starch level (10 vs. 20 and 30%). These results could be either species related or most possibly could be attributed to the higher inclusion levels of carbohydrates included compared to the diets of the current study.

Increased values were found for liver glycogen in H compared to L diets, but these were significant only for CP diets, most probably due to the digestible starch increase (Kim and Kaushik, 1992). Elevated carbohydrate inclusion level in diets has also been shown to increase liver glycogen in salmon (Refstie and Austreng, 1981; Arnesen, et al., 1995), tilapia (Wang, et al., 2005), rainbow trout (Austreng et al., 1977; Bergot, 1979) and seabass (Moreira et al., 2008). HSI values did not show any significant difference among treatments in seabream as also has been found for salmon (Arnesen et al., 1995) for different carbohydrate levels, but this is in contrast to work of other

authors. Specifically, significant differences in HSI were found in trout (Kim and Kaushik, 1992), silver perch (Stone et al., 2003), tilapia (Wang et al., 2005) and seabass (Moreira et al., 2008). No correlation was found between HSI and liver glycogen content, which is in contrast to results published for several other species (Hemre et al., 2002). Whilst increased glycogen deposition seems to induce liver enlargement (Stone et al., 2003) this was not shown here. Lanari et al. (1999) associated liver fat content with high carbohydrate inclusion in diets for seabass, but this was not supported by the present research either. This might be explained by the lower starch inclusion levels in the present study.

6.4.3 Haematological parameters

Hemre et al. (2002) in their review of carbohydrates in fish nutrition concluded that most teleost fishes have wide limits of glucose levels that could be attributed to differences among species or different life stages of the same species and it could also be feed related. Serum glucose values revealed a significant interaction between starch inclusion level and legume type included in tested diets showing that glucose levels in seabream could be affected both by the level and the type of starch. Chickpeas and peas resulted in decreasing values when included at high levels, while faba bean starch did not show any differences. Diet PL significantly increased glucose in serum compared to the wheat based diet, as well as CPL and beans when included at a high level. In contrast, raw pea starch at a $30g100g^{-1}$ level was found not to affect plasma glucose in Australian silver perch (Stone et al., 2003) compared to different carbohydrate sources at the same percentage, and the same was found for salmon fed oat and maize meals as carbohydrate sources (Arnesen et al., 1995). However glucose levels for pea starch were measured 3h after feeding and such values are not comparable to present values

measured 40h after last feeding. Moreira et al. (2008) found no differences in plasma glucose 6 hours after feeding seabass with 10, 20 or 30% of gelatinized starch in 25°C.

Cholesterol levels in blood plasma have been related to NSP content of plant materials (Levrat et al., 1996; Favier et al., 1997) in diets for both mammals or humans and are associated with lower plasma cholesterol levels (Wester, 2000). Hung et al. (1989) found that different carbohydrate sources could affect differently lipogenic activity, total cholesterol and triacylglycerol levels in blood plasma when included in sturgeon diets. Cholesterol and triacylglycerol levels in the current experiment were reduced with increasing levels of B and P, while wheat starch showed similar results with BL and CPL for cholesterol and lower values for triacylglycerols. The results are not in agreement with Hemre and Hansen (1998) who found no effect of carbohydrate source on these parameters. Furthermore, no correlation was found among serum cholesterol, triacylglycerols and HSI, liver and carcass fat content and neither between liver glycogen and NSP inclusion level. This was not unexpected due to the low differences in NSP contents of the present diets.

6.4.4 Immunology

Immunological studies in fish related to dietary legumes are scarce in the literature. Esparza et al. (1996) found that mice fed diets including legumes showed immunological responses ascribed to the ANF present in the diets. Soybean meal has been shown to cause immune system disturbances in both salmon (Krogdahl et al., 2000) and trout (Burrells et al., 1999). However, in seabream or for the legumes tested in the present study no data are available. It is possible that the diets containing the higher levels of ANF showed lowest growth and immunostimulatory evidence, but most of the values did not show significant differences and therefore further trials are

required for firm conclusions. Nevertheless, according to the present values, chickpeas were more appropriate as a wheat replacer for seabream diets than peas and faba beans.

6.4.5 Histology

High levels of soybean in the diets have been implicated in inducing abnormalities of hind gut in salmon (Baeverfjord and Krogdahl, 1996; Refstie et al., 2000; Aslaksen et al. 2007) and rainbow trout (Refstie et al., 2000), whereas there is some evidence that peas and faba beans (in 18 and $22g100g^{-1}$ respectively) in salmon diets do not cause histological abnormalities (Aslaksen et al., 2007). In this respect histological findings could be expected for other legumes or/and other fish species. Gilthead seabream seem to be resistant when exposed to plant materials as histological changes were only found when fish were fed diets containing 100% plant proteins (Sitja-Bobadilla et al., 2005). Results of the present study confirm this fact because no pathological effects were found in hindgut. The rest of the organs examined had a normal form, but in kidney tissue some melanomacrophage centres were found for all treatments, that could be either normal or attributed to the parasite infection (Ferguson, 2006) during the experiment.

6.5 Conclusions

None of the legume diets improved growth performance of seabream, compared to the control diet, however, the differences observed were not significant for growth parameters. Among the legume diets, CPL could be considered as the most efficient because of the higher PPV value. Carcass fat was affected by the carbohydrate source, with the faba bean diet resulting in reduced fat content. Liver glycogen was increased with increasing starch levels, but HSI was not affected. Enteritis was not induced in seabream fed diets including low or high levels of legumes.

Chapter 7. General Discussion and Conclusions

7.1 General

The present study aimed to investigate grain legumes, namely peas, chickpeas and faba beans, as potential feed ingredients in extruded diets for European seabass and gilthead seabream as these are the most important aquaculture species for Mediterranean countries. Each legume was processed and analyzed before inclusion in diets at up to 35% as both an energy source replacing wheat and as a protein source partially replacing various protein sources in each experiment. Specifically, in Chapter 3 the effects of different processing conditions were examined on the whole seed flours of tested ingredients. Nutritional and ANF of legumes and physical characteristics of experimental diet pellets including high and low levels of each legume were evaluated. In Experiment I the effects of wheat substitution by legumes were investigated on nutrient digestibility, gastrointestinal evacuation rate and serum glucose response in European seabass. In Experiment II growth, digestibility, haematological parameters, histological effects and fillet organoleptic characteristics as well as the interaction between starch inclusion level (low and high) and legume type were evaluated when tested legumes replaced wheat in European seabass diets. Lastly, in Experiment III growth, haematological parameters, histological effects and the interaction between starch inclusion level (low and high) and legume type were evaluated when tested legumes replaced wheat in gilthead seabream diets.

7.2 Nutrient digestibility

Nutrient digestibility was satisfactory for all diets at both 18ºC (Experiment I) and 25ºC (Experiment II), including either low or high legume levels and/or low or high starch levels. At low temperatures absolute digestibility values were lower than those observed at higher temperatures. However, fishmeal inclusion level was higher

(doubled) and plant inclusion lower in Experiment I. Temperature differences may explain digestibility differences as digestive enzymes are more efficient at 25ºC than at 18ºC (Papoutsoglou and Lyndon, 2005). In addition, digestibility evaluation in juvenile seabass at 25ºC and 18ºC showed increased starch but not protein and energy ADCs at the higher temperature (Moreira et al., 2008). Lower water temperature could also reduce feed intake and thus the endogenous gut losses can represent a greater proportion of the faecal waste resulting in a decrease of ADCs (Bureau et al., 2002), but this hypothesis requires further investigation. Combination of wheat and legume starch at low temperatures improved starch digestibility. An improvement in protein and starch digestibility was determined as GET increased indicating a possible positive effect of longer feed passage time on nutrient absorption.

7.3 Gastrointestinal evacuation time

Gastrointestinal evacuation rate has proven to be a very important parameter for modelling daily feed intake (Jobling, 1981) as it controls fish appetite (Riche et al., 2004). Inclusion of legumes in diets for seabass clearly increased GET of feed with faba bean having the strongest effect. This might be attributed to higher NSP inclusion level (Knudsen, 2001) as indigestible particles of feed are the last to remain in the stomach after the digestible part of the diet has been expelled and their evacuation is slow (Bromley, 1987). Different extruded starch sources, wheat and corn, resulted in different GER for gilthead seabream (Venou et al., 2003). It is possible that the structure of the extruded starch molecules of the tested legumes could result in this delay.

7.4   Seabass and seabream growth

In general, the results of the present dissertation showed a better response of seabass compared to seabream to legumes in the diets. Seabass showed improved growth performance when fed low level compared to the control, while seabream showed an overall decreased, although not significantly, for all diets tested. Among legume diets, chickpeas at a low level were the most efficient with increased SGR values in seabass and PPV in seabream indicating a better protein sparing effect of chickpea starch. Growth parameters interactions between starch levels and legume type were only found for seabass, while seabream was not affected by starch level or legume starch type. A negative effect was found in high level diets for seabass but this was significant only for chickpea.

7.5   Seabass and seabream carcass proximate composition

European seabass and gilthead seabream in experiments II and III showed no negative effects on carcass proximate composition of either low (PL) or high (PH) field pea containing diets when compared to the control. Pea seed meal was successfully included in diets for juveniles seabass (Gouveia and Davies, 1998; Gouveia and Davies, 2000) and gilthead seabream (Pereira and Oliva-Teles, 2002) with equivalent results. In particular seabass and seabream showed higher carcass protein when fed diet CPH, however, this was only significant for seabass, while seabream significantly increased carcass protein with increased faba bean level. Carcass fat was not affected by diet for seabream, but seabass showed significantly reduced carcass total lipid with increasing bean and chickpea starch (BH, CPH).

7.6 Seabass and seabream liver characteristics

Liver fat and HSI values were all higher for seabass than seabream, while glycogen level did not differ much. Liver glycogen levels were affected by starch level for both species, with higher starch levels resulting in higher glycogen concentration, although significance was found only for seabream. Increasing carbohydrate levels have been also associated with high glycogen contents in rainbow trout (Austreng et al., 1977; Bergot, 1979), salmon (Refstie and Austreng 1981; Arnesen et al., 1995), tilapia (Wang et al., 2005), and seabass (Moreira et al., 2008).

Seabass showed increased HSI for high starch level diets and a strong correlation between HSI and glycogen levels, while seabream did not show any differences among treatments and no correlation between HSI and liver glycogen. Correlation between these characteristics has been found also for other species as reviewed in Hemre et al. (2002). Extended glycogen deposition seems to induce liver enlargement (Stone et al., 2003), while Arnesen et al. (1995) did not find significant differences in HSI of salmon fed different levels of carbohydrates.

A significant interaction was found for seabream (BH lower than CPH, PH and CPL higher than PL, BL) liver fat but not for seabass. Liver fat was not affected by diet treatment for seabass and seabream when comparison was between H and L diets in legume pairs (PL vs. PH, CPL vs. CPH and BL vs. BH). Lanari et. al. (1999) found that liver fat content is associated with high carbohydrate contents in diets for seabass but this was not supported by any of the present experiments. Seabass H diets resulted in lower liver fat content than L diets, however, this difference could be explained by the lower inclusion levels of starch in the present study.

7.7 Seabass and seabream haematological characteristics

Different carbohydrate sources have been proven to affect differently lipogenic activity in white sturgeon (Hung et al., 1989) that is in agreement with the present study. Lipogenic activity differences reflecting on cholesterol and triacylglycerol levels seabass and seabream serum were found. Cholesterol levels decreased when both species fed high faba bean levels Triacylglycerols decreased for BH and PH only for seabream, while seabream showed no differences for any of the dietary treatments.

Glucose values were contradictory for the two species and the different legumes. Significant interactions for glucose were observed for seabream, with increasing glucose levels when fish were fed pea seed meal and L diets (excluding faba beans). Significant interactions for glucose levels were observed as well for seabass, with ascending glucose levels for PH, BH and CPH.

7.8 Immunology and histology

7.8.1 Immunology

Measurement of chemiluminescence as an immunological parameter did not show systematic disturbance with the use of legumes in seabass and seabream diets. However, faba bean diets showed an immunosuppressive tendency in seabass at the high inclusion level (BH). In seabream high chickpea level (CPH) had an immunosuppressing effect, while PH and BH showed imunostimulating effects. Inclusion of legume such as soybean in diets has been associated with immunological disturbances in both salmon (Krogdahl et al., 2000) and trout (Burrells et al., 1999).

7.8.2   Histology

Faba beans, chickpeas and peas did not cause enteritis in seabass or seabream hindgut when fed at low or high inclusion level in the diets. However, absorptive vacuoles were seen in increased quantities. Abnormal size and arrangement of vacuoles were found only in the distal intestine of seabass fed the control diet and to a lesser extent diet PH. Findings for the control diet could be attributed to the $17g100g^{-1}$ soybean inclusion level, while in seabream none of the tested diets caused any pathological effects in hindgut. High levels of soybean in the diets have been implicated in inducing hind gut abnormalities in salmon (Baeverfjord and Krogdahl, 1996; Refstie et al., 2000; Aslaksen et al., 2007) and rainbow trout (Refstie et al., 2000), whereas there is some evidence that peas and faba beans (in 18 and $22g100g^{-1}$ respectively) in salmon diets do not cause histological abnormalities (Aslaksen et al., 2007). The results of the present study also confirm the findings of Sitja-Bobadilla et al. (2005) that gilthead seabream is tolerant when fed diets including plant materials up to 75% without causing histological changes.

7.9   General conclusions

The results of Chapter 3, suggest that:

- extrusion processing could improve the nutritional value of the ingredients by significant reduction of TI.

- Phytate was not eliminated by extrusion, but problems caused by phytate could be possibly overcome by mineral supplementation (Porres et al., 2004).

- Total tannin levels were not affected by extrusion, but their levels in legumes were comparatively low compared to those known to affect fish growth performance (Francis et al., 2001)

- Peas, chickpeas and faba beans are raw materials that could be used in seabass and seabream diets without causing negative effects on pellet physical characteristics.

- Levels up to $35g100g^{-1}$ of each legume are acceptable for producing pellets that could be manufactured, transferred and stored.

The results of Experiment I, suggest that:

- All three legumes tested proved to have potential as feed ingredients for European seabass, mainly as carbohydrate replacers for wheat.

- Digestibility coefficients showed satisfactory values for all diets either including low or high levels of legumes.

- Starch digestibility was slightly improved for the diets that combined wheat and legume starch source.

- GET was significantly delayed by the inclusion of chickpeas,

- Foregut evacuation rate was reduced for all legume diets with faba beans showing the strongest effect.

- Glucose levels in seabass serum was also affected by the type of carbohydrates ingested with wheat starch showing more rapid increase and decrease of glucose

compared to fish fed pea and chickpea diets, while faba bean starch resulted in a lower glucose peak.

The results of Experiment II, suggest that:

- The inclusion of any of the three legumes up to 17% in seabass diets proved to be beneficial in terms of growth with chickpea diet giving the best growth values, but also the higher carcass fat content.

- Fish fed diets including 35% legumes showed decreasing growth trends compared to diets including 17% legumes.

- Partial substitution of commonly used plant proteins by the tested legume proteins was beneficial for seabass growth performance.

- In pathological terms, enteritis was not induced to any of fish groups and only high inclusion of pea seed meal caused increased numbers of absorptive vacuoles in hindgut.

- Organoleptic characteristics did not differ among steam cooked fillets of seabass fed either control or high legume level diets.

The results of Experiment III, suggest that:

- None of the legume diets improved growth performance of seabream, compared to the control diet. However, the differences observed were not significant for growth parameters, result that could have been also affected by the parasitic infection during the trial.

- Among the legume diets, CPL could be considered as the most efficient because of the higher PPV value.

- Carcass fat was affected by the carbohydrate source, with the faba bean diet resulting in reduced fat content.

- Liver glycogen was increased with increasing starch levels, but HSI was not affected.

- Enteritis was not induced in seabream fed diets including low or high levels of legumes.

The results of Experiments II and III, suggest that:

- In conclusion, all three legumes tested had better potential as feed ingredients for European seabass than for gilthead seabream, mainly as carbohydrate replacers for wheat and to a lesser extent for protein substitution. Further research is required to include growth trials to fully evaluate the nutritional value of these ingredients in practical diets for these species.

7.10 Future perspectives

Legumes like faba beans, chickpeas and field peas are valuable potential sources of both protein and starch for European seabass and gilthead seabream feeds. In this respect these three legumes offer flexibility to the feed manufacturer as they can partially replace both energy (cereals like wheat that are also used as binding agents) and protein (such as soybean or other plant and animal protein) (Fraser, 2005). All these crops are cultivated in Mediterranean countries as well as in the rest Europe. This could

be an advantage with a positive effect on the final price of the product due to minimization of transport costs, and also in development of agricultural production with vertical integration between seed and fish feed production.

Environmental protection is also of current interest and these legumes could contribute to this. Cultivations of legumes can decrease nitrogen pollution through their ability to naturally fertilize poor fields that for years have been cultivated with cereals, and can also increase total yields through rotation (Grain legumes, 2006).

Further research is required to fully evaluate nutritional benefits or drawbacks. Specifically:

- Long term experiments using faba beans, chickpeas and peas at low and high temperatures in farm cages could give safe information for the inclusion level of these legumes in practice. Small differences observed in the present study could be more clear after a whole growth period in intensive conditions

- Concentrated proteins of peas, chickpeas, and faba beans possibly have a good potential to replace soybean protein. Air classification is a cheap and easy method to produce concentrated proteins of plant grains.

- Dehulled or pre-extruded legumes or bio-ethanol byproducts of these legumes could also be tested in diets for seabass and seabream for their effects on digestibility and growth performance

- Although much research has been conducted on carbohydrates in fish nutrition, there are also many things that are still not clear about metabolism, interactions with

other materials and the specific effects of ANF on nutritional performance. Measurement of carbohydrate fractions and in vitro starch and protein digestibility of raw and extruded legumes could be also investigated to give further information for these materials.

- Evaluation of digesta viscosity in the intestine and digestive enzyme or microbe activity in each part of the intestine after feeding could also give valuable information about carbohydrate metabolism in these two species.

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Appendix: Presentations and publications from the project

Ia. Publications in progress (in peer-reviewed journals)

- Adamidou, S., Nengas, I., Henry M., Rigos, G., Grigorakis, K., Nikolopoulou, D., Kotzamanis, Y., Karacostas, I., Bell, J.G. and Jauncey, K. 2008. Growth, digestibility performance and blood serum parameters of European seabass (*Dicentrarchus labr*ax) fed extruded diets including low and high levels of three different legumes; peas (*Pisum sativum*, L.), chickpeas (*Cicer arietinum*, L.) and faba beans (*Vicia faba*, L.). *To be submitted for publication.*

- Adamidou, S., Nengas, I., Alexis, M.N., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, I., Rigos G. and Jauncey, K. 2008. Apparent nutrient digestibility and gastrointestinal time evacuation in European seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. *To be submitted for publication.*

Ib. Presentations in peer-reviewed conferences

- Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Tsakona, A., Campbell, P., Karakostas, I. and Jauncey, K. 2004. Apparent digestibility of nutrients and gastrointestinal evacuation time by seabass (*Dicentrarchus labrax*) fed diets containing different levels of legumes. XII International Symposium on Fish Nutrition & Feeding. Biarritz, France, May 28th – June 1st, 2006. Poster presentation.Adamidou, S., Nengas, I., Grigorakis, K., Karacostas, I. and Jauncey, K. 2008. Growth performance and digestibility in European seabass (Dicentrarchus labrax) fed extruded diets including low and high levels of three legumes; peas (Pisum sativum, L.), chickpeas (Cicer arietinum, L.) and faba beans (Vicia faba, L.). XIII International Symposium on Fish Nutrition & Feeding. Florianopolis, Brazil, June 1st – 5th, 2006. Poster presentation.