New species for EU aquaculture

Deliverable Report

Deliverable No:	D13.5		**Delivery Month:**	61
Deliverable Title	Evaluate and maximize the dietary incorporation of a non-GMO soybean meal			
WP No:	13		**WP Lead beneficiary:**	P4.IOLR
WP Title:	Larval Husbandry – grey mullet			
Task No:			**Task Lead beneficiary:**	P4.IOLR
Task Title:	Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.			
Other beneficiaries:	P2. FCPCT	P13. UNIBA	P15. ULL	
Status:	Delivered		**Expected month:**	60
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Objective: The effect of replacing poultry meal with soybean meal on growth, intestinal morphology and inflammation, peroxidation, innate antioxidant mechanisms and intestinal pathology.

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1.0 Introduction

Due to its high protein content and digestibility as well as a balanced amino acid profile, soybean meal (SBM) is widely used as a protein meal replacement in the growing of carnivorous and herbivorous species (Storebakken et al. 2000). On the other hand, soy protein products contain antinutrient factors, such as proteinase inhibitors and agglutinating lectins, but they can be inactivated by heat treatment. However, there are compounds such as phytic acid, glucosinolates, saponins, tannins, soluble non- starch polysaccharides and gossypol which are heat resistant and can interfere with protein digestion or chelate nutritionally essential elements including Ca, Zn and Fe (Hardy, 2010).

Importantly, the use of significant levels of soybean meal in fish diets can cause an inflammatory response in the distal intestinal epithelium resulting in enteritis and has been reported in salmonid and other carnivorous fish (Zhou et al., 2005; van den Ingh et al., 1991; Refstie et al., 2000; Refstie et al., 2001; Baeverfjord & Krogdahl, 1996; Rumsey et al., 1995) as well as omnivores such as carp (Urán 2008). Typical symptoms of

soybean meal induced enteritis are a shortening of the mucosal folds with reduced absorptive capacity of the enterocytes lining the epithelium (Urán 2008) with subsequent loss of the normal supranuclear vacuolization. There is a thickening of both lamina propria and sub-epithelial mucosa with a severe infiltration of inflammatory cells, particularly of macrophages and eosinophilic granulocytes as well as the presence of increased numbers of goblet cells in the epithelium. Fish enteritis leads to higher moisture levels in feces resulting in a more rapid passage through the gut, with less time available for digestion and absorption. Inflammation is generally associated with oxidative stress, which affects the innate antioxidant system comprised of the enzymes; superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH in the intestine and enterocytes (Sitjà-Bobadilla et al., 2005).

The soy protein and poultry meal levels in the **P4.IOLR** diet for the grey mullet are *ca.* 12% and 13% DW diet, respectively. However, poultry meal is prohibited in fish feeds in Europe (IRIDA, person. communication). Alternatively, poultry meal can be replaced with fish meal but the Diversify consortium is endevouring to produce a sustainable, fish meal-free diet for this species. On the other hand, if the poultry meal is replaced with additional soybean meal, which would then represent *ca.* 25% of the DW diet, it is unknown if this will have adverse effects on fish performance due to the presence of inflammation and oxidative stress. As a result, the aim of this study was to evaluate the effect of further increasing the level of soybean meal in the diet, which contains other plant based proteins, such as wheat and rapeseed, on juvenile grey mullet performance and the presence of inflammation. In addition, the effect of dietary supplementation of powdered *Haematococcus* algae (containing *ca.* 3% astaxanthin), as an effective antioxidant to reduce oxidative stress and the upregulation of the innate antioxidant system, was tested.

2.0 Materials an Methods

Juvenile F2 grey mullet (50 dph) spawned from **P4.IOLR** broodstock were stocked (100 fish tank^{-1}) in twelve 400 l V-tanks where UV treated, filtered (10 µ), ambient seawater (diluted to 25 ppt) at 25±0.5 ^{o}C, entered the tank from the bottom and exited through 500 µm mesh filters near the top with an exchange rate of 9.5 exchanges day^{-1}. The average fish wet weight among replicates and between treatments were not significantly (P>0.05) different (**Table 1**). The experimental set up allowed the testing of three dietary treatments (A, B, C) with 4 replicate tanks treatment^{-1}. Treatment A was the IOLR formulated grey mullet control diet and contained *ca.* 13% DW poultry meal, which was the only source of animal protein. Treatment B replaced the poultry meal fraction with soybean meal, so that the diet was isonitrogenous with the control. Treatment C was identicle to Treatment B but was supplemented with dried microalgae (*Haematococcus* sp.), which contains *ca.* 3% astaxanthin, resulting in 25 ppm of this carotenoid being added to the diet. The fish were fed the 1.4 mm pelleted experimental diets from 50 to 90 dph at a ration size of 6% wet body weight day^{-1} equally distributed over 4 meals. The experimental system was illuminated under a 12L:12D photoperiod with a light intensity of 500 lx.

At the beginning of the experiment 4 fish were taken before stocking in the experimental tanks, sacrificed in excess MS-222, and then individually weighed and standard length measured. Similarly, at the end of the study, 4 fish from each experimental tank were taken, sacrificed in excess MS-222, weighed individually and standard length measured. All sampled fish at the end of the study were treated identically in the following manner. The digestive tracts (DT) from the fish were dissected out and measured. The midgut was considered to be between 4-6 cm posteriorly from the junction of the stomach and foregut while the hindgut was considered to be between 10-12 cm from the end of the midgut towards the anus. When these sections were dissected out, they were placed in Petrie dishes where any remaining digesta was removed in phosphate buffer solution (PBS). The DT sections from two sampled fish from each tank were placed in Eppendorf tubes containing 10% buffered neutral formalin and stored at -32 ^{o}C for later histological analysis. In parallel, the DT sections of two fish from each tank were placed in Ependorff tubes containing RNAlater and stored at -32 ^{o}C for later RNA extraction and gene expression of the innate antioxidant system. For histology,

DT samples were dehydrated and embedded in paraffin blocks where 5 µm sections were stained wih hematoxylin and eosin and the slides examined under a microscope.

Table 1 Average fish wet weight (g) per treatment (A, B, C) at stocking of replicate (R) tanks (n=4). Treatment fish wet weights were not significantly different at the start of the experiment (P=0.1410).

Treatments	**A**	**B**	**C**
R1	1.4±0.3	1.5±0.3	1.4±0.3
R2	1.4±0.3	1.4±0.4	1.5±0.3
R3	1.4±0.3	1.4±0.3	1.5±0.4
R4	1.3±0.3	1.5±0.3	1.4±0.3
Average	1.4±0.01	1.5±0.01	1.4±0.01

3.0 Results

In **Fig. 1** juvenile grey mullet fed the control diet A, which contained poultry meal, gained 15.5 and 25.1% more weight (P<0.05) than fish fed the diets B and C, respectively. Diet A fish also exhibited markedly (P<0.05) longer and heavier digestive tracts (**Fig. 2** and **Fig. 3**, respectively) than fish fed diets B and C which, in general, differed little from each other. In addition, fish fed Diet A produced higher numbers of larger fish (>5 g) than fish fed Diets B and C, which was significant (P<0.05) in the latter (**Fig. 4**). Conversely, there was a tendency for Diet C to produce larger numbers of smaller fish, particularly in the <3-5 g range. There was no dietary effect on general digestive tract health, mucosa cell length and the presence of inflammation (**Fig. 5**) nor was there any mortality found among all tanks in the experiment during the course of the experiment.

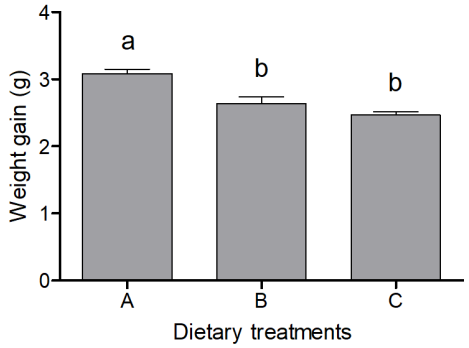

Figure 1 The effect of dietary treatments (A, B, C) on average fish wet weight gain (g). Bars having different letters were significantly (P<0.05) different.

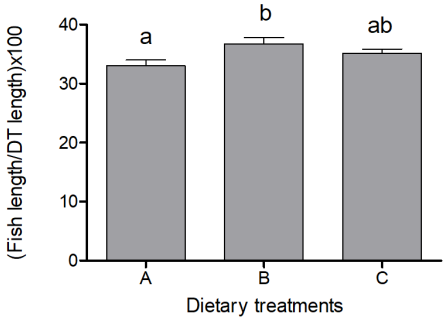

Figure 2 The effect of dietary treatments (A, B, C) on the ratio of the average fish length/digestive tract (DT) length x 100. Bars having different letters were significantly (P<0.05) different.

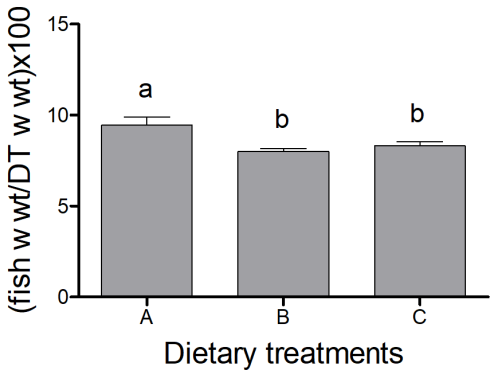

Figure 3 The effect of dietary treatments (A, B, C) on the ratio of the average fish wet weight (w wt)/digestive tract (DT) wet weight (w wt) x 100. Bars having different letters were significantly (P<0.05) different.

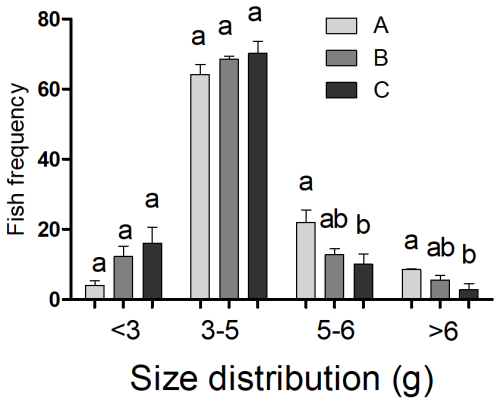

Figure 4 The effect of dietary treatments (A, B, C) on the size distribution among replicate populations in each of the treatments. Bars having different letters, within a size group were significantly (P<0.05) different.

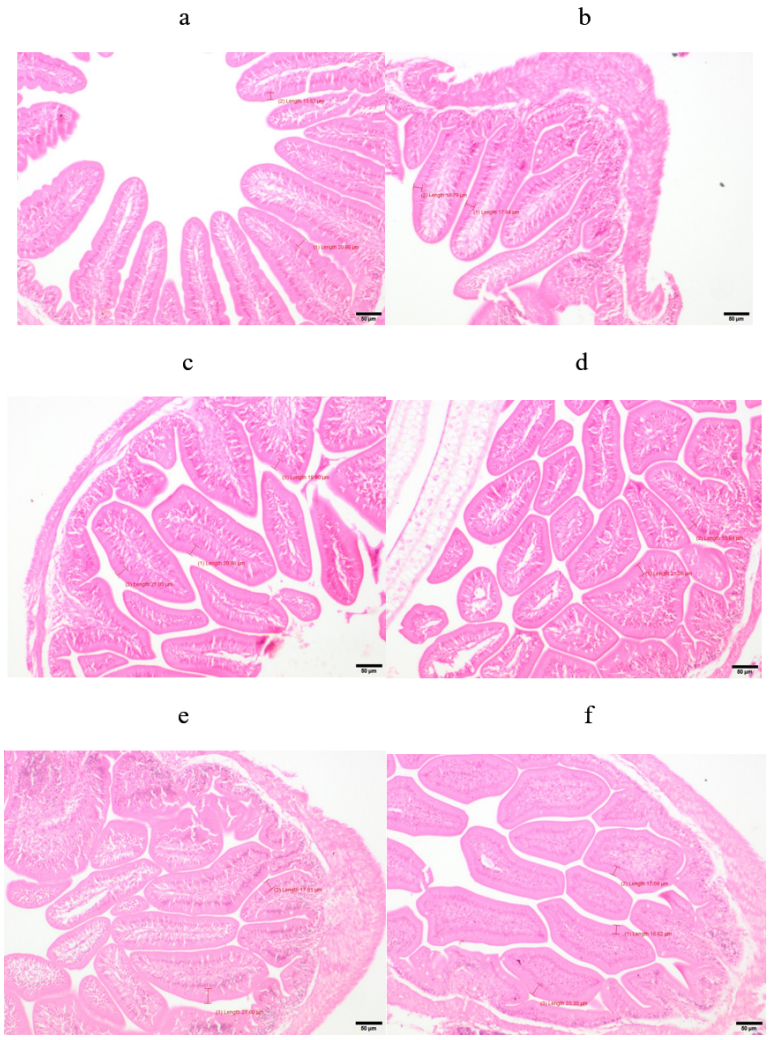

Figure 5 Representative histological sections (5 µm) of the midgut and hind gut of fish fed the diets A, B, C and D (a and b, c and d, e and f, respectively). No obvious pathology or inflammation was observed in any of the histological sections (n=48) taken from fish at the end of the experiment.

4.0 Discussion

Soybean meal (SBM) is commonly used as a partial fish meal replacement in the mariculture of carnivorous fish such as the European sea bass (*Dicentrarchus labrax*) (Tibaldi et al., 2006), gilthead sea bream (*Sparus aurata*) (Sitjà-Bobadilla et al., 2005) and to a greater degree in omnivorous species such as carp (Storebakken et al. 2000). This is primarily due to its high protein content, digestibility, market availability as well as a balanced amino acid profile. However, untreated SBM can contain antinutrient factors, such proteinase inhibitors and agglutinating lectins, which can markedly affect fish performance but can be inactivated by heat treatment. Nevertheless, there are compounds including phytic acid, glucosinolates, saponins, tannins, soluble non- starch polysaccharides and gossypol which are heat resistant (Hardy, 2010) and can interfere with protein digestion or chelate nutritionally essential elements including Ca, Zn and Fe. Moreover, higher levels of dietary SBM, in both carvorous (Baeverfjord & Krogdahl, 1996) as well as

omnivorous (Urán, 2008) fish, can cause an inflammatory response in the distal intestinal epithelium, which affects fish health, reduces intestinal nutrient absorption and somatic growth (van den Ingh et al., 1991; Refstie et al., 2000; Refstie et al., 2001). Inflammation, indendent of the cause, is frequently associated with oxidative stress and the upregulation of the genes involved in the innate antioxidation system (Sitjà-Bobadilla et al., 2005). In this study, there was no indication at all of inflammation, in terms of shortened mucosal folds or thickening of the lamina propria and sub-epithelial mucosa. In fact, DT samples from all fish exhibited healthy tissue with no signs of disease and presumably oxidation stress.

Although there was a significant improvement in the performance of fish fed the control-poultry meal diet, the change was not large nor due to triggering an inflammation response. There are differences in nutrient composition between soybean and poultry meal which may suggest an alternative hypothesis to the moderate effect of animal based meals. Plant based meals do not contain taurine, while it is found in considerable levels in animal protein. Taurine is an amino sulfonic acid that plays an array of critical roles in bile salt synthesis, anti-oxidative defense, cellular osmoregulation, as well as contributing to visual, neural and muscular function and development (Abdel-Tawwab et al., 2018; Fang et al., 2002; Omura and Inagaki, 2000). It has been shown to be a dietary essential in a number of fish species (Koven et al. 2018). Consequently, its absence in the all plant based protein diets B and C might explain the benefit of poultry meal (Wu et al., 2017). Another potential deficiency is the sulphur containing essential amino acid methionine, which is often the first limiting nutrient and is generally at lower levels in plant based proteins such as soybean meal while considerably represented in the protein fraction in poultry meal (Niu et al. 2016).

Taken together, the results suggest that there is a significant improvement in grey mullet juvenile performance when using animal based proteins, such as poultry meal, at about 13% DW diet in the IOLR formula. On the other hand, this advantage may be modulated by the supplementation of essential amino acids such as methionine and the sulfonic amino acid, taurine.

5. References

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Deviations

This deliverable was originally propoaws more than 5 years ago to compare the effect of non-GMO and GMO soybean meals on their effect on the inflammation of the distal digestive tract, as well as the expression of genes coding for the innate antioxidant system. In retrospect, it is unlikely that there are any

differences between a non-GMO and a GMO soybean meal on gut health, inflammation and the innate antioxidant system in grey mullet. A more relevant question concerns new European regulations that prohibit the use of poultry meal in animal and fish feeds, which would severely curtail any use of the IOLR grey mullet formula in grey mullet aquaculture. Consequently, it was decided to test the effect of replacing the poultry meal with additional soybean meal (increasing it to 25% of DW diet) and evaluate its effect on fish performance, intestinal pathology and inflammation as well as the gene expression of the innate antioxidant system. However, as there was clearly no pathology or inflammation in this study to suggest an effect on the innate antioxidant system, the RNA samples will not be analyzed for this purpose. Instead, these samples will be analyzed in the future for the gene expression of other critical digestive tract proteins such as the mucosa membrane-bound transporter of dipeptides and tripeptides; peptide transporter 1(Pept1).


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