TRAINING COURSE ON
FISH NUTRITION RESEARCH: RECENT ADVANCES AND PERSPECTIVES

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FISH NUTRITION RESEARCH:
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The present volume collects the contributions of lecturers at the **Training Course on Fish Nutrition Research: Recent Advances and Perspectives**, organized by the *Nutrigenomics and Fish Growth Endocrinology Group of Institute of Aquaculture Torre de la Sal* (IATS-CSIC). The Course was sponsored by **USSEC** and directed to R+D personnel of the aquaculture sector working in fish nutrition and health. From June 13-15 2017, conferences were held at Hotel Voramar in Benicàssim by researchers of **IATS-CSIC** (Nutrigenomics and Fish Pathology Groups), **INRA** (Drs. S. Kaushik and G. Corraze), **ULPGC** (Profs. M. Izquierdo and J. Afonso) and Sparos Lda (Dr. L. Conceição).

Topics covered a wide range of nutrition-mediated issues: larval nutritional programming, nutrient x genotype interactions, resistance to bacterial and parasite infections, food safety, and long-term consequences of low fish meal/fish oil diets in fish species of interest in aquaculture (rainbow trout, European sea bass, gilthead sea bream).

Main results and conclusions are from the **ARRAINA** EU Project. Current research on ongoing H2020 Projects (**ParaFishControl** , **AQUAEXCEL**^{2020}) is also presented.

The Course was completed with a visit to IATS-CSIC facilities on June 16.

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PRESENTATION OF CSIC ACTIVITIES

J. Pérez-Sánchez

The Spanish National Research Council (CSIC) is the largest public institution dedicated to research in Spain and the third largest in Europe. It belongs to the Spanish Ministry of Economy and Competitiveness through the Secretary of State for Research.

CSIC research is driven by its centres and institutes, which are spread across all the autonomous regions, and its more than 15,000 staff, of whom more than 3,000 are staff researchers and the same number again are doctors and scientists who are still training.

CSIC has 6% of all the staff dedicated to Research and Development in Spain, and they generate approximately 20% of all scientific production in the country.

Its multidisciplinary and multisectorial nature means CSIC covers all fields of knowledge from basic research to technological development, being organised in eight scientific-technical areas:

- Area 1. Humanities and Social Sciences
- Area 2. Biology and Biomedicine
- Area 3. Natural Resources
- **Area 4. Agricultural Sciences**
- Area 5. Physical Science and Technologies
- Area 6. Materials Science and Technology
- Area 7. Food Science and Technology
- Area 8. Chemical Science and Technology

The **Area of Agricultural Sciences** covers a wide area of research with a clear social impact, encompassing lines of research that study the land, plants and livestock farming, including fish. The Aquaculture research of **CSIC** is conducted in four Institutes with a wide geographic distribution.

● Instituto de Acuicultura Torre de la Sal, IATS (Castellón)
● Instituto de Investigaciones Marinas, IIM (Vigo)
● Instituto de Ciencias del Mar, Barcelona (ICM)
● Instituto de Ciencias del Mar de Andalucía, Cádiz (ICMAN)

The IATS represents the 50% of critical mass of CSIC in Aquaculture research, covering different aspects of Fish Nutrition and Physiology, Pathology, Reproduction, Ecotoxicology and Food Safety in collaboration with the Institute of Pesticides and Water Resources (IUPA) of the University Jaume I of Castellón,

Over the last decade, the IATS leaded the participation of CSIC in Fish farming EU Research Projects with a role as Coordinators, Work Package leaders or Research leaders of CSIC in the Project, using modern methodologies based on omic and integrative approaches.

This important contribution is exemplified by ARRAINA Project (F7P), but also by current EU Projects within the H2020 program.

PARAFISHCONTROL. Advanced tools and Research Strategies for Parasite Control in European farmed fish. Call SFS-2014-2A, grant no. 634429

AQUAEXCEL^{2020}. AQUAculture infrastructures for EXCELlence in European fish research towards 2020. Call INFRAIA-1-2014-2015, grant no. 652831

PERFORMFISH. From production to consumption: Integrating innovative approaches and sustainable performance across the Mediterranean aquaculture value chain. Call H2020-SFS-2016-2017, topic SFS-23-2016, grant no. 727610

DEVELOPING FEEDS WITH VERY LOW LEVELS OF FISH MEAL AND FISH OIL: ISSUES AND EFFORTS MADE AS PART OF ARRAINA

S. Kaushik

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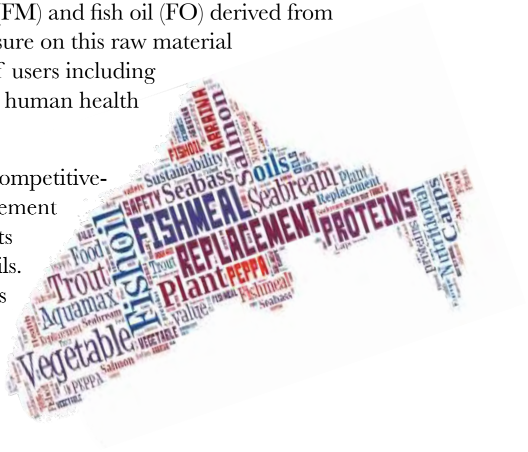
ERA Chair, EcoAaqua, ULPGC, Las Palmas, Spain.

Half of our seafood supply already comes from aquaculture. To keep up with the consumer demand for seafood, which could reach about 155 million tons by 2050, aquaculture production needs to double the current output. That translates into 2-3 million tons of extra fish feed per year. To achieve this, the sector needs to make more efficient use of resources. Traditionally, the feeds used for rearing fish have been relying highly on fishmeal (FM) and fish oil (FO) derived from capture fisheries. There is increasing pressure on this raw material due to growing demands from a variety of users including the expanding aquaculture sector and the human health market (e.g. fish oil food supplements).



We recognize that the sustainability and competitiveness of aquaculture depend on the replacement of FM and FO with alternative ingredients such as plant-based protein sources and oils. Over the past twenty years, much progress in developing feeds with low levels of FM or FO has been made. But we knew little about the long-term impact on the full life cycle (from egg to harvest) of farmed fish raised on such alternative feeds.

The FP7 project, ARRAINA was initiated to respond to this need by developing feeds with low levels of FM and FO for the full life cycle of five species of fish commonly reared in Europe and for measuring the long-term effects of such low FM / FO feeds. Research teams and the aquaculture industry joined hands in this project to bridge that knowledge gap and to address practical issues. The objective was to contribute towards increasing the productivity and performance of the aquaculture sector leading to competitive advantage of the whole sector at a global level.

The project has delivered practical tools and solutions to help boost the uptake of alternative fish feed across Europe. The tools include technical databases, guidelines and assurances covering a range of breeds and processes.

The first of the three-part series of technical booklets summarises the key ARRAINA outputs and provide "user-friendly resources" for understanding specific feed ingredients in aquaculture, and to help raise awareness of the very latest findings in the sector. Going beyond nutrient composition of feedstuffs, this booklet provided details on the functional properties, the effects on feed processing conditions, and the physical quality of feed pellets.

The second booklet in the series focused on specific nutrient requirements of Atlantic salmon, rainbow trout, carp, sea bass and sea bream, when fed diets low in FM and FO to raise healthy fish during various growth stages.

The third booklet dealt with biomarkers of interest to use as pertinent criteria to evaluate the effects of nutrients as well as to link them to specific biological processes. This is also complemented by an online database of key biomarkers for several fish species, providing information about nutrient requirements and changes related to specific biological/metabolic processes. This also introduces integrated food safety measurement tools to predict the effects of alternative aquaculture feeds on farmed fish.

General information on the project, publications and factsheets can be downloaded from the ARRAINA website, www.arraina.eu.

RAINBOW TROUT: LONG TERM FEEDING WITH LOW FM AND FO FEEDS INCLUDING BROODSTOCK

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Although it is possible today to replace a large amount of fish meal (FM) and/or fish oil (FO) by terrestrial plant products in alternative aquaculture feeds for several species, it is recognized that there is still a lack of information regarding the effects of such dietary changes throughout the whole life cycle of fish. This study aimed to determine the long-term effects of FM/FO free feeds from first feeding onwards, with specific focus at key points along development (juveniles, ongrowing, large size, broodstock).

Rainbow trout alevins (IBW: 0.14 g) from the same cohort were fed from the first feeding three different diets : a marine diet (M) based on FM and FO, a commercial-like diet (C) with an equal mixture of marine ingredients (FM-FO) and plant products, and a diet containing only plant-products (V). Fish were fed by hand until apparent satiation (gradual decrease of daily meal number: 8 at first feeding to twice from 3 months onwards). Fish were reared at 7ºC until 6 months (juveniles), when they were transferred to another experimental fish farm at 18ºC for continuing the feeding trials until fish reach 1.5kg (large size, 26 months feeding). After that, between 20 and 25 females from each group were selected and transferred to the other fish farm at 7ºC until reproduction (8 additional months feeding).

Survival was significantly lower with the dietº V from first feeding until the 10th week of the trial (cumulative rate 28%) then stopped. During the first 3 months of the trial the growth rate of fish fed the diet V was lower than that of the other 2 groups, mainly due to a lower feed efficiency (-10%), and then increased. Consequently after 7 months of feeding at 7ºC, body weight and daily growth index of juveniles were not significantly different between groups.

When fish were transferred at 18ºC, we observed a reduction in growth performance with diet V compared to the other 2 groups and after 13 months feeding, mean body weight was 30% lower (234 g vs ~330g). Feed efficiency was slightly lower in this group (-10%) even not significant. There was no difference in survival among the groups (96 vs 98%).

These differences in growth performance still remained in ‚Äúlarge trout‚Äù after 26 months feeding with a mean body weight of 1.5kg for fish fed diets M and C and 1.1kg for fish fed diet V.

As regards to whole body composition, in juveniles a decrease in dry matter and an increase in lipid content was noticed in fish fed the V diet whereas in ongrowing fish (13 months feeding) no significant differences in body composition were observed between the groups with around 15% lipids. In large size fish, lipid content was higher in trout fed the totally plant based diet (17% vs 13% in the M group).

Fatty acid profile of body lipids reflected that of the diets. However our results showed that rainbow trout was able to synthesize eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids from dietary 18C precursor. In trout fed diet V although dietary intake of LC-PUFA n-3 was null, around 0.9% EPA and 2% DHA were recovered in body lipid of fish at all developmental stages studied. Similar changes were also observed in muscle; but the proportions of EPA and DHA in fish fed the V diet were higher in muscle lipids (around 1.2% EPA & 3.8% DHA) than in whole body. The incorporation of plant ingredients led to a decrease in lipid peroxidation products in muscle, in agreement with the lower proportion of LC-PUFA and the lower FA unsaturation index.

Metabolic consequences of plant-based diets were assessed in liver and intestine of juveniles by a transcriptomic approach and by RT-qPCR in ongrowing fish (13 months feeding). Transcriptome analyses on intestine showed a down-regulation of genes involved in protein catabolism, carbohydrate metabolism and transport in fish fed the plant-based diets (C and V compared to M). These results were not fully confirmed on some selected genes in ongrowing fish. In the liver, the main diet-induced effects observed at 7 months involved genes of energy pathway and lipid/cholesterol metabolism. The enhanced expression of genes involved in cholesterol biosynthesis found in fish fed the C and V-diet was confirmed by RT-qPCR at both stages. In liver and intestine, n-3 LCPUFA biosynthesis pathway was globally up-regulated in fish fed the plant-based diets, in both juveniles and ongrowing fish.

Reproductive performances were followed individually on 6 females from each group at the end of the trial (34 months feeding total). Mean body weight of females fed the V diet was 20% and 30% lower than that of C and M group respectively. Gonadosomatic index was also lower in these females compared to the other 2 groups (M=C>V). Average ovum and larvae weight were lower in V and C-fed females compared to the M-fed females (15 and 13% reduction respectively). Survival rates at eyed stage and hatching were strongly reduced in the V group (70%) compared to M and C groups (90%).

Fatty acid composition of the eggs reflected that of the diets. However substantial amounts of EPA, DHA and ARA were recovered in ova from females fed the V diet, confirming the ability of trout to synthetize LC PUFA and to incorporate them into ova.

MAIN CONCLUSIONS

This long term trial showed for the first time that rainbow trout reared on a plant-based diet completely devoid of marine ingredients throughout the entire life cycle (from 1st feeding until reproduction) was able to grow and to produce viable ova and fry, even growth and reproductive performance were lower.

Rainbow trout was able to synthetize n-3 LC-PUFA and cholesterol when fed a totally plant-based diet, however the up-regulation of these pathways is not sufficient to counteract the absence of n-3 LC-PUFA and cholesterol inputs as shown by the modifications of tissue FA profiles and plasma cholesterol levels.

The information gained through the long term trial conducted on rainbow trout opens up avenues for further reduction of marine ingredients in trout diets including the whole rearing cycle. However to optimise growth, reproductive performance and flesh composition finer adjustments of feed formulations are still required.

ACKNOWLEDGMENTS

This work was funded by EU (ARRAINA, FP7-KBBE-2011-5-288925) and by the “Région Aquitaine”.

SEA BASS: IMPACTS OF LOW FM AND FO FEEDS ON HEALTH AND GROWTH PERFORMANCE

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The possibility of feeding European sea bass (Dicentrarchus labrax) with feeds based on plant-based ingredients replacing dietary fish meal (FM) or fish oil (FO) has been investigated by several authors. Replacement of about 80% of FM by a single protein source led to a reduction in fish weight gain and feed efficiency. Nevertheless, it is possible to have an almost total replacement of FM (up to 5%) by a blend of different plant protein sources when diets are adequately supplemented with essential nutrients and relatively high levels (15-20%) of FO. As regards FO, replacing 60% of the dietary FO by either a single or a mixture of VO did not adversely affect fish growth or tissue lipid content after 3 months of feeding, if a minimum content of essential fatty acids was provided in the diet containing about 35% of FM. Nevertheless, longer feeding periods of 8 months with 60% FO replacement by rapeseed oil or larger FO replacement levels (80% linseed oil) significantly reduced final body weight. However, the effects of a combined reduction of FM and FO down to very low inclusion levels have been little studied in European sea bass. Low FM / FO feeds can be deficient in n-3 long chain polyunsaturated fatty acids (LCPUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), essential for marine fish which have a reduced ability to synthetize LC-PUFAs from their C18 precursors present in plant oils. These n-3 LC-PUFAs are mostly produced in the lowest marine trophic levels, and are practically absent in terrestrial environments. Consequently, complete replacement of FO by vegetable oils will also necessitate appropriate supplementations with these essential fatty acids, such as those produced from microalgae.

The aim of this study was to evaluate the combination of maximum replacement of FM and FO by alternative meals (AM) and oils (AO) to develop nutritious, but more ecofriendly diets for European sea bass juveniles. European sea bass of 9.8±1.5 g and 9.1±0.5 cm were fed nine isonitrogenous (45% crude protein) and isolipidic (21%) diets containing gradually reduced levels of FM and FO as follows (%FM/%FO): 58/15, 20/6, 20/3, 10/6, 10/3, 5/6, 5/3 and 0/0. Another diet, 0/0+, was similar to the 0/0 but supplemented with LC-PUFA from microbial and marine sources. Diets were fed for 552 days.

After only 90 days of feeding, European sea bass fed the 0/0 diet showed reduced ($P<0.05$) final weight, final length, condition factor, perivisceral fat index and viscero-somatic index as well as feed efficiency, whereas addition of LC-PUFA significantly improved daily growth index (DGI) (0/0+ diet). Reduction of FM/FO down to 5/3 did not significantly affect fish survival or condition factor, whereas reduction to 5/6 did neither affect sea bass length or feed conversion and reduction down to 10/3 did not affected body weight, DGI and perivisceral fat or visceral weight. FM reductions down to 5% significantly reduced growth rate. FO substitution by VO led to reduced growth and increased liver to body weight ratios, liver lipid deposition and hepatocellular size. In liver and muscle, FO substitution increased n-6 fatty acids (FA) and n-6/n-3 ratio and reduced saturated FA and n-3 LCPUFA, whereas monounsaturated FA were not increased in proportion to dietary contents. Moreover, FO reduction increased the products from Δ 6 desaturase and elongases, Elovl6 and Elovl5.

After 90 days of feeding, fish were subjected to either an in vivo exposure to a sub-lethal dose of V. anguillarum via anal inoculation to evaluate GALT immune response to bacterial infection or to an ex vivo exposure to V. anguillarum, in order to assess the capacity of the pathogen to cross the intestinal epithelium. Additionally, inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNFα) gut pattern of immunopositivity was studied by immunohistochemistry.

Immunoreactivity to anti-iNOS and anti-TNFα was stronger in basal than on the apical fold region, with a gradient of increased immunopositivity towards the anus. Strong positive anti-TNFα isolated enterocytes were observed in the anterior gut in relation to low levels of dietary FM/FO and TNFα was strongly immunolabelled in the rectum apical epitelial cells compared to the rest of the intestinal segments, regardless of the dietary treatment. Submucosa and lamina propria immunoreactivity grade was related to the amount of leucocyte populations infiltrated and goblet cells presented immunopositivity to anti-iNOS but not to anti-TNFα.

European sea bass fed a diet with 3%FO exhibited higher (P<0.05) ex vivo gut bacterial translocation than those fed a diet with 6%FO, irrespective of the level of dietary FM (1). We observed a similar trend during the in vivo challenge test, however in this case, fish fed 5%FM diets also had lower mortality rates than fish fed 20%FM diets. At the end of the feeding trial, low dietary FM levels led to an up-regulation (P<0.05) of interleukin-1β (IL-1β) and TNFα, major histocompatibility complex-II (MHCII) and cyclooxygenase-2 (COX2) in the posterior gut. However, 24h post infection, fish fed 5%FM diets exhibited a lower GALT response tan fish fed 20%FM diets. At 48h post infection, European sea bass fed low dietary FM/FO diets showed an up-regulation of major histocompatibility complex-I (MHCI) and T-lymphocytes CD4+ and CD8+ gene expression related to high infection rates. After 7 days of challenge, the results obtained point to a reduced capacity to recover gut homeostasis in European sea bass fed low dietary FM levels.

Increase in VM and VO markedly affected anterior and posterior gut morphology and fatty acid composition,which mirrored the dietary fatty acid profile. FO replacement by VO increased lipid deposition in anterior gut lamina propria, denoting an effect in lipid metabolismand lipoprotein synthesis (2). In posterior gut, FM replacements by TM induced an engrossment of the submucosa, an up-regulation of the proinflammatory cytokines IL-1β and TNFα genes, a relatively high mucus production and changes in mucosal microbiota profiles. Finally, supplementation of 0%FM-0%FO diets with LC-PUFA contributed to maintain gut homeostasis in terms of GALT response and microbiota profiles alterations.

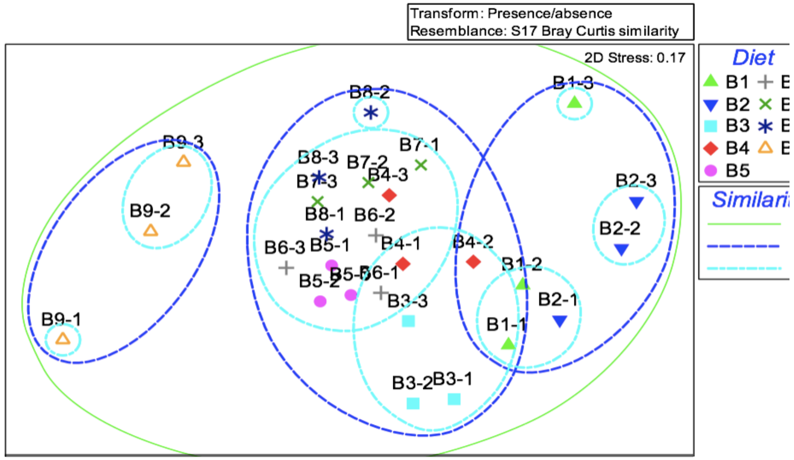

Figure 1. *Principal component analysis of microbiota present in posterior gut of seabass fed different FM and FO levels.*

In conclusion, it is possible to reduce dietary FM and FO levels down to 10% and 3% in mostly plant-based diets without affecting growth performance of European sea bass. However, further reduction of FM down to 5 or 0% significantly reduced growth and upregulated expression of GALT related genes. Thus, FM replacements by alternative meals lead to an increased submucosa cellular GALT response in posterior gut, altering the cytokine microenvironment and modulations of the mucosa microbial species populations, together with a trend to higher mucus production. Besides, FO replacement by VO increased lipid deposition in the anterior intestinal lamina propria. Finally, supplementation of 0% FM/0%FO diets with LC-PUFA contributed to maintaining gut homeostasis by ameliorating the effects of total FM/FO replacement in terms of GALT response and microbiota profiles alterations.

Acknowledgments

This work was funded by EU (ARRAINA, FP7-KBBE-2011-5-288925) project. Acknowledge is done to the Coordinator, Dr. Kaushik, the partners and all the contributors to this work.

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SEA BREAM: IMPACTS OF LOW FM AND FO FEEDS ON HEALTH, GROWTH PERFORMANCE, AND SEX POPULATION RATIO

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The availability of wild fishery-derived raw materials is finite and the rapid and sustained growth rate of global aquaculture have forced the industry to explore alternative and more sustainable feed ingredients. Much attention has been focused on plant ingredients and there is now accumulating evidence for a large and combined replacement of FM and FO in a wide range of fish species, including Atlantic salmon, rainbow trout, Atlantic cod, turbot European sea bass and gilthead sea bream. However, the long-term metabolic consequences of feeding very low FM and FO diets (with less than 10% inclusion levels of raw marine ingredients) is still under debate.

The nutritional and quality characteristics of edible fish matter are important aspects to consider. Certainly, high levels of n-3 LC-PUFA are important quality factors in human foods, and farmers are facing increasing pressures to include high levels of EPA (20:5n-3) and DHA (22:6n-3) in the finishing diets of salmonids and freshwater fish, which do not have specific requirements for n-3 LC-PUFA. This is more evident in marine fish due to their limited capacity to elongate and desaturate C18 FAs into long chain C20 and C22 PUFAs. However, importantly, this metabolic constraint facilitates the multi-species predictive modelling of fillet FA composition year-round using a dummy regression approach with gilthead sea bream as the reference subgroup category. This tool is public accessible through an easy-to-use-web interface www.nutrigroup-iats.org/aquafat to guarantee a relatively high content of n-3 LC-PUFAs in marine fish meat, ensuring that the human health benefits of consuming farmed fish are retained.

Early growth studies have also proven that FO can be totally replaced by a blend of VOs in practical gilthead sea bream diets with a 35% inclusion level of FM. Alternatively, up to 65–70% of FO can be replaced in diets with a 15–20% inclusion level of FM without detrimental effects or changes in growth performance (1) or FA composition of phospholipids (2), which are highly regulated to preserve cell integrity and function. However, to further proceed with low FM inclusion levels, fish feeds should be adequately fortified or supplemented with essential nutrients.

In the ARRAINA project, four plant protein-based diets were formulated and delivered by BioMar. FM was added at 25% in the control diet (D1) and at 5% in the other three diets (D2-D4). Added oil was either FO (D1 control diet) or a blend of vegetable oils (VO) (rapeseed oil: palm oil, 1/1), replacing the 58% (D2) and the 84% (D3-D4 diets) of FO. A commercial sodium butyrate preparation (NOREL, BP70) was added to the D4 diet at 0.4%. The nutritional composition of diets D2-4, including amino acids, phosphorus, phospholipids and cholesterol, was balanced to the control diet. EPA (20:5n-3) plus DHA (22:6n-3) content on dry matter basis was 2.9% (D1), 1.4% (D2) and 0.6-0.7% (D3, D4).

All fish in the trial grew fast from early life stages to completion of sexual maturation with overall SGRs of 1.4%, 0.7% and 0.51% for fish harvest at 300g, 1kg and 1.5-1.7kg, respectively. Fish also grew efficiently with an overall feed efficiency that decreased progressively from 1 in 300 g fish to 0.8-0.7 in 1-1.7kg fish. No signs of histopathological damage were found in any experimental group through all the life cycle despite of some transient increases of hepatosomatic indices in fish fed the lowest FM/FO diet (D3).

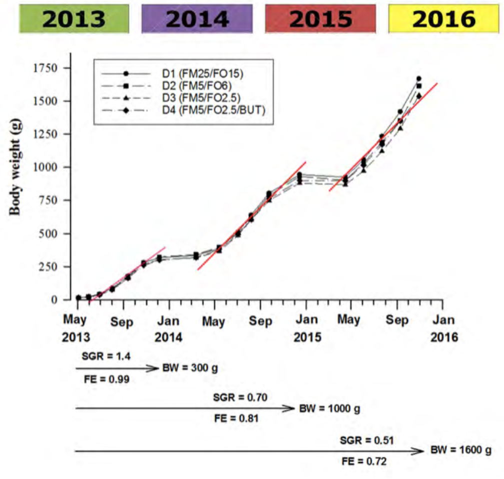

Figure 1. *Growth performance of gilthead sea bream fed ARRAINA diets from early life stages to completion of sexual maturation.*

Data on haematology, blood biochemistry and gene expression profiling of liver and skeletal muscle also support a very high replacement of FM and FO (3) However, extreme diet formulations (less than 10% of marine feedstuffs) might require a short adaptive period to avoid initial and transient detrimental effects on growth performance. Apparently, butyrate supplementation did not alter this time course, although it was able to restore the normal circulating concentration of haemoglobin and cholesterol levels. Other butyrate-mediated effects included the increase of plasma levels of choline and IGF-I over the course of the summer growth spurt. At the same time, butyrate supplementation was able to reverse the increased expression of inflammatory cytokines and muscle markers of cellular morphogenesis and protein breakdown in fish fed the extreme FM/FO diet.

In three-year old fish, a clear sexual dimorphism was found for body weight and hepatosomatic index when all sampled fish were considered as a whole regardless of nutritional background. A sexual dimorphism was also found for sexual steroids with a peak of estradiol in the females sampled in October-December, whereas the peak of 11-ketosterone was delayed in males to December. Plasma levels of testosterone were similar in both sexes. The two first components of principal component analysis (PCA) explained more than 90% of total variance of plasma levels of sexual steroids. The displacement along X-axis clearly separated males and females, whereas the movement along Y-axis was related to sampling time. Multivariate analysis also evidenced an androgenic effect in the steroid plasma profile of fish fed low fish FM/FO diets, which was especially evident for the extreme diet formulation (D3). However, this effect was apparently reversed by butyrate supplementation and the female/male ratio of D4 fish did not differ from that of control fish, whereas the proportion of females in D2+D3 fish was higher (P<0.06) than in control fish (64% vs 50%).

Altogether suggests the alteration of the biological reproductive clock of fish fed low FM/FO diets. Gilthead sea bream is a protandric hermaphroditic fish were most individuals reverse to the female sex at the age of two years through development and maturation of the ovarian portion of the gonad and regression of the testicular area. However, in contrast to fish exhibiting a true sequential hermaphroditism (complete replacement of the gonad of one sex by the other), the two types of gonads are adjoined and form an ovotestis in gilthead sea bream, where the male sex reversal is a highly-regulated process dependent not only of individual determinism, but also of environmental, social and nutritional conditions. The ultimate mechanisms require further studies, because our results indicate that vegetable diets (D2, D3) exerted an androgenic rather than estrogenic effect, which was apparently reversed by butyrate supplementation.

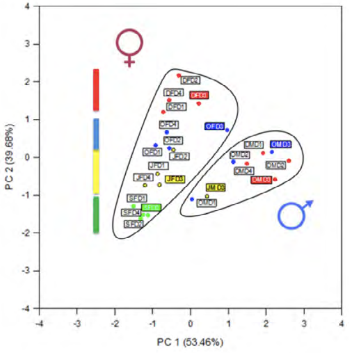

Figure 2. *Multivariate analysis of sexual sex steroids in three year-old fish from September 2105 to January 2016.*

Other drawbacks effects of extremely low FM/FO diets are related to intestinal health. This is first exemplified by changes in the gut transcriptome and intestinal epithelia integrity assessed by means of a pathway-focused PCR-array and electrophysiology, respectively (4). This is confirmed by integrative omic approaches that revealed changes in the composition of intestinal mucus proteome highly related to a reduced microbiota biodiversity, followed by a fast progression of disease outcomes in fish challenged with the myxosporean parasite Enteromyxum leei (see the communication of Estensoro et al., in this course).

Main conclusions

The replacement of marine feed ingredients is highly feasible in terms of growth performance in gilthead sea bream from early life to completion of sexual maturation. This assumption is supported by data on histopathological, biochemical and molecular biomarkers.

Data on key performance indicators from fish fed ARRAINA diets are reliable reference values (gold standard) for farmed sea bream.

Population sex ratio is nutritionally regulated in the protandric hermaphroditic sea bream, displaying fish fed low FM/FO diets a lowered female signal that seems to be restored by dietary butyrate.

The drawback effects of extreme diet formulations on gut transcriptome, mucus proteome, epithelia integrity, microbiota diversity & progression of disease outcomes are reverted by dietary butyrate.

Acknowledgments

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LARVAL NUTRITION AND PROGRAMMING IN MARINE FISH

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Nutrition during peri-conception and early development can modulate metabolic routes to prepare the offspring for adverse conditions. This phenomenon, known as nutritional programming deserves more attention in fish production. Environmental influences (including diet and feeding conditions) experienced during early development are increasingly thought to modify mammalian phenotype, in ways that are predictive of future conditions during adulthood. Therefore, nutritional programming is a nutritional experience at certain critical periods in life that can programme an organism development, metabolism and health for the future. Epigenetic mechanisms may be involved in nutritional programming. Epigenetics is the study of potentially heritable changes in gene expression that does not involve changes in the DNA sequence, thus it implies changes in the phenotype without changes in the genotype. Using seahorses as a model, we have found that in fish n-3 long-chain polyunsaturated fatty acids (n-3-LC-PUFA) in diets for males during gametogenesis, affects offspring fatty acid composition, growth, morphology and feeding (1, 2).

The complete removal of fishmeal (FM) and fish oil (FO) is required to promote the sustainable development of aquaculture and for that, fast growing high quality fish that are fed without FM and FO are necessary. Early nutritional programming may allow the production of fish better adapted to utilize diets with vegetable meals (VM) and oils (VO). A series of studies have been conducted feeding gilthead sea bream (Sparus aurata) broodstock with diets containing four different replacement levels of FO by linseed oil (LO) and their effect on fecundity and spawn quality, egg composition, Δ-6-desaturase (Δ6D) gene expression, progeny growth performance and their growth response to a challenge with diets low in FO and FM, but high in VO and VM were studied. The results showed that feeding gilthead sea bream broodstock with high LO diets had very long-term effects on the offspring (3). Thus, FO replacement by LO up to 80–100% in broodstock diets for gilthead sea bream not only reduced fecundity and spawn quality, but also growth of 45 dah and 4-month-old juveniles, as well as Δ6D gene expression. However, when the 4 month-old juveniles were fed with a low FM and FO diet, even those from broodstock fed only 60% replacement of FO by LO showed a higher growth and feed utilization than juveniles from parents fed FO. These results demonstrate the interesting potential of early nutritional programming of marine fish by broodstock feeding to improve long-term performance of the progeny. Further studies are being conducted to determine optimum nutrient levels in the broodstock diets and the molecular mechanisms implied to develop effective nutritional intervention strategies for this species.

Moreover, 16-month-old juveniles obtained from broodstock fed different FO/LO diets and fed at 4 months either a high or low FO/FM diet were challenged with a low FM FO diet for 2 months (4). The results showed that when 4-month-old juveniles had been challenged with a (low FM/FO diet) vegetable meal and oil based diet, at 18 months-old offspring growth was higher than for fish whose parents had been fed only FO, suggesting that the nutritional challenge at 4 months may acted as a "reminder" effect added of the parental programming. Thus, fish coming from parents fed intermediate levels of LO grew significantly better when fed low FM/FO diets and showed better FCR. No significant effect was found on proximate composition, whereas both parental feeding and "reminder" diet had a significant effect on liver fatty acid composition, suggesting the modulation of the lipid metabolism in the liver. Molecular studies denoted that LO inclusion in broodstock diets down-regulated lpl, elovl6, and cpt1 in the liver of 18 months-old gilthead sea bream. Besides a positive correlation was obtained between ppar-alpha and cox2 expression in fish liver. The results of this study denote that nutritional programing though broodstock-feeding affects liver metabolism for a long-term. Besides, feeding a "reminder" diet during juvenile stages in combination with broodstock feeding persistently improved low FM/FO diets utilization, growth and gene expression along the fish life-cycle.

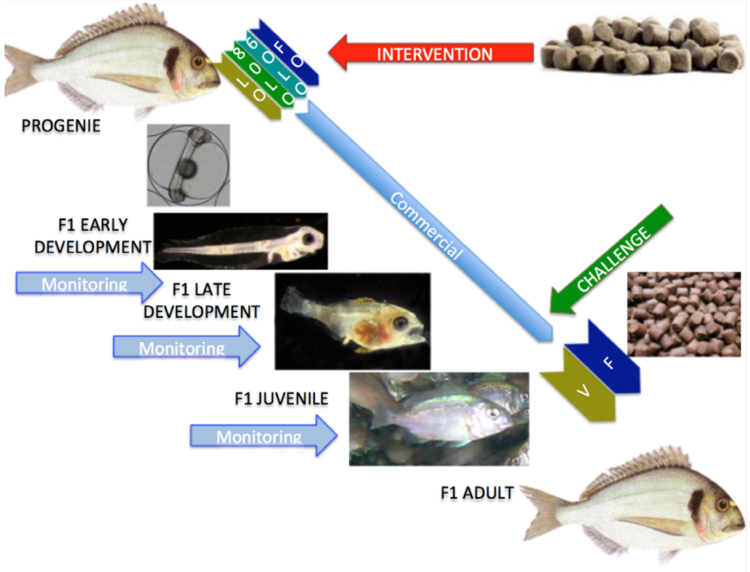

Figure 1. *Standard design for a nutritional programing study through parental diet: Dietary intervention with parental diets with different FO/LO ratios; feeding the offspring a commercial protocol during larval development and juvenile stages and challenging the fish with a low FM/FO diet for a short period to determine if the broodstock diet had an effect on the ability of the fish to use low FM/FO diets.*

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IMPACT OF PARASITE INFECTIONS IN SEA BREAM AND SEA BASS. THE PARAFISHCONTROL APPROACH

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Aquaculture remains one of the fastest-growing food-producing sectors and now provides almost half of all fish for human food. This share is expected to rise to 62% by 2030 (1). However, pathogens are among the main limiting factors of aquaculture as they can produce financial losses estimated to be about 20% of the total production value. Among them, it is estimated that the world annual grow-out loss due to parasites in finfish farming ranges from 1% to 10% of harvest size, with an annual cost that can reach up to $9.58 billion (2).Therefore the integration of different mitigating, preventive and management solutions is essential for the future increase of the aquaculture industry. This is what the European H2020 project ParaFishControl is trying to achieve, by improving understanding of fish-parasite interactions and by developing innovative solutions and tools for the prevention, control and mitigation of the major parasites affecting Atlantic salmon, rainbow trout, common carp, European sea bass (ESB), gilthead sea bream (GSB) and turbot (www.parafishcontrol.eu). The current situation for the parasitic diseases in the Mediterranean species will be described.

GSB and ESB culture represents today 98% of the total farming production in the Mediterranean region. The massive establishment of net cages has contributed to the tonnage increase, but it is also responsible in part for the extension and dispersal of parasitic diseases. Net cages offer favourable conditions for parasite dispersal: 1) non-controlled water, 2) introduction of fish from other geographical areas, 3) access to invertebrate intermediate hosts, 4) contact with wild fish (acting as natural or reservoir hosts), 5) a good surface for biofouling, and therefore for the settlement and attachment of parasitic stages, and 6) non-controlled fish effluents (mucus casts, faeces, dead animals, escapees) which allow parasite life cycles keep going.

Parasites inflict not only direct mortalities, but also morbidity, poor growth (due to lower feed intake and/or lower feed conversion rate), parasitic castration, higher susceptibility to opportunistic pathogens, poor marketability, and lower resistance to stressful conditions, such as size sorting, transport, vaccination, stormy weather, low dissolved oxygen, etc.

Monogeneans and crustaceans are among the most prevalent ectoparasites in sea cages, whereas flagellates and ciliates are responsible for disease outbreaks mainly in earth ponds and recirculating systems. **Table 1** gathers the most relevant ones. All of them have direct life cycles, and therefore are easily transmitted from fish to fish, especially under high density farming conditions. The most threatening ectoparasite for GSB farming is the gill fluke Sparicotyle chrysophrii, which is widely distributed along Mediterranean waters. It produces mortalities, lethargy, emaciation, severe anaemia and histopathological gill damage. It is favoured by high densities and net biofouling, as eggs can attach easily to nets. In ESB cage farming, the isopod Ceratothoa oestroides is the most serious pathogen, though with an uneven distribution in the Mediterranean basin.

Group/Species	Host site	Host
MONOGENEA		
Diplectanum aequans	Gills	ESB
Furnestinia echeneis	Gills	GSB
*Sparicotyle chrysophrii**	Gills	GSB
CRUSTACEA		
Anylocra physodes	Gills, skin	ESB, GSB
*Caligus minimus**	Oral cavity, skin	ESB, GSB
*Ceratothoa oestroides**	Oral cavity	ESB, GSB
*Lernanthropus kroyeri**	Gills	ESB
Nerocila orbingnyi	Gills, skin	ESB
PROTOZOA		
*Amyloodinium spp.**	Skin, gills	ESB, GSB
Brooklynella hostilis	Gills	ESB, GSB
Cryptobia spp.	Gills	ESB, GSB
Cryptocaryon irritans	Gills	GSB
Neoparamoeba spp.	Gills	ESB, GSB
Trichodina spp.	Gills	ESB, GSB

***Table 1**. Main ectoparasites found in farmed gilthead sea bream and European sea bass. * indicates species included in the ParaFishControl project.*

Table 2 gathers the most prevalent endoparasites, being myxozoans and coccidians the most relevant. The gastrointestinal tract is the target host site with the highest biodiversity of parasites, followed by the urinary system, although some species are systemic and can invade all organs. The life cycle is unknown for most of them. In GSB, the most threatening endoparasite is the myxozoan Enteromyxum leei (3). It has been found in many countries of the Mediterranean coast, Red sea and Japanese waters. It has been described to infect up to 60 different fish species, though with different degrees of pathogenicity. It causes anorexia, emaciation and caquexia and finally death.

In the last years, some parasites have up welled as new emerging diseases, which in some cases are real threats for the cultures. This is the case of the microsporean Enterospora nucleophila in GSB, which produces great growth impairment in juveniles and trickling mortalities (4).

The fight against all these parasites has two main bottlenecks: 1) the scarcity of antiparasitic preparations registered for use in Mediterranean fish, including the difficulty of the application of some ectoparasitic treatments for high amounts of fish and the lack of commercial treatments for endoparasites; 2) the lack of commercial vaccines, though much effort has been done in the last years to understand the fish immune response against some of these parasites (5). Strategies based on the exploitation of the fish immune system are starting to be used, such as the use of immunostimulators included in aquafeeds, but their efficacy against parasites has not been fully determined.

MAIN CONCLUSIONS

Modern intensive aquaculture procedures (both in recirculating systems and sea cages) and fish stock movements in the global market favour parasite dispersal. The scarcity of antiparasitic preparations and the lack of commercial vaccines are the main refrains for the control of these parasitic infections. The future improvement of these problems needs the application of integrated pest management strategies (IPMS), which gather the knowledge of the host-parasite system, prevention, monitoring, intervention and finally evaluation and planning (6).

Group/Species	Host site	Host
DIGENEA		
Cardicola aurata	Gills	GSB
APICOMPLEXA		
Cryptosporidium molnari	Stomach	GSB, ESB[1]
Eimeria dicentrarchi	Intestine	ESB
Eimeria sparis	Intestine	GSB
Goussia sparis	Intestine	GSB
MICROSPORIDIA		
Glugea sp.	Muscle	GSB
*Enterospora nucleophila**	Intestine	GSB
Pleistophora sp.	Muscle	GSB
MYXOZOA		
Ceratomyxa labracis	G. bladder	ESB
Ceratomyxa diplodae	G. bladder	GSB
Ceratomyxa sparusaurati	G. bladder	GSB
*Enteromyxum leei**	Intestine	GSB, ESB[1]
Kudoa sp.	Muscle	GSB
Kudoa iwatai	Systemic	GSB
Leptotheca sparidarum	Trunk kidney	GSB
Ortholinea auratae	U. bladder	GSB
Sphaerospora dicentrarchi	Systemic	ESB
Sphaerospora (ex. Polysporoplasma) sparis	Trunk kidney	GSB
Sphaerospora testicularis	Testis	ESB

***Table 2.** Main endoparasites found in farmed gilthead sea bream and European sea bass. * indicates species included in the ParaFishControl project.1 Minor pathogenic effect for this fish species.*

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NUTRITIONALLY-MEDIATED EFFECTS ON BACTERIA AND PARASITE INFECTIONS IN GILTHEAD SEA BREAM

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The expanding aquaculture industry has been studying the use of alternative feeding strategies for reducing the costs of fish feeds. Among these strategies, the substitution of fish meal (FM) and fish oil (FO) with vegetable ingredients is one of the most promising. However, low FM and FO inclusion levels were associated to intestinal inflammation and poor growth and survival. Studies on gilthead sea bream demonstrated that FO could be replaced in plant-protein based diets up to 66% (66VO) without detrimental effects on growth, immunocompetence or intestinal and hepatic architecture. In addition, supplementation with sodium butyrate showed promising results reversing potential detrimental effects of extreme vegetable diet formulations. With such background, the next step was to test whether plant proteins and VO optimized diets, with or without butyrate supplementation, could affect the immune system and disease outcome when animals face a pathogenic threat.

Enteromyxum leei is among the most important pathogens that affect gilthead sea bream farming. It is a widely spread myxosporean parasite that invades slowly and progressively the intestine of the host producing signs like anorexia, cachexia and eventually death. This disease can be fish-to-fish transmitted by cohabitation with infected animals or exposure to contaminated water effluents and there are no preventive or curative treatments. The use of such a parasitic model, intestinal and slow progressing, is an optimal model to test whether different diets can affect the intestinal health of the animals, especially when no detrimental changes are observed in non-pathogenic conditions. In fact, when fish fed 66VO diets for a long term were infected with E. leei, the progression of the infection and the severity of the disease signs were higher. 66VO fed fish showed lower feed intake, growth, condition factor and hematocrit and lower serum levels of the antimicrobial molecules nitric oxide and lysozyme (1). These results manifested that VO replacement is a predisposing cause that worsens the disease outcome when fish face a parasitic threat and further research was conducted to unravel the cause.

Mucins are large, filamentous, highly glycosylated glycoproteins secreted by goblet cells or membrane bound to the epithelium. They are one of the first immune defense barriers that an intestinal pathogen encounters. The 66VO diet produced a significant decrease in goblet cells with neutral and acidic mucins in anterior and middle intestinal segments, which correlated with higher prevalence and intensity of infection when fish were exposed to the parasite (2). A different study measuring mucin gene expression in different intestinal segments showed that parasitic infection induced a general down-regulation of all intestinal mucins, regardless of the diet. However, the expression of I-Muc, a novel mucin characterized in gilthead sea bream only expressed in the posterior segment of the intestine, was significantly down-regulated with the 66VO diet and its expression was even lower upon infection (3).

A very important factor in the immune response against pathogens are immunoglobulins (Ig), produced and secreted by B cells. In fish intestines, two Ig isotypes were described to be important in the defense against pathogens: the highly expressed IgM, more involved in systemic responses, and the fish specific IgT, related to mucosal responses. Plant based diets did not have any effect on B cell numbers or Ig gene expression under normal conditions. However, upon parasitic infection the number of IgM+ cells and IgM gene expression was much higher in the posterior intestines of 66VO fed fish (4). Interestingly, these diets induced a complete inhibition of the IgT up-regulation normally induced by the parasite (5). These results can be interpreted as an attempt to compensate the lack of IgT by increasing IgM levels. Nonetheless, it is clear that this is not enough to overcome the worse disease outcome in 66VO fed fish.

Clearly, the differences in disease susceptibility are induced by changes in multiple factors. Thus, more massive approaches were performed to characterize the effect of vegetable ingredients on gilthead sea bream intestinal health. A customized microarray of 7,500 genes showed no differences in the transcriptome of healthy fish fed FO or 66VO diets. However, upon E. leei infection 66VO fed fish showed strong up-regulation of immune related genes and down-regulation of complement and xenobiotic metabolism genes (6).

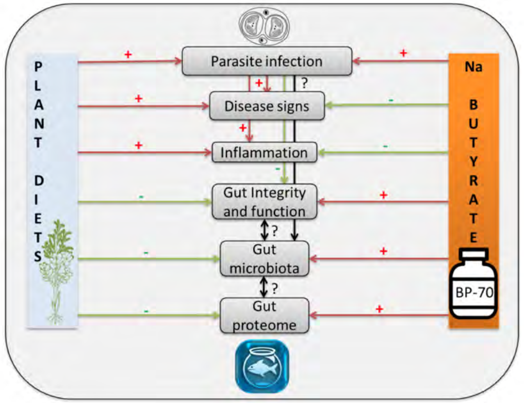

Figure 1. *Main results obtained when feeding gilthead sea bream with extreme vegetable diets or supplementing them with sodium butyrate. Red arrows (+) indicate increasing effect and green ones (-) decreasing effect. Black arrows (?) indicate possible relationship to be further explored in future studies.*

Aiming to compensate the drawbacks of these vegetable diets, the addition of sodium butyrate was considered and tested. The effect of plant based diets with even higher levels of substitution (up to 84%), with or without butyrate supplementation, was assessed on gilthead sea bream intestinal transcriptome, proteome and microbiome.

Transcriptomic profiling showed that extreme substitution with plant ingredients induced important changes in the anterior intestine, up-regulating the expression of inflammatory markers, in coincidence with a higher presence of granulocytes and lymphocytes in the submucosa, decreased trans-epithelial electrical resistance and dysregulation of genes involved in antioxidant defenses, epithelial permeability and mucus production. Interestingly, most of these effects returned to control values with butyrate supplementation (7). The anterior intestine mucus proteome was also affected. Proteins related to digestion, transport, cell signaling and cellular morphology were regulated by highly substituted diets and returned to control levels upon butyrate supplementation. Plant-based diets also induced a shift in the intestinal microbiota composition involving a decrease in microbial diversity and a significant increase of the genus Photobacterium. Butyrate supplementation reversed these effects by increasing the diversity even more than in the control fish (8).

As expected, high inclusion levels of plant ingredients induced a proinflammatory profile in fish intestines with some signs of epithelial damage and dysregulation of the normal microbiota, but butyrate supplementation helped reverse most of these changes. In fact, upon E. leei infection, fish fed butyrate-supplemented diets were able to cope better with the disease, and although still infected, they showed improved disease signs, even better than control diet fed fish (8). Butyrate also increased the survival upon bacterial challenge (8).

MAIN CONCLUSIONS

Plant based diets can show promising results in terms of growth and, depending on the level of inclusion, with no significant effects on the general health of the animals. However, under pathogenic conditions higher susceptibility can be found. This can be due to alterations in the mucin barrier and number of goblet cells as well as dysregulation of immunoglobulin production or immune gene expression, among other factors. Butyrate supplementation showed to be a promising strategy to overcome the drawbacks of plant-based diets in a carnivorous marine fish.

ACKNOWLEDGMENTS

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AQUAGENOMICS: CSD2007-00002; AGL2009-13282-C02-01; Mi2-Fish: AGL2013-48560-R; Prometeo 2010/006 and PrometeoII/2014/85; PIE project no. 201740E013; MINECO FPDI-2013-15741; APOSTD/2016/037 grant by the “Generalitat Valenciana”) projects.

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MODELS AND TOOLS FOR MEASURING ENVIRONMENTAL IMPACTS

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The current drive for sustainability in the aquaculture industry has pushed feed formulators towards a reduction in the use of fish meal, replacing it with alternative protein sources, often of plant vegetable origin. Besides the effect on protein digestibility and retention efficiency, the introduction of plant vegetable ingredients in feed formulations can have a major impact on phosphorus outputs to the environment, given the low digestibility of the major form of phosphorus present in plants (i.e. phytic acid), and also on nitrogen outputs as the plant protein tend to have amino acid imbalances. It is therefore of the utmost importance to assess and quantify the effect that these changes in feed formulations can have on the environmental impact of aquaculture activities.

Despite the popularity and usefulness of mass budget (or bioenergetic) approaches in these type of estimations, most implementations disregard the fact that the parameters used for estimation are affected by some level of uncertainty and simply use point estimates for each of the required parameters. Moreover, interactions between major driving factors for environmental impact such at feed intake, water temperature and proximal composition are difficult to assess without a major experimentation effort. Also marginal deficiencies in amino acids are often overlooked by such bioenergetics models. Furthermore, many of the people that would be interested in such estimations (e.g. fish farmers) might not have all the information that would normally be required to perform the calculations (e.g. N and P digestibility, fish whole body composition).

Here we introduce two tools that attempt to address these issues: 1) the WASTEst tool which allows the estimation of P, N and total waste output uncertainties based on (input) performance variability, combined with statistical models that provide plausible confidence intervals in the case of unavailable (optional) inputs; and 2) the FEEDNETICS dynamic simulation model, which predict the effect of changes in feed formulation, feeding and physical parameters (e.g. temperature) on downstream performance (e.g. growth, FCR, feed costs), environmental impact (e.g. total released nitrogen and phosphorus) and consumer-value parameters (e.g., fatty acid profile) of fish.

In WASTEst (http://www.sparos.pt/wastest), calculations are based on typical distributions for parameters such as protein digestibility, phosphorus digestibility and whole body compositions were obtained through Dirichlet regression of gilthead seabream, seabass, carp and salmon data present in the scientific literature and technical sheets of fish feeds. The tool itself consists of a web application with a front-end in PHP (that processes user input) and a back-end in R (that processes the data and generates plots).

In a practical hypothetical scenario where two feeds (“Low Cost” and “Premium”, both with 45% CP, 10% ash and 1,1% phosphorus) are compared against each other by WASTEst on the basis of their performance (i.e., fish initial weight, final weight and FCR) in a typical fish trial (3 tank replicates per feed), shows that, even when incomplete information is provided to the tool (in this particular case, no information was provided on whole body composition and apparent digestibility was only roughly estimated), it is still possible to perform some quantitative predictions in regards to their relative environmental impact. It can be seen in Figure 1, that there is a significant difference in terms of N waste (at a 5% level) for the two diets, given the lack of overlap of the respective 95% confidence intervals, with diet “Low Cost” (i.e. Scenario 1) displaying higher values than diet “Premium” (i.e. Scenario 2). It can also be seen that these observed differences cannot plausibly be explained simply in terms of digestibility differences, as diet “Low Cost” shows differences both at the level of fecal and metabolic N losses.

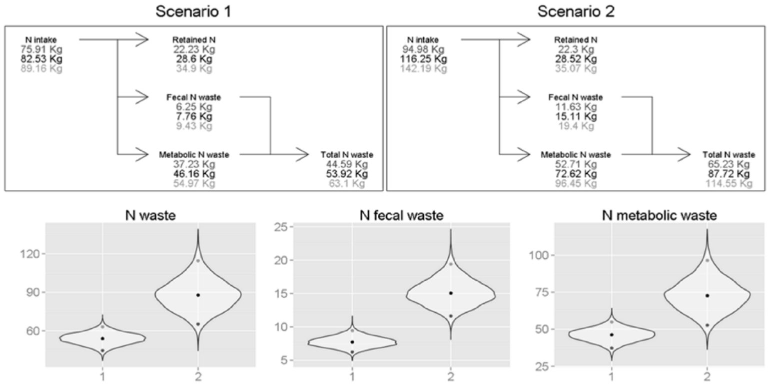

Figure 1. *On the top panel, a comparison of the N mass budget for the two scenarios; besides the typical value, an upper and lower bound of a 95% confidence interval is also provided. On the bottom panel, distribution plots of the same parameters. Point estimates (median) are depicted as a black dot, while probability densities are proportional to object width. As on the panel above, the 95% confidence interval is depicted for each output. All values are in “kg waste per metric ton of biomass produced”.*

The FEEDNETICS dynamic simulation model has been calibrated/parametrized to model the long-term behavior of gilthead seabream in an aquaculture context. Specifically, it consists of a quantitative mechanistic model of fish metabolism, where the complete set of metabolic fluxes is expressed as a system of differential equations. The model is implemented in the PowerSim Studio 10 software package, which runs under a user-friendly interface specifically developed for FEEDENTICS. This model can predict the interactive effect of changes in feed formulation, including marginal amino acid deficiencies, feeding level and water temperature on growth performance, FCR, and total released nitrogen and phosphorus.

As an example, three diet formulations were simulated in FEEDNETICS, and chosen to be realistic representations of: a fish meal based diet (FM); a diet where 60% of the fish meal is replaced by vegetable ingredients (PP60); and a diet where 100% of the fish meal is replaced by vegetable ingredients (PP100). The three diets were set to have similar composition (14% carbohydrates, 20% crude lipid and 50% crude protein) and apparent digestibility coefficients (30% for carbohydrates, 90% for crude lipid and 90% for crude protein), basically just differing in terms of amino acid profile. Common parameters, fish initial body weight (176 grams), trial duration (90 days), feeding regime (approximately 0.75% BW/day) and temperature (22 ºC) were set to be comparable to an experimental trial performed at CCMAR/University of Algarve (Portugal).

Simulations with FEEDNETICS showed reasonable values for growth rate and N gain (around 150 mg/kg/day). Specifically, the growth performance obtained for the diet with 60% replacement of fish meal by vegetable ingredients (around 80% of the FM growth performance) is on the same order as the results obtained in one of our fish trials (where a PP60 diet displayed around 85% of the FM growth performance). As expected, the most aggressive diet (PP100) showed an even greater degree of growth performance loss together higher N losses, showing how even small differences in amino acid profile can have a significant impact on growth performance and waste output. In terms of fish body composition, the model predicts that the levels of crude protein and fat are, for all three cases, within the expected range for gilthead seabream, with body energy density values also displaying very small differences between groups.

Main conclusions

The two tools here presented seem useful to assess and quantify the effect of changes in feed formulations on performance and environmental impact of aquaculture activities.

WASTEst provides waste output estimates based on incomplete data, but also by making the best of the data obtained from replicated experiments. It also gives reliable confidence intervals on estimations once more detailed data on nutrient digestibility are available. It is a valuable tool to compare environmental impacts of alternative feed formulations.

FEEDNETICS is able to generate a realistic prediction of concrete scenarios, including the interactions between feeding level, feed formulation and water temperature. In addition it accurately predicts performance differences due to dietary amino acid imbalances. It may be used to optimize feed formulations and feeding regimes.

Acknowledgments

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BIOMARKERS FOR FISH NUTRITION RELATED ISSUES

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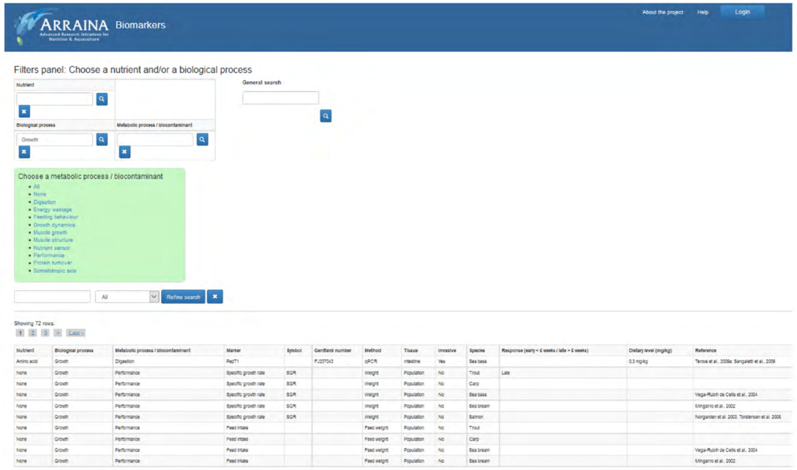
Biomarkers are defined as measurable biological characteristics that can be evaluated as indicators of biological conditions, such as growth, health, or any other productive trait. On the basis of the potential application of biomarkers in aquaculture, we can classify them in three categories: diagnostic (assessment about disturbances in a trait of interest), prognostic (prediction about if there will be any disturbance or not) and therapeutic (indicative of the restoration in a trait of interest after corrective actions). In order to assess the prediction, causes, diagnosis, progression or outcome of treatments in aquaculture practice, several tools and technologies are applied for key biomarkers determination. Ideal biomarkers should be easy to handle measurements taken in the less invasive manner, that showed a highly sensitive and specific response when used alone or in combination with other biomarkers, and within an affordable cost. In addition, they should be consistent across the fish developmental stage, season, reproductive and nutritional condition, genetic background, etc.

One of the main objectives of the EU FP7 ARRAINA Project has been to identify and validate biomarkers for key traits of interest in the main cultured fish species in Europe (Atlantic salmon, rainbow trout, common carp, European sea bass and gilthead sea bream). The discovery of new biomarkers has also been conducted, by means of untargeted approaches using transcriptomic, proteomic and metabolomic techniques. As a result, several sets of biomarkers have been identified by ARRAINA partners to assess nutrient requirements and to metabolically phenotype the nutritionally mediated effect of feeds on health and performance status in cultured fish species. These results, combined by other from bibliography, making a total of more than 800 independent entries, have been grouped for public consultation in a database web page (www.nutrigroup-iats.org/arraina-biomarkers), designed for single or combined searches of biomarkers providing information on nutrient requirements and specific biological processes or metabolic pathways, including those related to health and welfare, growth and reproductive performance, quality at harvest, safety, and stress responses (**Fig. 1**). This web tool, that undergoes continuous updating by interested partners, is intended to constitute a reference database for R+D professionals in the aquaculture sector in order to determine the most informative biomarker, detection methods and reference values for each trait and species of interest.



Biological process: Growth

Showing 72 rows.

Nutrient	Biological process	Metabolic process / biocontaminant	Marker	Symbol	GenBank number	Method	Tissue	Invasive	Species	Response (early < 6 weeks / late > 6 weeks)	Dietary level (mg/kg)	Reference
Amino acid	Growth	Digestion	PepT1		FJ237043	qPCR	Intestine	Yes	Sea bass		0.3 mg/kg	Terova et al., 2009a, Sangaletti et al., 2009
None	Growth	Performance	Specific growth rate	SGR		Weight	Population	No	Trout	Late		
None	Growth	Performance	Specific growth rate	SGR		Weight	Population	No	Carp			
None	Growth	Performance	Specific growth rate	SGR		Weight	Population	No	Sea bass			Vega-Rubin de Celis et al., 2004
None	Growth	Performance	Specific growth rate	SGR		Weight	Population	No	Sea bream			Mingarro et al., 2002
None	Growth	Performance	Specific growth rate	SGR		Weight	Population	No	Salmon			Norgarden et al. 2003, Torstensen et al. 2008
None	Growth	Performance	Feed intake			Feed weight	Population	No	Trout			
None	Growth	Performance	Feed intake			Feed weight	Population	No	Carp			
None	Growth	Performance	Feed intake			Feed weight	Population	No	Sea bream			Vega-Rubin de Celis et al., 2004
None	Growth	Performance	Feed intake			Feed weight	Population	No	Sea bream			Mingarro et al., 2002

Figure 1. *Front page of the ARRAINA biomarkers database with the output of a consultation and metabolic process options to refine the search.*

Many (almost 50%) of the biomarkers present in the ARRAINA database are reported to be routinely assessed by the determination of tissue gene expression. These omic techniques together with more conventional biometric and biochemical approaches contribute to define the normal range of variation for selected markers of fish performance. This molecular approach often requires the nucleotide sequences of the genes of interest, and some of them are usually not available in public repositories apart than for research model species like zebrafish or Fugu. This has been the reason of the construction of nucleotide databases with a high coverage of actively transcribed and protein-codifying RNAs for the two main species cultured in the Mediterranean area, the gilthead sea bream (1) and the European sea bass (2). This information is accessible through easy-to-use web interfaces hosted at www.nutrigroup-iats.org/seabreamdb and www.nutrigroup-iats.org/seabassdb that contain data from assembled, annotated and manually or semi-automatically curated sequences. These web tools offer the possibility for data requisition by several Blast options or direct word searches for annotated names or gene ontology terms. Both transcriptomes are the basis for the design of specific oligo-microarrays and targeted qPCR-array layouts, and can also serve as a reference for proteomic and RNAseq analysis.

The analytical power of the gilthead sea bream microarray is evidenced through the expression profiling of larvae sampled every 3 h through a 24 h period, revealing a pronounced circadian rhythm with more than 3000 unique genes differentially expressed Importantly, the two first components of PCA explained more than 80% of total variance that would drive three major steps: i) the anticipatory food response before the morning onset of lights, ii) the afternoon protein accretion and tissue damage repair, and iii) the night reset/stand-by of biological processes before the onset on a new rhythm of extremely fast grow and high feed intake.

At intestinal level, microarray gene expression revealed in both gilthead sea bream and European sea bass a highly regulated gut transcriptome from anterior to posterior intestine sections. Experimental evidence also indicated a large regulation of gilthead sea bream gut transcriptome in a seasonal basis, with changes in the gene expression profile of several thousands of genes, but regardless of season and feed intake ARRAINA feed formulations did not drive massive changes in the gut transcriptome of sea bream. This multi-species approach is key for the determination in each intestinal segment of nutrient transporters that could potentially serve as robust candidate markers of feed intake or feed efficiency.

Pathway-focused chip PCR arrays have also proven to be powerful tools for the assessment of transcriptomic profiling of lipid metabolic disorder in liver, adipose tissue and skeletal muscle during fasting and re-feeding in gilthead sea bream and European sea bass (3, 4). A "growth-chip" highlighted highly regulated molecular signatures that were specific of tissue and nutrient deficiencies in fish fed diets formulated for specific nutrient deficiencies. More gilthead sea bream PCR-array chips are now available for wide transcriptional profiling of the intestine (5) and mitochondria (6, 7).

Main conclusions

Although there is not a unique "magic biomarker" that gives by itself an entire screening of the fish performance status, the use of molecular tools in combination with other omics approaches are arising as a powerful tool of diagnostic and predictive value.

Wide and targeted transcriptomics analysis considered in an integrative manner is contributing to fill the gaps on aquaculture biomarkers.

Circadian clocks are proposed as robust markers of fish quality and nutritional condition at early life stages.

The functional specialization across the intestine transcriptome is by itself a robust biomarker that helps to explain differences in key performance indicators within and among farmed fish species.

Sequential pathway-focused PCR-arrays lead to define early biomarkers of nutrient deficiencies and their reference values.

Acknowledgments

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PREDICTING FATTY ACID PROFILES OF FISH FED DIFFERENT FAT SOURCES

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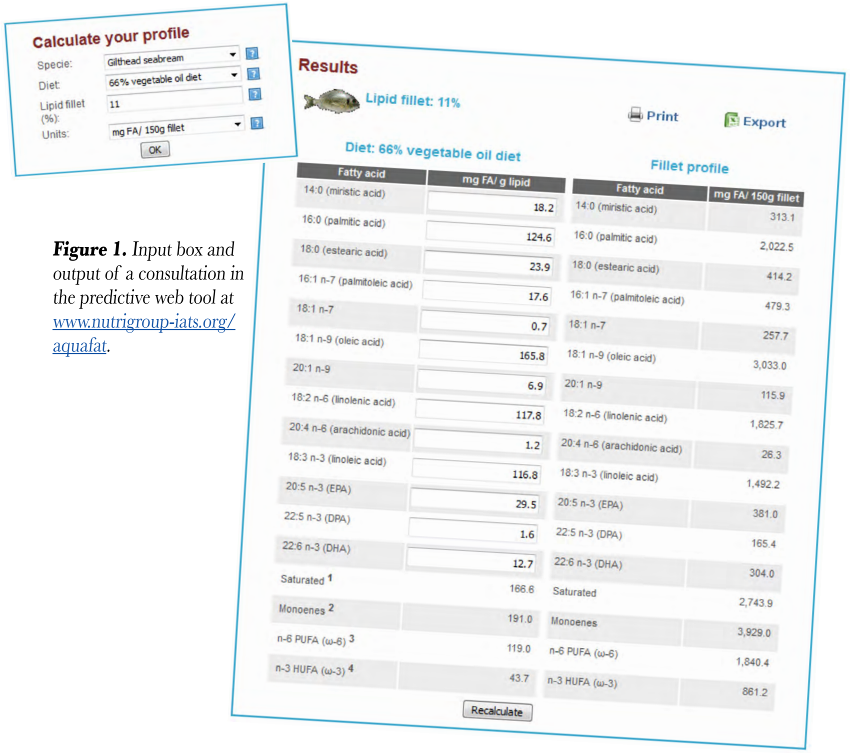
Fish are the most important source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) in the human diet, especially eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids. The FA composition of cultured fish fillets is highly dependent of that of the fish feeds, and this feature is especially critical in marine fish, as they have a limited ability for biosynthesis of LC-PUFA due to deficiencies in the PUFA desaturation/elongation pathways. Over the course of the last years the inclusion level of marine feedstuffs in fish diets is decreasing, and the most used alternative sources for new feed formulations are raw materials of vegetable origin. As vegetable oils are devoid of n-3 LC-PUFAs, fish fed high replacement diets are prone to show reduction in fillet EPA and DHA content. In this context, research in the Nutrigenomics group of IATS has been conducted to develop a predictive tool for modelling the fillet FA profile according to a given dietary composition in several marine fish species with relevance in aquaculture, which is placed at www.nutrigroup-iats.org/aquafat.

The tool has been constructed on the basis of regression models coming from empirical and published results. In a first step, a regression modelling approach served for correlating the fillet FA profile of gilthead sea bream fed a commercial diet during a two-year production cycle with the dietary FA composition and the fillet lipid level at each sampling time. This model was further validated with results on market-size fish available in the literature and by a trial with experimental diets based in plant protein and fish oil (**1**). Then, data on gilthead sea bream served as the reference subgroup category for fitting dummy regression equations of fillet FA composition of two flatfish species, turbot and sole, sampled at different stages of the production from fingerlings to harvest size in fish farms (2). This approach takes advantage of the background data on gilthead sea bream for making more consistent and reliable the set of regression equations. Furthermore, equations are scalable by addition of new dummy variables for the inclusion of more fish species of interest. The system was recently updated with the addition to the dummy regression equation of data from European sea bass cultured over a 20-months productive cycle at IATS facilities (**3**). In this species, the predictive values derived from data on fish reared at laboratory scale were correlated with those obtained from fish harvested at commercial size in a fish farm in order to up-scale and validate the equations and the web tool, and the regression plot with 12 representative FA showed a coefficient of determination near to equality (r2=0.98).

The predictive web tool requires from the user to indicate the fish species of interest (to date, gilthead sea bream, turbot, sole and European sea bass), the fillet lipid content (as percentage of wet weight) and the FA composition of the diet, which can be picked from a pre-determined list (FO diet, 33%VO diet, 66%VO diet, VO diet) or fully customized by entering the FA profile (mg FA/g lipid). As a result, the obtained output will show the detailed FA profile of fillet as mg FA per g of lipid or per 150 g of fillet (**Fig. 1**). Results output allows edition of the diet FA composition for further recalculations.



Calculate your profile

Specie: Gilthead seabream
Diet: 66% vegetable oil diet
Lipid fillet (%): 11
Units: mg FA/ 150g fillet

Results

Lipid fillet: 11%

Diet: 66% vegetable oil diet

Fatty acid	mg FA/ g lipid
14:0 (miristic acid)	18.2
16:0 (palmitic acid)	124.6
18:0 (estearic acid)	23.9
16:1 n-7 (palmitoleic acid)	17.6
18:1 n-7	0.7
18:1 n-9 (oleic acid)	165.8
20:1 n-9	6.9
18:2 n-6 (linolenic acid)	117.8
20:4 n-6 (arachidonic acid)	1.2
18:3 n-3 (linoleic acid)	116.8
20:5 n-3 (EPA)	29.5
22:5 n-3 (DPA)	1.6
22:6 n-3 (DHA)	12.7
Saturated [1]	166.6
Monoenes [2]	191.0
n-6 PUFA (ω-6) [3]	119.0
n-3 HUFA (ω-3) [4]	43.7

Fillet profile

Fatty acid	mg FA/ 150g fillet
14:0 (miristic acid)	313.1
16:0 (palmitic acid)	2,022.5
18:0 (estearic acid)	414.2
16:1 n-7 (palmitoleic acid)	479.3
18:1 n-7	257.7
18:1 n-9 (oleic acid)	3,033.0
20:1 n-9	115.9
18:2 n-6 (linolenic acid)	1,825.7
20:4 n-6 (arachidonic acid)	26.3
18:3 n-3 (linoleic acid)	1,492.2
20:5 n-3 (EPA)	381.0
22:5 n-3 (DPA)	165.4
22:6 n-3 (DHA)	304.0
Saturated	2,743.9
Monoenes	3,929.0
n-6 PUFA (ω-6)	1,840.4
n-3 HUFA (ω-3)	861.2

Figure 1. *Input box and output of a consultation in the predictive web tool at www.nutrigroup-iats.org/aquafat.*

In summary, dummy regression has served for the construction of a powerful multispecies tool for predictive modelling the nutritionally tailored fillet FA composition of marine fish. This application will be of utility for a wide range of stakeholders, including fish farmers, fish producers and consumers in order to face the nutritional human recommendations in LC-PUFAs and meet daily human requirements in EPA and DHA (500 mg/day), as well as produce more tailored and healthy fish products for human consumers.

ACKNOWLEDGMENTS

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OCCURRENCE AND POTENTIAL TRANSFER OF "CLASSICAL" & "NEW" ORGANIC CONTAMINANTS BY USE OF PLANT-BASED AQUAFEEDS

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Limited access to fish meal and fish oil for the rapidly growing aquaculture has accelerated the need of aquafeeds with lower reliance on marine sources. Plant oils and proteins represent the main reliable fish feed alternatives for the major farmed fish species in Europe. More than ten years ago, sea bream and Atlantic salmon feeding was based on around 85-90 % of marine ingredients. Research carried out for its cultivation with alternative plant-based diets led to replace as a minimum of 80% of the added marine ingredients (**Table 1**). This recent development of plant-based sustainable feed introduces new challenges in the field of fish safety that were not previously relevant by use of traditional marine feed ingredients.

Ingredients (% of total)	**Atlantic salmon**	**Sea bream**
Vegetable meal mixture	65	73.84
Wheat	*6*	*6.64*
Wheat gluten	*15*	*7.3*
Corn gluten	*4*	*25*
Soybean protein	*18*	*25*
Pea protein	*13*	
Rapeseed meal		*9.9*
Field peas	*9*	
Vegetable oil mixture	15.8	13.0
Linseed oil	*2.2*	
Palm oil	*4.8*	*6.5*
Rapeseed oil	*8.8*	*6.5*
Marine sources	14.4	7.5
Fish meal	*8*	*3*
Fish oil	*4.4*	*2.5*
Krill meal	*2*	
Fish protein		*2*
Vitamin and mineral supplementation	4.8	5.66

Table 1. *New trend compositions of alternative feed reared on plant sources.*

To be coherent with the current feed compositions and, to deal with contaminants introduced by new alternative plant sources but, at the same time, covering traditional contaminants from marine ingredients, advanced analytical methodology should be developed and validated (1-5). This purpose includes the detection, reliable identification and quantification of persistent organic pollutants (POPs), polybrominated diphenyl ethers (PBDEs), pesticides, polycyclic aromatic hydrocarbons

(PAHs) and mycotoxins, as the main groups. The use of hyphenated techniques like gas chromatography (GC) and liquid chromatography (LC) coupled to low and high resolution mass spectrometry (MS and HRMS) were world-wide accepted techniques of choice to preserve feed safety, fish welfare and public health (Fig. 1).

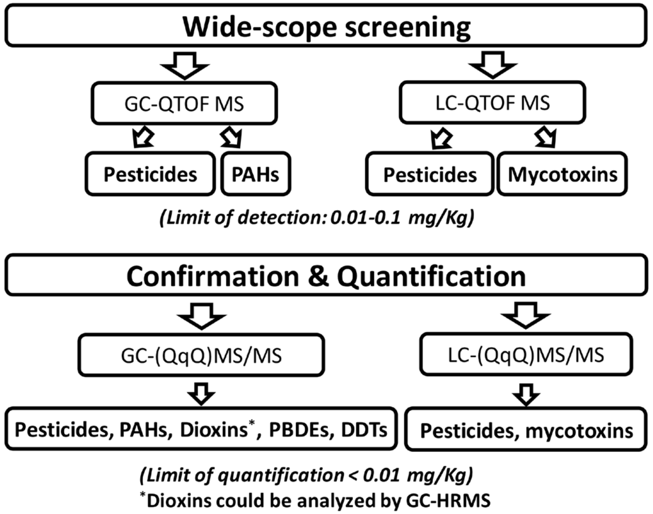

Figure 1. *Overview picture of the analytical methodology strategy developed.*

This comprehensive strategy allowed the assessment of the presence of relevant contaminants in plant & marine feed ingredients, feeds based on these ingredients, and Atlantic salmon and gilthead seabream cultured on the basis of these feeds.

Of the novel contaminants identified in feed ingredients and feed, the pesticides chlorpyrifos-methyl, pirimiphos-methyl, boscalid and azoxystrobin, and the mycotoxins deoxynivalenol and fumonisins (B1+B2+B3) were detected (1-4). PAHs, and especially the light (≤ 4 ring), were found in all samples but benzo(a)pyrene equivalents (BaPe) were around 20-fold below maximum residue level established (2 µg/kg) (2,5). The new pesticides and mycotoxins were not detected in fish fillet.

The transfer of contaminants from feed to public foodstuffs is essential for an appropriate human risk assessment of feed contaminants as well as for the harmonization of legislation of contaminants throughout the aquaculture production chain. One example of the "new" considered contaminants is mycotoxins, which are world-wide found in cereal grains and animal feed. Mycotoxins are produced by fungi that pre-harvest infect agricultural crops (field mycotoxins) or post-harvest agricultural commodities stored under certain temperature and humidity conditions (storage mycotoxins) (3). A second group of novel contaminants are polar pesticides, which are currently used on crops (soya, wheat etc.) that are used as aquafeed ingredients (1, 2, 4, 5). These "new" pesticides are not organochlorine pesticides (OCP), such as DDT and HCB, which have mostly been banned for agricultural use. Additionally, PAHs are a widespread contaminants associated along the aquaculture food chain (2, 5). These contaminants comprise several groups of compounds with a wide variety of physico-chemical characteristics. It is important to remark that only PAH transfer factors were calculated under the ARRAINA trials as only PAHs were found in both feed and fish fillet. Transfer factors for salmon were in the range of 0.04-0.29 while sea bream factors were in the range of 0.03-0.05. In all cases, these factors were higher than those values calculated when marine ingredients were at higher percentage in feed.

MAIN CONCLUSIONS

No transfer to edible fillets of relevant contaminants such as polar pesticides, traditional POPs and mycotoxins were observed when using plant-based feed. PAHs were the only contaminant family transferred to the edible fillets, but however BaPE were around 20-fold below the maximum residue level established (2 µg/kg). The safety risks are mainly associated with plant derived contamination and the subsequently feed-to-fillet transfer of PAHs as well as the potential transfer of other new emerging contaminants like mycotoxins.

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SHARING EU AQUACULTURE RESEARCH FACILITIES (AQUAEXCEL & AQUAEXCEL2020)

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In order to support the development of a sustainable production of high quality seafood with reduced environmental impact, the European Union is promoting since 2011 the coordinated use and development of major aquaculture experimental facilities through two research Projects, AQUAEXCEL (Aquaculture Infrastructures for Excellence in European Fish; 2011-2015, FP7) and AQUAEXCEL2020 (2015-2020, Horizon 2020). An important effort has been put to make available to the research community (public and private) the transnational access (TNA) to a number of aquaculture experimental facilities across Europe. The facilities have the capacity to undertake experimental trials on a selection of commercially important aquaculture fish species and system types. The TNA program allows external teams to access to the Project partners' facilities via submission of research proposals in periodical calls, which are funded based on the evaluation by an independent selection panel. Access to the research infrastructures and associated travel and subsistence expenses are covered by the Project budget.

During the lifetime of AQUAEXCEL, 136 applications were received over nine calls with a total of 97 TNA projects carried out. The greatest number of applications and approvals was in the area of nutrition, followed by physiology, welfare and health. Our institution (CSIC) was represented by IATS, and it offered two types of access for experimental (IATS-EXP) and analytical (IATS-ANA) works. IATS-EXP offered access to about 250 holding tanks located in different units at IATS, with different shapes and capacities (from 3,000 l to 30 l), together with the associated wet labs and sampling rooms. Access to IATS-ANA included nine analytical laboratories with the scientific appliances and devices to conduct most of the techniques and analyses involved in research in aquaculture: microscopy, histology, histochemistry, ISH, immunoassays, gas and liquid chromatography, PCR arrays other molecular techniques, in vitro cell and eukaryotic culture, isotopic assays, micromanipulation, etc. IATS installations are adequate for conducting experiments in most of the disciplines involved in aquaculture research: fish pathology and immunology (parasite and bacteria challenges), physiology, reproduction, nutrition and growth, live prey and larval rearing. Experimental studies can be conducted with a great variety of species: gilthead sea bream, European sea bass, sole, turbot, Artemia, with access to one of the largest Artemia Cysts collection available in Europe.

The ongoing AQUAEXCEL2020 offers to aquaculture researchers from both academia and industry the opportunity to access to 39 unique research infrastructures from 19 of the participating organizations. The available facilities cover the entire range of production systems, environments, scales, fish species and fields of expertise. Access is available to EU and Associated States' research teams, industry, and small and medium-sized enterprises. Access is also available to third countries (countries outside the EU) on a limited basis (up to 20%). Researchers from any EU or Associated State can apply for access to research infrastructures in a different country to that of the lead researcher. Proposal calls are established to be open every 3 months during the Project, and a calendar of call status and calendar of upcoming calls can be found at the AQUAEXCEL2020 website (www.aquaexcel2020.eu).

Criteria for selection of the proposals are based on the scientific quality, degree of innovation, relevance, exploitation potential and ethical soundness of the studies. In considering the exploitation potential, attention is given to how the work contributes to meet the objectives of the European Aquaculture Technology and Innovation Platform, as defined in the thematic Strategic Research and Innovation Agenda (www.eatip.eu). The results of TNA works are required to be made available to the scientific community by publications in research journals and communications in Congresses and other forums.

The CSIC offer in the AQUAEXCEL2020 TNA program includes IATS-EXP and IATS-ANA accesses like in the previous Project, and it has been extended to the experimental facilities of Instituto de Investigaciones Marinas (IIM-EXP) in Vigo (Atlantic coast of Spain), with large experience in bacteria and virus challenges. A year and a half after the start of the Project, the four proposals submitted for access to IATS facilities have been approved, the first two are finished, and the results of the first (Dr. Juan Fuentes from Portuguese CCMAR, "Electrophysiological testing of intestinal function and integrity on sea bream with different nutritional background, INTEBREAM", May-June 2016) are part of a large study about the role of dietary butyrate on the restoration of intestinal status of gilthead sea bream fed extreme diets low in fish meal and fish oil (1).

In summary, the TNA program of AQUAEXCEL2020 provides, through periodical calls, an excellent opportunity for the establishment of contacts and collaborations of public and private research groups with key aquaculture infrastructures in order to conduct experiments and analysis for further advance and development of the European aquaculture.

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SEA BREAM GENETIC IMPROVEMENT. NUTRIENT X GENOTYPE INTERACTIONS

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Gilthead sea bream (Sparus aurata L.), is the most important marine species in the Mediterranean aquaculture. To maintain its competence status, against other species, companies are introducing genetic improvement programs (GIPs) as essential and complementary tool to minimize costs, increase benefits or keep the turnovers. GIPs provide continuous, cumulative, permanent and extensible growth to the industry. However, GIPs' implementation requires innovative ideas to avoid additional handicaps. Thus, GIPs adapted to the business model of companies are much more successful over time, when those report their benefits in medium and long time periods. In gilthead sea bream, there are different GIPs operating in the Mediterranean industry, evaluating traits related with growth, morphology, disease resistance, product quality and feed efficiency. Nowadays, the cultured selected seed of gilthead sea bream represents more than thirty percent of the total production, with a mean genetic progress per generation around 17%. A major question to be considered under a GIP, is related with the market expansion of companies. To do it, environment-genotype interactions (GE) have to be measured in order to guarantee the production level expected by buyers. Scarce GE interactions have being described in gilthead sea bream at industrial scale for important traits related with growth, carcass, fish and meat quality, under framework of PROGENSA® (Spanish National GIP). On the other hand, marine fish feeds are optimized by substitution of fish oil and fish meal by vegetable sources, constituting new scenarios which could involve nutrition-genotype interaction (GN). Nowadays, several experiments are being conducted under two projects, PROGENSA® and PERFORMFISH, to study the importance of GN interaction in gilthead seabream, at experimental and industrial scales, respectively. In PROGENSA, tracing and validating of growth and intermediary metabolism biomarkers between families with different genetic background, which could be implemented in GIPs for a more efficient evaluation of broodstocks. In PERFORMFISH, studying the feed efficiency on a wide number of families fed with diets with different growth efficiency degree. Results of all these aspects are reviewed.

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