



ARRAINA

Advanced Research Initiatives for
Nutrition & Aquaculture

UNDERSTANDING BIOMARKERS IN FISH NUTRITION

TECHNICAL BOOKLET



Technical content prepared by CSIC with contributions from other ARRAINA project partners

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UNDERSTANDING BIOMARKERS IN FISH NUTRITION

TECHNICAL BOOKLET

Reliable use of biomarkers and methodologies for assessing nutritional status, and metabolic and health conditions, in fish fed with diets based on plant ingredients.

FEED INGREDIENTS IN AQUACULTURE

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INTRODUCTION

ABOUT THIS BOOKLET

This booklet is the final in a series of three technical booklets on the nutrition and feeding of fish farmed in Europe, and has been produced under the framework of the **European Union FP7 funded ARRAINA project (Advanced Research Initiatives for Nutrition & Aquaculture)**. The aim is to make these booklets widely available both to targeted stakeholders and society in general, in order to raise awareness of the science-based knowledge supporting the development of high-quality, safe, and environmentally sustainable aquaculture feeds.

This booklet is specifically aimed at **scientists and feed producers**, but it also targets other industrial segments (e.g. fish farmers, feed additives companies, retailers) and individuals interested in gaining further knowledge of the physiological consequences of the raw materials that are currently used in the feeds of farmed fish. The species focussed on by the **ARRAINA** project are: Atlantic salmon, rainbow trout, common carp, European sea bass and gilthead sea bream.

The objective of this booklet is to provide a reliable set of biomarkers and associated methodologies to assess the nutritionally mediated effects on growth performance, metabolic homeostasis, stress responsiveness and health condition of fish which are fed

new diet formulations from early life stages to completion of production cycle and sexual maturation. Of particular value are predictive and non-invasive biomarkers available at a relatively low cost, although the combination of **conventional and omics approaches** is emerging as a user-friendly option.

The first two **ARRAINA** technical booklets were focused on **feed ingredients** and **nutrient requirements**, and are available at www.arraina.eu.

ABOUT AQUACULTURE FEEDS IN EUROPE

Fish feeds are produced using a large variety of feed ingredients that have different nutritional and physical properties. They are formulated on the basis of solid scientific knowledge, contributing to the development of an environmentally-friendly aquaculture sector and to produce high quality nutritious food for humans¹⁻³.

The production of environmentally sustainable aquaculture feeds starts with the selection of high-quality raw materials that may need to be supplemented with specific nutrients (amino acids, phospholipids, vitamins, macro and micro minerals) to support maximum growth, health and well-being of fish. Given that the nutrient profiles of marine feedstuffs and plant protein-sources can vary greatly, one of the aims of the **ARRAINA** project was to identify which

micronutrients, and in which form, need to be supplemented to plant-based diets to support maximum growth and production of farmed fish from early life stages to completion of sexual maturation.

The **ARRAINA** project brings together different competences for developing tools and methodologies based on targeted and non-targeted biomarker approaches in the five species of interest for the assessment of nutrient requirements or status of fish, going further than just identifying differences in growth parameters.

Additionally, the project considers the potential presence and prevalence of contaminants associated with plant-based feedstuffs on fish fillets for human consumption, developing targeted and broad screening methods for fast and sensitive analyses.

ABOUT THE ARRAINA PROJECT

It is well recognised that the sustainability and competitiveness of aquaculture depend strongly on the replacement of capture fishery-derived fish meals and oils with alternative feedstuffs of plant origin. **ARRAINA** has been responding to this need by measuring the long-term effects that these changes in diet composition will have on the full life cycle of fish for which presently little is known.

INTRODUCTION CONTD.

One major aim of **ARRAINA** is to develop applicable tools and solutions of technological interest to the European fish feed industry. In collaboration with SMEs, ARRAINA strengthens the links between the scientific community and the EU feed industry and its contribution could increase the productivity and performance of the aquaculture sector leading to competitive advantage at a global level.

For general information on the project, you may download the project factsheet from the **ARRAINA** website www.arrassain.eu. For more information, please contact the **ARRAINA** Project Coordinator, Sadasivam Kaushik (sadasivam.kaushik@inra.fr) or the Project Manager, Vincent Troillard (vincent.troillard@inra.fr).

¹ Benedito-Palos L.; Navarro J.C.; Sitjà-Bobadilla A, Bell J.G.; Kaushik S.; Pérez-Sánchez J. (2008). High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. *British Journal of Nutrition* 454, 8-18.

² Nasopoulou C.; Zabetakis L. (2012). Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. *IWT Food Sci. Technol.* 47, 217-224.

³ Tacon S.G.J.; Metian M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285, 146-158



ARRAINA BIOMARKERS DATABASE

Based on the literature and **ARRAINA** project results, several sets of biomarkers have been identified to assess nutrient requirements and to metabolically phenotype the nutritionally mediated effects of feeds on health and performance status in the five fish species of the project (Atlantic salmon, rainbow trout, common carp, European sea bass and gilthead sea bream). Examples of use include histopathological, biochemical and molecular markers of tissues and body fluids to be indicative of a wide range of biological/metabolic processes linked with protein, carbohydrate and lipid metabolism.

Relevant information from **ARRAINA** is periodically uploaded to the online **ARRAINA** biomarkers database (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.

At this stage, the **ARRAINA** biomarkers database contains more than 700 independent entries submitted by all the partners of the project. The database is periodically updated with new partner inputs, and especially valuable are the reporting data to establish the normal range of variation and robustness of selected markers in nutritionally challenged fish.

IDEAL BIOMARKERS:

- ✓ Non-invasive
- ✓ Easy to measure
- ✓ Cost effective
- ✓ Highly sensitive and specific when used alone or in combination with other biomarkers
- ✓ Consistent across developmental stage, season, reproductive and nutritional condition, genetic background, etc.

FISH SPECIES IN FOCUS:

- ✓ Atlantic salmon (*Salmo salar*)
- ✓ Common carp (*Cyprinus carpio*)
- ✓ European sea bass (*Dicentrarchus labrax*)
- ✓ Gilthead sea bream (*Sparus aurata*)
- ✓ Rainbow trout (*Oncorhynchus mykiss*)

USER-FRIENDLY DATABASE OF BIOMARKERS IN FISH NUTRITION

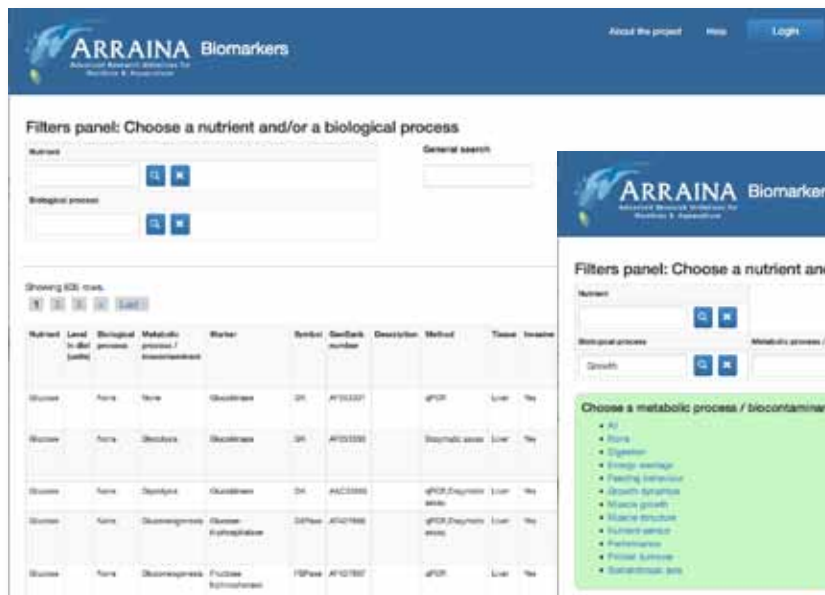
www.nutrigroup-iats.org/arraina-biomarkers

EXAMPLE OF USE

1

2

3



ARRAINA Biomarkers
Advanced Research Initiatives for Nutrition & Aquaculture

Filters panel: Choose a nutrient and/or a biological process

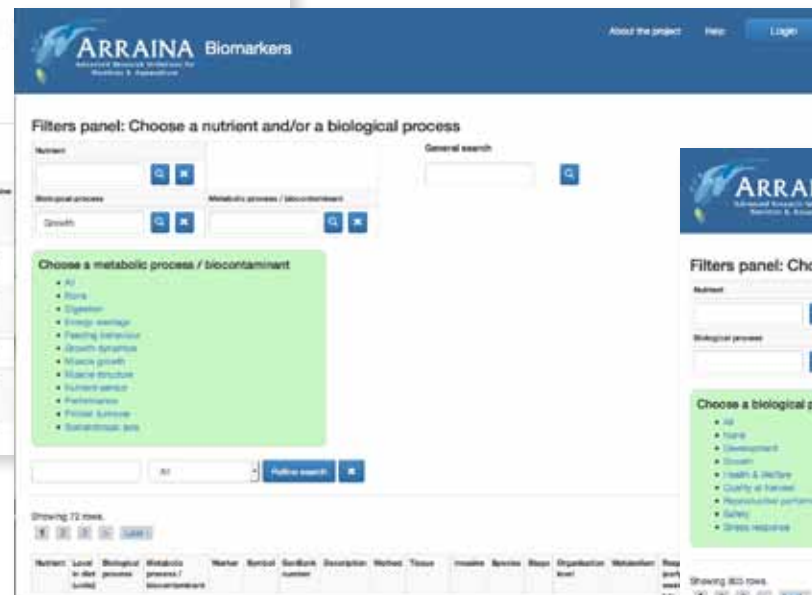
Nutrient:

Biological process:

General search:

Showing 100 items.

Nutrient	Level in diet process (unit)	Biological process / Metabolite	Marker	Symbol	SciBank number	Description	Method	Tissue	Species
Glucose	None	None	Glucose	GL	AT02207	gPCR	Liver	Yes	
Glucose	None	Glucose	Glucose	GL	AT02208	Spectrophotometry	Liver	Yes	
Glucose	None	Spontaneous	Glucose	GL	AT02209	gPCR/Enzymatic assay	Liver	Yes	
Glucose	None	Glucose	Glucose	GL	AT02210	gPCR/Enzymatic assay	Liver	Yes	
Glucose	None	Glucose	Glucose	GL	AT02211	gPCR	Liver	Yes	



ARRAINA Biomarkers
Advanced Research Initiatives for Nutrition & Aquaculture

Filters panel: Choose a nutrient and/or a biological process

Nutrient:

Biological process:

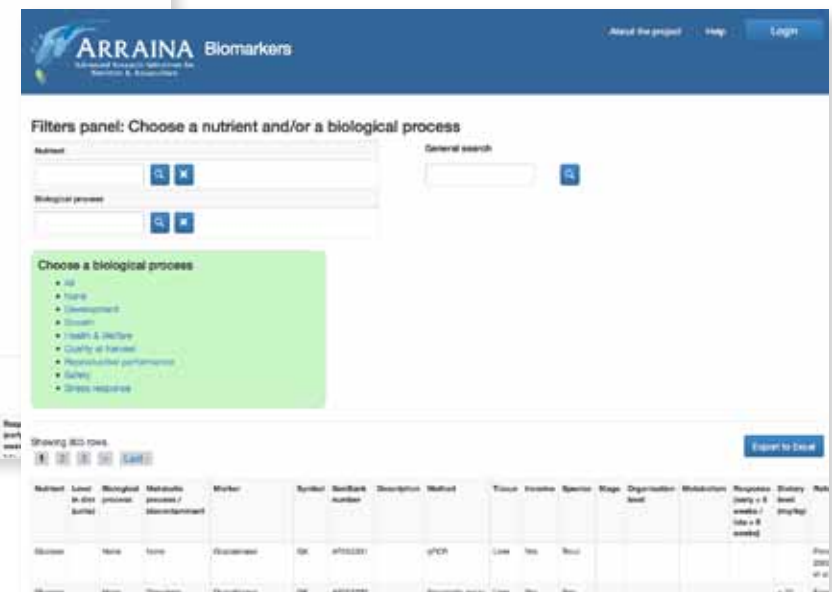
General search:

Choose a metabolic process / biocontaminant

- All
- Acid
- Alkaline
- Energy storage
- Feeding behaviour
- Growth dynamics
- Mucus glands
- Muscle structure
- Stress response
- Performance
- Protein synthesis
- Synthetic

Showing 10 items.

Nutrient	Level in diet process (unit)	Biological process / Metabolite	Marker	Symbol	SciBank number	Description	Method	Tissue	Species	Researcher	Organization	Publication	Response time (days / weeks / months)	Stability	Ref.
Glucose	None	None	Glucose	GL	AT02207	gPCR	Liver	Yes	Yes						



ARRAINA Biomarkers
Advanced Research Initiatives for Nutrition & Aquaculture

Filters panel: Choose a nutrient and/or a biological process

Nutrient:

Biological process:

General search:

Choose a biological process

- All
- Acid
- Alkaline
- Development
- Glucose
- Health & Welfare
- Quality of farmed
- Reproductive performance
- Safety
- Stress response

Showing 100 items.

Nutrient	Level in diet process (unit)	Biological process / Metabolite	Marker	Symbol	SciBank number	Description	Method	Tissue	Species	Researcher	Organization	Publication	Response time (days / weeks / months)	Stability	Ref.
Glucose	None	None	Glucose	GL	AT02207	gPCR	Liver	Yes	Yes						

TOOLS TO PREDICT FLESH FATTY ACID PROFILES

Fillet fatty acid composition of fatty fish and, to a lesser extent, lean fish, highly reflects the composition of diet. The replacement of fish oil with vegetable oils can compromise the high nutritive value of farmed fish as an important source of omega-3 long-chain poly-unsaturated fatty acids (PUFA) in the human diet. This is more evident in marine fish due to their limited capacity to elongate and desaturate C18 fatty acids into long chain C20 and C22 PUFA.

To ensure the compatibility of fish feeds with healthy fish for human consumers, an interactive and publicly **accessible web interface (www.nutrigroup-iats.org/aquafat)** has been developed within the framework of **ARRAINA** and national projects (AQUAFAT) to predict the fatty acid composition of farmed fish flesh.

The tool has been initially developed and validated for **European sea bass, gilthead sea bream, turbot, and sole**. The model is based on dummy-regression equations, and it is able to **predict the proportion of saturated, mono-unsaturated and poly-unsaturated fatty acids in a fish fillet** when key data on diet composition and body fat content are provided.




BIOMARKERS OF MINERAL AND VITAMIN REQUIREMENTS

ZINC AND BONE DEVELOPMENT IN FRESHWATER AND SEAWATER SALMON

Zinc (Zn) is a mineral that functions as a cofactor in several enzyme systems and is crucial for normal bone development. However, high levels of dietary Zn increase the cost of feed, increasing also the release of minerals to the aquatic environment. Therefore, optimal additions of Zn to the feed are needed to secure normal fish development with minimal environmental impact.

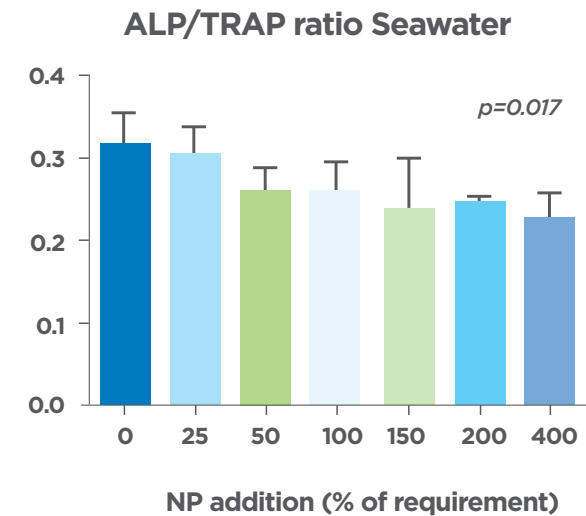
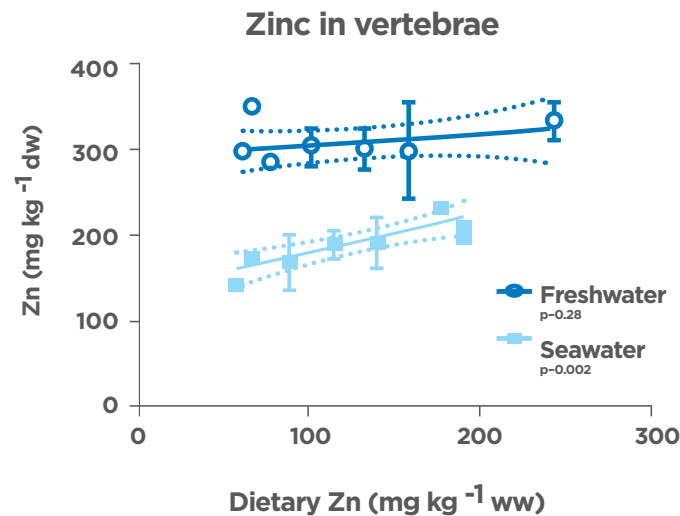
ARRAINA studies indicate that total and vertebral Zn content of seawater fish is lower than in

freshwater fish. In fresh water, dietary levels of <62 mg Zn/kg were sufficient for a vertebral and whole fish Zn concentration comparable to normal levels in Atlantic salmon. This is in line with data on Zn requirement for Atlantic salmon reared in fresh water (Maage et al. 1993). However, in salmon reared in seawater, 160 mg Zn/kg diet resulted in vertebral and whole fish Zn concentrations comparable to normal levels found in seawater-reared Atlantic salmon.

Regarding Zn-dependent enzymes involved in bone metabolism, the ALP/TRAP¹ ratio in freshwater fish did not change when extra Zn

was added to their diets. This supports the recommendations made by Maage et al. (1993) (37-67 mg Zn/kg diet) for freshwater Atlantic salmon.

In seawater grown salmon, the ALP/TRAP ratio was significantly reduced when more Zn was added to the diet. Along with the increases in whole fish and vertebral Zn content, this provides strong evidence that the dietary Zn addition to seawater salmon should be around 160-190 mg/kg to support healthy bone development.



¹ Alkaline phosphatase (ALP) – marker of bone forming cells, osteoblasts - Tartrate resistant acid phosphatase (TRAP) – marker of bone-resorbing cells, osteoclasts - Maage A.; Julshamn K. (1993). Assessment of zinc status in juvenile Atlantic salmon (*Salmo salar*) by measurement of whole body and tissue levels of zinc. Aquaculture 117, 179-191.

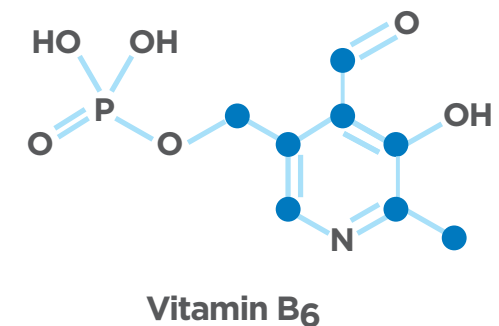
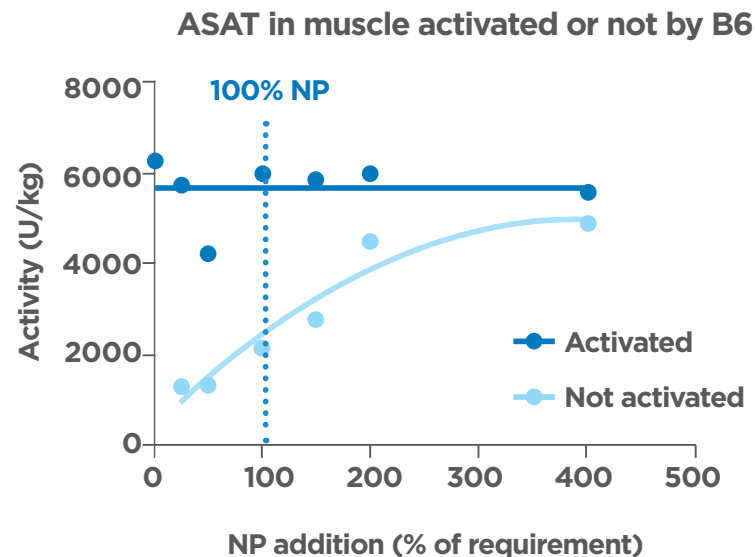
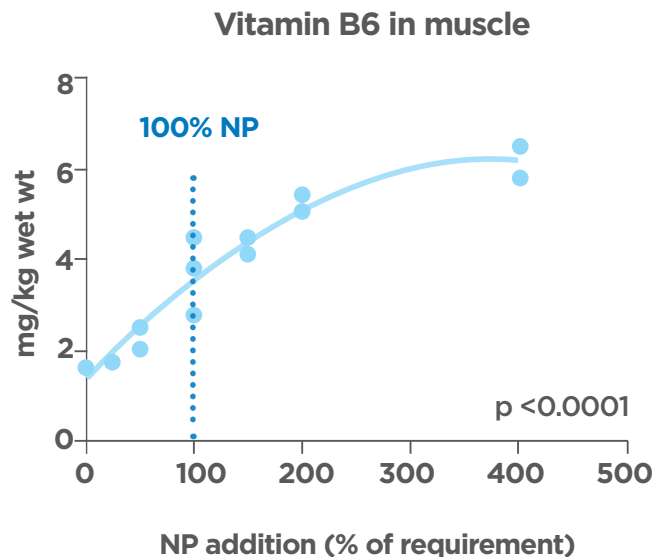
MARKERS OF VITAMIN B6 REQUIREMENTS IN FRESHWATER AND SEAWATER SALMON

The use of alternative ingredients as fish meal replacers in feeds for farmed fish requires increased focus on water soluble vitamin supplies. Fish meal is rich in most B vitamins, whereas other ingredients may contain variable amounts both of these and other chemical forms that differ in bioavailability.

ARRAINA biomarkers to determine vitamin B6 (pyridoxine) requirements include classic muscle tissue measures of B6 vitamin status and the

corresponding activity of **ASAT**¹ in muscle, a metabolic enzyme that uses vitamin B6 as cofactor. Alternatively, vitamin B6 takes part in the 1-C metabolism through the transsulfuration pathway, and the resulting metabolic outcome (organ lipid accumulation, metabolite profiles and gene expression patterns) should be used as a marker of vitamin B6 deficiency, although it is unspecific.

The results showed that the **current recommendations for vitamin B6 in practical plant-based feeds for Atlantic salmon are too low**, both in freshwater and seawater.



¹ Aspartate aminotransferases (ASAT) uses vitamin B6 as cofactor and its activation is a good marker of vitamin B6 status.

MARKERS OF METHIONINE METABOLISM IN RAINBOW TROUT

The sustainability of the growing aquaculture industry depends on the progressive reduction of wild-fishery derived feedstuffs in fish feeds. Plant proteins have therefore been proposed as sustainable alternatives to fish meal. However, the essential amino acid profile in plant proteins differs from that of fish meal. As a result, plant-based diets do not generally meet the essential amino acid requirements for some fish species, rainbow trout for example, and often necessitate the addition of one or several crystalline amino acids in order to meet the essential amino acid requirements. Methionine is considered one of the most limiting essential amino acids in many plant-protein sources.

Studies performed in the framework of the **ARRAINA** project indicate that methionine deficiency negatively affects growth performances but also impacts cellular mechanisms regulating metabolism and growth. In juvenile trout, methionine deficiency increased proteolytic pathways as indicated by the higher white muscle levels of autophagy-related markers at both

protein (**LC3-II and beclin 1**) and gene expression levels (**ATG4b, ATG12 I, UVEAG, SQSTM1, Mu1 and Bnip3**). Several genes related to proteasome proteolytic system (**Fbx32, MuRF2, MuRF3, ZNF216 and Trim32**) were also significantly upregulated by methionine limitation.

Regarding hepatic metabolism, the **ARRAINA** studies highlighted cholesterol metabolism as a target of methionine deficiency with a reduced expression of genes involved in the synthesis of cholesterol* in the liver of female broodstock and an enhanced expression of genes related to cholesterol elimination** (**CYP7A1, ABCG8**) in young fry.

Together, these results extend our understanding of mechanisms regulating the reduction of muscle growth induced by dietary methionine deficiency, and provide valuable information on biomarkers of the effects of plant-based diets.

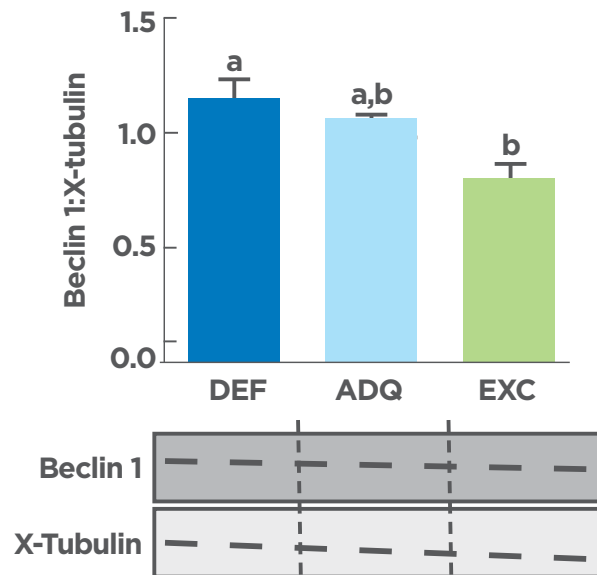


MARKERS OF CHOLESTEROL METABOLISM AFFECTED BY DIETARY METHIONINE IMBALANCE:

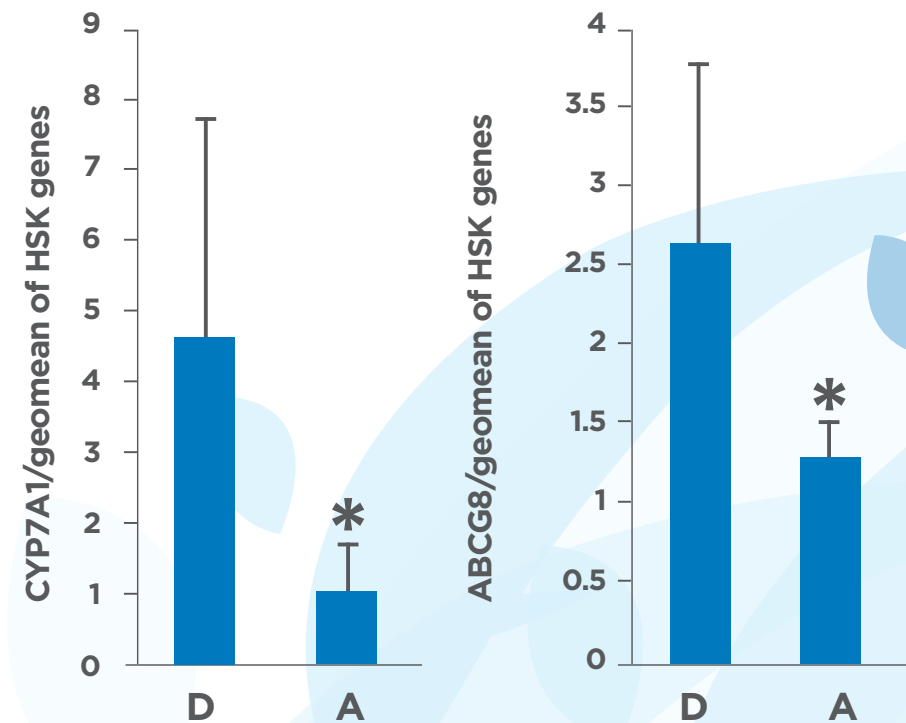
*Cholesterol synthesis: HMGCS, HMGCR, DHCR7 and CYP51

**Cholesterol elimination: CYP7A1 and UGT1A3 (bile acid synthesis) and ABCA1, ABCG8 and LXR (cholesterol efflux and excretion)

Beclin protein level in white muscle of trout fed diet with deficient (DEF), adequate (ADQ) or excess (EXC) levels of methionine



Hepatic gene expression level of CYP7A1 and ABCG8 in trout fed deficient (D) or adequate (A) methionine diets



MARKERS OF LIPID METABOLISM AND GROWTH PERFORMANCE IN EUROPEAN SEA BASS AND GILTHEAD SEA BREAM

Experimental evidence indicates that tissue histopathological scoring, and measurement of circulating levels of electrolytes, metabolites and hormones accurately reflect impaired growth performance, stress condition, and disease outcomes in European sea bass and gilthead sea bream. However, the use of these analyses as diagnostic or predictive tools is not yet well established. This is mostly due to the lack of reliable information on reference values, which is especially evident for the new biomarkers emerging from “omic” approaches.

The **ARRAINA** project aims to fill some of these knowledge gaps, focusing on the definition of normal range values for the most robust and informative biomarkers with emphasis on the liver, skeletal muscle, adipose tissue and the intestine. This is a meta-analysis approach with complex data mining of results from different feeding/ growth trials:

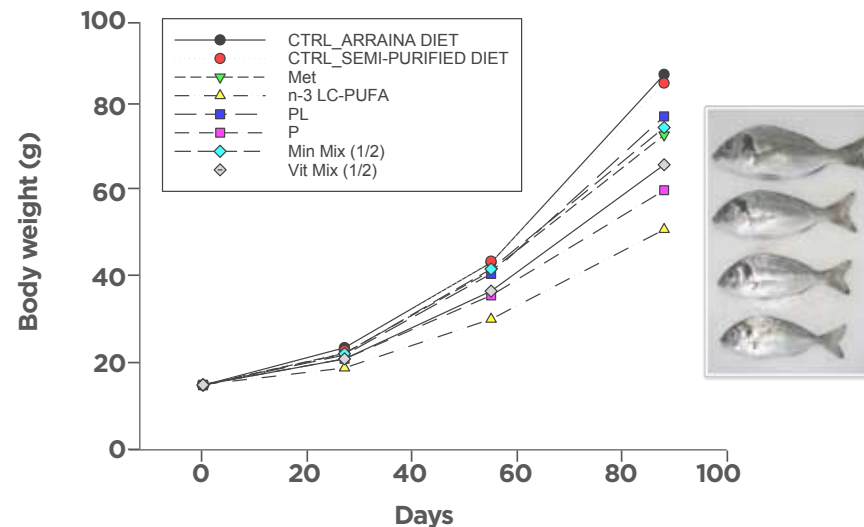
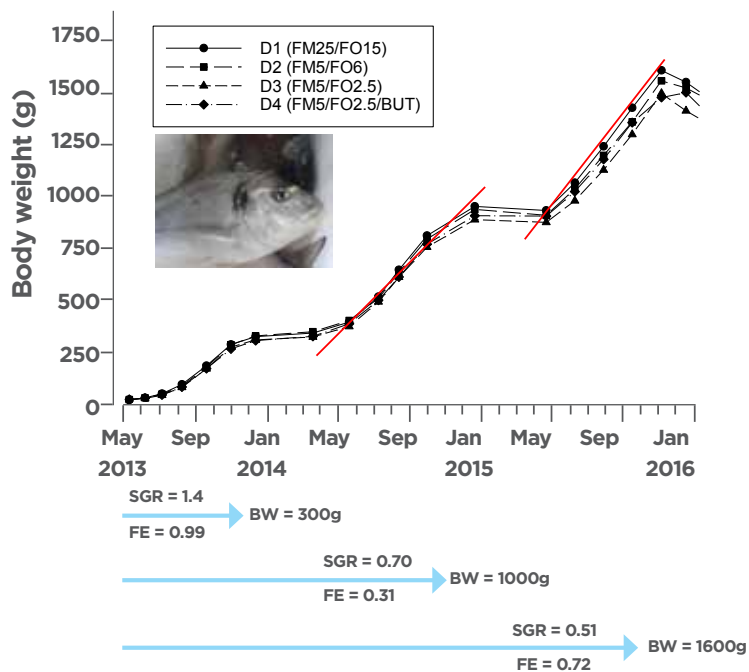
- **Fasting and re-feeding** of fish which have been fed commercial diet formulations.
- **Short-term trials with semi-purified diets** formulated for specific nutrient deficiencies in

methionine, essential fatty acids, phospholipids, phosphorous or minerals.

- **Long-term trials** with **ARRAINA** diets formulated by BIOMAR (industrial partner within the project) with different proportions of marine feedstuffs and plant ingredients.

EXPECTED RESULTS

- Definition of the most informative markers and tissues for the functional assessment of a given nutrient deficiency and metabolic status.
- Identification of the normal range of variation for selected markers in fish which have been fed both standard feed and sustainable feed formulations based on plant ingredients.



MOLECULAR MARKERS OF LIPID METABOLISM IN EUROPEAN SEA BASS AND GILTHEAD SEA BREAM

Customised LIPID-chip PCR arrays with more than 40 selected markers have been used in the **ARRAINA** project for the transcriptomic profiling of lipid metabolic disorders in both European sea bass and gilthead sea bream

In the **liver**, up to **24 differentially expressed (DE) genes** (including desaturases, elongases, phospholipases and enzymes of lipoprotein and cholesterol metabolism) have been initially recognised as robust markers of lipid metabolic disorders in juveniles of gilthead sea bream with clear signs of deficiencies in essential fatty acids or phospholipids. Among them, five genes are unspecific markers of lipid metabolic disorders, whereas the rest are emerging as robust and specific markers of fatty acid (15) or phospholipid (4) deficiencies.

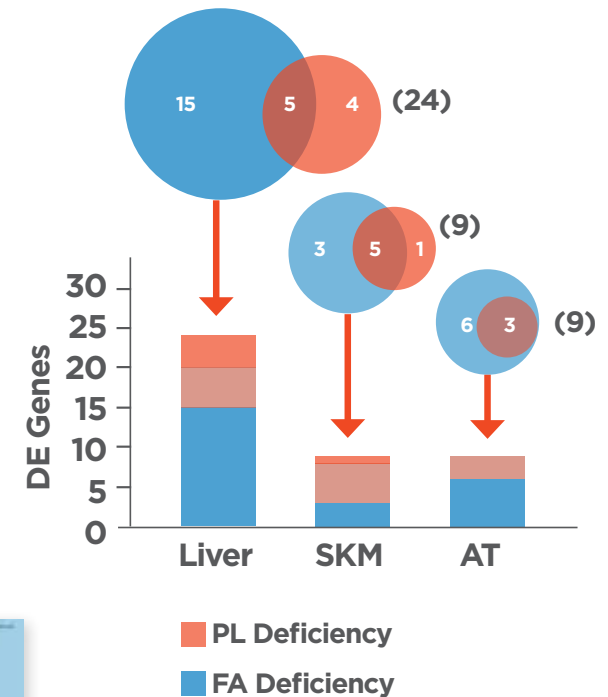
The same trend was found in the **skeletal muscle and adipose tissue**, although the number of **DE genes** decreased from 24 to 9.

This different tissue responsiveness is even more evident in the **brain**, with a gene expression pattern that remains mostly unaltered during fasting and refeeding in both European sea bass and gilthead sea bream.

LIPID-CHIP

- ✓ 43 genes
- ✓ Elongases, 4
- ✓ Desaturates, 3
- ✓ PL metabolism, 10
- ✓ Lipase-related genes, 9
- ✓ β -oxidation, 4
- ✓ Cholesterol & lipoprotein metabolism, 10
- ✓ Transcription factors, 3

Benedito Palas et al., 2013; 2014
Rimoldi et al., 2016

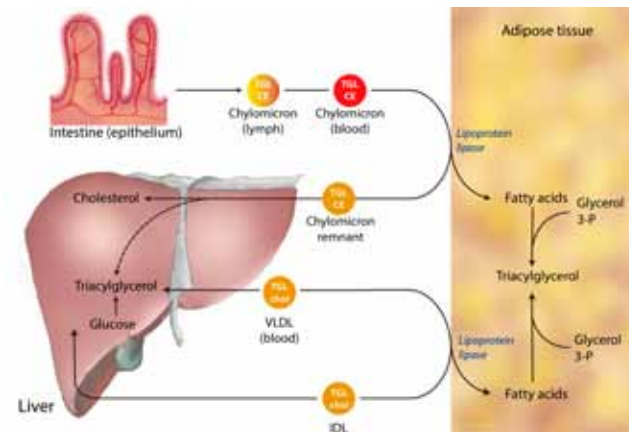


BIOMARKERS OF LIPID METABOLISM IN GILTHEAD SEA BREAM

The number of **DE genes** was practically reduced by half when the **LIPID-chip PCR-array** was used to assess the differential gene expression pattern in the liver of gilthead sea bream fed **ARRAINA** diets supporting maximum growth from early life stages.

Overall, the **magnitude of changes** was also reduced consistently.

Together, all these observations contribute to refine the search for **early and robust** biomarkers of lipid metabolism, helping to establish their **normal range of variation** in fish fed with different dietary lipid sources.



MAIN SIGNS OF FATTY ACID DEFICIENCIES*

- ✓ Anaemia
- ✓ Increased erythrocyte fragility
- ✓ Hypocholesterolemia
- ✓ Hypoproteinemia
- ✓ Increased respiratory burst
- ✓ Liver GH-desensitization
- ✓ Hepatic steatosis
- ✓ Abundance of intestine lipid droplets
- ✓ Reduced adipose tissue mass
- ✓ Changes in the staining pattern of goblet cells
- ✓ Inflammation of intestinal submucosa
- ✓ Changes in the hepatic expression pattern of elongases, desaturases, phospholipid- and lipase- related genes

MAIN SIGNS OF PL DEFICIENCIES[†]

- ✓ Hypoproteinemia
- ✓ Hepatic steatosis
- ✓ Abundance of intestine lipid droplets
- ✓ Liver GH-desensitization
- ✓ Increased number of intestinal rodlet cells
- ✓ Changes in the hepatic expression pattern of elongases, desaturases, and phospholipid-related genes

ROBUST MARKERS OF FATTY ACID* OR PL[†] DEFICIENCIES

- ✓ ELOVL5[†]
- ✓ ELOVL6*
- ✓ SCD1a/1b*
- ✓ FADS2^{††}
- ✓ PLA2G6*
- ✓ PLA2G15[†]
- ✓ LPL*
- ✓ Apo B-100^{††}
- ✓ DHCR7*
- ✓ CYP46A1*

ELONGASE
 DESATURASE
 PHOSPHOLIPASE
 LIPOPROTEIN METABOLISM
 CHOLESTEROL METABOLISM

BIOMARKERS OF GROWTH PERFORMANCE AND SPECIFIC NUTRITIONAL STATUS

Most growth regulatory events are mediated at hormonal level by the **somatotropic axis**, keeping pituitary growth hormone (**GH**) secretion and hepatic/extra-hepatic insulin-like growth factor (**IGF**) production under control. Hence in farmed fish, and gilthead sea bream in particular, changes in circulating levels of **GH** and **IGF-I** highly reflect the growth disorders arising from crowding and mishandling stress, reduced ration size, and nutrient imbalances, caused by replacing marine ingredients with plant ingredients.

The **ARRAINA** project took advantage of this knowledge and circulating levels of **GH** and **IGF-I** were measured in juveniles and adults of gilthead sea bream, to establish their normal range of variation under different growth and nutrient conditions.

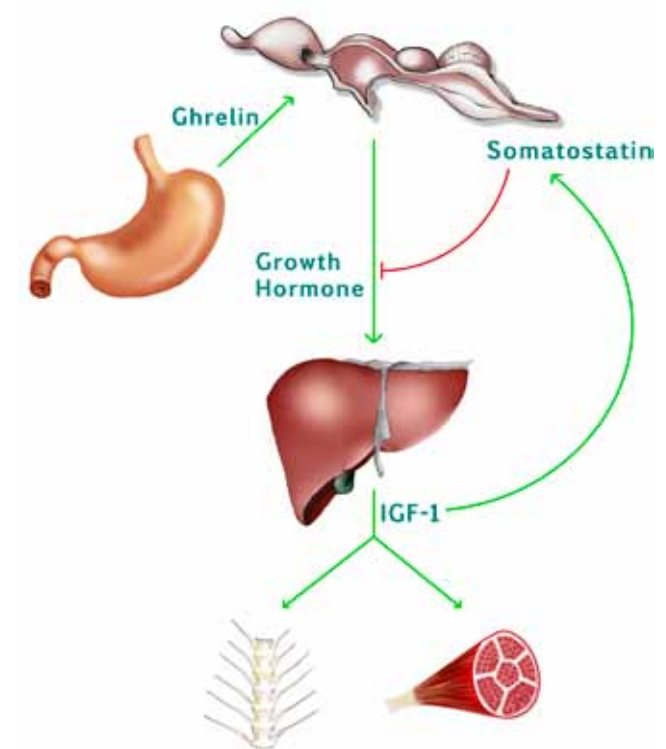
At the molecular level, the assessment of growth status was completed by liver and muscle gene expression profiling with a customised **GROWTH-chip PCR-array**: IGFs, IGF binding proteins & GH/IGF/insulin receptors (12), markers of muscle cell growth and differentiation (16), protein breakdown, folding and assembly (30), inflammatory and anti-inflammatory response (13), energy sensing

(5) and oxidative phosphorylation and respiration uncoupling (10).

Transcriptomic analyses revealed that more than 40 markers of growth performance were differentially regulated by specific deficiencies in amino acids, fatty acids, phospholipids or macro- and micro-nutrients. This is in fact a nutritional and tissue-specific regulated process, and the expression ratio of some markers has been proposed as indicator of both the type and the magnitude of a given nutrient deficiency.

GROWTH-CHIP

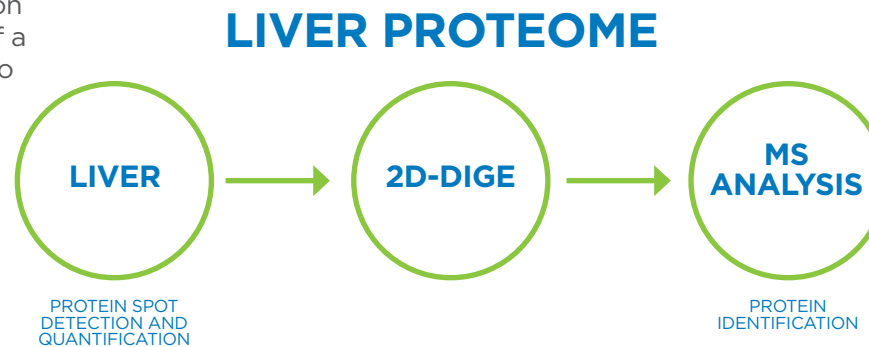
- ✓ 88 genes
- ✓ GH/IGF system, 12
- ✓ Muscle growth & differentiation, 16
- ✓ Protein breakdown, folding and assembly, 30
- ✓ Inflammatory & anti-inflammatory response, 13
- ✓ Energy sensors, 5
- ✓ OXPHOS & respiration uncoupling, 10



Attention is focused on the gene expression ratio of GHRs (**GHRI/GHR-II**), IGFs (**IGF-I/IGF-II**), myogenic growth factors (**MSTN/FSN, MyoD1/MyoD2**), molecular chaperones (**mtHsp70/GRP-170**) and metabolic energy sensors (**SIRT1/SIRT3**)

BIOMARKERS OF GROWTH PERFORMANCE AND SPECIFIC NUTRITIONAL STATUS CONTD.

Nutritionally mediated effects are also translated at the protein level, and **liver proteome mapping** also contributed to the discovery and validation of new biomarkers informative of the status of a given nutrient package, especially in relation to **phosphorus and essential fatty acid** deficiencies.



PERFORMANCE INDICATORS

PROTEIN IDENTIFICATION	BIOLOGICAL PROCESS
Group XIIB secretory phospholipase	Lipid catabolism
Retinol-binding protein 2	Retinoid metabolism
Delta (3,5)-delta (2,4)-dienoyl-CoA isomerase, mitochondrial	Fatty acid β -oxidation
Transcription factor HIVEP2	Regulation of transcription

SPECIFIC INDICATORS OF NUTRIENT DEFICIENCIES

✓ PHOSPHORUS DEFICIENCY	
3-hydroxyanthranilate 3,4-dioxygenase	Quinolate biosynthetic process
Trypsinogen 1	Proteolysis
✓ ESSENTIAL FATTY ACID DEFICIENCY	
Warm temperature acclimation-related 65 kDa protein	Cellular iron ion homeostasis

MARKERS OF INTESTINAL FUNCTION AND ARCHITECTURE IN EUROPEAN SEA BASS AND GILTHEAD SEA BREAM

The **gastrointestinal (GI) tract** is involved not only in digestion and feed absorption but also in water and electrolyte balance, nutrient sensing and immunity. This diversity is now starting to be elucidated and molecular approaches are informing about the many vital functions conducted in the **GI tract** in the context of integrative and comparative physiology.

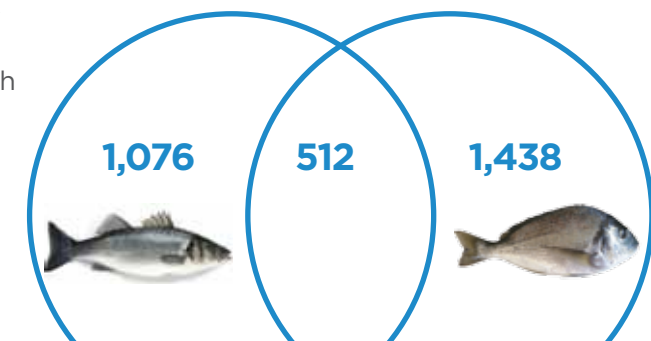
The fish **GI tract** is a major immunological site acting as physical and chemical barrier against invading organisms, and the cells within the intestine produce a large number of chemical substances to enhance barrier function, innate immunity and humoral immune responses.

Nutrient availability is also essential for the generation and maintenance of a protective effector immune system. For instance, deficiencies in **tryptophan, arginine and cysteine** reduce immune cell activation. The nutritionally mediated effects in the intestinal transcriptome and function are, however, highly fish species-specific. Both in European sea bass and gilthead sea bream, **short-chain fatty acids identified** in studies by the **ARRAINA** project are a clear example of how host diets and the gut microbiota shape the immune response and the integrity and permeability of the fish **GI tract**.

This is further supported by data from the **wide-proteomic profiling** of intestinal mucus of gilthead sea bream by means of **i-TRAQ methodology**. This gel-free proteomic approach does not allow the study of post-transcriptional modifications in gilthead sea bream, although it has been shown to be very powerful in revealing the proteome of complex samples when this methodology is used in combination with a homologous protein database for protein matching.

In this context, the **ARRAINA** microarray gene expression profiling revealed a marked functional specialisation of the **GI tract** of European sea bass and gilthead sea bream with more **than 2,000 DE genes** from anterior to posterior intestine segments. Among them, **512** were **DE genes** in both species. This set of genes mostly reflects a conserved **distribution pattern of nutrient transporters** across the intestine, which is proposed by itself as a marker of intestinal functionality in nutritional studies.

3,026 DE genes are mapped with the IPA software



DE genes across the intestine of active feeding European sea bass and gilthead sea bream

NUTRITIONALLY MEDIATED EFFECTS ON THE INTESTINAL TRANSCRIPTOME

Microarray and RNA-seq gene expression profiling of the GI tract of gilthead sea bream also revealed a marked specialisation on a temporal basis with more than 6,000 **DE genes** (2,000 after a fold-change cut-off of 2) from anterior to posterior intestine segments when comparing active (summer growth) and non-active feeding fish (overwintering).

This **spatial and temporal plasticity** of intestinal gilthead sea bream transcriptome contrasts with the high intestinal refractoriness of fish which were fed the large range of **ARRAINA** diets.

A targeted PCR-array approach (**GUT-chip**), with more than 80 markers of intestinal function, architecture and permeability, also supported the high preservation of intestinal function in fish fed **ARRAINA** diets. However, changes in the expression pattern of genes involved in antioxidant defence, epithelial permeability and mucus production indicates some pro-inflammatory conditions in fish fed diets with extremely low levels (less than 7%) of marine feed ingredients.

This was confirmed with histological observations, but most of these effects were reversed to the wild phenotype of fish fed marine-based diets with the supplementation of extreme diets with a

commercial sodium butyrate preparation (0.4%, BP-70[®]Norel) of an industrial partner within the project. This is indicative of the pleiotropic action of this organic acid that also modifies histone acetylation and the expression of interleukin 10 in the intestine of European sea bass.

Together with changes on the intestinal transcriptome, butyrate supplementation helps to restore the nutritionally mediated changes on **gut microbiota composition, intestinal mucus proteome** and **trans-epithelial electrical resistance** (surrogate markers of epithelial integrity). This would support the **improved disease outcome** in experimentally infected fish with the intestinal parasite *Enteromyxum leei*.



 PLOS ONE

GUT-CHIP

- ✓ **86 genes**
- ✓ Cell differentiation and proliferation, 13
- ✓ Intestinal architecture and permeability, 18
- ✓ Enterocyte mass and epithelial damage, 11
- ✓ Immune surveillance, 19
- ✓ Pattern recognition receptors, 14
- ✓ Mitochondria function and biogenesis, 11

Pérez-Sánchez et al., 2015; 2016;
Terova et al., 2016

MONITORING OF INTESTINAL HEALTH & FUNCTION "ALL IN ONE"

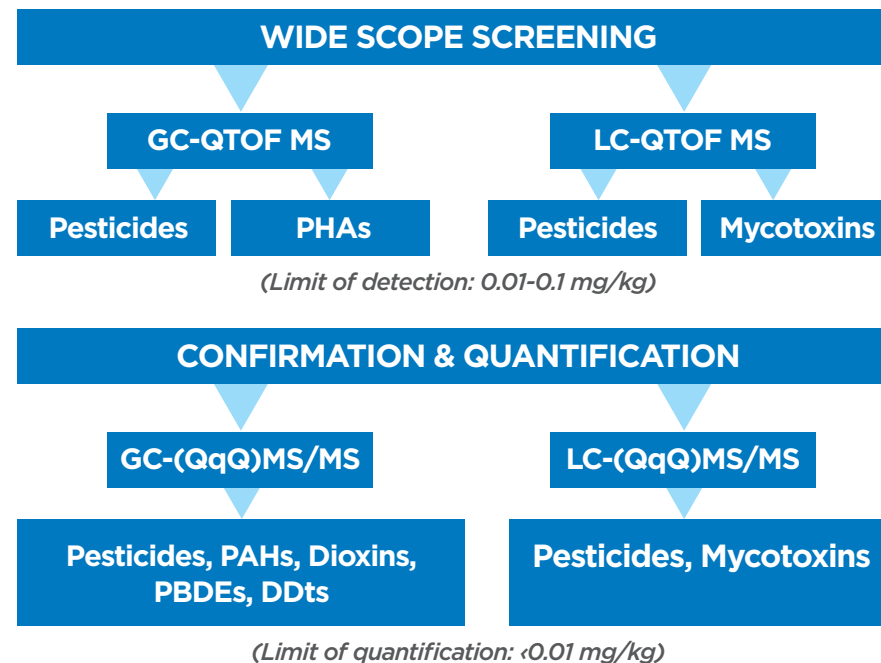
- **GI transcriptome**
- **Mucus proteome**
- **Metagenome**
- **Electrical resistance**

DETERMINING THE POTENTIAL PRESENCE AND PREVALENCE OF CONTAMINANTS IN FISH FILLETS FOR HUMAN CONSUMPTION

A number of contaminants, including **poly-aromatic hydrocarbons** (PAH), **pesticides** and **mycotoxins** (toxic substances produced by fungi) are associated with non-marine based ingredients used in aquaculture feeds.

The **ARRAINA** project, based on past national (AQUAMAX) and EU projects, recognised the necessity of developing universal screening methods for fast and sensitive detection of a wide range of contaminants, independent of their chemical properties.

LC/GC-MS analyses were carried out through the production cycle to identify the potential presence of contaminants in feed ingredients, **ARRAINA** fish feeds and, most importantly, in fish fillets for human consumers.



KEY RESULTS:

- ✓ In **ARRAINA** diets, **mycotoxins** were, in all cases, below the maximum permitted levels in products for animal feeding (European Commission Recommendation 2006/576/EC).
- ✓ No regulated **mycotoxins** were detected in fillets of gilthead sea bream or Atlantic salmon which were fed **ARRAINA** diets.
- ✓ **Pesticides** were found in feed ingredients and fish feeds, but not in edible fillets of gilthead sea bream or Atlantic salmon.
- ✓ **Light PAHs** were found in feed ingredients, feeds and below regulated levels in edible fillets.

CONCLUDING REMARKS

How nutrients or dietary components modulate fish performance and a wide range of biological processes (e.g. lipid and fatty acid metabolism, muscle growth, oxidative stress, health and immune response) affecting fish performance has been explored in **ARRAINA**. Data generated within the project indicate that the most important European farmed fish species, which were the focus of **ARRAINA**, are able to grow with plant-based diets without any, or with a very limited supply (< 7%), of marine feed ingredients. This data covers the complete life cycle from first life stages to completion of sexual maturation, producing ova and viable larvae and juveniles. Significant progress has also been made on the different metabolic pathways and processes affected by high dietary levels of plant proteins and/or vegetable oils.

The **ARRAINA** project selected appropriate panels of measurements for routine and clinical assessment of fish performance. The multi-

nutrient dietary approach chosen in **ARRAINA** dictates an organismal approach on fish health and fish performance, integrating both classic measurements of single nutrient status (e.g. tissue nutrient levels) with measurements of physiological status. This approach has delivered new insights into assessment of nutrient requirements in practical diets.

A challenge has been not only to screen and make the best use of tools available already, but also to integrate new knowledge arising from transcriptomics (gene expression analysis), proteomics (protein expression analysis), metabolomics (metabolite profiling) and metagenomics (microbiota genome analyses) in order to define a reliable and healthy phenotype of fish.

Of particular value are biomarkers that precede the onset of metabolic disturbance or predict

the capacity of the animal to cope with dietary, environmental and age-related stresses. Currently, we are still far from establishing the normal range of variation for most valuable biomarkers through the full productive cycle, but the **ARRAINA** project contributed significantly to progress in this area. This third **Technical Booklet** aims to highlight the different methodologies and approaches carried out to build efficient integrative tools that combine targeted and non-targeted approaches.

It is difficult to generalise due to differences in growth performance, nutrient requirements and life cycle of the five key commercial species of European aquaculture, although significant advances have been made in the development of interactive tools, including new genomic resources, such as transcriptomic gilthead sea bream and European sea bass databases.

www.arrassina.eu

www.nutrigroup-iats.org/arrassina-biomarkers

www.nutrigroup-iats.org/aquafat

www.nutrigroup-iats.org/seabreamdb

www.nutrigroup-iats.org/seabassdb

ARRAINA REFERENCES

- Ballester-Lozano GF, Benedito-Palos L, Riaza A, Navarro JC, Rosel J, Pérez-Sánchez J (2014). Dummy regression analysis for modelling the nutritionally tailored fillet fatty acid composition of turbot and sole using gilthead sea bream as a reference subgroup category. *Aquaculture Nutrition* 20:421-430.
- Ballester-Lozano GF, Benedito-Palos L, Estensoro I, Sitjà-Bobadilla A, Kaushik S, Pérez-Sánchez J (2015). Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets. *British Journal of Nutrition* 114:713-726.
- Ballester-Lozano GF, Benedito-Palos L, Mingarro M, Navarro JC, Pérez-Sánchez J (2016). Up-scaling validation of a dummy regression approach for predictive modelling the fillet fatty acid composition of cultured European sea bass (*Dicentrarchus labrax*). *Aquaculture Research* 47:1067-1074.
- Belghit I, Skiba-Cassy S, Geurden I, Dias K, Surget A, Kaushik S, Panserat S, Seiliez I (2014). Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (*Oncorhynchus mykiss*). *British Journal of Nutrition* 112:493-503.
- Benedito-Palos L, Calduch-Giner JA, Ballester-Lozano GF, Pérez-Sánchez J (2013). Effect of ration size on fillet fatty acid composition, phospholipid allostasis and mRNA expression patterns of lipid regulatory genes in gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition* 109:1175-1187.
- Benedito-Palos L, Ballester-Lozano GF, Pérez-Sánchez J (2014). Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene* 547:34-42.
- Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz A, Calduch-Giner JA, Pérez-Sánchez J (2016). Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry, and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454: 8-18.
- Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J (2014). Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*): implications on the physiological significance of UCP1-3 variants. *Fish Physiology and Biochemistry* 40:751-762.
- Calduch-Giner JA, Sitjà-Bobadilla A, Davey GC, Cairns MT, Kaushik S, Pérez-Sánchez J (2012). Dietary vegetable oils do not alter the intestine transcriptome of gilthead sea bream (*Sparus aurata*), but modulate the transcriptomic response to infection with *Enteromyxum leei*. *BMC Genomics* 470:13.
- Calduch-Giner JA, Bermejo-Nogales A, Benedito-Palos L, Estensoro I, Ballester-Lozano G, Sitjà-Bobadilla A, Pérez-Sánchez J (2013). Deep sequencing for de novo construction of a marine fish (*Sparus aurata*) transcriptome database with a large coverage of protein-coding transcripts. *BMC Genomics* 14:178.
- Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J (2016). Gene expression profiling reveals functional specialization along the intestinal tract of a carnivorous teleostean fish (*Dicentrarchus labrax*). *Frontiers in Physiology* Vol 7, article 359.
- Estensoro I, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J, Sitjà-Bobadilla A (2012). Modulation of the IgM gene expression and IgM immunoreactivity cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa). *Fish & Shellfish Immunology* 33:401-410.
- Estensoro I, Redondo M^ªJ, Salesa B, Kaushik S, Pérez-Sánchez J, Sitjà-Bobadilla A (2012). Effect of nutrition and *Enteromyxum leei* infection on gilthead sea bream (*Sparus aurata*) intestinal carbohydrate distribution. *Diseases of Aquatic Organisms* 100:29-42.

- Estensoro I, Ballester-Lozano GF, Benedito-Palos L, Grammes F, Martos-Sitcha JA, Mydland LT, Calduch-Giner JA, Fuentes J, Karalazos V, Ortiz A, Øverland M, Sitjà-Bobadilla A, Pérez-Sánchez J (2016). Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. PLoS ONE 11: e0166564.
- Hamre K, Sissener NH, Lock E-J, Olsvik PA, Espe M, Torstensen BE, Silva J, Johansen J, Waagbø R, Hemre G-I (2016) Antioxidant nutrition in Atlantic salmon (*Salmo salar*) parr and post-smolt, fed diets with high inclusion of plant ingredients and graded levels of micronutrients and selected amino acids. PeerJ (in press).
- Hansen A-C, Waagbø R, Hemre G-I (2015). New B vitamin recommendations in fish when fed plant-based diets. Aquaculture Nutrition 21: 507-527.
- Hemre, G.-I, Lock EJ, Olsvik PA, Hamre K, Espe M, Torstensen B, Silva J, Hansen A-C, Waagbø R, Johansen J, Sanden M, Sissener NH (2016) Atlantic salmon (*Salmo salar*) require increased dietary levels of B-vitamins when fed diets with high inclusion of plant based ingredients. PeerJ 4:e2493, 1-28.
- Nácher-Mestre J, Ibáñez M, Serrano R, Pérez-Sánchez J, Hernández F (2013). Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry. Journal of Agriculture and Food Chemistry 61:2077-2087.
- Nácher-Mestre J, Serrano R, Portolés T, Berntssen MHG, Pérez-Sánchez J, Hernández F (2014). Screening of pesticides and polycyclic aromatic hydrocarbons in feeds and fish tissues by gas chromatography coupled to high-resolution mass spectrometry using atmospheric pressure chemical ionization. Journal of Agriculture and Food Chemistry 62:2165-2174.
- Nácher-Mestre J, Serrano R, Beltrán E, Pérez-Sánchez J, Silva J, Karalazos V, Hernández F, Berntssen MHG (2015) Occurrence and transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients. Chemosphere 128:314-320.
- Olsvik P.A., Hemre G.I. & Waagbø R. (2013) Exploring early micronutrient deficiencies in rainbow trout (*Oncorhynchus mykiss*) by next-generation sequencing technology – from black box to functional genomics. PLoS ONE 8:e69461.
- Pérez-Cordón G, Estensoro I, Benedito-Palos L, Calduch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J (2014). Interleukin gene expression is strongly modulated at the local level in a fish parasite model. Fish & Shellfish Immunology 37:201-208.
- Pérez-Sánchez J, Borrel M, Bermejo-Nogales A, Benedito-Palos L, Saera-Vila A, Calduch-Giner JA, Kaushik S (2013). Dietary oils mediate cortisol kinetics and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (*Sparus aurata*) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. Comparative Biochemistry and Physiology 8D:123-130.
- Pérez-Sánchez J, Estensoro I, Redondo MJ, Calduch-Giner JA, Kaushik S, Sitjà-Bobadilla A (2013). Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. PLoS ONE 8:e65457.
- Pérez-Sánchez J, Benedito-Palos L, Estensoro I, Petropoulos Y, Calduch-Giner JA, Browdy CL, Sitjà-Bobadilla A (2015). Effects of dietary NEXT ENHANCE®150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). Fish & Shellfish Immunology 44:117-128.
- Portolés T, Ibáñez M, Garlito B, Nácher-Mestre J, Karalazos V, Silva J, Alm M, Serrano R, Pérez-Sánchez J, Hernández F, Berntssen MHG (2016). Comprehensive strategy for qualitative and quantitative analysis of pesticides through the production cycle of gilthead sea bream and Atlantic salmon. Food Chemistry (under revision).

- Prabhu PAJ, Schrama JW, Kaushik SJ (2014). Mineral requirements of fish: a systematic review. *Reviews in Aquaculture* 6:1-48.
- Rimoldi S, Benedito-Palos L, Terova G, Pérez-Sánchez J (2016). Wide-targeted gene infers tissue-specific molecular signatures of lipid metabolism in fed and fasted fish. *Reviews in Fish Biology and Fisheries* 26:93-108.
- Simó-Mirabet P, Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J (2016). Sirtuin energy-sensing at the molecular level. Tissue-specific gene expression and regulation by nutrient scarcity in gilthead sea bream (*Sparus aurata*). *J Comp Physiol B* DOI 10.1007/s00360-016-1014-0.
- Skiba-Cassy S, Geurden I, Panserat S, Seilliez I (2016). Dietary methionine imbalance alters the transcriptional regulation of genes involved in glucose, lipid and amino acid metabolism in the liver of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 454:56-65.
- Terova G, Díaz N, Rimoldi S, Ceccotti C, Gliozheni E, Piferrer F (2016). Effects of sodium butyrate treatment on histone modifications and the expression of genes related to epigenetic regulatory mechanisms and immune response in European sea bass (*Dicentrarchus labrax*) fed a plant-based diet. *PLoS ONE* 11:e0160332.



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