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# Sample size matters in dietary gene expression studies—A case study in the gilthead sea bream (*Sparus aurata* L.)

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ABSTRACT

One of the main concerns in gene expression studies is the calculation of statistical significance which in most cases remains low due to limited sample size. Increasing biological replicates translates into more effective gains in power which, especially in nutritional experiments, is of great importance as individual variation of growth performance parameters and feed conversion is high. The present study investigates in the gilthead sea bream *Sparus aurata*, one of the most important Mediterranean aquaculture species. For 24 gilthead sea bream individuals (biological replicates) the effects of gradual substitution of fish meal by plant ingredients (0% (control), 25%, 50% and 75%) in the diets were studied by looking at expression levels of four immune-and stress-related genes in intestine, head kidney and liver. The present results showed that only the lowest substitution percentage is tolerated and that liver is the most sensitive tissue to detect gene expression variations in relation to fish meal substituted diets. Additionally the usage of three independent biological replicates were evaluated by calculating the averages of all possible triplets in order to assess the suitability of selected genes for stress indication as well as the impact of the experimental set up, thus in the present work the impact of FM substitution. Gene expression was altered depending of the selected biological triplicate. Only for two genes in liver (*hsp70* and *tgf*) significant differential expression was assured independently of the triplicates used. These results underlined the importance of choosing the adequate sample number especially when significant, but minor differences in gene expression levels are observed.



## 1. Introduction

For gene expression studies today, a wide range of methods is available. Each of the methods comprises advantages as well as disadvantages. For low throughput expression analysis the use of quantitative PCR (qPCR) has been widely applied and the technique has become an important technology in the area of functional genomics (Vandesompele et al., 2002). In order to obtain statistical significance in qPCR (i) PCR efficiency, (ii) selection of suitable reference genes as well as (iii) the number of biological replicates are of importance (Bustin, 2008). While in most qPCR publications the PCR efficiency as well as the selection of appropriate reference genes are investigated (e.g., De Santis et al., 2011), the number of required biological replicates are not discussed (Karlen et al., 2007; Kitchen et al., 2010). Statistical power in biological as well as in medical studies is, independently of the method used, of great importance (Button et al., 2013). Nevertheless in most cases statistical power remains low, due to low number of independent biological samples, which in the majority of gene expression studies is limited to three as increasing sample size leads to more cost and increased laboratory work. The use of three replicates means that the results have a large confidence range (confidence interval) and a low confidence level. Assuming for example a confidence level of 95% and a confidence interval of 10 the required sample size would be 96. Thus, increasing biological replicates translates into a more effective gain in power, which, especially in nutritional experiments, is of importance as individual variation of growth performance parameters and feed conversion is high (Jobling and Baardvik, 1994; McCarthy et al., 1992). Consequently, fine tuning of expression analysis may be of great importance especially for nutritional studies in aquaculture where fish welfare is highly dependent on adequate feed provision.

Protein requirements in teleost species depend on the availability of protein sources, their amino acid (AA) profile and dietary energy level. For carnivorous fish, fish meal (FM) is the main pro-

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tein source in aquaculture feeds and provides them with nitrogen required for the synthesis of non-essential AA (Bakke, 2011). However, due to the limited availability of FM as well as its high cost, FM replacement levels by plant proteins in feeds have increased steadily over the past years. On the other hand, the use of high levels of plant ingredients as FM alternatives have been proven to affect fish health in several fish species (Lilleeng et al., 2009; Olsen et al., 2007; Urán et al., 2008a,b; Kokou et al., 2012). First evidence of altered immune system in marine fish due to vegetable oils was published in Atlantic salmon (Bransden et al., 2003). Among potential substitutes soy products are the main alternatives added to the fish diet although that, at high plant protein (PP) concentrations in the diets, alterations in the immune system have been observed, as well as significant changes in intestinal morphology and immune-related gene expression levels (Baeverfjord and Krogdahl, 1996; Urán et al., 2008a,b). It is further known that the fish intestine is of importance not only for the absorption of nutrient and osmoregulation, but also for its role as immune tissue preventing certain pathogens to penetrate into the organism (Cain and Swan, 2010; Rombout Jan et al., 2011). Hence, the intestine and particularly the distal intestine is considered as one of the major tissues to investigate the effects of nutrition on the immune system (Baeverfjord and Krogdahl, 1996). For studying possible immune response another important tissue is the head kidney due to the central hematopoetic role (Rise et al., 2004). During the last decade also liver was recognized as a major organ of the immune system due to its key role as a mediator of systemic and local immunity presenting an important site for immune regulation (Castro et al., 2014).

The gilthead sea bream (*Sparus aurata* L.) along with the European sea bass (*Dicentrarchus labrax*) belongs to the main aquaculture species in the Mediterranean. The genomic toolbox of the gilthead sea bream has been significantly enriched in the recent years, facilitating the identification and characterization of genes. Furthermore high throughput studies have been performed in sea bream showing on the one hand that no transcriptome response occurred in the intestine after FM substitution and on the other hand that plant meal substitution influenced the intestine transcriptome after exposure to a bacterial pathogen (Bonaldo et al., 2008; Calduch-Giner et al., 2013; Montero et al., 2010, 2008). Yet, only little is known about how FM substitution affects gene expression of immune related genes. In this line, the present study selected as case study the effects of three diets on distal intestine, head kidney and liver of the gilthead sea bream. The diets comprised gradual fish meal substitution by plant ingredients (0% (control), 25%, 50% and 75%) in 24 individuals per diet. Expression levels of four immune-and stress-related genes i.e. heat shock protein 70 (*hsp70*), beta-2 microglobulin (*b2m*), transforming growth factor beta 1 (*tgf-β1*) and cathepsin S (*catS*) in all 24 biological replicates were assessed in head kidney, intestine and liver. In addition, to investigate the possible usage of only three independent biological replicates the averages of all possible triplets were calculated and the impact of the experimental set up was assessed, i.e. in the present work the impact of FM substitution.

## 2. Methods

### 2.1. Feeding trial: experimental diets and tissue sampling

Animal care was carried out according to the "Guidelines for the treatment of animals in behavioral research and teaching."

The experiment was held in triplicate groups of sea cages ($6 \times 6 \times 6$ m) in the fish farm of Platia (Saronic Gulf). In each cage 5.000 graded fish (A-class) were placed after hand-counted and weighed in the beginning and at the end of the experiment. Average initial weight of the fish was approximately 125 g. To estimate fish weight 10% of the population was weighed in the beginning, while in the end of the experiment fish were harvested and the total population was weighed. The duration of the trial was 236 days (9th September–4th May) and temperature, oxygen concentration and mortalities were recorded daily. Average water temperature was 18.9 °C and the range was 14.6–27.1 °C. Feeding rate was predicted by feeding tables according to the temperature and estimated biomass increase. The diets were produced by BioMar Hellenic S.A. (Volos, Greece) and labeled as "RH" (Research Hellas). Standard bream diet (Efico Sigma 463 4.5 mm) was used as the control diet (20% FM) and compared to three experimental diets with decreasing levels of fishmeal, a diet with 15% FM (RH30), a diet with 10% FM (RH31) and a diet with 5% FM (RH32). Thus, the experimental diets corresponded to 25% (RH30), 50% (RH31) and 75% (RH32) of FM substitution compared to the control diet. Main ingredients are shown in Table 1. The experimental period was 6 months and fish reached the size of 350 g prior to tissue sampling. At the end of the experiment out of 24 fish per dietary treatment (in total 96) the distal intestine, head kidney and liver were removed and stored in RNAlater at −80 °C (in total 288 samples) for gene expression studies.

**Table 1**
Diet composition of the experimental diets.

| Ingredients (g/kg) | Feed code | | | |
|---|---|---|---|---|
| | Control | RH30 | RH31 | RH32 |
| Fish meal (std. 70%) | 200.00 | 140.00 | 90.00 | 40.00 |
| Fish hydrolysate (CPSP 90) | 10.00 | 10.00 | 10.00 | 10.00 |
| Rapeseed oil | 40.00 | 40.00 | 40.00 | 41.00 |
| Soya cake (48%) | 150.00 | 150.00 | 150.00 | 150.00 |
| Soya protein concentrate (60%) | 100.00 | 120.00 | 135.00 | 160.00 |
| Corn gluten (60%) | 162.00 | 196.00 | 235.00 | 265.00 |
| Wheat | 60.22 | 65.03 | 64.58 | 65.49 |
| Sunflower cake (37%) | 150.00 | 150.00 | 150.00 | 150.00 |
| Fish oil std. | 40.00 | 40.00 | 40.00 | 41.00 |
| Fish oil trimmings (salmon) | 79.00 | 80.00 | 81.00 | 81.00 |
| Premix & additives | 6.78 | 12.97 | 18.42 | 23.51 |
| Nutrients | | | | |
| Moisture (%) | 9.14 | 7.77 | 6.87 | 5.69 |
| Protein (%) | 44.00 | 44.00 | 44.00 | 44.00 |
| Fat (%) | 19.00 | 18.82 | 18.57 | 18.40 |
| Ash (%) | 5.48 | 5.33 | 5.16 | 5.03 |
| Cellulose (%) | 4.21 | 4.32 | 4.40 | 4.54 |
| Starch (%) | 5.91 | 6.58 | 7.00 | 7.43 |
| Digestible energy (MJ/kg) | 17.13 | 17.15 | 17.12 | 17.14 |

RH = Research Hellas.

### 2.2. RNA extraction and reverse transcription

RNA was extracted using the Trizol protocol in combination with Lysing Matrix tubes D (Mp Biomedicals, Santa Ana, California, USA). RNA quantity was measured using Nanodrop Spectophotometer (NanoDrop Technologies Inc., Wilmington USA) and quality was assessed by agarose gel as well as by DNAnalyzer (Agilent, Santa Clara, California). 1 μg total RNA was reverse transcripted using reverse transcriptase (200 U/μl) (Invitrogen,/VWR, Tromsø, Norway). Dilutions of 1:50 for each of the 288 samples were used for further analysis.

### 2.3. Gene identification and primer design

Genes involved in immune response were selected based on current literature and their transcripts were retrieved out of the non-redundant (nr) and expressed sequence tags (est) database of NCBI (Table 2). Each sequence was evaluated using Blastx search of NCBI. Primer pairs for qPCR were designed using a combination of different software programs: NetPrimer, Primer3 and Beacon. All primers were standardized and tested by agarose gel and the melting temperature.