



New species for EU aquaculture

Deliverable Report

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Objective: Report on the prevention/treatment of Systemic Granulomatosis in meagre: Based on the results from the study of the systemic granulomas, this deliverable will comprise a set of recommendations for the prevention and treatment of the disease.



One of the objectives of Work Package 24 – Fish health –meagre (WP24) was to test the two hypotheses that have been raised regarding the aetiology of Systemic Granulomatosis (SG) in meagre. The first is that it may be a metabolic disorder similar to the systemic granulomas observed in other cultured fish species, and the second that it is caused by bacterial pathogens most likely *Nocardia* spp.



Metabolic disorder hypothesis

The metabolic disorder hypothesis (Ghittino et al., 2004; Katharios et al., 2011) have been raised due to the similar systemic granulomas observed in other cultured fish species such as gilthead sea bream (*Sparus aurata*) (Paperna, 1987), rainbow trout (*Salmo gairdneri*) (Balouet and Baoudin Laurencin, 1986) and turbot (*Scophthalmus maximus*) (Baudin Laurencin and Messenger, 1991). In all cases, the development of the disease has been associated with nutritional imbalance in minerals and vitamins due to inadequacy of plant protein sources or problems related to long-term stored formulated feeds or frozen fish (Herman, 1996).

On task 24.1 we ran several feeding trials to assess the effect of vitamin D and Ca/P levels in feeds (HCMR), the effect of plant ingredients (HCMR), as well as the effect of minerals and vitamins levels (FCPCT)

Trial 1. The effect of vitamin D₃ inclusions in diets in the development of SG in meagre (HCMR)

For vitamin D₃ trial, four experimental diets with increasing levels of vitamin D₃ were prepared at HCMR (Athens, Greece). Meagre juveniles of 4 g average weight (n=600), produced in May 2014 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the feeding trial. Three replicates were used for each diet. The feeding trial lasted 93 days (July 2014-October 2014). At the end of the feeding trial samples were taken for granuloma evaluation and histology, estimation of specific biomarkers (CYP27, CYP24 enzymes), antioxidant enzymes activity and plasma analysis.

A semi-quantitative method was developed in order to assess fish status regarding the presence of granulomas. The method was based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. Each fish was dissected and internal organs were examined macroscopically. Fresh squash preparations of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney were assessed under a stereoscope. For the general state of each individual, the sum of the scores from the various tissues was calculated. The assessment scale used was according to the following scoring system shown in **Table 1**.

Table 1. The assessment scale used for the evaluation of granulomas.

Score 0	No granulomas
Score 1	Granulomas visible only with microscopy
Score 2	Granulomas visible macroscopically
Score 5	Tissue calcification

Main result: Supplementation with vitamin D₃ had no effect on the development of SG.

The full description of the work and results has been provided in Deliverable 24.1: The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre.

Trial 2. The effect of Ca:P ratio in the diet on the development of SG (HCMR)

For this trial, nine experimental diets with different levels of Ca and P were formulated at the SKRETTING Aquaculture Research Centre (SARC), Norway. The basal diet was formulated to contain about 53% crude protein and 15% crude lipid. P was supplemented separately to the basal diet of the mixture to obtain various concentrations of P, while the amount of Ca that was supplemented in the basal diet was calculated to be either equal or double the amount of P. Meagre juveniles of 1 g average weight (n=1350), produced in May 2015 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the feeding trial. Three replicates were allocated to each diet. The



feeding trial lasted 4 months (July 2015- November 2015). At the end of the feeding trial samples were taken for granuloma evaluation and histology, body and mineral composition, estimation of specific biomarkers (CYP27, CYP24 enzymes) and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in Table 1.

Main result: The high P content in the diets (15 g kg⁻¹) ameliorated the severity of granulomatosis.

The full description of the work and results has been provided in Deliverable 24.2: The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre.

Trial 3. The effect of high plant protein diets in the development of SG (HCMR)

The purpose of the third trial was to examine whether fish meal (FM) replacement by Plant Protein (PP) sources affects the development of SG. Furthermore, due to the results obtained in D24.2 we also investigated whether P supplementation in PP diets has any effect on SG. Four experimental diets were formulated at the SKRETTING Aquaculture Research Centre (SARC) with 60% (FM) and 14% (PP) and increasing levels of P in the diets with 14% fishmeal (PP+medium P, PP+high P). Meagre juveniles of 2 g average weight (n=600), produced in June 2016 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the feeding trial. The feeding trial lasted 3 months (August- November 2016). At the end of the feeding trial samples were taken for granuloma evaluation, histology and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in Table 1.

Main result: Plant proteins in the diets of meagre were found to affect negatively SG while P supplementation in the PP diets did not affect the overall condition but had a positive effect in the liver of the fish.

The full description of the work and results have been provided in Deliverable 24.5: The effect of high plant protein diets in the development of Systemic Granulomatosis in meagre.

Trial 4. The combined effect of vitamins E, C and carotenoids in the development of SG (FCPCT)

Six experimental diets were prepared by adding different levels of vitamin E, C and astaxanthin. Meagre juveniles of 79 g average weight (n= 900) were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain). The feeding trial lasted for 135 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in Table 2. The score was organ dependent, because the number of granulomas in each organ was variable.

Table 2. Severity score of granulomas in liver, kidney and heart

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Main result: The combination of a high dietary content of the antioxidants vitamin E and C ameliorates the severity of SG, decreasing the incidence and number of fish with higher severity of SG.



The full description of the work and results has been provided in Deliverable 24.11: Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis in meagre.

Trial 5. The effect of Zn, Mn and Se in the development of SG. (FCPCT)

Five isolipidic and isoproteic fish meal and fish oil based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se. Meagre juveniles of 15 g average weight (n= 2100) were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain). The feeding trial lasted for 90 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in Table 2.

Main result: The addition of target minerals did not ameliorate the granuloma incidence or severity, but recommended levels of minerals are: 40 mg·kg⁻¹ of Mn, 200 mg·kg⁻¹ of Zn, and 1.5 mg·kg⁻¹ of Se.

The full description of the work and results has been provided in Deliverable 24.11: Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis in meagre.

Pathogen hypothesis

The presence of granulomas in fish is a common immune response to a wide range of stimuli, including bacterial infections such as those caused by *Nocardia* spp. and *Mycobacterium* spp., as well as by the Mesomycetozoon *Ichthyophonus hoferi* (Roberts, 2012). In 2013, there was a report suggesting that the causative agent of SG in meagre was *Nocardia* sp. (Elkesh et al., 2013). Therefore, the aim of task 24.4 was to monitor meagre from various locations in Greece and try to identify and isolate *Nocardia* spp., or other granuloma-associated pathogens and to assess whether these bacteria and fungi represent an actual hazard for the species.

For this purpose a large number of healthy fish and fish exhibiting disease signs from various locations of Greece was sampled and examined using microbiological, molecular and histological techniques to test the “pathogen” hypothesis. General and selective nutrient media were used for isolation of bacteria or fungi. Molecular detection of target pathogens including *Nocardia* spp., *Mycobacterium* spp. and *Ichthyophonus hoferi* was performed with PCR using specifically designed primers. Histology was performed using standard and special staining techniques including Ziehl-Neelsen for acid-fast bacteria and Grocott stain for fungi.

In most fish examined from various locations of Greece, no bacterial growth was observed on the solid media used. In total we purified 25 isolates from various organs. None of the isolated bacteria had phenotypes consistent to *Nocardia* spp. Sequencing confirmed that none of the isolates belonged to the *Nocardia* genus. Moreover, the identified bacteria have not been reported as causative agents of disease and thus they are more likely environmental strains. In addition to the bacteria isolated in solid media, PCR analysis was performed directly on SG-affected tissues and organs using specific primers against the suspected pathogens, *Nocardia* spp., *Mycobacterium* spp., and *Ichthyophonus hoferi*. All samples assayed with this method were negative for all 3 pathogens surveyed, except 2 fish from West Greece which were PCR-positive for *Nocardia* in 4 out of the 6 different organs examined. For these samples, 16s rRNA sequencing showed 100% identity with *Nocardia seriola*. Histological analysis of the *Nocardia*-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* spp. in meagre (Elkesh et al., 2013). Ziehl-Neelsen stain was weakly positive in the colonies located in the skin lesions. The bacterial colonies were not demarcated by a granulomatous formation. Granulomas consistent to SG not containing bacteria were simultaneously present in all tissues examined and were distinctively different.

The full description of the work and results has been provided in ***Deliverable 24.4: Isolation and characterization of Nocardia from infected meagre.***



General conclusions and recommendations for SG in meagre

- Nocardiosis is present in Greece, most probably in a confined geographical region; however it is not the cause of SG.
- Vitamin D₃ supplementation did not affect the development of the SG.
- High P content in the diet seems to improve the condition.
- Plant protein replacement affects negatively the progression of the SG.
- High dietary content of the antioxidants vitamin E and C ameliorates the severity of SG, decreasing the incidence and number of fish with higher severity of SG.
- The addition of Zn, Mn and Se did not ameliorate the granuloma incidence or severity.

Taken together, the improvement of SG by a change in the diet together with the absence of pathogens in SG-affected population suggests that the metabolic hypothesis is more probable. The occurrence of only a single case of nocardiosis with different characteristics enforces this hypothesis.

However, the aetiology is still unknown and other nutritional metabolic factors have to be tested.

Taking into account all the above results our recommendations for prevention of SG in meagre are:

- A combined diet with high percentage of fishmeal (60%), high dietary content of P (15 g kg⁻¹) and high content of antioxidants vitamins E and C.
- Since there is no data available about the reversibility of SG, we recommend to start feeding with this diet when the fish weight is about 2 g.

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