RECENT ADVANCES IN THE STUDY OF SYSTEMIC GRANULOMATOSIS IN MEAGRE (*ARGYROSOMUS REGIUS*)

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Introduction

One of the most important bottlenecks of meagre (*Argyrosomus regius*) production is Systemic Granulomatosis (SG), a pathological condition affecting the majority of farmed populations. SG is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. The aetiology of the disease is unknown, however two hypotheses have been raised. The first is that it may be a metabolic disorder (Katharios et al., 2011) similar to systemic granulomas observed in other cultured fish species (Herman, 1996) and the second hypothesis is that it is caused by bacterial pathogens most likely *Nocardia* spp. (Elkesh et al., 2013). The aim of this study was to test these two hypotheses; for this we have run 3 feeding trials to identify potential nutritional causes of SG and monitored meagre populations farmed in various locations in Greece over the past 3 years in order to isolate and identify *Nocardia* spp., or other granuloma-associated pathogens.

Materials and methods

Three different feeding trials were conducted to assess the effect of vitamin D_3, Ca/P levels and plant ingredients in the feeds in the development of SG. For the vitamin D_3 trial, four experimental diets with increasing levels of vitamin D_3 were prepared at HCMR (Athens, Greece). For Ca/P trial, nine experimental diets with different levels of Ca and P were formulated at SKRETTING Aquaculture Research Centre (SARC), Norway, while for the trial with plant ingredients four experimental diets were prepared at SARC, with 60% and 14% fishmeal and increasing levels of P in the diets with 14% fishmeal. For each trial we used 3-month old (0.5-2 g) meagre produced at HCMR (Crete, Greece). The fish were placed into 500-l cylindrical tanks at an initial density of 50 fish per tank using three replicates for each diet. Every trial lasted approximately 3 months. Granulomatosis was assessed using a semi-quantitative ordinal-scale scoring system. Internal organs were dissected, examined macroscopically and assessed under a stereoscope. General state of each individual was assessed by the sum of the individual organs' scores and blood plasma biochemistry. In addition, expression of genes related to vitamin D_3 metabolism and antioxidant stress was measured with qPCR. 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.

Results

Histologically, several stages of the granuloma formation were identified, ranging from immature granulomas, multilayer mature granulomas to big areas of dystrophic calcification circumscribed by fiber tissue. In several cases, the initial stages of the granulomas were located at the blood vesicles resembling vasculitis. In most cases, there was an involvement of rodlet cells which were present in large numbers in all tissues. The overall pathology was not different to that described by Katharios et al., (2011). SG was not prevented either by vitamin D_3 or the different Ca:P ratio. On the other hand, high P content diets (15 gkg^{-1}) were found to improve the condition of granulomatosis. The overall condition in terms of the sum of the individual organs' scores of the fish was significantly better in the group fed the high P content while plant proteins were found to negatively affect SG. Fish fed 60% fishmeal were in significantly better state regarding the total score of granulomas in all tissues. Phosphorus supplementation (14 gkg^{-1}) in the plant proteins diets did not affect the overall condition of the fish. 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 Astakos, 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 seriolae*. 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.

Conclusions

Vitamin D_3 supplementation did not affect the development of the disease, high P content in the diet seems to improve the condition and plant protein replacement affects negatively the progression of the disease. Taken together the improvement of SG by change in the diet with the absence of pathogens in SG-affected population we believe 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.

References

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