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Histological assessment of Systemic Granulomatosis progression in meagre (Argyrosomus regius) during cage ongrowing phase

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THIS IS THE PEER REVIEWED VERSION OF THE FOLLOWING ARTICLE: **HISTOLOGICAL ASSESSMENT OF SYSTEMIC GRANULOMATOSIS PROGRESSION IN MEAGRE** (*ARGYROSOMUS* REGIUS) DURING CAGE ONGROWING PHASE WHICH HAS BEEN PUBLISHED IN FINAL FORM AT *DISEASES OF AQUATIC ORGANISMS* DOI: doi.org/10.3354/dao03606. THIS ARTICLE MAY BE USED FOR NON-COMMERCIAL PURPOSES IN ACCORDANCE WITH WILEY TERMS AND CONDITIONS FOR USE OF SELF-ARCHIVED VERSIONS.

1	Note
1	Note

2	Histological assessment of Systemic Granulomatosis progression in meagre (Argyrosomus
3	regius) during cage ongrowing phase.
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14	
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16 17 18 19	Running head: Systemic granulomatosis in meagre. Abstract: Meagre (<i>Argyrosomus regius</i>) is a potential candidate for Mediterranean aquaculture
20	diversification, although several health issues still represent important bottlenecks for its sustainable
21	production, including the Systemic Granulomatosis (SG). To evaluate the SG progression in meagre
22	during a ten-month period of cage ongrowing, a histopathological investigation has been carried out
23	on 108 meagre fed three different diets (commercial pellet, hydrated commercial pellet and defrosted
24	sardines). Histological sections of gills and visceral organs were examined and lesions referable to
25	SG scored from 1 to 3 according to the severity of the granulomatosis. The kidney and the liver were
26	the most affected organs, showing highest percentage of positivity for granulomas and severity of the
27	lesions along the whole observation period. By statistical analysis, an effect of diet and temperature
28	was found using a statistical mixed model (GLMM) followed by odds ratio analysis: the severity of

liver and digestive tract SG scores decreased in Cage 3 group (defrosted sardines), and with the increasing of temperature (p<0.05, negative estimates, and odds ratio <1). These observations, in accordance with literature, suggest that SG in meagre could be related to nutritional-metabolic factors with the possible influence of environmental factors such as temperature.

5

6 Keywords: systemic granulomatosis; meagre; Argyrosomus regius; aquaculture

7

8 **1. Introduction**

9 The progressive global unsustainability of fishery has promoted the increase of aquaculture 10 (Harvey et al. 2017, FAO 2020). Species diversification represents an important factor for replying 11 to market saturation and overproduction of the species currently farmed worldwide. In the 12 Mediterranean Sea, several new fish species have been subjected to farming trials to provide 13 alternatives, encountering many zootechnical and health issues (Harvey et al. 2017, FAO 2020, 14 https://www.diversifyfish.eu/about-diversify.html). Among others, meagre (Argyrosomus regius) is 15 one of the most promising fish species as candidate to be farmed on a large-scale production in the 16 Mediterranean (Monfort 2010, https://www.diversifyfish.eu/about-diversify.html), based on its rapid 17 growth and robustness. Although the productive performances of this fish species seem to be very 18 interesting, currently there are still several nutritional, environment-dependent and health bottlenecks 19 to be solved for optimization of meagre production. The Systemic Granulomatosis (SG) represents a 20 threat of primary importance, showing high prevalence rate (up to 100% in several farms), high 21 morbidity and low mortality rates (Katharios et al. 2011, Tsertou et al. 2018). Fish affected by SG 22 show growth reduction and nodular lesions in visceral organs, sometimes so evident making fish 23 unacceptable for consumers (Soares et al. 2018). Multiple granulomas in internal organs of affected 24 meagre are described in the few reports available in literature (Ghittino et al. 2004, Katharios et al. 25 2011, Tsertou et al. 2018, Ruiz et al. 2019a, Carvalho et al. 2019, Tsertou et al. 2020). The 26 etiopathogenesis influencing SG occurrence in the meagre is not yet completely clarified, although a nutritional/metabolic origin has been proposed (Katharios et al. 2011, Tsertou et al. 2018; Ruiz et al.
 2019a, Ruiz et al. 2019b, Ruiz et al. 2019c), as already assessed for SG in farmed sparids (Paperna
 1987, Colorni & Padrós 2011).

In order to study the progression of SG in farmed meagre during a cage ongrowing period in
relation to feed and temperature a histopathological investigation has been carried out in a Croatian
farm where outbreaks of SG had been observed in farmed meagre during the two previous production
years.

8

9 2. Materials and Methods

10 Twenty thousand fry (mean weight 6 g) coming from a foreign hatchery were divided into two cages of 225 m³ located 150 meter from coast, and having 20 m of water column from sea bed. with 11 12 10,000 thousand fry each and fed respectively a commercial pellet formulated for meagre (Cage 1) 13 or the same pellet but hydrated with 10% of water (Cage 2) from August 2015 to May 2016. Ten fry 14 were fixed in 10% neutral buffered formalin before their stocking in the cages. The gross composition 15 of the commercial pellet is reported in Table 1. In December 2015, 500 meagre were transferred from 16 Cage 1 into a third close cage (Cage 3) to be fed defrosted sardines (Sardina pilchardus) from Adriatic 17 Sea for the last 6 months left. The purpose was to have a third extemporaneous diet protocol for 18 studying SG evolution in relation to a natural diet in the winter ongrowing period. The third cage was 19 set only after 4 months to have meagre able to eat sardines.

The feeding protocol was consistent with a normal farm production cycle for meagre, thus no real experimental setting and no replicates were done. The field nature of this experiment did not permit to set diet replicates. The three cages were all in the same environmental conditions and the fish coming from the same hatchery batch; the age of fish was thus the same among the three cages at starting of the trial. Thus, it would be expected minimal differences in presentation of lesions between cages outside of diet. The only difference among cages was the administration of different diet protocols *ad libitum*.
 Cage 3 was fed the control diet of Cage 1 before the starting of the sardines feeding, as the size of
 fishes did not permit this type of feeding before.

During the 10-month-feeding period, a total of 108 meagre were randomly sampled monthly from each cage (Table 2) and subjected to necropsy on-site. Temperature and mortality were daily collected. Portions of gills and visceral organs (liver, stomach and intestine, kidney, spleen, heart) were fixed in 10% neutral buffered formalin. Samples were then processed for routine histology to obtain 5-μm-thick sections. Different staining techniques were performed to exclude other possible etiological causes of the granulomatous lesions observed: Hematoxylin and Eosin (HE), Periodic Acid Schift (PAS), Giemsa (G), and Ziehl-Nielsen (ZN), and Fite-Faraco (FF).

11 The severity of granulomatosis (SG) was scored for each organ following indications provided 12 by Gibson-Corley et al. (2013) as: mild granulomatosis (score 1) = 1-3 granulomas observed in the 13 whole tissue section; moderate granulomatosis (score 2) = 4–6 granulomas observed in the whole 14 tissue section; severe granulomatosis (score 3) = >6 granulomas observed in the whole tissue section. 15 Statistical analysis was performed with R software (Version 3.6.1). Data were firstly tested for 16 normality by Shapiro-Wilk test. A Generalized Linear Mixed-Effects Models (GLMM) was used to 17 test the effect of diet (Cage), temperature and age on the SG score. In the model, the SG score was 18 used as dependent variable, the diet (Cage) and the temperature as fixed factors, and the age as random 19 effect, following the procedures by Vitali et al. (2020). The GLMM was then followed by odds ratio 20 analysis. Differences were considered statistically significant with p < 0.05.

21

22 **3. Results**

Mortality ranged from 3.69 to 8.47% during the study period and did not differ among diet groups. The temperature ranged from 24.66 °C in August as highest value to 13.27 °C in March as lowest value (Table 2). The juvenile meagre sampled for histology upon arrival at the farm already presented scattered multifocal granulomas in different organs, mainly in the kidney. Out of the totality of sampled meagre (108) during the entire study period, granulomas were observed in 101 (93.5%) fish. The granulomas were observed in the different organs with the following frequency: kidney 93.5%, liver 39.8%, digestive tract (stomach, intestine, pancreas and perivisceral fat) 13.0%, heart 9.3%, spleen 1.9%, gills 0.9%.

All the lesions observed in all animals and during the entire trial period were referred to Systemic
Granulomatosis (SG) syndrome, since no acid-fast bacteria nor fungi or other etiological agents were
observed within the granulomas by the specific staining methods used at histological examination.

10 The granulomatous inflammation had different severity scores in each organ examined: 11 granulomas were either focal or multifocal to coalescing or disseminated throughout the whole 12 section, representing a systemic granulomatous inflammation (Fig. 1-3). In the kidney, especially, the 13 area occupied by the granulomatous inflammation reached the 50% of the whole section in the most 14 severe cases.

15 In general, granulomas appeared in various stages of development. Morphologically, the 16 granulomas varied in size and complexity: in early stages, they were composed mainly by aggregates 17 of epithelioid histiocytes and macrophages arranged in concentric layers, and few peripheral 18 lymphocytes, plasma cells and mast cells but no fibroblasts at the periphery (Fig. 4a). In more 19 advanced lesions, the central core of the granuloma was often characterized by coagulative or 20 colliquative necrosis with presence of amorphous eosinophilic material and karyorrhectic debris (Fig. 21 4b), and granulomas became surrounded by numerous concentric layers of fibroblasts (Fig. 4c), which 22 in some cases produced thick bands of collagen (fibrosis). In severe cases, granulomas became 23 coalescent with the formation of large inflammatory foci (Fig. 4d).

By considering each organ separately, in the kidney the center of several granulomas was characterized by granular basophilic aggregates corresponding to dystrophic mineralization (Fig. 4c); in the liver, a macrovacuolar steatosis was also constantly observed and sometimes the granulomas were centered on pancreas glandular tissue (Fig. 2); in the gastrointestinal tract, granulomas were observed in lamina propria, submucosa, *tunica muscolaris*, and frequently in the associated perivisceral fat (Fig. 3); in the cardiac muscle, granulomas were uncommon, and mainly located in the ventricle; the spleen was rarely affected and granulomas were observed in two fish only. In the gills, only one granuloma in a single fish was observed.

6 GLMM statistical analysis followed by odds ratio showed a significant effect of both diet (Cage)

7 and temperature on liver and digestive tract SG scores, as presented in Table 3. For both organs, the

8 severity of SG scores decreased in the group fed defrosted sardines (Cage 3) and with the

9 increasing of temperature (p < 0.05, negative estimates, and odds ratio <1), as reported in Table 3.

10

11 **4. Discussion**

12 The Systemic Granulomatosis (SG), already well known in farmed gilthead seabream Sparus 13 aurata (Paperna et al. 1980, Paperna 1987), is currently recognized as an important disease also in 14 farmed meagre and it represents a limiting factor for the promising production of this fish species 15 (Ghittino et al. 2004, El-Shebly et al. 2007, Monfort 2010, Kružić et al. 2016). Similar non-infectious 16 syndromes have been reported also in salmonids (Herman 1996, Good et al. 2016) and turbot 17 Scophthalmus maximus (Tixerant 1984). In gilthead seabream and turbot SG syndrome, a diet-related 18 hypertyrosinemia causing needle-like tyrosine crystals deposit within granulomas has been described 19 related to vitamin C deficiency (Paperna et al. 1980, Tixerant 1984, Baudin Laurencin et al. 1989). 20 Recent literature has suggested vitamins deficiency as possible nutritional cause of SG also in meagre 21 (Ruiz et al. 2019a). Furthermore, Carvalho et al. (2019) found that high levels of n-3 long chain PUFA 22 (up to 3.5%) in diet of meagre fingerlings decreased the incidence of hepatic granulomatosis and 23 steatosis, thus suggesting a possible relation between essential fatty acid deficiency and hepatic 24 granulomatosis in this species. Tsertou et al. (2020) demonstrated that granulomatosis in juveniles 25 meagre was improved by high fishmeal (60%) and high P dietary content (15 g/kg) with respect to 26 plant proteins-based diets. This seems to support our results where the diet made of defrosted sardines 1 (Cage 3) seemed to improve the SG score of the liver and the digestive tract with respect to the
2 commercial pellet diet (Cage 1) having only 25% fishmeal and 6.5% fish oil.

3 At histology, the lesions observed in the current study are similar to those reported by Ruiz et al. 4 (2019a) but differ from the ones described by Ghittino et al. (2004), who observed granulomas mostly 5 localized in the perivascular areas of the various visceral organs, associated with a peripheral 6 inflammatory reaction, hemorrhaging, and not surrounded by a connective capsule. In our study, the 7 kidney and the liver were the most affected organs, showing highest percentage of positivity for 8 granulomas and severity of the lesions along the whole observational period, in line with the previous 9 reports on meagre (Tsertou et al. 2018). However, SG score in kidney seemed not to be improved by 10 a particular feed, whereas the liver and the digestive tract (including perivisceral fat) seemed to be 11 affected by the diet, particularly defrosted sardines.

These findings could be explained by the fact that the administration of defrosted sardines, similar to wild meagre natural feeding, could have a beneficial effect on meagre nutrition and metabolism and, thus, mitigate the pathological changes established in the early stages of life. Other authors, in fact, reported that the onset of granulomatosis in meagre starts from the larval stage as result of nutritional deficiency or imbalance (Ruiz et al. 2019b, Ruiz et al. 2019c).

Regarding a possible relationship with the temperature, the results obtained in this study confirm the presence of a significant effect of the temperature on the severity of SG in liver and digestive tract, particularly the increasing of temperature was inversely correlated with the SG severity. At this regard, the literature hypothesized a possible metabolic distress in meagre under low temperature as observed in a study by Antonopoulou et al. (2020), where higher temperature (23-26°C) seemed to improve growth parameters and to lower several markers of oxidative stress (i.e. the Heat Shock Proteins) with a tissue-specific response.

The results taken together seem to highlight a role of temperature and diet on the pathogenesis of granulomatosis in meagre; the liver and the digestive tract were found the most affected by temperature and diet, leading to support the hypothesis of a metabolic dysfunction under thiscondition in meagre.

Although recent studies (Ruiz et al. 2019a, Ruiz et al. 2019b, Ruiz et al. 2019c) have demonstrated that SG in meagre can be mitigated since the larval stage by applying a proper feeding sequence and high dietary antioxidants, this pathology is still considered one of the main health issues in farmed meagre (Soares et al. 2018), especially when the fish are placed in the on-growing sectors with a SG already established since the early larval phase as observed in this study.

8 Despite some limits, the absence of diet replicates due to the field nature of the trials, the present 9 histopathological study aims to analyze the evolution of Systemic Granulomatosis in farmed meagre 10 under the influence of different factors like diet and temperature in different feeding regimes of a 11 normal on-growing production cycle, giving a contribution to better understand the course of this 12 important disease in field conditions. . The three cages were all in the same environmental conditions, 13 the fish coming from the same hatchery batch, and the age of fish was the same among the three cages 14 at starting of the trial, thus it would be expected minimal differences in presentation of lesions 15 between cages outside of diet. Further systematic studies are needed to increase the knowledge about 16 this health issues and overcome the SG as a possible bottleneck in the production of this fish species.

17

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- 12 **Table 1.** Gross composition of commercial pellet for meagre.

Nutrients	Content
Crude proteins (%)	48.0
Crude lipids (%)	16.0
Nitrogen free extract (%)	19.6
Crude cellulose (%)	2.6
Ashes (%)	8.1
Total phosphorus (%)	1.1
Gross energy (MJ/Kg)	20.8
Digestible energy (MJ/Kg)	18.7
Digestible Protein/Digestible Energy (g/MJ)	24.8
Added Vitamin A (I.U/Kg)	15000
Added Vitamin D3 (I.U/Kg)	1500
Added Vitamin E (mg/Kg)	260

Table 2. Number of meagre sampled from each cage, mean weight per sampling time, and

Sampling date	Temperature °C	N.	fish/	cage	e Mean weight (g)			
		1	2	3	1	2	3	
03/08/15	24.66	3	3	-	22.2 ± 5.8	18.9 ± 5.2	-	
02/09/15	23.10	3	3	-	50.7 ± 15.1	43.9 ± 15.3	-	
02/10/15	19.98	3	3	-	81.5 ± 30.0	63.2 ± 24.9	-	
02/11/15	18.43	3	3	-	100.3 ± 42.8	103.5 ±33.9	-	
04/12/15	16.28	4	4	4	158.2 ± 54.3	127.4 ± 45.9	141.7 ± 61.6	
07/01/16	14.40	4	4	4	153.4 ± 60.9	139.0 ± 55.8	209.9 ± 94.3	
10/02/16	13.38	5	5	5	171.5 ± 64.9	139.9 ± 55.5	225.2 ± 76.5	
11/03/16	13.27	5	5	5	194.4 ± 78.1	142.6 ± 59.5	259.3 ± 91.0	
08/04/16	15.33	5	5	5	172.6 ± 82.8	148.9 ± 64.4	283.0 ± 91.5	
06/05/16	17.21	5	5	5	195.1 ± 76.9	191.13 ± 69.3	350.1 ± 118.2	

2 registered temperature (- = animal not sampled since the cage 3 was not present yet).

Table 3. Significant effect of diet (Cage) and temperature on SG scores.

		Die	et (Cage)		Temperature			
	Estimate	P-value	OR	CI 97.5%	Estimate	P-value	OR	CI 97.5%
Liver	-0.54504 (Cage 3)	0.0453	0.5798185	0.9885318	-0.25112	0.0023	0.7779324	0.9141558
Digestive tract	-1.9954 (Cage 3)	0.0081	0.1359647	0.5948054	-0.5274	0.0032	0.5901422	0.8378513

2 OR=odds ratio; CI= confidence interval; NS=no significance.





Figure 1. (a) Kidney, score 1. Three granulomas characterized by concentric layers of epithelioid
cells are evident in the renal interstitial tissue. Bar = 100µm. (b) Kidney, score 2. Four
granulomas showing central dystrophic mineralization in three of them. Bar = 100µm. (c)
Kidney, score 3. Disseminated granulomas (up to 11) throughout the entire section. Bar = 500µm.
(d) Kidney, score 3. Large granulomatous inflammatory foci. Granulomas are coalescing, with
large central necrosis and circumscribed by several concentric layers of fibroblast and collagen
bands. Bar = 500µm. H&E stain.



Figure 2. (a) Liver, score 1. A single granuloma in the liver parenchyma which shows also a
diffuse macrovacuolar steatosis. Bar = 100µm. (b) Liver, score 1. Three granulomas showing
several concentric layers of epithelioid cells and fibroblasts. Bar = 100µm. (c) Liver, score 3.
Disseminated to coalescing granulomas with central necrosis. Bar = 500µm. (d) Liver, score 3.
Disseminated to coalescing granulomas also centered around biliary ducts. Bar = 500µm. H&E
stain.



Figure 3. (a) Digestive tract, score 1. A single granuloma in the intestinal submucosa. Bar = 500 μ m. (b) Digestive tract, score 1. Two coalescing granulomas in the lamina propria. Bar = 100 μ m. (c) Digestive tract, score 1. A single granuloma in the gastric mucosa. Bar = 100 μ m. (d) A single granuloma in the intestinal *tunica muscolaris*. Bar = 100 μ m. (e) Digestive tract, score 2. Multiple granulomas located in the gut-associated perivisceral fat. Bar = 100 μ m. (f) Digestive tract, score 1. A single early phase granuloma on perivisceral pancreatic tissue. Bar = 100 μ m.



2 Fig. 4: A) Heart: granuloma at early stage with eosinophilic amorphous necrotic center surrounded 3 by epithelioid cells; B) Kidney - granuloma with coagulative necrotic center characterized by 4 karyorrhectic cells surrounded by epithelioid cells, lymphocytes and, externally, fibroblasts; C) 5 Kidney: granuloma with central core of necrotic tissue debris and basophilic matter referable to 6 calcifications surrounded by concentric layers of epithelioid cells, macrophages, fibroblasts and peripherally located lymphocytes and eosinophilic granular cells / mast cells; D) Liver: two 7 8 coalescing granulomas surrounded by thick layers of concentric cellular layers of fibrous connective 9 tissue presenting an onion-skin appearance (H&E). Scale bars: 200µm

- 10
- 11