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

Note

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

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Running head: Systemic granulomatosis in meagre.

Abstract: Meagre (*Argyrosomus regius*) is a potential candidate for Mediterranean aquaculture diversification, although several health issues still represent important bottlenecks for its sustainable production, including the Systemic Granulomatosis (SG). To evaluate the SG progression in meagre during a ten-month period of cage ongrowing, a histopathological investigation has been carried out on 108 meagre fed three different diets (commercial pellet, hydrated commercial pellet and defrosted sardines). Histological sections of gills and visceral organs were examined and lesions referable to SG scored from 1 to 3 according to the severity of the granulomatosis. The kidney and the liver were the most affected organs, showing highest percentage of positivity for granulomas and severity of the lesions along the whole observation period. By statistical analysis, an effect of diet and temperature 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.

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

1. Introduction

The progressive global unsustainability of fishery has promoted the increase of aquaculture (Harvey et al. 2017, FAO 2020). Species diversification represents an important factor for replying to market saturation and overproduction of the species currently farmed worldwide. In the Mediterranean Sea, several new fish species have been subjected to farming trials to provide alternatives, encountering many zootechnical and health issues (Harvey et al. 2017, FAO 2020, https://www.diversifyfish.eu/about-diversify.html). Among others, meagre (*Argyrosomus regius*) is one of the most promising fish species as candidate to be farmed on a large-scale production in the Mediterranean (Monfort 2010, https://www.diversifyfish.eu/about-diversify.html), based on its rapid growth and robustness. Although the productive performances of this fish species seem to be very interesting, currently there are still several nutritional, environment-dependent and health bottlenecks to be solved for optimization of meagre production. The Systemic Granulomatosis (SG) represents a threat of primary importance, showing high prevalence rate (up to 100% in several farms), high morbidity and low mortality rates (Katharios et al. 2011, Tsertou et al. 2018). Fish affected by SG show growth reduction and nodular lesions in visceral organs, sometimes so evident making fish unacceptable for consumers (Soares et al. 2018). Multiple granulomas in internal organs of affected meagre are described in the few reports available in literature (Ghittino et al. 2004, Katharios et al. 2011, Tsertou et al. 2018, Ruiz et al. 2019a, Carvalho et al. 2019, Tsertou et al. 2020). The 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.

2. Materials and Methods

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

The severity of granulomatosis (SG) was scored for each organ following indications provided by Gibson-Corley et al. (2013) as: mild granulomatosis (score 1) = 1–3 granulomas observed in the whole tissue section; moderate granulomatosis (score 2) = 4–6 granulomas observed in the whole tissue section; severe granulomatosis (score 3) = >6 granulomas observed in the whole tissue section.

Statistical analysis was performed with R software (Version 3.6.1). Data were firstly tested for normality by Shapiro-Wilk test. A Generalized Linear Mixed-Effects Models (GLMM) was used to test the effect of diet (Cage), temperature and age on the SG score. In the model, the SG score was used as dependent variable, the diet (Cage) and the temperature as fixed factors, and the age as random effect, following the procedures by Vitali et al. (2020). The GLMM was then followed by odds ratio analysis. Differences were considered statistically significant with $p < 0.05$.

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.

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

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

GLMM statistical analysis followed by odds ratio showed a significant effect of both diet (Cage) and temperature on liver and digestive tract SG scores, as presented in Table 3. For both organs, the severity of SG scores decreased in the group fed defrosted sardines (Cage 3) and with the increasing of temperature ($p<0.05$, negative estimates, and odds ratio <1), as reported in Table 3.

4. Discussion

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

(Cage 3) seemed to improve the SG score of the liver and the digestive tract with respect to the commercial pellet diet (Cage 1) having only 25% fishmeal and 6.5% fish oil.

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

Despite some limits, the absence of diet replicates due to the field nature of the trials, the present histopathological study aims to analyze the evolution of Systemic Granulomatosis in farmed meagre under the influence of different factors like diet and temperature in different feeding regimes of a normal on-growing production cycle, giving a contribution to better understand the course of this important disease in field conditions. . The three cages were all in the same environmental conditions, the fish coming from the same hatchery batch, and the age of fish was 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. Further systematic studies are needed to increase the knowledge about this health issues and overcome the SG as a possible bottleneck in the production of this fish species.

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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

Added Vitamin C (mg/Kg)	150

1

2

Table 2. Number of meagre sampled from each cage, mean weight per sampling time, and registered temperature (- = animal not sampled since the cage 3 was not present yet).

Sampling date	Temperature °C	N. fish/cage			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

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

	Diet (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

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

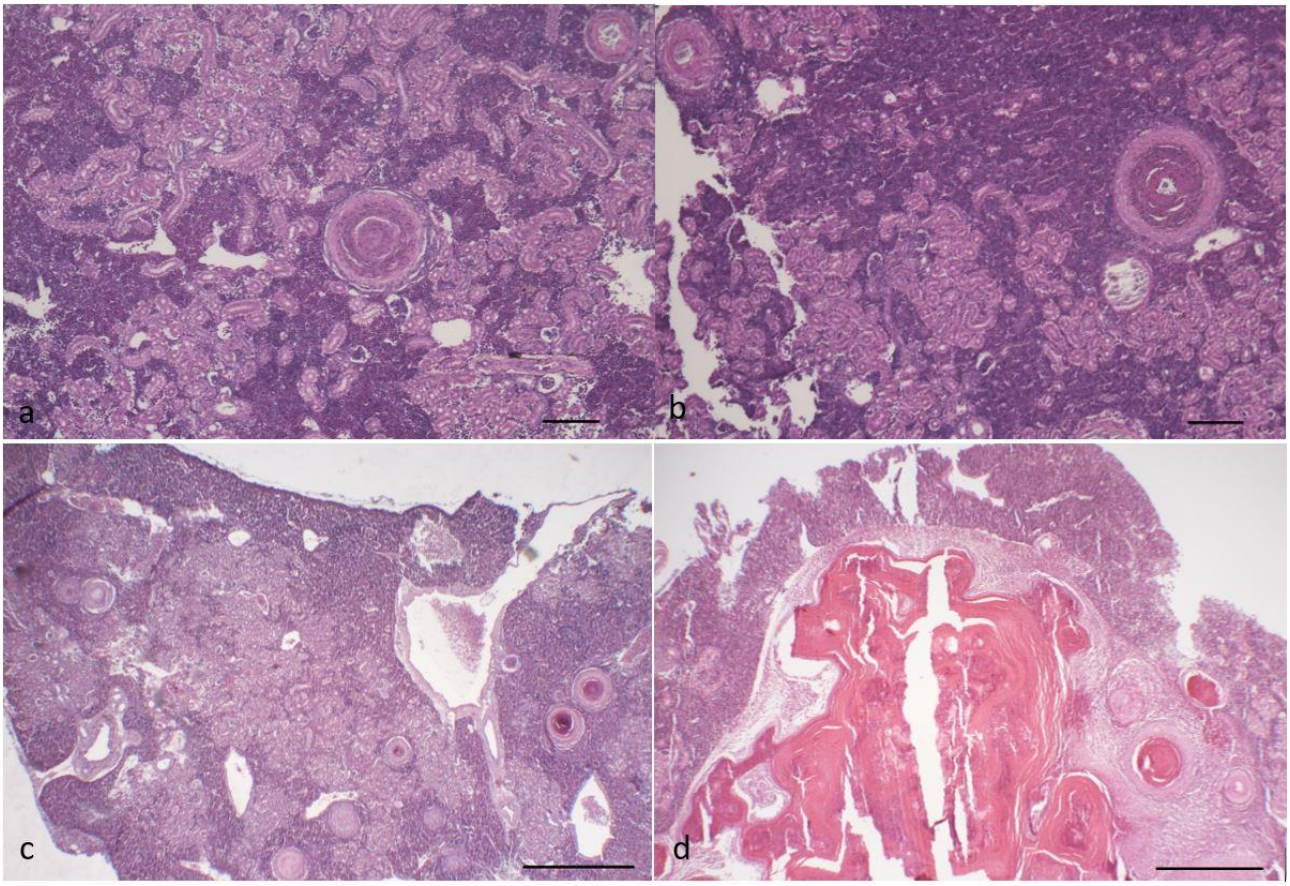

Figure 1. (a) Kidney, score 1. Three granulomas characterized by concentric layers of epithelioid cells are evident in the renal interstitial tissue. Bar = 100µm. (b) Kidney, score 2. Four granulomas showing central dystrophic mineralization in three of them. Bar = 100µm. (c) Kidney, score 3. Disseminated 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.

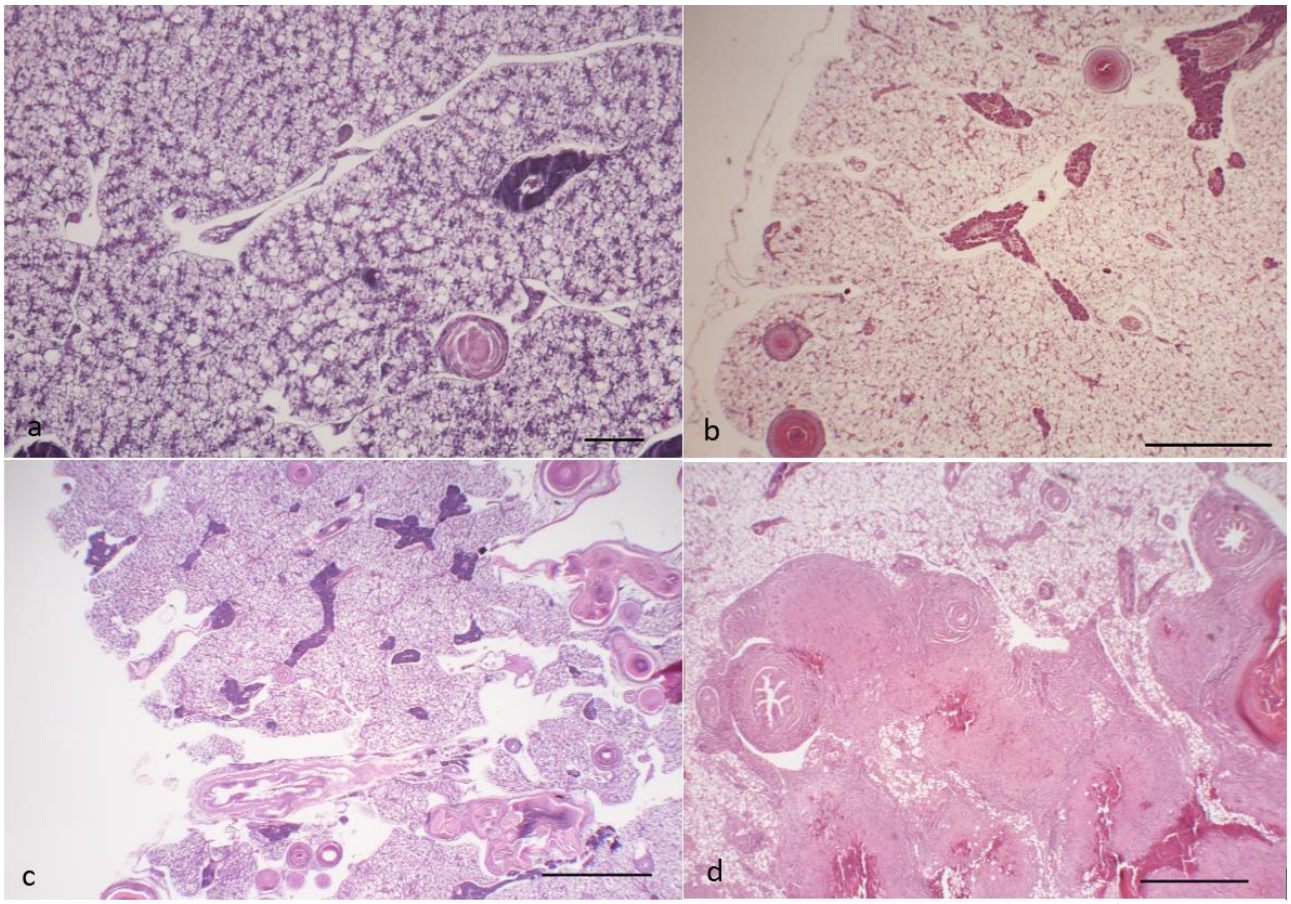

Figure 2. (a) Liver, score 1. A single granuloma in the liver parenchyma which shows also a diffuse macrovacuolar steatosis. Bar = 100µm. (b) Liver, score 1. Three granulomas showing several concentric layers of epithelioid cells and fibroblasts. Bar = 100µm. (c) Liver, score 3. Disseminated to coalescing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

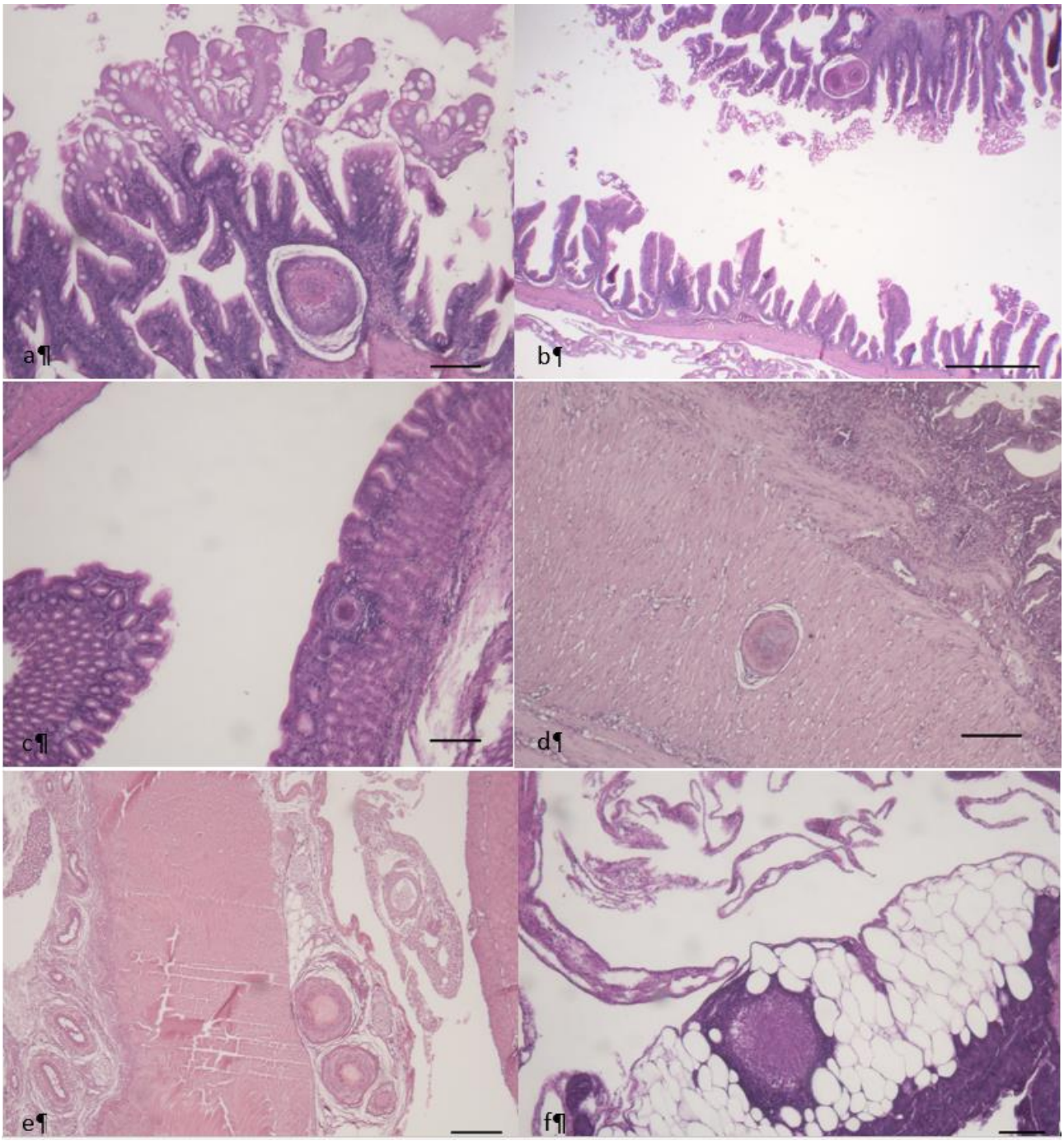

Figure 3. (a) Digestive tract, score 1. A single granuloma in the intestinal submucosa. Bar = 500µm. (b) Digestive tract, score 1. Two coalescing granulomas in the lamina propria. Bar = 100µm. (c) Digestive tract, score 1. A single granuloma in the gastric mucosa. Bar = 100µm. (d) A single granuloma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

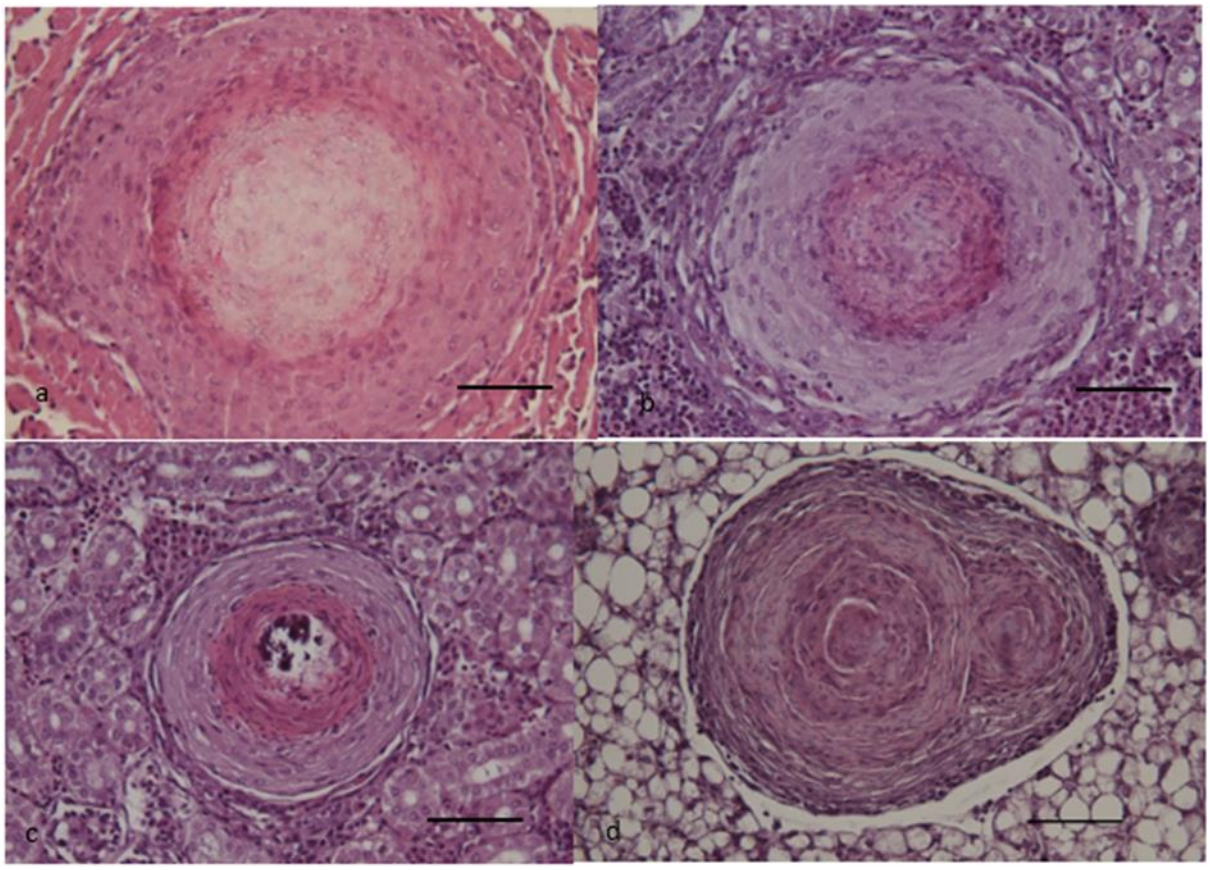

Fig. 4: A) Heart: granuloma at early stage with eosinophilic amorphous necrotic center surrounded by epithelioid cells; B) Kidney - granuloma with coagulative necrotic center characterized by karyorrhectic cells surrounded by epithelioid cells, lymphocytes and, externally, fibroblasts; C) Kidney: granuloma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