Deliverable Report

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Objective: The objective of this task is to investigate the possible causes of Systemic Granulomatosis. This report refers to the effect of Ca:P ratio in the diet on the development of Systemic Granulomatosis in meagre (trial 2).

Introduction

Inorganic elements or minerals are required to maintain metabolism and growth in fish. In contrast to most terrestrial animals, fish can absorb a number of inorganic elements like calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe), zinc (Zn), copper (Cu) and selenium (Se) from their external aquatic environment in both freshwater and seawater. Phosphates and sulphates, however, are more effectively obtained from feed sources (NRC 1993; Lall 2002).

Minerals, based on relative dietary requirements, are classified into two groups: macro or major minerals that are required in large amounts (from a few tenths of a gram to over a gram per day), and micro or trace minerals that are required in very small amounts (from micrograms to milligrams per day) (Webster & Chhorn 2015). Their main functions include the formation of skeletal structure, electron transfer, regulation of acid-base equilibrium and osmoregulation. Minerals are also important components of organic compounds such as proteins and lipids, and act as enzyme activators (NRC 1993; Lall 2002; Webster & Chhorn 2015).

Ca and phosphorus (P) are closely related to the development and maintenance of the skeletal system but they are also involved in various physiological processes including the maintenance of acid-base equilibrium (Lall 2002). Ca is one of the most abundant cations in the body of a fish. Ca requirement

of fish is mainly fulfilled by direct absorption from the aquatic environment. In terrestrial animals, bone is the major site of Ca regulation, but in fish gas exchange across gills, fins and oral epithelia provides them with continuous access to an unlimited Ca reservoir. From those structures, gills are considered the most important site for calcium regulation in both freshwater and marine fish (Flik et al. 1995; NRC 1993; Lall 2002). Furthermore, fish scales are an important site of Ca metabolism and deposition (Lall 2002). In addition to bone formation and maintenance of skeletal tissue, Ca ions are widely distributed in soft tissues. Other Ca functions include muscle contraction, blood clot formation, nerve transmission, maintaining the integrity of the cell membrane, and activation of several important enzymes. In the cell membrane, calcium is closely bound to phospholipid, where it controls the permeability of the membrane (NRC, 1993, Lall, 2002).

In contrast to Ca, dietary P supplies most of the phosphate required for growth and metabolism of fish because the concentration of phosphate is low in natural waters (NRC 1993; Lall 2002). P is an essential mineral for fish as a constituent of bones and scales but it also serves as a buffer to maintain optimal pH in body fluids and has an integral role in cellular fraction, as it is a key component of nucleic acids, phospholipids, phosphoproteins, ATP and several key enzymes (Coote et al. 1996; Lall 2002; Webster & Chhorn 2015). Ca deficiency is not common in fish while P deficiency results in reduced growth, decreased feed efficiency, reduced bone mineralization and skeletal abnormalities (Lall & Lewis-McCrea 2007; Lall 2002).

The ratio of Ca:P, should always be considered together with the specific dietary levels of those minerals, otherwise excess Ca or P may cause problems in the fish body or remain unabsorbed (Porn-Ngam et al. 1993; Hossain & Yoshimatsu 2014; Coote et al. 1996). Ca is considered an important element for P utilization because Ca-binding protein is a carrier for both Ca and P from the intestine (Hossain & Yoshimatsu 2014). In some cases, when there is an excess of Ca in comparison with P, P is not absorbed by the intestine because it is combined with the Ca to form calcium phosphates that are not biologically available (Andrews et al. 1973; Chavez-Sanchez et al. 2000). Most fish maintain a constant ratio of Ca:P in bone as well as plasma, so a ratio of Ca:P needs to be maintained in fish feeds also (NRC 1993; Hossain & Yoshimatsu 2014).

As we described in D24.1, the most important bottleneck of meagre *Argyrosomus regius* production is Systemic Granulomatosis (SG) because it affects the majority of farmed populations. The aetiology of the disease is still unknown however there is evidence that it is related to nutrition (Katharios et al. 2011). SG is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. It has been shown that dietary factors, such as imbalances among Ca, P and Mg, are considered important for the development of SG in fish (Herman 1996). Paperna (Paperna et al. 1977) described ectopic calcification in gilthead bream *Sparus aurata* fed on a diet with high mineral content, which demonstrated nodular granulomata throughout the body. Furthermore, Dunbar & Herman (1971) indicated a direct relationship of visceral granulomas in brook trout *Salvelinus fontinalis* with P levels suggesting that a dietary mineral imbalance can disturb metabolism, causing precipitation of Ca phosphate or Ca carbonate.

One of the objectives of Work Package 24 – Fish health –meagre (WP24) is to identify the causes of SG via several feeding trials. The first trial concluded that vitamin D_3 has no effect on the development of SG. However, a number of studies indicate the effects of vitamin D_3 and its major metabolites (e.g. cholecalciferol (25(OH)D), calcitriol ($1,25(OH)_2D$)] on Ca and P metabolism in freshwater teleosts and a few in marine species (Avila et al., 1999; Devara Sunita & Raghuramulu 1999; Vielma et al., 1999; Lall et al., 2007). Circulated levels of Ca and P and $1.25(OH)_2D$ metabolite feedback possibly influence Ca and P excretion, absorption and re-absorption and 1a-hydroxylase activity (Lall et al., 2007). Thus, the purpose of this second trial was to examine whether different levels of dietary Ca and P and Ca/P ratios affect the development of SG but also to investigate its role in growth performance, body and mineral composition and serum biochemistry.

Materials and methods

Experimental diets

Nine experimental diets with different levels of Ca and P were formulated using calcium carbonate and monoammonium phosphate as sources of Ca and P, 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 (**Table 1**).

Table 1. Formulation and chemical analysis of the experimental diets (% dry weight)

Raw Material (%)	Q	R	S	T	U	W	X	Y	Z
Wheat	17.45	15.39	14.32	15.67	13.9	10.35	12.34	9.47	6.26
Corn gluten	12.23	15	13.94	13.19	14.15	15	15	15	15
Wheat gluten	15.05	14.16	14.5	14.74	14.43	14.59	14.16	14.79	19.77
Soya	25	25	25	25	25	25	25	25	19.39
Fishmeal	20	20	20	20	20	20	20	20	20
Fishoil	9.99	9.85	9.9	9.94	9.89	9.89	9.85	9.9	9.84
Phosphorus	0	0	0	1.18	1.21	1.26	3.37	3.18	3.33
Calcium	0	0.32	2.05	0	1.13	3.63	0	2.38	6.13
Vit Premix	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28

Chemical Analysis	Q	R	S	T	U	W	X	Y	Z
Dry matter	92.8	89.79	92.8	92.8	92.8	93	92.8	93	93
Moisture	7.2	10.21	7.2	7.2	7.2	7	7.2	7	7
Protein	52.3	52.3	52.3	52.3	52.3	52.3	52.3	52.3	52.3
Fat	15	15	15	15	15	15	15	15	15
Ash	5.31	5.56	7.31	6.43	7.57	10.05	8.55	10.66	14.17
Calcium (gKg^{-1})	5.49	6.44	12.83	5.49	10	20	5.55	15	30
Phosphorus (gKg^{-1})	6.44	6.44	6.42	10	10	10	15.58	15	15
Ca:P	0.85	1.00	2.00	0.55	1.00	2.00	0.36	1.00	2.00

Experimental fish and feeding trial

Meagre of about 1 g, produced in May 2015 at the facilities of tP1. HCMR were used for the feeding trial. In total, 1350 fish were weighed and placed into 27 500-l cylindrical tanks at a density of 50 fish

per tank (0.47 kg m^{-3}). Three replicates were allocated to each diet. The feeding trial lasted 4 months (1 July 2015- 27 October 2015).

Tanks were supplied with borehole water (salinity 35‰, pH 7.5) and all had similar light conditions and temperature (20°C). Airstones in each tank provided aeration and nets were placed over the tanks in order to prevent fish escape. The fish were hand-fed to visual satiety twice a day for 7 days a week, while feed intake was recoded daily.

Sampling and analytical methods

Before every sampling, the fish were starved for 24 h to reduce handling stress and allow digestion and tract evacuation. Fish were then anaesthetized with 2-phenoxyethanol and weighed.

Every month, 3 fish from each tank (9/treatment) were sampled for granuloma evaluation and histology.

At the end of the feeding trial samples were taken for body and mineral composition, estimation of specific biomarkers (CYP27, CYP24 enzymes), histology and plasma analysis. The following indices were calculated:

Condition factor (K)	$K = \frac{BW\ (g)}{TL\ (cm)^3}$
Specific growth rate (SGR)	$SGR\% = 100\ x\ \frac{\ln(Final\ BW - Initial\ BW)}{\Delta T}$
Feed conversion ratio (FCR)	$FCR = \frac{feed\ intake\ (g)}{wet\ weight\ gained\ (g)}$
Hepatosomatic Index (HSI)	$K = 100\ x\ \frac{LW\ (g)}{BW\ (g)}$

BW: body weight, TL: Total Length, ΔT: (Time, days), LW: Liver weight

Whole-body composition analysis

For a whole-body composition analysis, 3 fish from each tank (9/treatment) at the end of the trial were randomly sampled and stored at -20°C. For the proximate composition analysis of whole-body samples, the following techniques were used. Moisture: dry at 90°C until constant weight, Ash: burn in a furnace at 600°C for 7 h; Crude protein: Dumas method (factor 6.25); Crude lipids: methanol/ chloroform extraction (Folch et al. 1957).

Mineral determination of whole body

At the end of the feeding trial, 3 fish from each tank (9/treatment) were randomly sampled and stored at -20°C until lyophilization. Analyses of Ca, P and Mg were performed at the Laboratory of Soil Science of the Mediterranean Agronomic Institute of Chania (MAICh) using an inductively coupled plasma optical emission spectrometer (ICP-OES).

Plasma analysis

Blood samples were collected from the caudal vasculature into heparinized syringes. After centrifugation for 15 min at 6000 r.p.m., the plasma was removed and stored at −80°C for further

analysis. Specifically, Ca, P, Mg, triglyceride (Trig), glucose (Glu) and total proteins (TP) were assessed using commercially available kits (Biosis, Greece). The samples were pooled by diet and by the presence of tissue calcification (no calcification and calcification). Analyses were performed at the Department of Biology, University of Crete.

Expression of CYP27A mRNA

The sequence of Cyp27 mRNA for meagre was unavailable and was partially determined using a Smarter RACE cDNA amplification Kit (Takara) according to manufacturer's recommendations and primers designed based on conserved regions of Cyp27 mRNA coding region in other fish species:
CYPF_1100-1120 GTGGCAGCCTCATCAGTCC,
CYPF_1100-1120_SC AATGCTATGTGCGGCTGACC,
CYPF_1840-1860 GAGACCCTTGTGTAGACTCCA,
CYPR_1500-1520 TGGGTCAGCCGCACATAGC,
CYPR_2000-2020 GGACCTCTGCAGGACCACG.

The sequence of Cyp24 mRNA was unavailable for meagre as well as for other fish species thus it was not possible to be determined.

RNA extraction and cDNA synthesis
Nine livers per treatment were frozen in dry ice and stored at -80^{o}C until extraction of RNA. RNA was extracted from, approximately, 20 µg of tissue per sample using the TRI© Reagent (Sigma) and according to manufacturer's instructions. To eliminate gDNA contamination, RNA preparations were treated with DNAse I (New England Biolabs) at 37^{o}C for 30 min, followed by heat inactivation of the enzyme and ethanol-precipitation with ammonium acetate, according to manufacturer's recommendations. RNA concentration and purity was assessed with a Quawell-Q5000 micro volume cuvette-free spectrophotometer. cDNA was synthesized from 500 ng total RNA per sample using the PrimeScript RT reagent kit TAKARA following the protocol recommended by the manufacturer for SYBR green assay.

Quantitative RT-PCR analysis
The relative transcript abundance of CYP27 was determined by quantitative (q) RT-PCR. 10 µl reactions with the equivalent of ca. 15 ng total RNA were performed in duplicate for each sample using the KAPA SYBR fast qPCR Universal Master Mix (KapaBiosystems), following the manufacturer's instructions, on an ABI 7500 real time PCR system (Applied Biosystems/Life Technologies). Expression levels of CYP27 for each sample were normalized by the corresponding expression of β-Actin. The primers used were: for β-Actin CGCGACCTCACAGACTACCT and AACCTCTCATTGCCGATG and for Cyp27 ACCCGTACAGCTTCATCC and TCGTATTGCTGCATTAACCTG. Primers for Cyp27 were designed based on a partial cDNA sequence, determined as described above, using the PerlPrimer software. β-Actin and Cyp27 primers were used at 200 nM and 400 nM final concentrations, respectively. Cycling conditions for both genes were as follows: a 3 min initial denaturation step at 95°C followed by 40 cycles of denaturation for 3 sec at 95°C and primer annealing and template extension for 30 sec at 60°C. PCR amplification efficiencies and Ct-quantity regression coefficient (R2) for β-Actin and Cyp27 were 95%/0.997 and 98%/0.996 respectively. Relative transcript abundance for each gene and cDNA sample was determined based on Ct values with the 7500 software v2.3 (Applied Biosystems, USA). Transcript relative quantity for CYP27 was normalized to the corresponding quantity of β-actin in each sample. Means and standard errors of normalized Cyp27 relative expression were determined per treatment.

Visual evaluation of granulomatosis

To assess fish status regarding the presence of granulomas, a semi-quantitative method was developed 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:

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

Histology

Samples of heart, liver, intestine, spleen and kidney were fixed in 4% formaldehyde: 1% glutaraldehyde. Subsequently they were dehydrated in gradually increased ethanol solutions (70-96%) and then embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections of 4 μm were obtained with a microtome (RM 2245, Leica, Germany). After drying, slides were stained with methylene blue/azure II/basic fuchsin according to Bennett et al. (1976) and examined under a light microscope.

Statistical analysis

Univariate Analysis of Variance (Factorial) was used to test the effects of the nine experimental diets. Where significant differences were found ($p<0.05$), the Tukey test was used to rank the groups. A Kruskal-Wallis test was performed to compare the severity of granulomatosis in the various organs among groups. Statistical analyses were made using IBM SPSS Statistics 20 software.

Results

Growth performance

Juvenile meagre grew from ~1 g to ~15 g over the course of the feeding trial. The P content in the diets didn't affect the weight of fish at any time point in the feeding trial. However, fish fed diets with lower or higher Ca than P exhibited significantly higher body weight and length than fish fed diets with equal Ca and P content during the last month of the feeding trial. Furthermore, the length was significantly higher in fish fed with medium and high P content than those fed the low medium content diets (**Figs 1 and 2**).

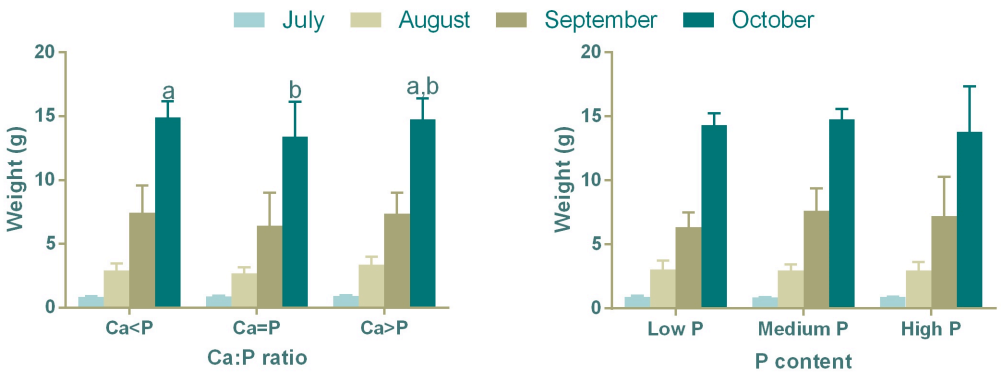

Figure 1. Average weight (g) of meagre fed diets with different levels of Ca:P and different levels of P in the beginning of the experiment and after 1, 2 and 3 months. Values are mean ± SD. Different letters indicate the statistically significant differences between the diets in each time point ($p<0.05$).

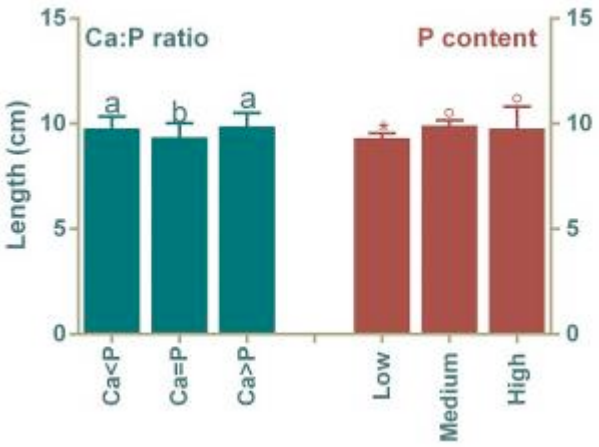

Figure 2. Average standard length (cm) of meagre fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets with different Ca/P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

SGR was significantly higher in fish fed diets with a lower Ca than P content compared with those fed diets with equal Ca to P content, while FCR did not differ significantly among diets with different Ca:P ratio. Concerning the P content in the diets, fish fed the medium and high P content diets had significantly higher SGR than fish fed low P diets, while FCR was lower in fish fed low and medium P (**Fig. 3**).

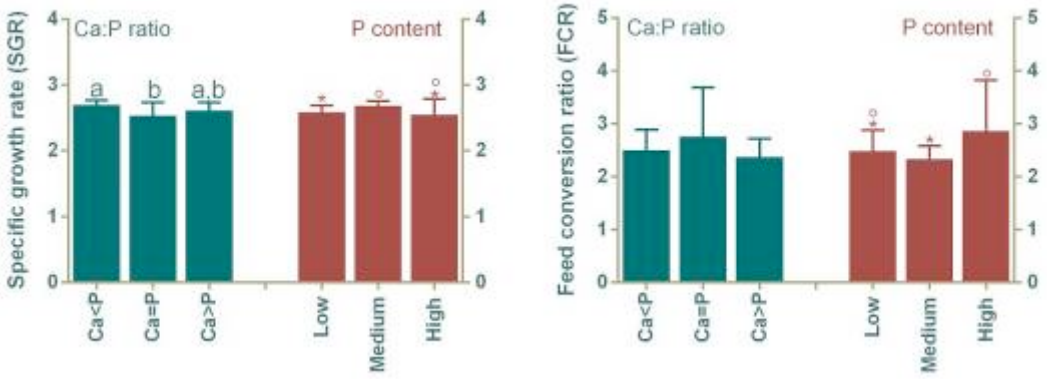

Figure 3. Specific growth rate (SGR) and feed conversion ratio (FCR) of meagre fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets with different Ca/P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

Condition factor (K) was significant higher in fish fed diets with lower or equal Ca:P levels while the HSI was higher in fish fed equal amounts of Ca and P. A higher condition factor was found in fish of the low P group, followed by the fish fed the medium and high P. HSI was higher in fish fed the high content of P (**Fig. 4**).

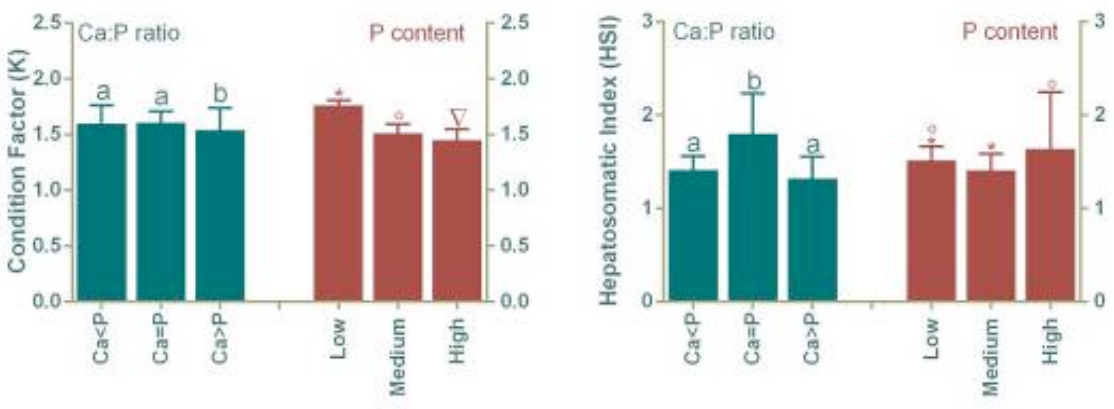

Figure 4. Average condition factor (K) and hepatosomatic index (HSI) of meagre fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets with different Ca/P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

Whole-body composition

Proximate analysis indicated that whole body moisture and ash was affected by the P content in the diets. The moisture was significantly lower in fish fed the high P content diets while the ash was significantly higher followed by the medium and low P content groups. The protein content of whole body was significantly higher in diets with Ca higher than P. The lipid content was unaffected by the increase in dietary P content or by the different Ca:P ratios (**Table 2**).

Table 2. Body composition in meagre fed with diets containing different levels of Ca:P ratio and different P levels. Values are mean ± SD. Different letters indicate the statistically significant differences between the diets with different Ca:P ratio. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

		Ca:P ratio			P content		
		Ca<P	**Ca=P**	**Ca>P**	**Low**	**Medium**	**High**
Moisture	Average	**76.30**	**76.76**	**76.46**	**77.19***	**76.91***	**75.31°**
	Stdev	1.09	1.04	2.06	1.05	0.96	1.64
Ash	Average	**3.08**	**3.21**	**3.42**	**2.41***	**3.00°**	**4.46▼**
	Stdev	0.93	0.93	1.03	0.28	0.38	0.48
Protein	Average	**19.56[a]**	**18.92[a]**	**20.95[b]**	**19.48**	**19.91**	**20.11**
	Stdev	0.65	1.30	1.68	0.94	0.96	2.45
Lipid	Average	**3.95**	**3.96**	**3.95**	**3.93**	**4.11**	**3.80**
	Stdev	0.37	0.41	0.51	0.46	0.41	0.38

Mineral determination of whole body

Table 3 shows the values of the examined whole body minerals of meagre fed various Ca:P ratios and P content in the diets. Ca:P ratio affected the concentration of P in the whole body. Specifically, fish fed diets with Ca equal or higher to P exhibited significantly higher levels of P and zinc compared to those fed diets with lower Ca than P. Ca, P and Mg concentration increased significantly with increasing levels of P in the diets.

Table 3. Mineral composition in meagre whole body, fed with diets containing different levels of Ca:P ratio and different levels of P. Different letters indicate the statistically significant differences between the diets with different Ca:P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

		Ca:P ratio			P content		
		Ca>P	**Ca=P**	**Ca>P**	**low**	**medium**	**high**
Ca	average	**320.709**	**309.454**	**336.363**	**231.697***	**344.827°**	**398.664▼**
	stdev	81.121	73.861	92.324	33.859	44.112	40.259
P	average	**26.369[a]**	**29.773[b]**	**31.717[b]**	**18.330***	**30.732°**	**40.350▼**
	stdev	11.805	7.129	10.142	4.379	1.294	3.447
Mg	average	**12.783**	**12.853**	**13.314**	**11.291***	**12.940°**	**14.962▼**
	stdev	0.274	0.263	0.304	0.146	0.174	0.108

Plasma analysis

Plasma metabolites of fish with no tissue calcification are presented in **Fig. 5**. Fish fed diets with more Ca than P exhibited statistically significant higher levels of plasma P, Mg, glucose and triglycerides compared with those fed lower or equal Ca:P levels. Ca and the total proteins of the plasma were not affected by the various Ca:P ratios. On the other hand, the amount of P in the diet affected plasma Ca, P, glucose and triglycerides. All these metabolites were significantly higher in fish fed the high P content diets. In contrast, plasma total proteins decreased significantly with increasing P content in the diet while Mg was not affected.

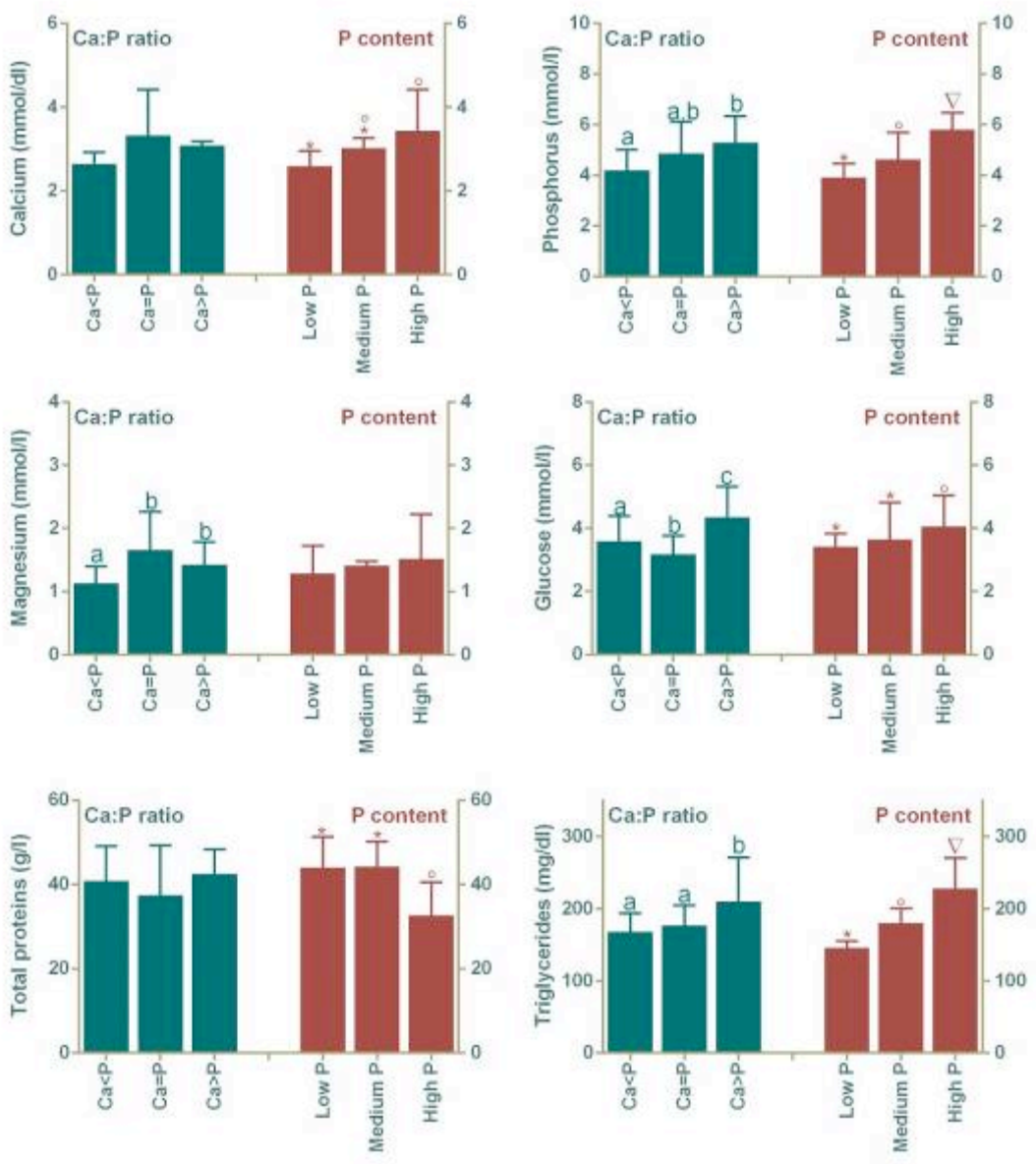

Figure 5. Plasma metabolites of meagre with no tissue calcification fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets with different Ca:P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

In fish with tissue calcification, plasma P was higher for those fed the diets with equal or higher Ca than P, while triglycerides were higher in fish fed diets with equal Ca and P content and lower in fish fed diets with higher Ca than P. The other metabolites were not affected by Ca:P ratios. Plasma Ca, glucose and triglycerides increased with increasing levels of P in the diet in fish with tissue calcification while total proteins decreased significantly. P and Mg in the plasma were higher in fish fed the medium P followed by those fed the high and low P content (**Fig. 6**).

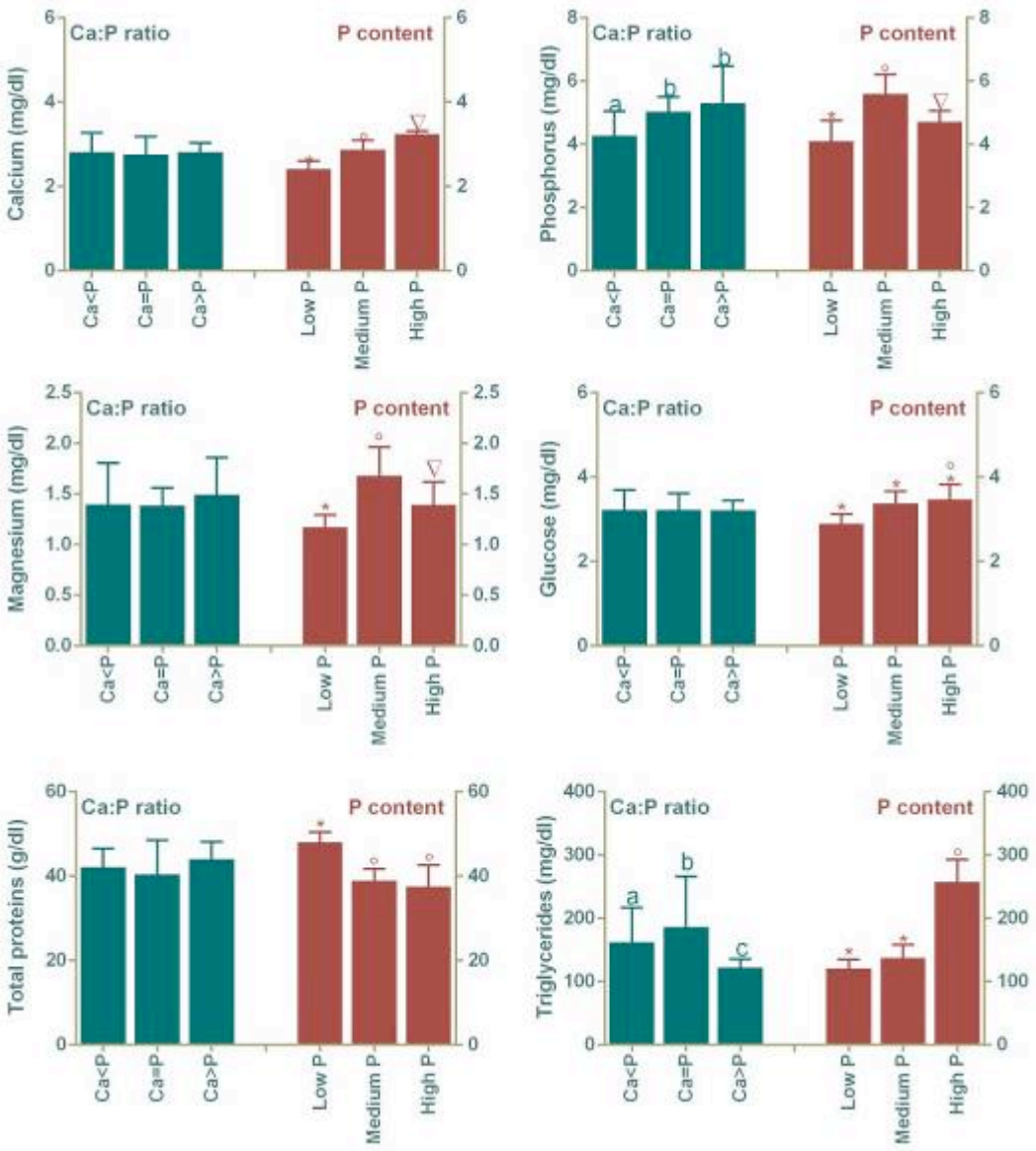

Figure 6. Plasma metabolites of meagre with tissue calcification fed diets with different levels of Ca:P ratio (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets with different Ca:P ratios. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

Expression of CYP27A mRNA

Results on relative expression of CYP27A mRNA are shown in **Fig. 7**. The expression of CYP27A did not differ significantly among diets with different Ca:P ratios, while diets with high P content lead to an upregulation of CYP27A expression in the liver compared with the low and medium P diets.

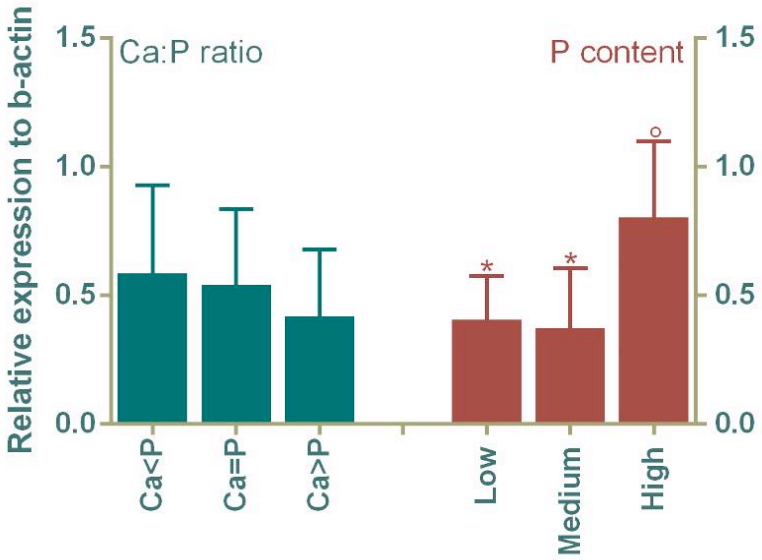

Figure 7. Relative expression of CYP27A in liver of meagre fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) at the end of the feeding trial. Values are means ± SD. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

Evaluation of granulomas

At the beginning of the feeding trial, fish did not exhibit granulomas in the tissues studied. After 3 months of feeding, granulomas were observed in all groups of fish. **Figures 8** and **9** show the boxplots of total scores of granulomas for the different diets at the end of the experiment. The medians of the groups with different Ca:P ratios are similar, which was confirmed by Kruskal–Wallis test ($H(2)=0.083$, $p=0.959$). On the other hand the medians of the groups with high and low P content are significantly different ($H(2)=10.077$, $p=0.006$).

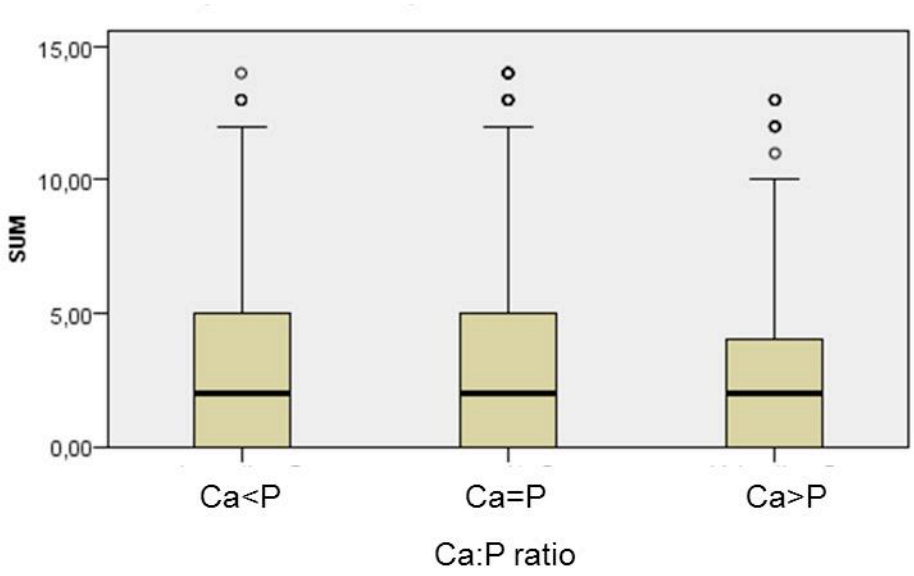

Figure 8. Boxplots of total score of granulomas in meagre fed with various Ca:P levels at the end of the experiment. Outliers are presented as circles. The medians of the groups with different Ca:P ratios are similar ($H(2)=0.083$, $p=0.959$).

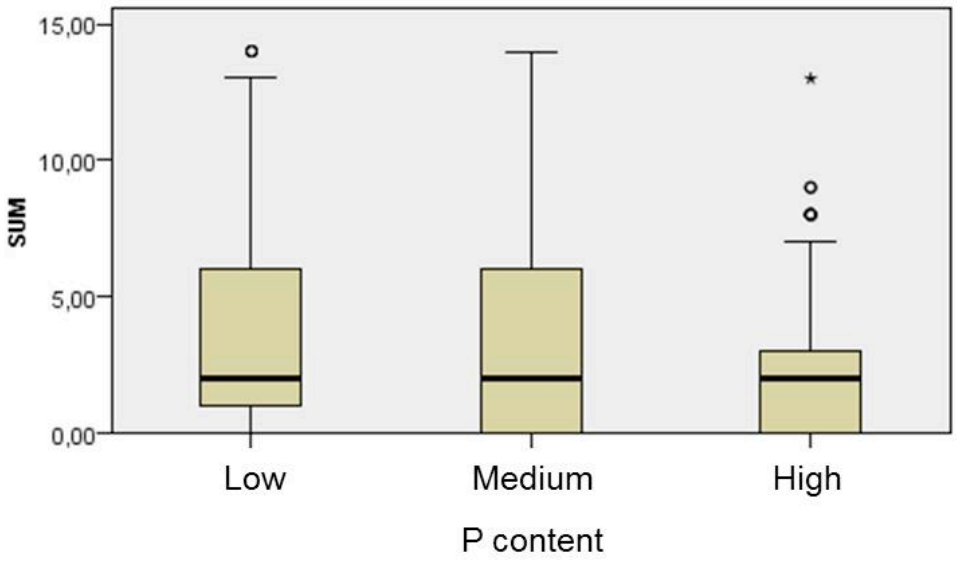

Figure 9. Boxplots of total score of granulomas in meagre fed with various P levels at the end of the experiment. Outliers are presented as circles and extreme scores as asterisks. The medians of the groups with high and low P content were significantly different (H(2)=10.077, p=0.006).

The tissues that appeared to be mostly affected by granulomatosis were the kidney and the liver, followed by the spleen. **Figures 10** and **11** show the percentage of fish in each of the 4 categories of the granulomas scoring system, at the end of the experiment for every examined tissue. Ca:P ratio did not affect the development of granulomatosis while on the contrary, statistically significant differences were observed between diets with different levels of P. Specifically, a statistically significant difference exists between the diets and the development of granulomatosis for the liver (p=0.006) and the kidney (p=0.004). Fish fed the diet with high P content, exhibited more tissues with no granulomas and less tissues with calcification compared to fish fed the diet with low and medium P content. Kidneys and livers from the fish of all diets presented all levels of granulomatosis severity: no granuloma, granulomas visible only by microscopy, granulomas visible macroscopically and tissue calcification. No statistically significant difference was observed in any other organ. No granulomas were observed in the peritoneum or the swim bladder in any of the fish examined irrespective of the diet.

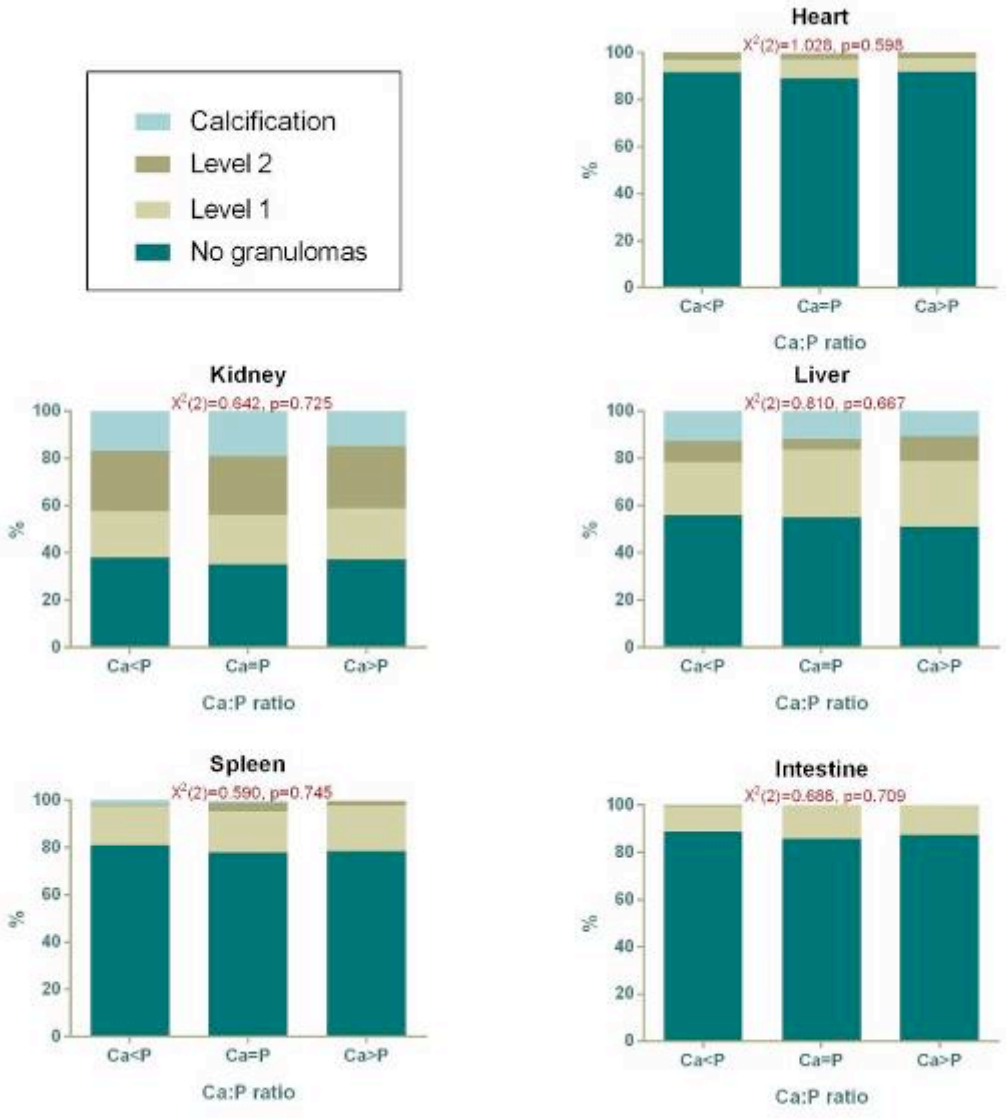

Figure 10. Percentage of the fish fed with various Ca:P levels in each of the 4 categories of the granulomas scoring system (no granuloma, Level 1:granulomas visible only by microscopy, Level 2: granulomas visible macroscopically and tissue calcification) for every examined tissue. Kruskal-Wallis test results are indicated with red letters.

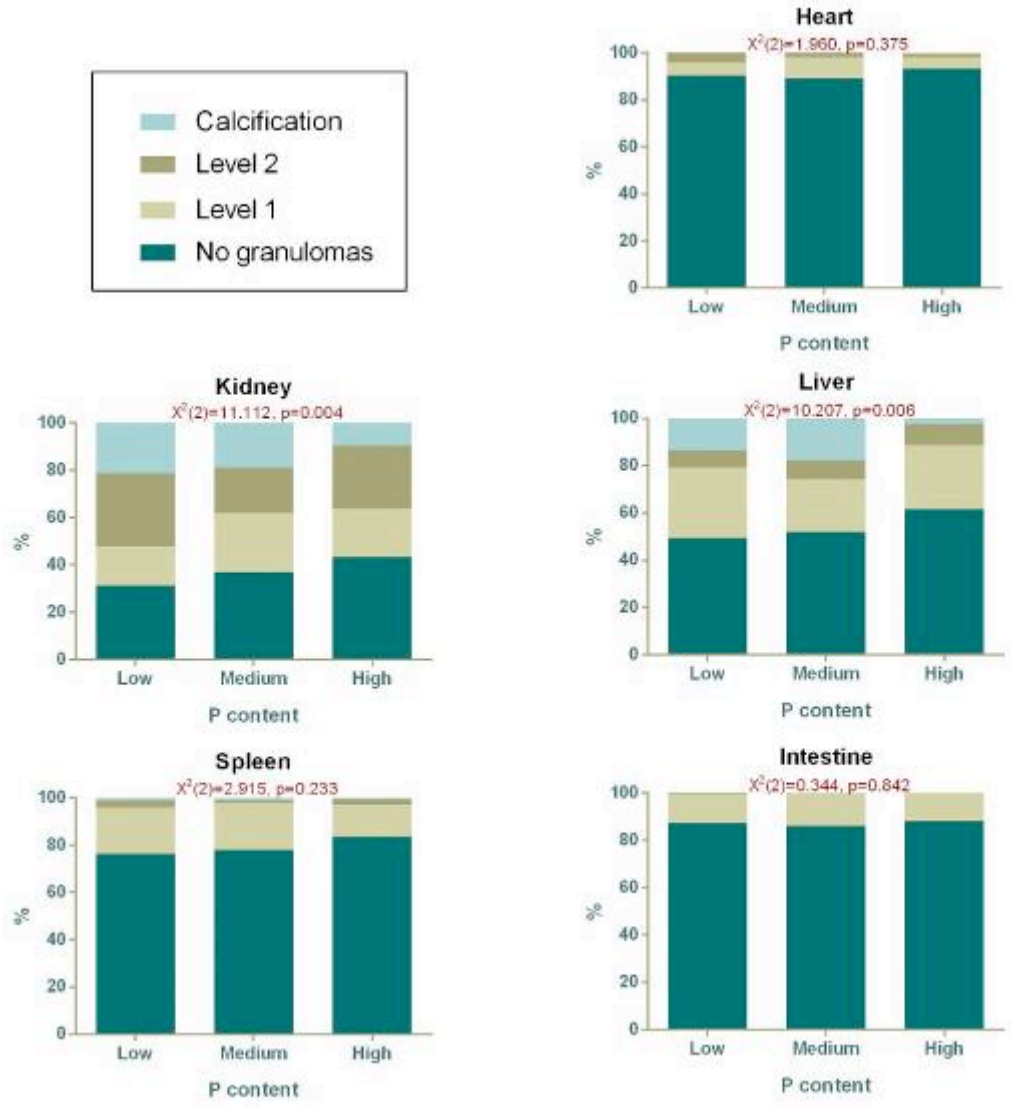

Figure 11. Percentage of the fish fed with various P levels in each of the 4 categories of the granulomas scoring system (no granuloma, Level 1: granulomas visible only by microscopy, Level 2: granulomas visible macroscopically and tissue calcification) for every examined tissue. Kruskal-Wallis test results are indicated with red letters.

Histology

The fish of all different experimental groups had similar lesions and the histological assessment was in accordance to the visual examination of the fresh preparations. The overall pathology was not different to that described by Katharios et al. (2011). Several stages of the granuloma formation with the characteristic epithelioid cells could be identified in the examined tissues ranging from immature granulomas, multilayer mature granulomas to big nodules, possibly a result of the merging of several adjacent granulomas that had big areas of central necrosis with dystrophic calcification circumscribed by fibrous tissue (**Fig. 12**). Rodlet cells were present in large numbers in all tissues. Rodlets are aligned like epithelial cells in the peritoneal membranes **(Fig. 12D)** but they are also found in liver, pancreas and intestine. Melanomacrophage centers were present in spleen of these fish (**Fig. 12C**). The kidney and liver were the organs mainly affected although granulomas were also found in heart and spleen. In most of the cases the granulomas do not contain necrotic areas, however in some cases necrosis was evident.

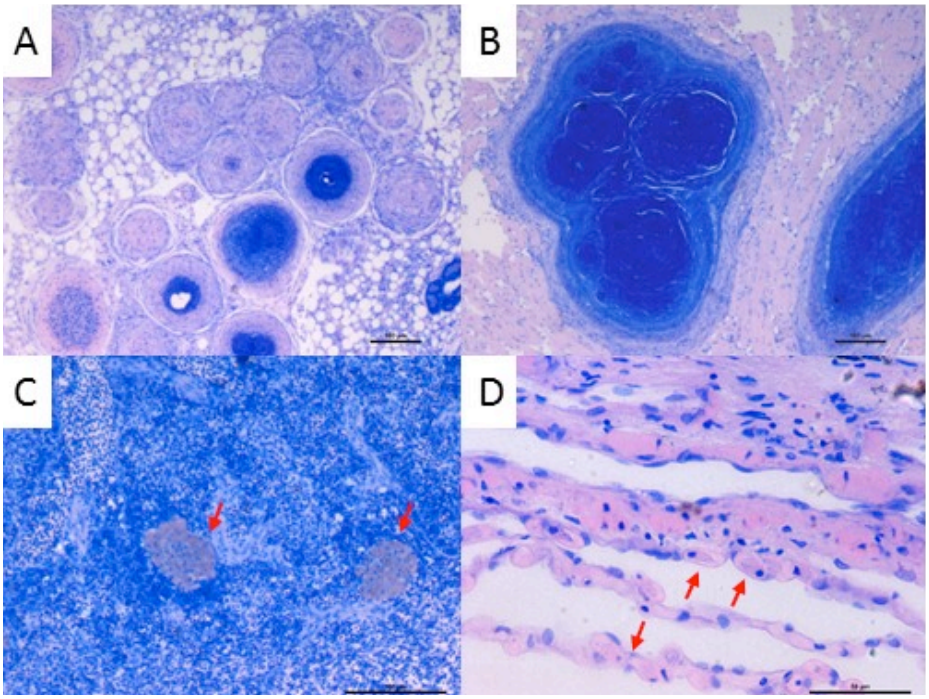

Figure 12. A. Multiple granulomas in the liver of a meagre. **B.** Merging of several adjacent granulomas in heart. **C.** Melanomacrophage centers in spleen. **D.** Numerous rodlets cells are evident in the peritoneal membrane.

Discussion

Although the fish of all groups exhibited granulomas, the present trial showed that the high P content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis. Fish of this diet group exhibited a significantly lower percentage of liver and kidney calcification, and there was a significantly higher percentage of fish with no granulomas in these organs compared to those fed the low and medium content of P. The organs mostly affected by granulomatosis were the kidney, the liver and the spleen. This result is in accordance with the hypothesis that granulomatosis could be a metabolic disorder or a nutritional disease.

For juvenile meagre, the optimum P content for improved growth was found to be 10 g kg^{-1}. Although no effect was observed in the final weight of the fish, this P level resulted in larger final length and better SGR and FCR compared with the 6.44 or 15 g kg^{-1} P content. The dietary P requirements for commonly cultured fish species had been reported to be between 3 and 15 g kg^{-1} of diet (Lall 2002). Higher growth and feed efficiency due to dietary P supplementation have also been reported in various cultured species such as the European seabass *Dicentrarchus labrax* (Oliva-Teles & Pimentel-Rodrigues 2004), gilthead seabream (Pimentel-Rodrigues & Oliva-Teles 2001), red seabream *Crysophrys majo*r (Sakamoto & Yone 1973), black seabream *Sparus macrocephalus* (Shao et al. 2008), grouper *Epinephelus coioides* (Ye et al. 2006), channel catfish *Ictalurus punctatus* (Andrews et al. 1973), Atlantic salmon *Salmo salar* (Albrektsen et al. 2009), American cichlid *Cichlasoma uraphthalmus* (Chavez-Sanchez et al. 2000) and haddock *Melanogrammus aeglefinus* (Roy & Lall 2003). In contrast, there was no significant effect of dietary P on growth of rainbow trout *Oncorhynchus mykiss* (Fontagné et al. 2009), Atlantic cod *Gadus morhua* (Kousoulaki et al. 2010) and Senegalese sole *Solea senegalensis* (Salas-Leiton et al. 2015).

On the other hand, Ca supplementation does not seem necessary for meagre, since no statistically significant difference was observed on growth indices between the groups with lower and higher Ca content, which is in agreement with the generally accepted view that most fish can absorb Ca from the

aquatic environment to meet their requirements (NRC 1993; Lall & Lewis-McCrea 2007). Calcium supplementation also had no significant effects on growth for grouper (Ye et al. 2006), red sea bream (Sakamoto & Yone 1973), Atlantic cod (Kousoulaki et al. 2010) and rainbow trout (Kalantarian et al. 2013; Fontagné et al. 2009).

It has been shown that bone ash is the most sensitive practical criterion for evaluating dietary P utilization and can provide a more accurate indication than values based on body weight changes or feed efficiency (Vielma et al. 2002; Lall 2002). According to Roy & Lall (2003) the ash content of a vertebrae represents P metabolized from various tissues as well as P absorbed from dietary sources. The present trial showed that whole body ash was significantly lower in fish fed diets without P supplementation, which indicates its necessity for bone mineralization. These results are in accordance with Roy & Lall (2003) in haddock, Andrews et al. (1980) in catfish, Chavez-Sanchez et al. (2000) in American cichlid, Fontagné et al. (2009) in rainbow trout, Kousoulaki et al. (2010) in Atlantic cod, Pimentel-Rodrigues & Oliva-Teles (2001) in gilthead seabream and Ye et al. (2006) in grouper. On the contrary, in European seabass there were no significant differences in whole body ash between basal and P supplemented diets (Oliva-Teles & Pimentel-Rodrigues 2004). In addition, no effect of P supplementation was observed on the body's lipid content, which is in agreement with our results on meagre. P supplementation did not affect the lipid content also in European sea bass and gilthead sea bream (Oliva-Teles & Pimentel-Rodrigues 2004; Pimentel-Rodrigues & Oliva-Teles 2001), whereas in other studies an increase of lipid content in fish fed P deficient diets was reported (Sakamoto & Yone 1973; Chavez-Sanchez et al. 2000; Albrektsen et al. 2009).

Histological assessment confirmed the results of the visual inspection performed on fresh preparations. There was a clear chronic inflammation manifested with the granulomatous lesions, however there were implications of an involvement of the vascular system and the enigmatic rodlet cells. The secretory nature of rodlet cells might be connected to the defense mechanism of meagre against infection, however this cannot be fully supported since there is no data on the presence of these cells in normal or wild specimens. As we described in *Deliverable 24.1 The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre*, in many cases, the granulomas of meagre seemed to be of an infectious origin and resembled the granulomas observed in mycobacteriosis. However, until now no infectious agent has been demonstrated in histological sections using various staining techniques, such as Ziehl-Neelsen and Giemsa and the results of bacterial cultures in various general and selective media and PCRs against various possible microbial agents (*e.g. Nocardia spp*, Mycobacteria, fungi etc) have been negative.

Measurement of plasma constituent levels can be an important indicator of the nutritional, physiological and clinical status of the fish. The advantage of this analysis is that it is easy to measure several samples in a short period using commercial kits, and also it can be carried out without killing the animal (Maita, 2007). However, the diagnostic value of this examination is questionable, especially in fishes, due to the lack of reliable references of the normal values. Various factors such as age, sex, water quality, temperature, handling and analytic methods may contribute to variability in hematological data, thus it is difficult to compare results from different studies or set normal ranges (Maita, 2007; Tavares-Dias and Moares, 2007). Lall (2002) suggested that plasma P can be used as a response criterion for estimating P requirement of fish, however since it can also reflect changes in absorption, deposition and urinary excretion of P, the use of this criterion as an index of P requirement is rather unreliable (Antony Jesu Prabhu et al. 2013; Shen et al. 2016). Many studies in various fish species observed that supplementation of dietary P led to an increase of plasma P concentration (Dougall et al. 1996; Vielma & Lall 1998; Vielma et al. 2002; Roy & Lall 2003; Yang et al. 2006). Our results are in agreement with these, since the high P content diets led to a significantly higher concentration of plasma P in meagre with no tissue calcification. It is noteworthy that in fish with tissue calcification the concentration of plasma P in the group with high P content is lower compared to the group with medium P content. This may be associated with the involvement of P in the formation of calcification in the soft tissues. On the other hand, Kousoulaki et al. (2010) in Atlantic cod and Skonberg et al. (1997) in rainbow trout did not observe differences in plasma P with increasing dietary P.

Supplementation of P also led to an increase of plasma Ca, glucose, Mg and triglycerides concentration and to a decrease of plasma total proteins regardless of the presence of tissue calcification. Concentrations of plasma Ca and Mg were not affected by dietary P treatment in silver perch *Bidyanus bidyanus* (Yang et al. 2006), in striped bass *Morone saxatilis* (Dougall et al. 1996) and rainbow trout (Skonberg et al. 1997). On the other hand, Vielma & Lall (1998) reported an increase of plasma Ca and a decrease of Mg with an increase in dietary Ca phosphate. Shao et al. (2008), in contrast to our results, showed that plasma triacylglycerol and total cholesterol decreased in fish fed diets supplemented with P suggesting that dietary P could influence immunological functions in black seabream, and has an effect on lipid metabolism. From the comparison of our results with values from the trial with Vitamin D_3 and other species (**Table 4**) we could see that Ca, P and Vitamin D_3 (reported in Deliverable 24.1) affects in different ways the plasma metabolites of meagre. Plasma P are higher in meagre fed diets with different levels of Vitamin D_3 in comparison with those fed the various levels of Ca and P, while Ca and Mg were lower. The results of the Ca/P trial are in the range recorded for other fish species.

Relative expression of CYP27A mRNA was affected by the P content, as an upregulation of CYP27A expression was indicated in liver of fish fed the high P content diets compared with those fed the low and medium P diets. Thus, we considered that P perhaps plays the role of a regulator for the 25 hydroxylase (CYP27A) and the 25(OH)D_3 metabolite. This is a new finding in the metabolism of D_3 in fish and can be added to the proposed scheme for the role of phosphorus suggested by Lall et al. (2007). It is well-known that vitamin D_3 is firstly converted to 25(OH)D_3 in liver by CYP27A, and that secondly 25(OH)D_3 is converted to 1*a*,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ in kidney, liver and several other tissues (Lock et al. 2010). The active form of vitamin D, 1*a*,25(OH)$_2D_3$ plays essential roles in calcium homeostasis. Specific binding of 1*a*,25(OH)$_2D_3$ to enterocyte basal lateral membrane (BLMs) preparations in rainbow trout when adapted to seawater decreased and correlated with inhibited intracellular Ca uptake (Larsson et al. 2003). Earlier, Sundh et al. (2007) had measured increased hepatic and renal production of 24,25(OH)$_2D_3$ in rainbow trout after a transfer to seawater. Lock et al. (2010) mentioned that the sensitivity of enterocyte BLMs to vitamin D_3 metabolites is part of the fish strategy to adapt to the calcium-rich environment of seawater (>10 mM Ca). In order to interpret these results further investigation is essential to establish a relationship between the CYP27A expression, vitamin D metabolites and Ca and P levels.

The body mineral content of meagre was significantly affected by P and Ca supplementation. Increased levels of dietary P led to an increase of P, Ca and Mg. In addition, when fish were fed diets with equal or higher Ca than P they exhibited higher levels of P. So, it is evident that dietary P is more important than Ca for the availability of other minerals in the body of the fish. The necessity of dietary P for deposition of other minerals, mainly Ca, P and Mg, was also suggested for other fish species like grouper (Ye et al. 2006), rainbow trout (Skonberg et al. 1997), Atlantic Salmon (Albrektsen et al. 2009), silver perch (Yang et al. 2006), red seabream (Sakamoto & Yone 1973), American cichlid (Chavez-Sanchez et al. 2000) and haddock (Roy & Lall 2003). In contrast, Vielma & Lall (1998) observed decreased Mg and zinc levels in Atlantic salmon fed high dietary P while Hardy & Shearer (1985) found reduced body zinc content in rainbow trout. Competitive inhibition of cations during intestinal absorption may be the reason for an observed decrease in the concentration of minerals with an increase in dietary P or Ca content (Roy & Lall 2003; Lall 2002). In fish, information on the effect of dietary P or Ca on availability of other elements is limited and further investigation is needed.

In summary, for juvenile meagre the optimum P content for improved growth was found to be 10 g kg^{-1}, while a Ca supplementation does not seem necessary, as fish can absorb Ca from the aquatic environment to meet their requirements. High P supplementation affects the development of granulomatosis at least in the kidney and liver of fish and leads to an upregulation of CYP27A expression in the liver. Phosphorus supplementation is also necessary for bone mineralization in meagre, as indicated by the body and mineral composition analysis.

Table 4. Plasma biochemical parameters in meagre (with or without tissue calcification) fed with diets containing different Ca:P ratios and different levels of phosphorus (Ca:P trial), in meagre fed four diets with 4550 (D0), 7000 (D1), 10000 (D2) and 20000 (D3) IU/Kg vitamin D_3, with no granulomas (0), granulomas (1) and calcification of even one tissue (5), in human and in different fish species (Ca, P, Mg, Glu=mmol l^{-1}, Trig=mg dl^{-1}, TP=g l^{-1})

Ca/P trial												
	no tissue calcification						tissue calcification					
	Ca<P	Ca=P	Ca>P	Low P	Medium P	High P	Ca<P	Ca=P	Ca>P	Low P	Medium P	High P
Ca	2.64±0.29	3.33±1.09	3.09±0.10	2.60±0.36	3.02±0.24	3.44±0.97	2.82±0.46	2.75±0.43	2.81±0.22	2.42±0.19	2.87±0.22	3.24±0.08
P	4.21±0.81	4.86±1.26	5.29±1.06	3.92±0.56	4.63±1.07	5.81±0.66	4.27±0.78	5.03±0.47	5.30±1.17	4.10±0.66	5.60±0.61	4.71±0.35
Mg	1.13±0.26	1.66±0.60	1.42±0.36	1.29±0.44	1.42±0.07	1.52±0.71	1.40±0.41	1.39±0.17	1.49±0.37	1.17±0.12	1.68±0.28	1.39±0.23
Trig	167.79±25.70	176.61±27.79	209.52±61.68	145.65±9.65	180.53±19.82	227.73±42.90	162.46±54.70	186.55±79.76	122.27±13.26	120.87±13.81	137.68±20.57	257.98±34.70
Glu	3.60±0.80	3.18±0.59	4.35±0.97	3.42±0.41	3.65±1.18	4.06±0.98	3.22±0.48	3.23±0.39	3.22±0.22	2.90±0.22	3.38±0.28	3.48±0.35
TP	40.90±8.23	37.45±11.86	42.53±5.80	44.05±7.26	44.22±6.01	32.60±7.93	42.13±4.38	40.47±8.05	44.07±4.11	48.04±2.39	38.91±2.81	37.55±5.12

Vitamin D_3 trial							
	D0	D1	D2	D3	no granuloma	Granuloma	Calcification
Ca	1.60±0.60	1.72±0.59	1.46±0.18	1.35±0.23	1.37±0.42	1.45±0.43	1.78±0.41
P	8.70±1.05	8.02±0.70	8.22±1.08	9.35±0.52	9±0.67	8.76±1.02	7.95±0.93
Mg	0.75±0.07	0.80±0.09	0.79±0.10	0.72±0.08	0.73±0.10	0.81±0.05	0.75±0.09
Trig	301.33±14.53	283±39.74	307±25.74	270.33±37.99	274.13±36.41	303.25±23.04	293.88±33.94
Glu	5.05±0.35	3.18±0.92	3.00±0.75	3.63±0.65	4.23±0.60	3.17±1.04	3.74±1.24
TP	57.5±6.86	59.5±10.89	56.33±11.09	50±8.58	58.38±11.21	61.38±6.48	47.75±4.17

	Human	Seabass	Seabream	Seabass	Oyster toadfish	Striped Seabream	Common Dentex	Gilthead Seabream	Acantopagrus latus	Epinephelus coioides	Rainbow trout
		Coz-Rakovac et al. 2005	Peres et al. 2013	Peres et al. 2014	Mensinger et al. 2005	Yildiz 2009			Akbary 2014		Charoo et al. 2014
Ca	2.25-2.75	-	3.8	4.0	2.3±0.05	2.65±0.11	3.49±0.11	2.82±0.09	19.80±2.97	16.80±2.34	9.98
P	0.97-1.45	-	3.7	2.7	-	3.08±0.12	4.56±0.21	2.90±0.08	2.35±0.40	2.40±0.57	2.87
Mg	0.73-1.48	-	1.2	1.5	-	-	-	-	-	-	2
Trig	4-200	59.3	289.1	405.5	49.1±4.8	-	-	-	102.02±11.82	68.10±15.34	-
Glu	4.44-6.66	3.7	6.0	7.2	-	4.06±0.11	4.25±0.08	4.65±0.43	2.46±0.90	2.43±0.56	9.32
TP (g/l)	63-80	36.0	45.0	49.0	49±5	-	-	-	43.1±3.2	39.2±3.6	33

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Deviations: This report was scheduled to be delivered in Mo 24, assuming that work on the project could start on Mo 1. As the project started on November, while the reproductive season of the species is in April-May, the juveniles for the experiment became available on Mo 21 (as opposed to Mo 12 as projected in the DOW). The experiment was completed in November (Mo 24) and the required analysis of the samples obtained was completed 6 months later. Therefore, an extension was requested and was approved by the coordinator.

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