New species for EU aquaculture

Deliverable Report

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Objective: Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis in Meagre (*Argyrosomus regius*): The deliverable will present evidence whether or not the balance of pro/anti-oxdiant nutrients in diet affects the appearance of Systemic Granulomatosis in meagre. A description of the occurrence of systemic Granulomatosis under the different dietary regimes is presented.

Description:

Systemic granulomatosis is a growing disease with a high morbidity that could be caused by a nutritional imbalance in meagre. The majority of farmed populations are affected by systemic granulomatosis (Ghittino *et al*., 2004). Systemic granulomatosis is characterized by the presence of multiple granulomas in internal organs, which progressively produce a necrotic centre surrounded by a layer of epithelial cells and macrophages. This disease mostly affects the spleen, kidney, liver and heart, where macroscopic nodules of varying diameter usually are observed (Ghittino *et al.,* 2004). Although granulomatosis is associated with the incidence of certain bacterial diseases (i.e. Nocardiosis), the inability to demonstrate pathogens associated to granulomas in other fish species has supported the hypothesis that a connection exists between systemic granulomatosis and a nutritional imbalance.

Different nutrients have been associated to the incidence of granulomatosis, including certain vitamins and minerals. In fish, it has been suggested that a deficiency of vitamin C causes an impairment of tyrosine catabolism, which leads to its precipitation in tissues and thereby cause development of the granulomas (Tixerant *et al.,* 1984). In addition, levels of other vitamins, such as vitamin K (Vidal *et al.,* 2016) or vitamin

D (Cotou *et al*., 2016) have also been associated with the incidence of granulomatosis in meagre. Granulomas of unknown aetiology have been related with mineral imbalances in salmonids, due to mineral deposits present in the central core of the granulomas (Good *et al*., 2015).

These findings may support a non-infectious diet-related origin of granulomatosis in this fish species. To elucidate the involvement of dietary micronutrients on the incidence of granulomas in meagre juveniles, two different experiments were carried out. The first was related with dietary vitamins E, C and astaxanthin, and the second was related with the addition of dietary Mn, Zn and Se and the addition of high levels of vitamin C on the appearance and incidence of systemic granulomatosis in meagre.

Material and methods

Experimental diets

Experiment I.
Based on an isocaloric (16% lipid) and isoproteic (50% protein) fish meal and fish oil-based diet, six experimental diets were prepared by adding different levels of vitamin E, C and astaxanthin. Diet 0 (no addition of vitamin E, C and astaxanthin), Diet A (500 $mg \cdot kg^{-1}$ A), Diet EC (300 $mg \cdot kg^{-1}$ E, 100 $mg \cdot kg^{-1}$ C), Diet ECA (300 $mg \cdot kg^{-1}$ E, 100 $mg \cdot kg^{-1}$ C, 500 $mg \cdot kg^{-1}$ A), Diet EECC (700 $mg \cdot kg^{-1}$ E, 600 $mg \cdot kg^{-1}$ C), Diet EECCA (700 $mg \cdot kg^{-1}$ E, 600 $mg \cdot kg^{-1}$ C, 500 $mg \cdot kg^{-1}$ A) (**Table 1)**. A subsample of each diet was taken and stored at -80ºC for the subsequent biochemical analysis.

Table 1. Composition of experiment I diets (% dry weight)

	O	**A**	**EC**	**ECA**	**EECC**	**EECCA**
Ingredients						
Wheat	15.97	15.97	15.97	15.97	15.97	15.97
Wheat gluten 12C	16.50	16.50	16.50	16.50	16.50	16.50
SPC 12C	16.64	16.64	16.64	16.64	16.64	16.64
Faba beans whole 12C	5.00	5.00	5.00	5.00	5.00	5.00
FM North-Atlantic 12C	35.00	35.00	35.00	35.00	35.00	35.00
Fishoil North-Atlantic	10.26	10.26	10.26	10.26	10.26	10.26
DL-Methionine	0.20	0.20	0.20	0.20	0.20	0.20
L-Lysine	0.02	0.02	0.02	0.02	0.02	0.02
Min mix 2/04 med jod	0.10	0.10	0.10	0.10	0.10	0.10
Plurivel 70%	0.21	0.21	0.21	0.21	0.21	0.21
Lutavit E-50	0.00	0.00	0.03	0.03	0.07	0.07
Lutavit C Aquastab 35%	0.00	0.00	0.01	0.01	0.06	0.06
Vit.mix no VK	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin K	0.0035	0.0035	0.0035	0.0035	0.0035	0.0035
Astaxanthin 10%	0.00	0.05	0.00	0.05	0.00	0.05

Experiment II.
The formulation, proximate composition and fatty acid content of the experimental diets are shown in **Table 2 & Table 3** respectively. Five isolipidic (16.7% lipid) and isoproteic (49.6% protein) fish meal and fish oil based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se. Diet C (100 mg·kg^{-1} C), Diet C+Mn/Zn/Se (100 mg·kg^{-1} C, 40 mg·kg^{-1} Mn, 200 mg·kg^{-1} Zn, 1.5 mg·kg^{-1} Se), Diet CC (600 mg·kg^{-1} C), Diet CCC (1200 mg·kg^{-1} C), Diet CCCC (3200 mg·kg^{-1} C). The analysed dietary content of vitamin E, C and K and minerals Mn, Zn and Se for each treatment is showed in **Table 2.**

Table 2. Raw material composition and analysis of the experimental diets

	Diet				
Raw Material (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
Wheat	17,39	17,37	17,22	17,03	16,38
Corn gluten	5,00	5,00	5,00	5,00	5,00
Wheat gluten	6,78	6,79	6,86	6,95	7,25
Soya concentrate	25,08	25,07	25,02	24,95	24,73
Fish meal	35,00	35,00	35,00	35,00	35,00
Fish oil	10,41	10,41	10,41	10,41	10,41
Phospahte	0,14	0,14	0,14	0,14	0,14
Vitamin E	0,03	0,03	0,03	0,03	0,03
Vitamin C	0,01	0,01	0,16	0,33	0,90
Premix vit min	0,10	0,10	0,10	0,10	0,10
Vitamin K	0,00698	0,00698	0,00698	0,00698	0,00698
Astaxanthin	0,05	0,05	0,05	0,05	0,05
Zinc sulphate	0,00	0,01111	0,00	0,00	0,00
Selenium Sodium selenite	0,00	0,00115	0,00	0,00	0,00
Manganese Manganese sulphate	0,00	0,00261	0,00	0,00	0,00
[VOLUME]	100,00	100,00	100,00	100,00	100,00
DRY_MAT	91,78	91,78	91,79	91,82	91,89
V MOIST	8,23	8,23	8,21	8,19	8,12
C PROT	50,00	50,00	50,00	50,00	50,00
C FAT	16,00	16,00	16,00	16,00	16,00
ASH	7,44	7,46	7,54	7,66	8,07
Zinc (mg/kg)	161,11	200,00	160,97	160,80	160,24
Manganese (mg/kg)	31,65	40,00	31,61	31,56	31,39
Selenium (mg/kg)	0,98	1,50	0,98	0,98	0,98
VIT E (mg/kg)	300,00	300,00	300,00	300,00	300,00
VIT K (mg/kg)	35,00	35,00	35,00	35,00	35,00
VIT C (mg/kg)	100,00	100,00	600,00	1200,00	3200,00
ASTA (mg/kg)	50,00	50,00	50,00	50,00	50,00

Table 3. Fatty acid composition (percentage of fatty acids) in diets of meagre. The data are expressed as means of three technical replicates per batch of diet. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	**C**	**C+Mn/Se/Zn**	**CC**	**CCC**	**CCCC**
14:00	7.97	7.52	7.64	7.55	8.12
16:00	22.49	21.49	20.88	20.28	22.20
18:00	4.32	4.14	4.06	3.98	4.30
20:00	0.30	0.38	0.33	0.32	0.35
Σ Saturated[1]	36.60	35.00	34.47	33.71	36.61
16:1n-7	7.76	7.44	7.60	7.64	8.11
18:1n-9	12.07	12.89	11.18	10.91	11.62
18:1n-7	3.14	3.06	3.01	3.00	3.19
20:1n-7	3.21	3.11	2.86	2.75	2.98
22:1n-11	4.59	4.38	4.02	3.84	4.15
Σ Monosaturated[2]	32.92	32.98	30.73	30.15	32.19
18:2n-6	6.14	6.72	5.57	5.14	5.28
18:3n-6	0.28	0.27	0.29	0.29	0.30
20:2n-6	0.23	0.23	0.23	0.23	0.25
20:3n-6	0.10	0.10	0.11	0.11	0.12
20:4n-6	0.73	0.74	0.81	0.85	0.87
Σ n-6PUFA[3]	7.64	8.21	7.18	6.81	1.71
18:3n-3	1.09	1.26	1.09	1.08	1.13
18:4n-3	1.77	1.82	2.08	2.19	2.19
20:3n-3	0.09	0.09	0.09	0.09	0.09
20:4n-3	0.49	0.49	0.55	0.58	0.59
20:5n-3	7.87	8.14	9.60	10.26	10.24
22:5n-3	0.91	0.94	1.11	1.19	1.19
22:6n-3	7.84	8.32	10.07	10.72	10.73
Σ n-3PUFA[4]	20.06	21.07	24.59	26.10	26.15
(n-3+n-6) PUFA	27.70	29.27	31.77	32.91	27.86
Total n-3 LC-PUFA[5]	1711	1789	2133	2275	2275
Proximate composition					
Proteins (%)	49.6	49.3	49.6	48.9	49.5
Lipids (%)	16.8	16.8	16.4	16.8	16.5
Moisture (%)	7.2	8.0	7.9	8.2	7.6
Ash (%)	7.1	6.9	7.0	7.0	7.4
Vitamin E (mg/kg)	228	242	243	241	255
Vitamin C (mg/kg)	98	96	586	1180	2835
Vitamin K (mg/kg)	23	23	23	22	23
Mn (mg/kg)	37	49	34	34	35
Zn (mg/kg)	130	180	130	140	140
Se (mg/kg)	1.1	1.6	1.1	1.1	1.2

Fish and feeding
The meagre juveniles for both experiments were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain).

Experiment I
The initial mean weight was 79.6 ± 0.34g, the juveniles were transferred to 18 fibre glass tanks of 500 L with 50 fish per tank at an initial stocking density of 7.9 $kg \cdot m^{-3}$. All tanks were covered with a net. The temperature and dissolved oxygen concentration were measured twice a week with values from 17.6 to 21.6 °C and 5.8 to 6.6 mg L^{-1}, respectively.
The meagre juvenile were fed 3 times per day (8:00, 11:30, 15:00), 6 days per week (Monday-Saturday), for 135 days. All the uneaten feed of each tank was collected daily and dried to calculate the daily feed intake. Dead fish were recorded daily and survival was determined.

Experiment II
The experiment was carried out in 21 fibre-glass tanks of 500 L with 100 fish/tank (3.20 kg/m^3) for all the diets. The initial mean weight was 15.75 ± 0.56g. Fish were reared under natural light conditions throughout the feeding trial. The juvenile meagre were fed 3 times per day (8:00, 11:30, 15:00), 6 days per week for 90 days with the different experimental diets. All the uneaten feed was collected daily from each tank and dried in order to calculate the daily feed intake. Dead fish were recorded daily and survival was determined. After 90 days of feeding samples were collected for histology, biochemical analysis and gene expression analysis.

Growth performance and feed utilization
The data were analysed according to the following equations:
Survival (%) = 100*(final number fish – initial number fish)/ initial number fish
Growth (%) = ((final mean weight – initial mean weight)/initial mean weight)*100.
Weight gain = (final mean weight- initial mean weight).
SGR (specific growth rate) = 100 x (ln final mean weight – ln initial mean weight)/ nº of days.
FCR (feed conversion ratio) = feed intake (g)/ weight gain (g).
K (condition factor (%)) = 100*(fish weight/ (fish length)3).
HSI (hepatosomatic index (%)) = 100*(liver weight / fish weight).
VSI (viscerosomatic index (%)) = 100* (fish weight - fish eviscerated fish weight)/fish weight.

Histology and histopathology
At the beginning of each trial, 50 meagre juveniles and at the end 23 fishes per diet were sampled for macroscopic evaluation of granulomas and samples of liver, kidney, heart and spleen were taken for histological analysis. The samples were fixed in 4% formalin, dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 µm, fixed to the microscope slide, heated and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN), the Fite-Faraco method and Gram stain.

The severity of granulomatosis was scored in each organ. A quantitative method was developed to classify the severity of granulomas in each organ. The score was organ dependent, because the number of granulomas in each organ was variable. The severity was classified in liver, kidney and heart according to the following criteria shown in **Table 4**.

Table 4.- Severity score of granulomas in liver, kidney and heart.

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

Biochemical analysis

Feed and fish biochemical composition analysis were conducted following standard procedures. Lipids in liver, heart, kidney and feeds were extracted with a choloroform-methanol (2:1 v/v) mixture following the Folch method. Protein content (Kjeldahl method), dry matter and ash were also determined. Fatty acids from total lipids were prepared by transmethylation. Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following standardized conditions of the FCPCT facilities. The concentration of vitamins in diets was analysed by HPLC. TBARs were measured in triplicate from extracted total fatty acids (10mg/ml) of liver, kidney and heart. The concentration of vitamin E & C was determined in diets and fish tissues (liver, heart and kidney) by HPLC analysis (Betancor *et al*., 2012).

Gene expression

Liver, kidney and heart were taken aseptically from 10 fish per tank at each final sampling and stored at -80 °C until further analysis. Total RNA was extracted from, approximately, 100 mg of sample using TRI Reagent® (Sigma). The cDNA was synthetized from 1 µg of total RNA using the iScript cDNA Synthesis Kit (BIORAD) in 20 µl reactions, which included 4 µl 5× iScript Reaction Mix, 1 µl iScript Reverse Transcriptase (BIORAD), 13 µl Milli-Q sterile water and 2 µl RNA (1 µg) of the sample. The reverse transcription was done in a thermal cycler (iCycler) at 25°C for 5 min, 60 min at 42°C and finally heating samples for 5 min at 85°C. The relative transcript abundance of the target genes glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT_ was determined by quantitative real time PCR (qPCR). Primer efficiency for each gene was previously evaluated to ensure that it was close to 100%. All PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate using 10 µl Thermo Scientific Luminaris Color Higreen qPCR Master Mix (Bio-Rad Hercules, California), 1 µl of forward and reverse primers (100 pmol·µl^{-1}), 6 µl water nuclease-free and 5 µl of a 1:10 dilution of the cDNA, with the exception of the reference genes, which were determined using 2 µL of cDNA, in a final volume of 20 µl. In addition, amplifications were carried out with a systematic negative control (NTC, non-template control) containing no cDNA. The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50°C for 2 min, a denaturation at 95°C for 10 min, followed by 35 cycles: 15 s at 95°C, 30 s at the annealing Tm and 30 s at 72°C. Expression level of each gene was normalized to the corresponding expression of β-Actin, Elongation factor 1α and Tubulin, which were chosen as the most stable house-keeping genes according to GeNorm.

Statistical analysis

All statistical analyses were done with Statgraphics. The normality was checked with the Kolmogorov Smirnov test. The homogeneity of variance was performed with the Levene test. The variables that satisfied the normality and homogeneity for? a parametric one-way (ANOVA) and Tukey test were used. For non-parametric variables, data which did not display a normal distribution and homogeneity of variance, were analysed for significance with a Kruskal-Wallis test. To compare two variables, a T-student test? was used for the variables with normality and a Mann-Whitney test for the non-parametric data. A significance level of 0.05 was used.

Results

Growth and biological parameters

Experiment I
Combination of different levels of dietary vitamin E, C or astaxanthin did not affect fish growth performance, feed utilization and biometric parameters of juveniles meagre over 104 days of feeding (**Table 5**). In this feeding period, meagre juvenile grew from ~ 79.6 g to 264.3 g. No significant difference among the final weight, length, weight gain, specific growth rate (SGR), survival, fish condition factor (K) and hepatosomatic and visceral indexes were found. It a good food conversion ratio (FCR) was obtained in all the diets (0.84-0.87) but there were no differences among the diets. After the 104 days of feeding, the number of fish per tank was changed from 50 to 25 to adjust the density. The mean initial weight was ~ 264.3 g and 59 days later (day 163) the mean weight was ~402.9g. There were significant differences in the final weight being higher in the diets O, ECA and EECCA. The FCR ranged from 0.91-1.01, and the SGR was 0.77-0.86 (**Table 6**).

Table 5. Growth of meagre within the first on-growing period (0-104 days).

0 - 104 experimental days						
Diets	Weight (g)	Weight gain (g)	Length T (cm)	FCR	SGR	Survival (%)
O	268.1±44.3	188.5±5.5	28.2±1.6	0.86±0.01	1.15±0.02	100±0.0
A	258.3±43.4	178.9±7.8	28.1±1.4	0.87±0.02	1.12±0.02	100±0.0
EC	262.8±45.6	183.2±2.5	27.8±1.6	0.87±0.02	1.14±0.03	98.0±2.0
ECA	265.1±42.3	185.9±11.5	28.2±1.5	0.87±0.02	1.15±0.02	96.7±4.2
EECC	260.7±38.5	180.4±11.2	28.2±1.3	0.87±0.03	1.12±0.03	97.3±4.62
EECCA	271.0±42.8	188.5±3.6	28.2±1.6	0.84±0.00	1.16±0.03	98.0±2.0

Table 6. Growth of meagre within the second on-growing period (104 - 135 days). Different letters denote significant differences ($p<0.05$).

104 -135 experimental days									
Diets	Weight (g)	Weight gain (g)	Length T (cm)	FCR	SGR	HSI	VIS	K	Survival (%)
O	413.4± 69.6 ^{bc}	128.9± 9.8	33.42 ± 1.79	0.92± 0.04	0.85 ± 0.04	1.68 ± 0.20 ^{b}	3.21 ± 0.23	1.10 ± 0.07 ^{b}	100±0.0
A	386.2± 73.7 ^{a}	114.1± 15.8	32.76 ± 1.92	0.98± 0.09	0.80 ± 0.08	1.68 ± 0.22 ^{b}	3.13 ± 0.31	1.09 ± 0.07 ^{b}	100±0.0
EC	394.3± 69.6^{ab}	113.8± 1.4	32.91 ± 1.96	1.01± 0.03	0.77 ± 0.03	1.52 ± 0.25 ^{ab}	3.09 ± 0.31	1.10 ± 0.07 ^{b}	100±0.0
ECA	414.7± 74.8^{bc}	130.9± 4.7	33.40 ± 2.09	0.91± 0.04	0.86 ± 0.06	1.70 ± 0.36 ^{b}	3.25 ± 0.45	1.10 ± 0.08 ^{b}	100±0.0
EECC	393.9± 65.5^{ab}	116.8± 8.2	33.17 ± 1.76	1.00± 0.14	0.80 ± 0.07	1.45 ± 0.23 ^{a}	2.96 ± 0.27	1.07 ± 0.05 ^{a}	93.3± 1.2
EECCA	414.7± 73.7^{c}	126.7± 4.0	33.32 ± 1.94	0.92± 0.04	0.83 ± 0.04	1.63 ± 0.30 ^{ab}	3.11 ± 0.33	1.10 ± 0.06 ^{b}	93.3± 1.2

Experiment II
Inclusion of different levels of dietary C and minerals Mn, Se and Zn did not affect meagre final weight, length and growth parameters **(Table 7).** Growth, SGR, FCR, and K factor were significant higher in the treatments with low density (100 fish/tank). Juvenile meagre grew from ~ 15.75 g to ~ 94 g in 90 days. A good food conversion ratio (FCR) was obtained among all the dietary treatments (0.75~0.80) but without significant differences.

Table 7. Meagre (*Argyrosomus regius*) growth performance after 90 days of feeding diets with different levels of vitamin C and Mn, Zn and Se. Different letters in a column denote significant differences ($p<0.05$). FCR, food conversion ratio; SGR, specific growth rate. (Mean ± SD).

Diet	Final weight (g)	Length (cm)	FCR	SGR	survival (%)
C	94.05 ± 21.05^{ab}	18.12 ± 1.63	0.75 ± 0.01^{a}	2.02 ± 0.02b	98.3 ± 3.6
C+ Mn/Se/Zn	94.94 ± 18.96^{b}	18.07 ± 1.25	0.76 ± 0.02^{a}	1.99 ± 0.05ab	97.8 ± 2.0
CC	94.66 ± 23.16^{ab}	18.07 ± 1.50	0.76 ± 0.01^{a}	2.00 ± 0.03ab	98.0 ± 2.0
CCC	96.83 ± 22.08^{b}	18.15 ± 1.40	0.75 ± 0.01^{a}	2.02 ± 0.06b	98.7 ± 3.1
CCCC	96.03 ± 23.75^{b}	18.24 ± 1.51	0.76 ± 0.01^{a}	2.00 ± 0.02ab	97.8 ± 3.2

Biochemical composition

Experiment I.
The dietary treatments did not affect the lipid content in the analyzed tissues, which were 34 %, 25.5 % and 2.6 % in kidney, liver and heart, respectively (**Tables 8, 9 and 10**). The fatty acid profile of the tissues of fish reflected the dietary fatty acid content. The highest levels of total monounsaturated fatty acids were observed in liver, followed by kidney and heart, whereas the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) were higher in the heart, followed by kidney and liver (**Tables 8, 9 and 10**, respectively). All the other fatty acids were similarly distributed in the three tissues. Significant differences were observed in the percentage of fatty acids in the heart - saturated fatty acids being lower in fish fed the diets EECC and EC compared with the rest of the diets.

Table 8. Selected fatty acid composition (% total fatty acids) and proximate lipid composition (%) of heart of meagre juveniles.

	0	A	EC	ECA	EECC	EECCA
Lipids (%)	2.7 ± 0.2	2.6 ± 0.1	2.6 ± 0.3	2.6 ± 0.8	2.8 ± 0.3	2.5 ± 0.2
Total saturated[1]	**29.5 ± 1.8^{ab}**	**32.5 ± 0.7^{b}**	**27.2 ± 0.8^{a}**	**32.3 ± 1.7^{b}**	**28.6 ± 0.4^{a}**	**32.1 ± 1.5^{b}**
Total monounsaturated	**18.2 ± 1.5**	**18.7 ± 0.5**	**19.1 ± 3.7**	**19.7 ± 1.5**	**17.3 ± 0.7**	**18.2 ± 0.5**
18:2n-6	6.1 ± 0.4	6.3 ± 0.2	6.3 ± 0.3	6.2 ± 0.1	5.8 ± 0.1	6.0 ± 0.2
20:4n-6	2.3 ± 0.2	2.4 ± 0.2	2.2 ± 0.3	2.2 ± 0.0	2.3 ± 0.3	2.3 ± 0.1
Total n-6 PUFA[3]	**9.7 ± 0.1**	**9.8 ± 0.3**	**9.4 ± 0.2**	**9.3 ± 0.1**	**9.1 ± 0.5**	**9.3 ± 0.2**
18:3n-3	0.6 ± 0.0	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0
20:5n-3	8.9 ± 0.8	8.2 ± 0.4	9.4 ± 0.8	7.9 ± 0.9	9.0 ± 0.6	8.5 ± 0.4
22:6n-3	27.4 ± 2.9^{ab}	24.7 ± 1.1^{ab}	28.4 ± 2.5^{ab}	24.4 ± 1.7^{a}	29.5 ± 0.6^{b}	25.7 ± 1.0^{ab}
Total n-3 PUFA[4]	**40.5 ± 2.5^{abc}**	**36.7 ± 1.2^{ab}**	**42.3 ± 3.0^{bc}**	**36.3 ± 2.7^{a}**	**42.9 ± 0.3^{c}**	**38.1 ± 1.0^{abc}**
Total PUFA[5]	**52.3 ± 2.3^{ab}**	**48.8 ± 1.1^{ab}**	**53.7 ± 3.2^{b}**	**48.0 ± 2.5^{a}**	**54.1 ± 0.6^{b}**	**49.7 ± 1.2^{ab}**
Total n-3 LC-PUFA	**38.7 ± 2.6^{ab}**	**35.0 ± 1.3^{a}**	**40.3 ± 3.3^{ab}**	**34.5 ± 2.7^{a}**	**41.1 ± 0.4^{b}**	**36.4 ± 1.0^{ab}**

Data are expressed as means ± SD (n=3). [1] Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Statistical differences were determined by ANOVA analysis ($p<0.05$). PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA.

Table 9. Selected fatty acid composition (% total fatty acids) and proximate lipid composition (%) of kidney of meagre juveniles.

	0	A	EC	ECA	EECC	EECCA
Lipid content (%)	37.8 ± 3.3	32.0 ± 0.6	33.2 ± 2.5	37.2 ± 3.1	34.2 ± 3.1	33.9 ± 0.9
Total saturated[1]	**28.1 ± 0.3**	**29.7 ± 0.5**	**29.3 ± 1.1**	**29.1 ± 1.6**	**29.5 ± 0.4**	**28.7 ± 0.9**
Total monounsaturated[2]	**32.8 ± 2.2**	**31.6 ± 3.7**	**30.9 ± 3.2**	**31.9 ± 4.7**	**30.3 ± 2.0**	**31.8 ± 4.0**
18:2n-6	6.8 ± 0.2	6.4 ± 0.4	6.4 ± 0.3	6.6 ± 0.6	6.3 ± 0.1	6.3 ± 0.3
20:4n-6	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.4	1.3 ± 0.2	1.3 ± 0.4
Total n-6 PUFA[3]	**8.7 ± 0.2**	**8.4 ± 0.2**	**8.4 ± 0.2**	**8.5 ± 0.3**	**8.4 ± 0.2**	**8.23 ± 0.1**
18:3n-3	1.3 ± 0.0	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	1.1 ± 0.2
20:5n-3	7.9 ± 0.3	8.1 ± 0.9	8.2 ± 0.3	8.1 ± 0.9	8.3 ± 0.4	8.4 ± 0.8
22:6n-3	15.7 ± 1.6	15.6 ± 2.4	16.5 ± 2.2	15.5 ± 2.7	16.8 ± 1.4	16.1 ± 2.4
Total n-3 PUFA	**28.9 ± 1.8**	**28.8 ± 3.1**	**29.8 ± 2.2**	**29.8 ± 3.1**	**30.2 ± 1.7**	**29.7 ± 2.9**
Total PUFA[4]	**39.1 ± 2.0**	**38.7 ± 3.2**	**39.8 ± 2.3**	**39.0 ± 3.1**	**40.1 ± 1.9**	**39.5 ± 3.1**
Total n-3 LC-PUFA	**25.6 ± 2.0**	**25.7 ± 3.4**	**26.7 ± 2.5**	**25.7 ± 3.5**	**27.2 ± 1.8**	**26.5 ± 3.3**

Data are expressed as means ± SD (n=3). [1]Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Statistical differences were determined by ANOVA analysis ($P<0.05$). PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA.

Table 10. Selected fatty acid composition (% total fatty acids) and proximate lipid composition (%) of liver of meagre juveniles.

	0	A	EC	ECA	EECC	EECCA
Lipid content (%)	26.7 ± 2.0	27.8 ± 1.1	24.1 ± 2.7	25.6 ± 2.8	25.5 ± 3.0	25.1 ± 1.6
Total saturated[1]	**31.8 ± 2.0**	**32.6 ± 1.5**	**32.7 ± 2.1**	**30.9 ± 1.3**	**31.2 ± 1.4**	**31.7 ± 2.6**
Total monounsaturated[2]	**48.3 ± 0.5**	**47.4 ± 1.4**	**48.3 ± 1.1**	**47.4 ± 0.3**	**47.3 ± 1.4**	**47.3 ± 0.7**
18:2n-6	6.1 ± 1.0	5.5 ± 0.8	5.6 ± 0.7	6.2 ± 0.7	6.3 ± 0.5	5.8 ± 1.0
20:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
Total n-6 PUFA[3]	**6.9 ± 1.1**	**6.3 ± 0.9**	**6.4 ± 0.9**	**7.1 ± 0.7**	**7.2 ± 0.6**	**6.7 ± 1.2**
18:3n-3	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.2
20:5n-3	2.8 ± 0.3	3.0 ± 0.4	2.8 ± 0.5	3.1 ± 0.0	3.1 ± 0.5	3.2 ± 0.6
22:6n-3	4.8 ± 0.5	5.4 ± 1.0	4.7 ± 0.8	5.9 ± 0.4	5.5 ± 0.9	5.5 ± 0.8
Total n-3 PUFA[4]	**11.9 ± 1.3**	**12.5 ± 1.9**	**11.4 ± 1.8**	**13.4 ± 0.4**	**13.1 ± 2.2**	**13.1 ± 2.1**
Total PUFA[5]	**20.0 ± 2.5**	**20.0 ± 2.8**	**19.0 ± 2.8**	**21.7 ± 1.1**	**21.5 ± 2.8**	**21.0 ± 3.3**
Total n-3 LC-PUFA	**9.4 ± 1.0**	**10.2 ± 1.6**	**9.2 ± 1.6**	**11.0 ± 0.3**	**10.6 ± 1.8**	**10.7 ± 1.7**

Data are expressed as means ± SD (n=3). [1]Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Statistical differences were determined by ANOVA analysis ($P<0.05$). PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA

Experiment II.

There were no differences in the tissue lipid content among the fish fed the different experimental diets, being for kidney around 4.5%, liver 16 % and heart 4.4%. The fatty acid profile of the tissues of fish reflected the dietary fatty acid content. The highest levels of total monounsaturated fatty acids were observed in liver, followed by kidney and heart, however the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) was higher in the heart, followed by kidney and liver. All the other fatty acids were similarly distributed in the three tissues (**Table 11, Table 12. & Table 13**).

Table 11. Hepatic fatty acid composition (percentage of fatty acids) of meagre fed different experimental diets. Data are expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
14:00	2.69 ± 0.19	2.70 ± 0.21	2.55 ± 0.15	2.58 ± 0.04	2.74 ± 0.14
16:00	23.66 ± 1.79	24.66 ± 2.59	24.93 ± 1.15	24.27 ± 0.66	23.33 ± 0.38
18:00	7.87 ± 1.21	7.71 ± 0.86	8.21 ± 0.33	7.54 ± 0.38	7.11 ± 0.23
20:00	0.25 ± 0.03	0.26 ± 0.02	0.25 ± 0.03	0.24 ± 0.02	0.23 ± 0.02
Σ Saturated[1]	35.11 ± 2.77	35.97 ± 3.41	36.55 ± 1.60	35.26 ± 0.92	34.11 ± 0.65
16:1n-7	9.44 ± 0.16	8.91 ± 0.39	9.30 ± 0.12	9.22 ± 0.38	9.46 ± 0.09
18:1n-9	21.92 ± 0.78	22.22 ± 1.38	22.33 ± 0.69	21.78 ± 0.89	21.45 ± 0.55
18:1n-7	3.24 ± 0.29	3.42 ± 0.19	3.36 ± 0.09	3.40 ± 0.12	3.49 ± 0.16
20:1n-7	2.93 ± 0.07	2.85 ± 0.26	2.74 ± 0.13	2.78 ± 0.02	2.83 ± 0.16
22:1n-11	2.09 ± 0.07	1.97 ± 0.23	1.98 ± 0.12	1.95 ± 0.05	2.01 ± 0.18
Σ Monosaturated[2]	41.75 ± 1.11	41.53 ± 1.19	41.79 ± 0.82	41.24 ± 1.33	41.44 ± 0.92
18:2n-6	5.54 ± 0.49	6.11 ± 0.66	5.36 ± 0.20	5.60 ± 0.37	6.12 ± 0.29
18:3n-6	0.15 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.03	0.18 ± 0.00
20:2n-6	0.24 ± 0.02	0.25 ± 0.03	0.24 ± 0.00	0.24 ± 0.01	0.26 ± 0.01
20:3n-6	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.11 ± 0.00
20:4n-6	0.50 ± 0.13	0.53 ± 0.12	0.48 ± 0.09	0.51 ± 0.11	0.58 ± 0.02
Σ n-6PUFA[3]	6.77 ± 0.78	7.40 ± 0.90	6.56 ± 0.35	6.86 ± 0.57	7.53 ± 0.29
18:3n-3	0.68 ± 0.13	0.79 ± 0.11	0.65 ± 0.06	0.68 ± 0.08	0.73 ± 0.00
18:4n-3	0.71 ± 0.26	0.71 ± 0.20	0.69 ± 0.14	0.73 ± 0.18	0.82 ± 0.04
20:3n-3	0.08 ± 0.03	0.08 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
20:4n-3	0.52 ± 0.18	0.56 ± 0.22	0.53 ± 0.10	0.56 ± 0.10	0.62 ± 0.02
20:5n-3	4.35 ± 0.74	3.83 ± 1.41	3.63 ± 0.89	3.88 ± 1.12	4.37 ± 0.32
22:5n-3	1.41 ± 0.24	1.26 ± 0.38	1.31 ± 0.11	1.29 ± 0.35	1.49 ± 0.08
22:6n-3	6.74 ± 1.45	6.00 ± 1.21	6.36 ± 0.79	7.53 ± 0.61	6.81 ± 0.56
Σ n-3PUFA[4]	14.49 ± 2.93	13.24 ± 3.52	13.25 ± 1.96	14.74 ± 1.53	14.92 ± 0.95
(n-3+n-6) PUFA	21.25 ± 3.69	20.64 ± 4.36	19.81 ± 2.28	21.61 ± 2.07	22.45 ± 0.67
Total n-3 LC-PUFA[5]	13.02 ± 0.45	11.65 ± 1.12	11.83 ± 1.03	13.26 ± 0.72	13.29 ± 0.53
Lipids (%)	17.39 ± 1.05	16.21 ± 1.72	15.88 ± 1.09	17.44 ± 1.80	14.07 ± 3.28
Proteins (%)	7.98 ± 0.31	8.29 ± 0.22	8.34 ± 0.37	8.17 ± 0.40	8.32 ± 0.30
Moisture (%)	62.89 ± 0.77	64.21 ± 1.82	63.61 ± 1.24	62.30 ± 2.36	64.03 ± 2.60
Ash (%)	0.67 ± 0.06	0.70 ± 0.23	0.65 ± 0.18	0.52 ± 0.07	0.71 ± 0.20

Table 12. Fatty acid composition (percentage of fatty acids) of kidney of meagre fed different experimental diets. Data expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
14:00	3.82 ± 0.38	3.80 ± 0.66	3.27 ± 0.65	3.89 ± 0.53	3.73 ± 0.42
16:00	20.07 ± 0.20	19.78 ± 0.94	20.90 ± 0.86	20.10 ± 0.77	20.26 ± 0.27
18:00	5.97 ± 0.32	5.66 ± 0.90	6.85 ± 0.78	5.72 ± 0.84	6.11 ± 0.74
20:00	0.26 ± 0.01	0.25 ± 0.00	0.26 ± 0.01	0.25 ± 0.01	0.26 ± 0.02
Σ Saturated[1]	31.07 ± 0.20	30.38 ± 1.31	32.13 ± 0.97	30.89 ± 0.96	31.27 ± 0.57
16:1n-7	5.17 ± 0.73	5.18 ± 0.92	4.36 ± 0.98	5.30 ± 0.68	5.07 ± 0.67
18:1n-9	11.48 ± 0.48	12.10 ± 0.96	10.51 ± 0.89	11.17 ± 0.38	11.06 ± 0.26
18:1n-7	2.99 ± 0.10	3.02 ± 0.10	3.16 ± 0.20	2.97 ± 0.03	2.99 ± 0.06
20:1n-7	2.37 ± 0.23	2.41 ± 0.27	2.14 ± 0.22	2.35 ± 0.24	2.26 ± 0.13
22:1n-11	2.55 ± 0.38	2.57 ± 0.61	2.01 ± 0.55	2.58 ± 0.52	2.37 ± 0.33
Σ Monosaturated[2]	26.30 ± 1.99	27.01 ± 2.78	23.72 ± 2.57	26.05 ± 1.94	25.38 ± 1.35
18:2n-6	6.32 ± 0.25	6.64 ± 0.35	6.02 ± 0.33	6.34 ± 0.53	6.20 ± 0.18
18:3n-6	0.14 ± 0.01	0.14 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
20:2n-6	0.27 ± 0.02	0.28 ± 0.03	0.31 ± 0.05	0.26 ± 0.02	0.27 ± 0.03
20:3n-6	0.13 ± 0.00	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.00	0.13 ± 0.00
20:4n-6	1.93 ± 0.34	1.83 ± 0.58	2.63 ± 0.80	1.92 ± 0.41	2.01 ± 0.25
Σ n-6PUFA[3]	9.30 ± 0.25	9.50 ± 0.32	9.81 ± 0.71	9.30 ± 0.20	9.27 ± 0.11
18:3n-3	0.85 ± 0.06	0.94 ± 0.16	0.69 ± 0.14	0.82 ± 0.12	0.79 ± 0.08
18:4n-3	1.19 ± 0.15	1.20 ± 0.28	0.93 ± 0.27	1.24 ± 0.28	1.15 ± 0.23
20:3n-3	0.10 ± 0.00	0.09 ± 0.00	0.10 ± 0.02	0.09 ± 0.02	0.08 ± 0.01
20:4n-3	0.52 ± 0.06	0.50 ± 0.08	0.47 ± 0.07	0.52 ± 0.08	0.51 ± 0.04
20:5n-3	9.56 ± 0.24	9.24 ± 0.76	10.26 ± 0.96	9.86 ± 0.13	9.78 ± 0.31
22:5n-3	1.67 ± 0.04	1.60 ± 0.09	1.74 ± 0.13	1.71 ± 0.10	1.69 ± 0.03
22:6n-3	16.70 ± 1.89	16.88 ± 1.72	17.11 ± 0.40	16.86 ± 1.63	17.33 ± 1.12
Σ n-3PUFA[4]	30.60 ± 1.90	30.44 ± 2.25	31.31 ± 1.04	31.11 ± 1.24	31.33 ± 0.64
(n-3+n-6) PUFA	39.90 ± 2.02	39.94 ± 2.47	41.12 ± 1.74	40.42 ± 1.27	40.60 ± 0.74
Total n-3 LC-PUFA[5]	28.45 ± 2.10	28.22 ± 1.21	29.58 ± 1.05	28.95 ± 2.30	29.31 ± 0.95
Lipids (%)	4.69 ± 0.23	4.18 ± 0.11	4.10 ± 0.29	4.53 ± 0.33	4.47 ± 0.45
Proteins (%)	14.43 ± 0.23	15.30 ± 0.23	15.14 ± 0.49	15.30 ± 0.30	14.61 ± 0.54
Moisture (%)	80.01 ± 0.32	80.12 ± 0.68	80.47 ± 0.24	80.27 ± 0.51	79.81 ± 0.24
Ash (%)	0.45 ± 0.03	0.47 ± 0.04	0.45 ± 0.05	0.45 ± 0.03	0.46 ± 0.03

Table 13. Cardiac fatty acid composition (percentage of fatty acids) of meagre fed different experimental diets. Data expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
14:00	1.36 ± 0.14	1.14 ± 0.22	1.36 ± 0.20	1.25 ± 0.08	1.48 ± 0.24
16:00	19.07 ± 0.25	18.62 ± 0.80	18.90 ± 0.20	18.75 ± 0.55	18.66 ± 0.30
18:00	9.54 ± 0.48	10.94 ± 1.58	9.47 ± 0.52	9.69 ± 0.33	9.08 ± 0.37
20:00	0.26 ± 0.01	0.24 ± 0.03	0.24 ± 0.00	0.25 ± 0.01	0.25 ± 0.01
Σ Saturated[1]	30.73 ± 0.36	31.63 ± 1.04	30.42 ± 0.47	30.39 ± 0.77	29.97 ± 0.44
16:1n-7	1.71 ± 0.19	1.35 ± 0.30	1.75 ± 0.38	1.47 ± 0.17	2.12 ± 0.39
18:1n-9	7.92 ± 0.33	7.33 ± 0.38	7.81 ± 0.64	7.33 ± 0.24	8.36 ± 0.60
18:1n-7	3.47 ± 0.14	3.48 ± 0.19	3.51 ± 0.09	3.52 ± 0.14	3.45 ± 0.09
20:1n-7	1.72 ± 0.04	1.62 ± 0.14	1.69 ± 0.10	1.61 ± 0.02	1.75 ± 0.11
22:1n-11	0.98 ± 0.08	0.86 ± 0.16	0.96 ± 0.12	0.90 ± 0.05	1.02 ± 0.16
Σ Monosaturated[2]	16.74 ± 0.54	15.59 ± 0.89	16.66 ± 1.29	15.76 ± 0.43	17.67 ± 1.28
18:2n-6	4.96 ± 0.20	5.01 ± 0.20	4.96 ± 0.21	4.82 ± 0.12	5.02 ± 0.29
18:3n-6	0.15 ± 0.08	0.17 ± 0.11	0.19 ± 0.01	0.15 ± 0.09	0.19 ± 0.01
20:2n-6	0.36 ± 0.00	0.37 ± 0.04	0.36 ± 0.02	0.36 ± 0.02	0.35 ± 0.01
20:3n-6	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00
20:4n-6	3.29 ± 0.19	3.52 ± 0.16	3.26 ± 0.17	3.44 ± 0.07	3.15 ± 0.19
Σ n-6PUFA[3]	9.90 ± 0.28	10.26 ± 0.26	9.89 ± 0.11	9.96 ± 0.18	9.82 ± 0.12
18:3n-3	0.49 ± 0.06	0.45 ± 0.06	0.47 ± 0.06	0.45 ± 0.01	0.50 ± 0.06
18:4n-3	0.26 ± 0.05	0.20 ± 0.08	0.28 ± 0.07	0.24 ± 0.06	0.32 ± 0.07
20:3n-3	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.00
20:4n-3	0.30 ± 0.01	0.27 ± 0.02	0.31 ± 0.03	0.36 ± 0.14	0.32 ± 0.02
20:5n-3	8.96 ± 0.04	8.59 ± 0.34	9.09 ± 0.15	8.87 ± 0.31	8.72 ± 0.47
22:5n-3	2.08 ± 0.07	1.99 ± 0.09	2.07 ± 0.06	2.05 ± 0.02	2.06 ± 0.05
22:6n-3	27.30 ± 0.56	27.84 ± 0.44	27.66 ± 1.28	28.67 ± 0.63	27.44 ± 1.99
Σ n-3PUFA[4]	39.49 ± 0.58	39.44 ± 0.34	39.95 ± 1.05	40.72 ± 0.54	39.45 ± 1.88
(n-3+n-6) PUFA	49.39 ± 0.36	49.70 ± 0.33	49.84 ± 1.07	50.68 ± 0.40	49.28 ± 1.85
Total n-3 LC-PUFA[5]	38.64 ± 2.10	38.69 ± 2.03	39.13 ± 1.52	39.95 ± 0.87	38.54 ± 1.32
Lipids (%)	4.47 ± 0.92	3.91 ± 0.78	4.41 ± 0.76	4.81 ± 0.89	4.04 ± 0.37
Proteins (%)	15.82 ± 1.47	15.58 ± 0.15	15.16 ± 0.50	15.48 ± 0.74	15.55 ± 0.25
Moisture (%)	82.70 ± 0.21	82.46 ± 0.30	82.53 ± 0.18	81.84 ± 0.30	82.26 ± 0.41
Ash (%)	0.35 ± 0.04	0.40 ± 0.03	0.33 ± 0.04	0.35 ± 0.05	0.40 ± 0.03

The level of lipid peroxides measured as malondialdehyde (MDA), as indicated by TBARS content (nmol g tissue-1), was not affected in liver by diet, but was significantly lower in heart and kidney of fish fed with the diet CCC (**Figure 1, Figure 2 & 3, respectively**).

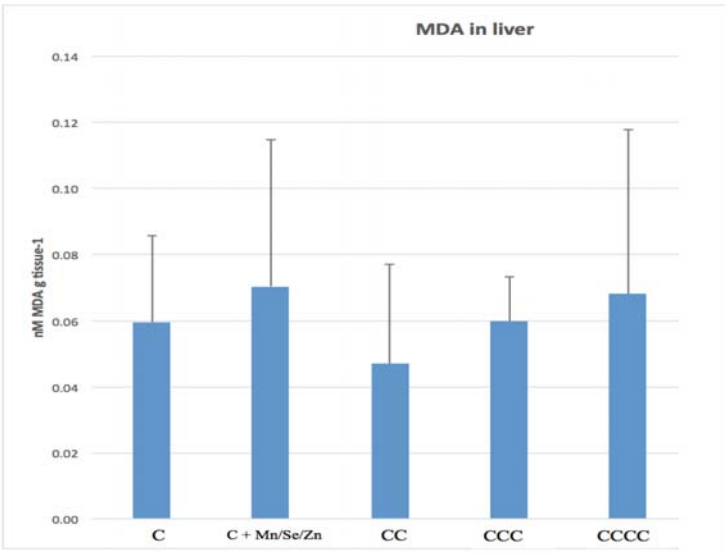

Figure 1. TBARS content in liver of juvenile meagre after 90 days of being fed with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences (P<0.05).

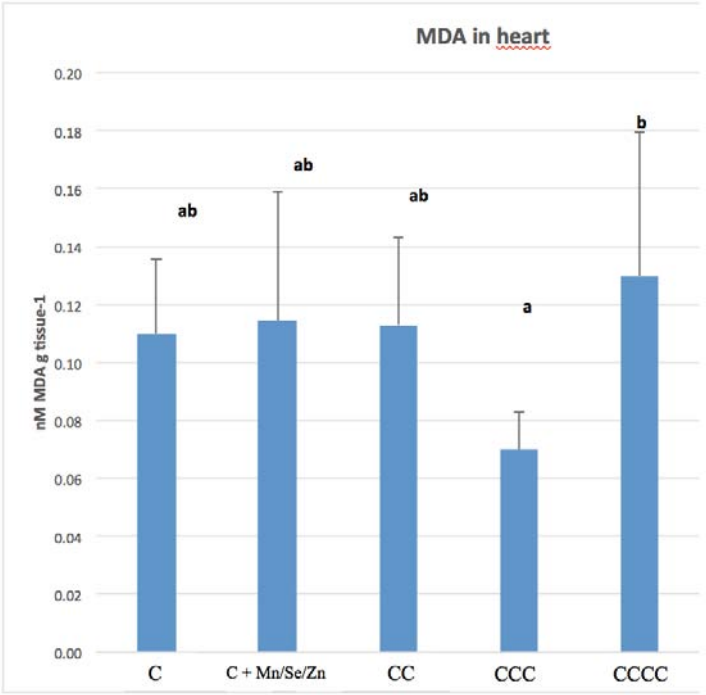

Figure 2. TBARS content in heart of juvenile meagre after 90 days of being fed with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences (P<0.05).

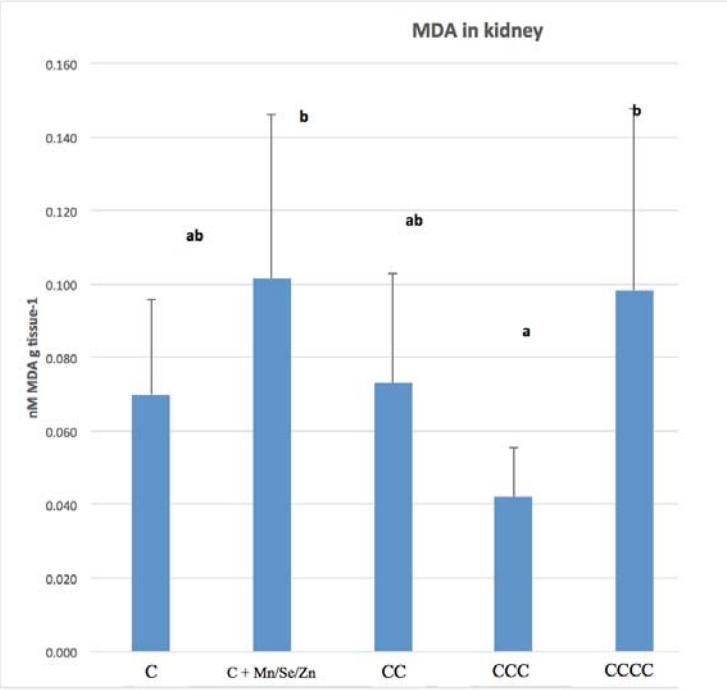

Figure 3. TBARS content in kidney of juvenile meagre after 90 days of being fed with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences ($p<0.05$).

Histology and histopathology

Experiment I

At the initial sampling, the percentage of fish presenting microscopic granulomas was 45 %. At the end of the feeding period, only 10 fish presented macroscopic granulomas, not being related to any particular dietary treatment. In the microscope observation for the evaluation of the tissues different stages of development of the granulomas could be observed. These granulomas begin with an accumulation of macrophages (**Figure 4.a**), which progressively produce a necrotic centre (**Figure 4.b**), and in some cases are surrounded by a layer of fibroblasts and inflammatory cells (**Figure 4.c**). In all cases, the Zielh-Neelsen and Fite -Faraco stains, which are being used to demonstrate acid-fast bacteria, were negative.

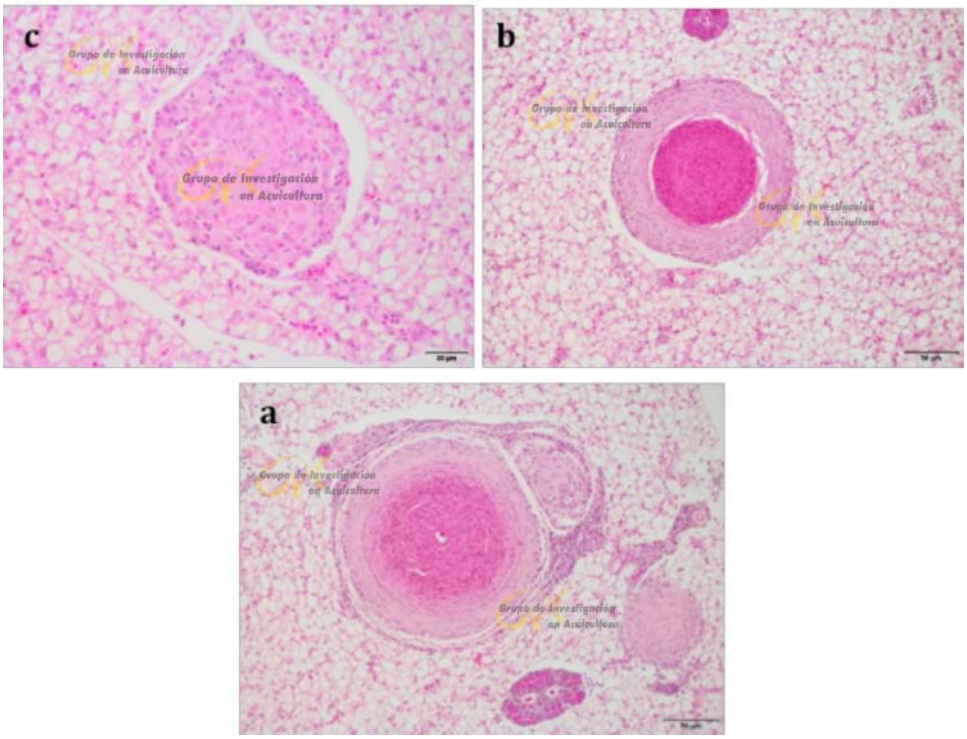

Figure 4. Different stages of granuloma development in liver of meagre juveniles. **a)** Irregular aggregates of macrophages. **b)** Granuloma with necrotic center. **c)** Well developed granuloma with necrotic center surrounded by a layer of inflammatory cells.

The most affected organ was the liver (up to 96% of fishes), followed by the kidney (~ 41%) and heart (~ 6%). No granulomas were found in spleen Significant differences were found in the prevalence of granulomatosis in the livers of the fish (**Table 14**), which tended to have less granulomas when astaxanthin is added to low levels of vitamin E and C, and when high levels of vitamins E and C with or without astaxanthin were included in diets for juvenile meagre (**Table 15**).

Table 14. Prevalence of Granulomatosis (microscopic granulomas) in the liver of the fish fed different diets ($p<0.05$).

Diets	% Fish affected with microscopy granuloma
O	96.33 ± 7.57^{ab}
A	96.00 ± 6.93^{ab}
EC	100.00 ± 0.00^{b}
ECA	81.57 ± 12.42^{a}
EECC	79.67 ± 10.97^{a}
EECCA	87.33 ± 1.15^{ab}

Similar results, although without significant differences, were obtained in the severity of granulomas **(Table 15)** and in the number of fish with a severity score of 3 points in liver and kidney **(Figure 5),** decreasing both

values when astaxanthin is added to low levels of vitamin E and C, and when high levels of vitamins E and C with or without astaxanthin are included.

Table 15. Average granuloma severity ($p<0.05$).

	Granuloma severity		
Diets	Liver	Kidney	Heart
O	1.43 ± 1.24	0.74 ± 1.01	0.00 ± 0.00
A	1.65 ± 0.83	0.70 ± 1.02	0.04 ± 0.21
EC	1.61 ± 0.99	0.74 ± 1.01	0.13 ± 0.34
ECA	1.39 ± 0.99	0.65 ± 0.88	0.30 ± 0.88
EECC	1.13 ± 0.97	0.74 ± 1.01	0.13 ± 0.63
EECCA	1.30 ± 0.88	0.39 ± 0.66	0.17 ± 0.65

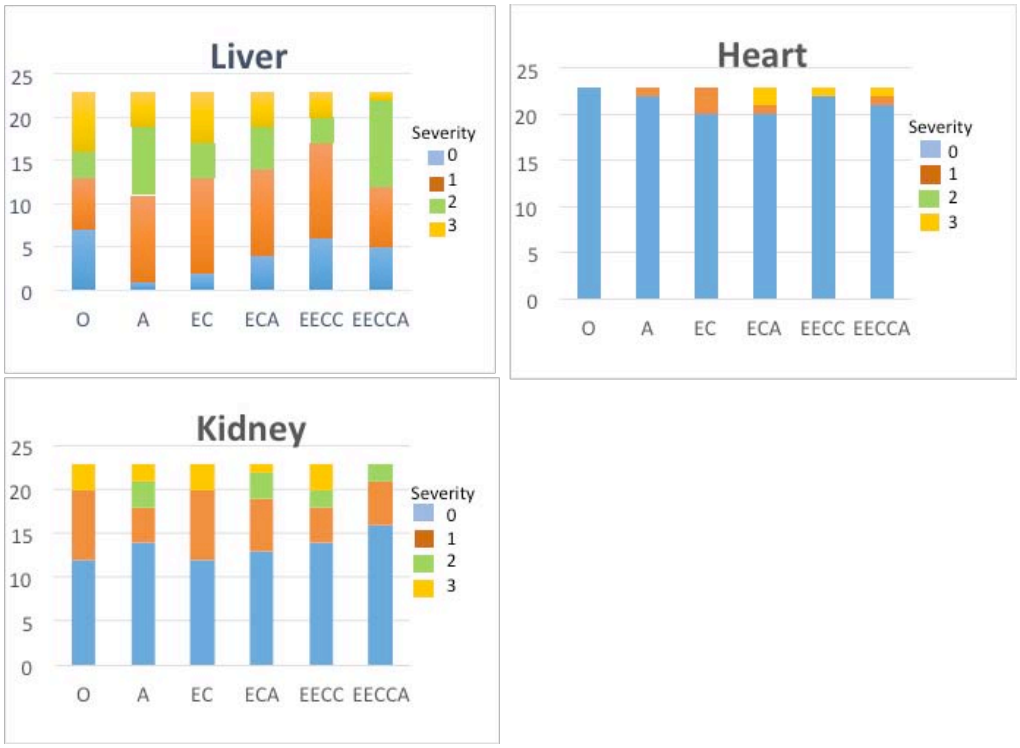

Figure 5. Number of individuals in each severity stage in liver, kidney and heart of fish fed different diets ($p<0.05$).

Experiment II.

Gross granulomas in tissues (liver, kidney and heart) were only observed in 2 fish, not being related to any dietary treatment. The histopathological evaluation revealed different stages of granuloma development (**Figure 6**). At initial stages, granulomas were observed as isolated and irregular aggregates of macrophages and some lymphocytes (**Figure 6a**) that later formed concentric layers (**Figure 6b**). These aggregates progressively led to a necrotic centre with an external layer of fibrocytes (**Figure 6c**). In the final stages, the

granuloma was completely composed of laminar material, especially observed in heart and kidney (**Figure 6d**).

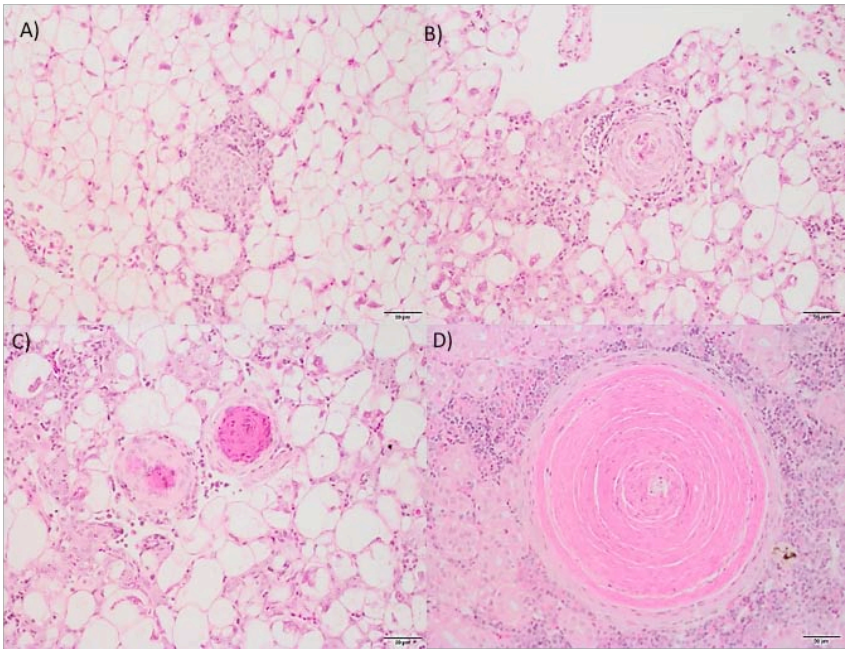

Figure 6. Different stages of granuloma development. **A)** Irregular aggregates of macrophages and inflammatory cells. **B)** Concentric layers of macrophages and inflammatory cells**. C)** Necrotic centre with an external layer of fibrocytes. **D)** Granuloma composed completely of laminar material in kidney).

Regarding microscopic granulomas, the most affected organ was the liver followed by the kidney and heart. A significantly lower number of fish with hepatic granulomas were observed when a high level of vitamin C was added to the feeds (CCC-CCCC diet) (**Table 16**).

Table 16. Percentage of affected liver, kidney and heart with granulomas, of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se after 90 days of feeding with experimental diets. Data are means ± SD. Values in each row with a different superscript are significantly different ($p<0.05$).

Diets	Liver	Kidney	Heart
C	76.7 ± 3.51^{cb}	60.0 ± 6.30	16.7 ± 20.82
C+Mn/Se/Zn	76.7 ± 2.89^{cb}	60.0 ± 0.00	6.7 ± 11.50
CC	83.3 ± 5.77^{c}	70.0 ± 5.00	10.0 ± 17.32
CCC	63.3 ± 1.53^{a}	66.7 ± 4.58	3.33 ± 0.58
CCCC	63.3 ± 1.15^{a}	56.7 ± 5.77	3.33 ± 0.58

The severity score did not show significant differences among fish fed the different dietary treatments in any tissue after 90 days of feeding, however there was a tendency to a decrease in the severity of granulomatosis in liver, for instance in liver the average score was 1.37 in diet C vs a score of 0.97 in diet CCC (**Table 17).**

Table 17. Average granuloma severity scored in liver, kidney and heart of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se after 90 days of feeding with experimental diets. Data are means ± SD. Values in each row with a different superscript are significantly different ($p<0.05$).

Diets	Liver	Kidney	Heart
C	1.37 ± 1.13	0.73 ± 0.78	0.17 ± 0.38
C+Mn/Se/Zn	1.40 ± 1.07	0.77 ± 0.97	0.07 ± 0.25
CC	1.33 ± 1.09	1.00 ± 0.87	0.10 ± 0.31
CCC	0.97 ± 1.03	1.03 ± 0.93	0.03 ± 0.18
CCCC	1.23 ± 1.28	0.87 ± 0.97	0.03 ± 0.18

No calcification was observed at any stage or analyzed tissue. The specific staining (Ziel-Neelsen, Fite-Faraco and Gram stain), were negative, discarding a possible infectious origin (**Figure 7).**

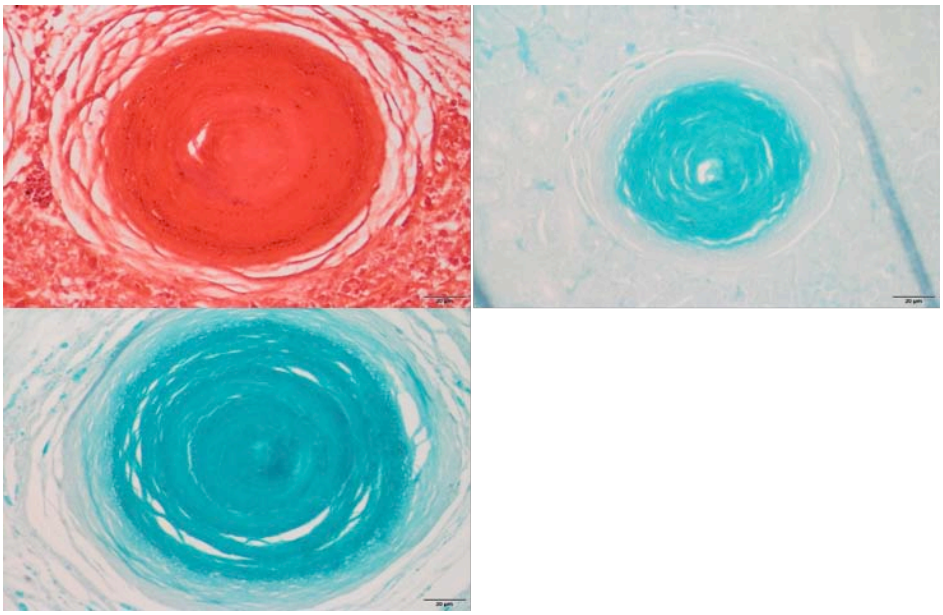

Figure 7. A) Gram stain of granuloma in liver, B) Ziel-Neelsen stain of granuloma in kidney, and C) Fite-Faraco stain in liver.

Gene expression analysis

Experiment I.
The hepatic expression of *cat* was significantly higher in the liver of fish fed the highest level of vitamin E than in fish fed diet 0. Significant differences were not found in the gene expression of *sod* ($p = 0.09$) or *gpx* ($p = 0.201$) in liver but fish fed diets supplemented with the lowest levels of vitamin E and C (EC diet) tended to show a reduced expression of these enzymes.
No significant differences in the gene expression of *cat*, *sod* and *gpx* were observed in kidney. There was an increase in the mRNA levels of *gpx* in fish fed high levels of vitamins E and C (EECC diet) albeit not significant ($p = 0.073$).
In heart, the gene expression of *sod* and *gpx* was significantly increased in fish fed with low levels of vitamins E and C (EC diet).

Experiment II.
There were significant differences in *cat*, *gpx* and *sod* expression in the liver; the expression of this enzyme was higher in fish fed high levels of vitamin C (CCCC diet) compared to fish fed diet C (**Figure 8, Figure 9 & Figure 10**).

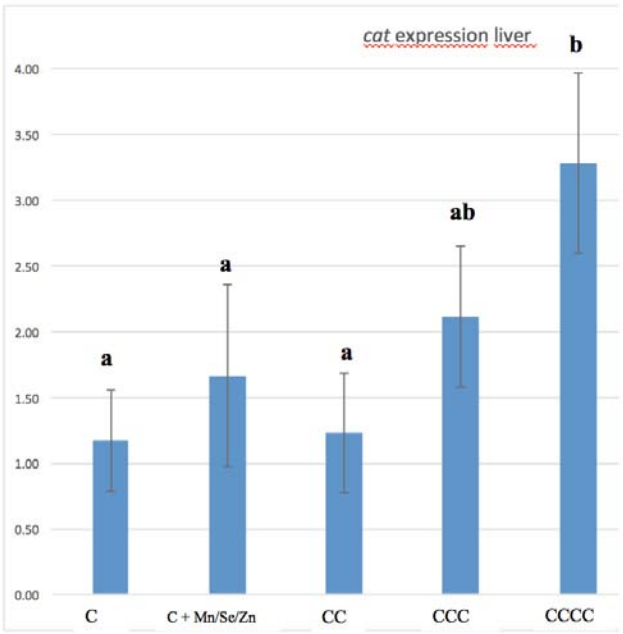

Figure 8. *cat* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($P<0.05$).

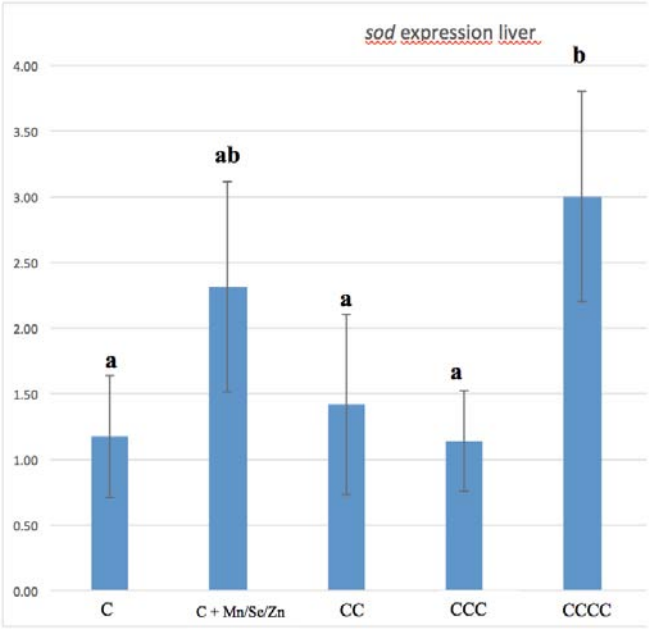

Figure 9. *sod* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences (P<0.05).

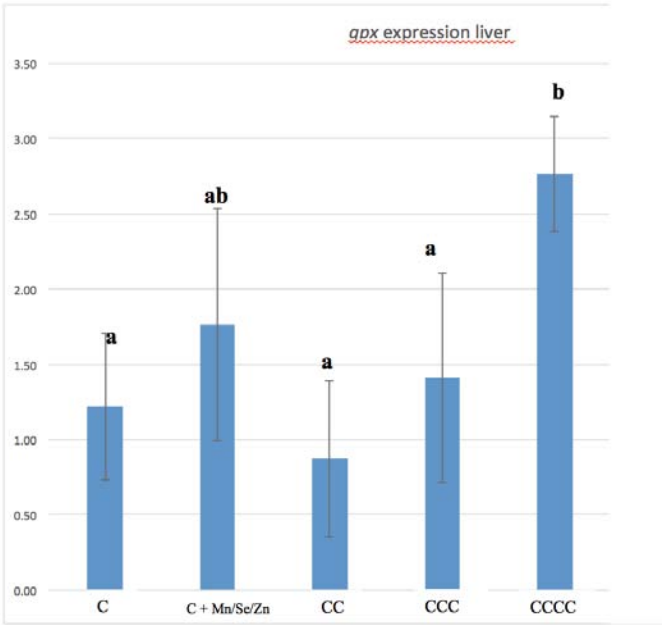

Figure 10. *gpx* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences (p<0.05).

Significant differences (p<0.05) were also observed in the gene expression of *cat* in kidney in fish fed diet CCC (**Figure 11**). No differences were observed in the expression of *sod* and *gpx* (**Figure 12 & Figure 13).**

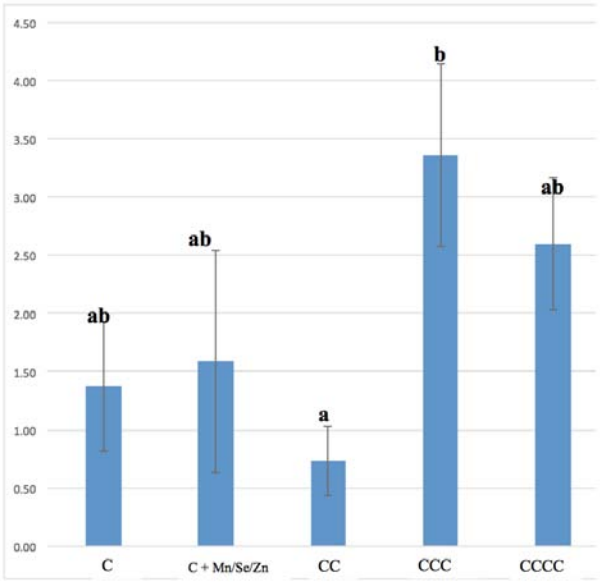

Figure 11. *cad* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($p<0.05$).

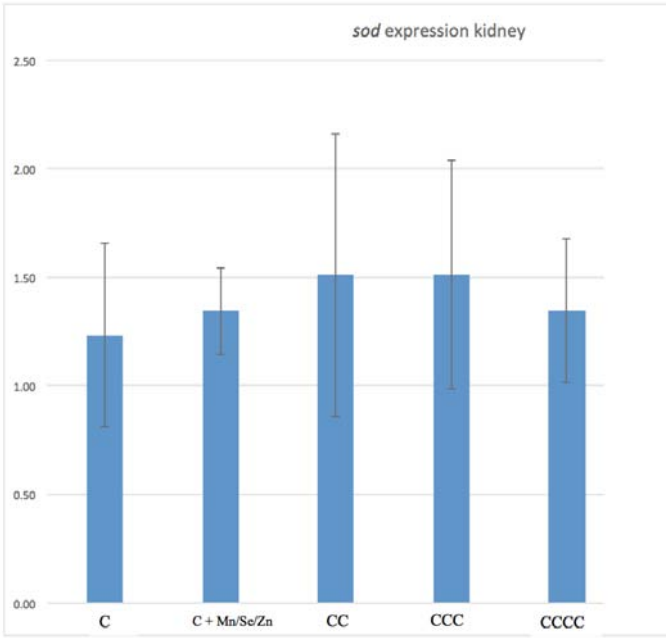

Figure 12. *sod* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($p<0.05$).

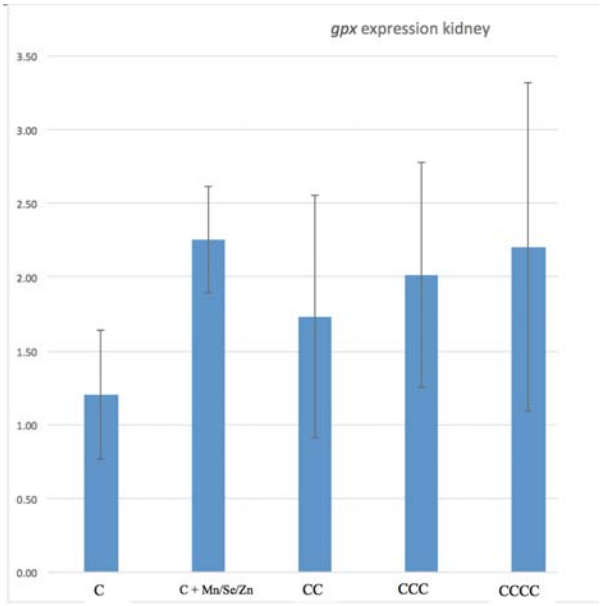

Figure 13 *gpx* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($p<0.05$).

The expression of *cat* in heart was not affected by the inclusion of different levels of vitamin C or Mn, Zn and Se (**Figure 14**). However, significant differences ($p<0.05$) were obtained in the gene expression of *sod* and *gpx* **(Figure 15 & Figure 16)**. The expression was increased in fish fed with high levels of vitamin C (CCCC).

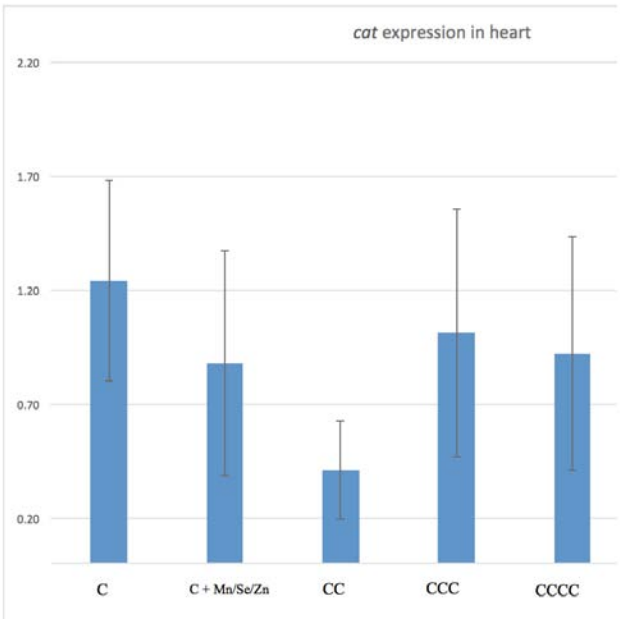

Figure 14. *cad* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($p<0.05$).

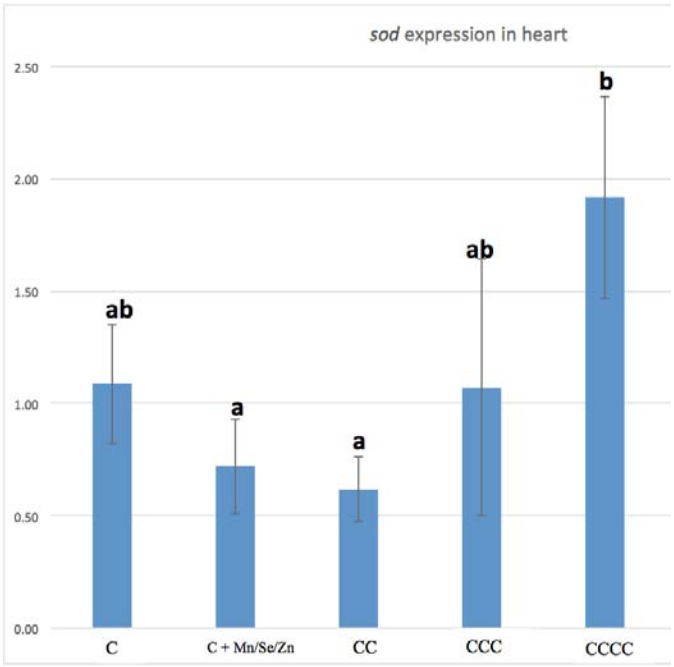

Figure 15. *sod* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($p<0.05$).

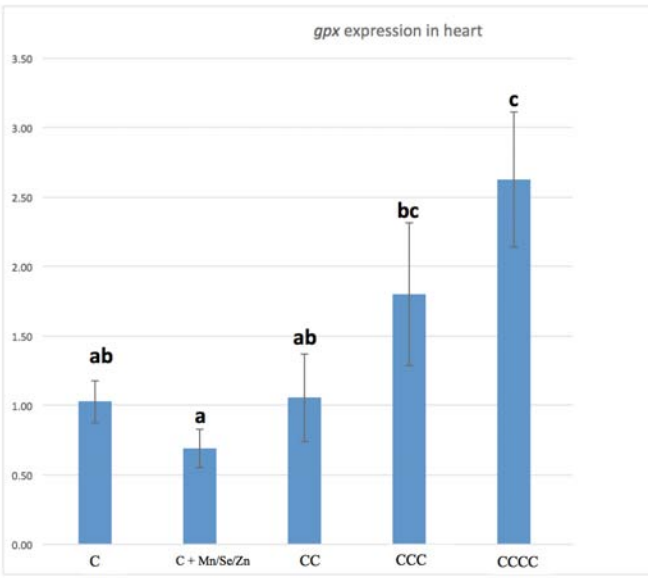

Figure 16. *gpx* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding with experimental diets. Different letters denote significant differences ($P<0.05$).

Granulomatosis in Meagre. Recommended levels of micronutrients.

The combination of a high dietary content of the antioxidants vitamin E and C increased the incidence and number of fish with lower severity of Systemic Granulomatosis. The addition of target minerals did not ameliorate the granuloma incidence or severity, but recommended levels of minerals are: 40 $mg \cdot kg^{-1}$ of Mn, 200 $mg \cdot kg^{-1}$ of Zn, and 1.5 $mg \cdot kg^{-1}$ of Se. This pathology could be mediated by nutritional factors and the antioxidant status of the tissues. The recommended levels of the different micronutrients is: 700 $mg \cdot kg^{-1}$ of Vitamin E, 1200 $mg \cdot kg^{-1}$ of Vitamin C and 500 $mg \cdot kg^{-1}$ of astaxanthin.

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Deviations: There were no deviations from the DOW.

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