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Maslinic acid as a feed additive to stimulate growth and hepatic protein-turnover rates in rainbow trout (*Onchorhynchus mykiss*)

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Abstract

Maslinic acid is a triterpene present in a considerable proportion in solid residues from olive-oil production. In the present work the effects of maslinic acid on growth, protein-turnover rates and nucleic-acid concentration on liver were investigated in the rainbow trout. Five groups of 120 fish of a mean body mass of 20 g were fed for 225 days with diets containing 0, 1, 5, 25 and 250 mg of maslinic acid per kg diet. At the end of the experiment, whole-body and liver weight and growth rate of trout fed with maslinic acid were higher than controls. The highest weight increase was registered for the group fed 250 mg kg^{-1}, representing a 29% increase over controls. The total hepatic DNA or liver cell hyperplasia levels in trout fed with 25 and 250 mg of maslinic acid kg^{-1} were 37% and 68% higher than controls. Also in these same groups of trout, fractional and absolute hepatic protein-synthesis rates were significantly higher than in control, and significant increments in hepatic protein-synthesis efficiency and protein-synthesis capacity were reported. In close agreement with these results, microscopy studies showed that trout fed on 25 and 250 mg kg^{-1} hepatocytes appeared to be more compact, with a larger rough-endoplasmic reticulum and larger glycogen stores than controls. These results suggest that maslinic acid can act as a growth factor when added to trout diet.

Keywords: Growth; Hyperplasia; Maslinic acid; Protein-turnover rates; Rainbow trout; Liver; Nucleic acid concentrations; Feed additive

1. Introduction

Maslinic acid (2-α,3-β-dihydroxyolean-12-en-28-oic acid), a 472.70 Da triterpenoid compound derived from oleanolic acid (3-β-hydroxyolean-12-en-28-oic acid), is widely distributed in plants, being particularly abundant in the surface wax on the fruits and leaves of *Olea europaea* (Bianchi et al., 1994). Maslinic acid, obtained from many plant species, is also present in considerable proportion in the solid waste from olive-oil production (García-Granados et al., 2000). An extraction process from this waste has been established by which large amounts of maslinic acid are obtained, enabling the exploitation of this compound (García-Granados, 1998a). Several research groups have tested maslinic acid activity as a protease inhibitor. This property has been employed in the treatment of several pathologies, such as those caused by human immunodeficiency viruses (Xu et al., 1996; García-Granados, 1998b; Vlietinck et al., 1998) or parasite of genus *Cryptosporidium* (García-Granados, 1998c). Both types of infectious agents have a similar action mechanism and produce specific proteases that allow them to penetrate host-cell walls and invade new cells. Maslinic acid inhibits the action of protease, thereby obstructing the invasion. Other biological activities of this compound are also being studied. In this sense, recent results have demonstrated the role of maslinic acid as a glycogen phosphorylase inhibitor (Wen et al., 2005, 2006). This compound inhibits the increase in plasma glucose induced in diabetic mice by adrenaline (Wen et al., 2005). Also antioxidant (Montilla et al., 2003), antiviral

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(Xu et al., 1996) and antitumor (Taniguchi et al., 2002) activities of this compound has been reported.

One of the commonest methods of fish farming worldwide is pond aquaculture, for which it is of prime importance to discover how to achieve the highest production rates within the shortest time and cost. One strategy is to incorporate new substances into fish diets to enhance food-conversion efficiency or improve general conditions for fish growth and maintenance. In this sense, compounds such as algae (Mustafa et al., 1995), steroids (Cheema and Matty, 1977; Santandreu and Díaz, 1994; Farrar and Rodnick, 2001), antibiotics (Black et al., 1991) or other compounds acting as immunostimulants (Cook et al., 2003) have been used. This study seeks to determine whether maslinic acid can be used an additive and whether or not it induces negative changes in tissues such as liver due to its direct action or accumulation. For a working hypothesis, we assume that maslinic acid could regulate tissue growth by acting on the protein-turnover rates and nucleic-acid concentrations. Thus, the purpose of the present study was to investigate the way in which the addition of maslinic acid to a standard fish diet influences growth, protein-turnover rates and nucleic-acid concentrations in the liver of rainbow trout (*Oncorhynchus mykiss*). We have focused our investigation on the liver because of its important role in regulating the energy metabolism in fish and directing nutrients to the rest of tissues. Moreover, it is the main site of ammoniagenesis (Walton and Cowey, 1977), fatty-acid synthesis (Henderson and Sargent, 1981), and gluconeogenesis (Mommsen, 1986). Because of all these functions the liver is necessarily involved in the regulation of fish growth. The control of the accumulation of structural proteins in tissues results largely from a balance between protein-synthesis and protein-degradation rates. The liver plays a central role in protein metabolism, serving as a short-term store for amino acids to compensate for the fluctuating supply from gastro-intestinal tract (Elwyn, 1970). The liver synthesises large quantities of export proteins, mainly plasma proteins, which function outside this tissue. Protein turnover provides a mechanism by which the tissue-protein pool can adapt to the liver's functional demands that is, a high protein-turnover rate in the liver acts as a response to changes in the plasma supply (Peragón et al., 1999).

2. Materials and methods

2.1. Chemicals

L-[2,6-^{3}H] Phenylalanine (37 MBq mL^{-1}) was supplied by Amersham Biosciences, UK. L-Phenylalanine, L-tyrosine decarboxylase, β-phenylethylamine, leucylalanine and pyridoxal 5-phosphate came from Sigma-Aldrich Chemical Co., USA. All other chemical compounds were bought from Fluka, Switzerland, and were of analytical grade. Maslinic acid was provided by Dr. A. García-Granados, Department of Organic Chemistry, University of Granada, Spain.

2.2. Fish and experimental design

Rainbow trout (*O. mykiss*) were obtained from a local fish farm (Loja, Granada, Spain). For adaptation to laboratory conditions, they were kept for 2 weeks in 360 L fibreglass tanks in fresh, continuously aerated water (1.5 L min^{-1}) at 15.0±0.5 °C under controlled lighting conditions (08:00–20:00) and given free access to a standard commercial diet. Five experimental groups were formed by randomly assigning them 180 fish each. All the fish were weighed individually to ensure a homogeneous sample at the beginning of the experiment. Each group of 180 fish was separated into three different tanks at 60 fish per tank.

Previously, 5 different diets were formulated for the experiment. All diets were made from a standard commercial diet (Dibaq-acuicultura, Segovia, Spain) that contained 44.0% protein, 30% lipids, 4% digestible carbohydrates and 21.16 MJ kg^{-1} diet of gross energy. The gross-energy content was calculated using metabolizable energy values of 19.6, 17.2 and 39.5 kJ g^{-1} for protein, carbohydrates and lipids, respectively (Brett and Groves, 1979). This standard diet was pulverized and mixed with the appropriate amount of maslinic acid in pure form to provide a final concentration of 0, 1, 5, 25 and 250 mg per kg of diet. Pellets were made by passing the diet mixture through an electric meat grinder fitted with disc of 1.5 and 3.0 mm hole size. After drying at 30 °C the diets were kept in opaque sacs and preserved in a refrigerated chamber at 2 °C. These diets were analysed for crude protein, total lipids and moisture using the Association of Official Analytical Chemist method (1984), and they were formulated to meet the requirements of the American Institute of Nutrition (1977, 1980). The inclusion of maslinic acid did not change the gross energy of the diet.

Each experimental group was fed twice daily for 225 days with one of the specific diets: control, 1, 5, 25 and 250 mg of maslinic acid kg^{-1}. This time period was sufficient for the trout of 20 g to reach commercial weight. Daily, from the beginning to the end of the experimental period, the fish were fed manually with a ration equivalent to 1.5% of total tank biomass. For each tank, the weight of total biomass was calculated by weighing the fish weekly and making the specific growth curve as indicated below. The feed consumption of all groups was recorded daily. The relative daily intake of the fish was calculated by dividing the absolute daily diet intake by the mean body weight plotted on the growth curve.

The mortality ratio over the experimental period in the different groups was 4.3% with respect to the initial pool of fish. No significant differences were found among the different treatments.

2.3. Growth curves

The evolution of whole-body and liver weight and liver-protein content over the experimental period in the different experimental groups were represented by the corresponding growth curve. For the plotting of these curves, all data of weights and liver-protein content for the experimental period were used. All the fish were individually weighed at the beginning of the experiment and this process was repeated every week to plot the growth curves. Initial samples of 12 fish from each experimental group, 4 from each tank were killed by cervical dislocation and the weight and protein content of their livers recorded. The protein concentration was determined

according to Lowry et al. (1951). These initial mean values were used for reference in the determination of liver-protein accumulation rate throughout the experiment. Every 2 weeks, 6 fish were sampled from each group to measure their whole-body and liver weights as well as liver-protein concentration. Also, growth curves included the weight of fish used at the beginning of the experiment and after 85, 150 and 225 days. After 225 days of the experiment, 6 fish from each group were killed and their liver removed for microscopic study. All the fish remaining at the end of the experiment were used to provide the final data for whole-body growth, liver-protein and liver-weight curves.

Over the experimental period, the individual whole-body growth rate (G_R) and liver-protein accumulation rate (K_G) were calculated as described in Peragón et al. (1998).

2.4. Protein-turnover rates

The fractional liver-protein synthesis rate was determined as described by Peragón et al. (2001) at 85, 150 and 225 days after the beginning of the experiment, in which the trout were fed 0, 25 and 250 mg kg^{-1} diets. These were the maslinic acid concentrations at which the greatest effects on growth were detected and thus in which protein-turnover rates and nucleic-acid concentrations were determined. The caudal-vein injection solution contained 150 mM L-phenylalanine including L-[2,6 ^{3}H] phenylalanine at 37.0 MBq mL^{-1} (100 μCi mL^{-1}) and a specific radioactivity of 1 640 dpm $nmol^{-1}$. The dosage was 50 μCi 100 g body $mass^{-1}$ at a volume per dosage of 0.5 mL 100 g body $mass^{-1}$.

For the determination of fractional protein-synthesis rate (K_S), 10 fish were used from each experimental group. Two fish were killed 2 min after the injection, and eight after 45 min. The experimental procedure used for K_S determination is described in Peragón et al. (2001).

The fractional protein-synthesis rate, K_S, expressed as % protein synthesised day^{-1}, was calculated as:

$$K_S(\%\ day^{-1}) = [(S_{Bt2} - S_{Bt1})/S_{A(t2-t1)}] \times [1440/(t2-t1)] \times 100$$

where S_{Bt1} and S_{Bt2} represent the specific protein-bound radioactivity at 2 and 45 min after injection; $S_{A\ (t2-t1)}$ stands for the average free pool of specific radioactivity over the period ($t2-t1$); 1440 is the number of minutes in a day.

The absolute protein-synthesis rate (A_S) was calculated as the product of K_S/100 multiplied by the total protein content of the liver and expressed as mg protein synthesised per day. The fractional protein-degradation rate (K_D) was taken as the difference between the protein-synthesis (K_S) and protein-accumulation (K_G) rates, calculated for a period of 43 min and expressed as a percentage per day. The absolute protein-degradation (A_D) and absolute protein-accumulation (A_G) rates were calculated in a manner similar to that for A_S.

Protein-retention efficiency (PRE), defined as the ratio between protein retained as growth versus total protein synthesised, was calculated as $(K_G/K_S)\times 100$.

2.5. Determination of hepatic DNA and RNA concentrations

The RNA concentration was determined by the method described by Munro and Fleck (1966), and applied by Peragón et al. (2001). In the same samples used for protein-turnover determination, RNA and DNA were separated, purified and quantified. After precipitation with 0.2 M $HClO_4$ the pellet was washed twice with 0.2 M $HClO_4$ and RNA was separated from the DNA and protein by basic hydrolysis with 0.3 M KOH at 37 °C for 1 h followed by acidification in 1.2 M $HClO_4$. The supernatant was diluted to 0.6 M $HClO_4$ and the RNA concentration was determined by spectrophotometry at 260 nm,

Table 1
Body growth, food intake and nutritional indexes for rainbow trout fed with different concentration of maslinic acid in the diet

	Control	1 mg kg^{-1}	5 mg kg^{-1}	25 mg kg^{-1}	250 mg kg^{-1}	*F*-ratio	*P*-value	*df*
Body mass (g) and whole-body growth rate (G_R)								
Initial	20.55±0.42^a	20.26±0.40^a	20.46±0.50^a	20.45±0.42^a	20.62±0.42^a	0.097	0.983	49
Final	184.32±2.15^a	209.85±2.98^b	207.37±2.61^b	219.43±3.35^c	238.19±2.95^d	47.610	0.001	49
G_R^1	0.98±0.01^a	1.04±0.01^b	1.03±0.01^b	1.06±0.01^c	1.09±0.01^d	73.068	0.001	49
Relative daily ingestion (mg · g $fish^{-1}$)								
Diet	13.89±0.10^a	13.78±0.10^a	14.91±0.10^a	13.80±0.09^a	13.65±0.12^a	0.766	0.553	49
Proteins	6.08±0.04^a	6.06±0.04^a	6.10±0.05^a	6.07±0.04^a	6.01±0.05^a	0.568	0.687	49
Lipids	4.17±0.03^a	4.14±0.03^a	4.16±0.03^a	4.14±0.03^a	4.10±0.04^a	0.753	0.561	49
Carbohydrates	0.56±0.01^a	0.55±0.01^a	0.55±0.09^a	0.55±0.01^a	0.55±0.01^a	1.259	0.300	49
Energy^2	0.29±0.01^a	0.29±0.01^a	0.29±0.01^a	0.29±0.01^a	0.29±0.01^a	0.201	0.937	49
Maslinic acid^3	0	0.014±0.001^a	0.07±0.001^b	0.35±0.01^c	3.41±0.04^d	11 271.517	0.001	49
Nutritional indexes								
FER^4	0.77±0.04^a	0.83±0.02^a	0.82±0.03^a	0.84±0.03^a	0.87±0.03^a	1.345	0.268	49
PER^5	1.75±0.09^a	1.73±0.11^a	1.87±0.07^{ab}	1.90±0.08^{ab}	1.98±0.06^b	1.655	0.177	49

The results are expressed as the mean±S.E.M of 10 data. The experimental time in all cases was 225 days. The terms "initial" and "final" indicate the average initial and final mass of all experimental trout. Data were treated with a one-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. Data in a row followed by different superscript letters are statistically different. ^1G_R, whole body growth rate, is expressed as % day^{-1}; ^{2}Relative gross energy ingestion is expressed as kJ g $fish^{-1}$; ^{3}Relative daily ingestion of maslinic acid is expressed as μg g $fish^{-1}$; ^{4}FER, feed-efficiency ratio, mass gain (g) diet intake $(g)^{-1}$; ^{5}PER, protein-efficiency ratio, mass gain (g) protein intake $(g)^{-1}$; *df*, degrees of freedom.

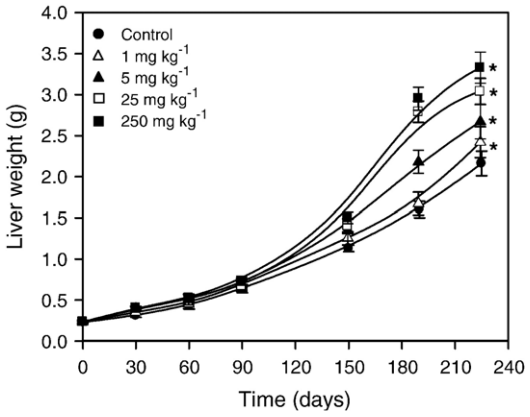

Fig. 1. Evolution of liver mass in trout fed with different concentrations of maslinic acid added to a standard diet. The results are expressed as the means±S.E.M. of 10 observations. Liver growth for each group is defined by the following equations: control: $Y=0.2372e^{0.0101X}$, $r=0.9982$; 1 mg kg^{-1}: $Y=0.2485e^{0.0103X}$, $r=0.9974$; 5 mg kg^{-1}: $Y=0.2576e^{0.0109X}$, $r=0.9962$; 25 mg kg^{-1}: $Y=0.2521e^{0.0117X}$, $r=0.9930$; 250 mg kg^{-1}: $Y=0.2500e^{0.0121X}$, $r=0.9952$. In all cases, Y=liver mass, X=time in days and $P<0.001$. In the last experimental time, the significant differences between the values for the different groups compared with control are marked as * ($P<0.05$).

comparing with a RNA standard curve plotted in the same way as the problem samples.

The DNA concentration was determined fluorometrically using the method described by Labarca and Paigen (1980). Liver homogenates were made in saline phosphate buffer at 1:20 w/v. A standard curve of calf-liver DNA was also made. Aliquots of 2 mL of both types of samples were incubated in darkness with 2.4 mL of Höechst 33258. After 10 min, fluorescence was measured at 450 nm after excitation at 350 nm.

The RNA and DNA concentrations were expressed as mg g tissue^{-1}. The protein:DNA and RNA:DNA ratios were calculated together with the total-protein, RNA and DNA contents.

The values of the protein-synthesis rate are to a great extent proportional to RNA concentrations, and thus protein-synthesis capacity (C_S) can also be defined as a ratio of RNA:protein and expressed as mg g^{-1}. Protein-synthesis efficiency (K_{RNA}) is defined as the amount (g) of protein synthesised per day and RNA unit (g) and is calculated as $[(K_S/C_S)\times 10]$. The protein-synthesis rate/DNA unit (K_{DNA}) is defined as the amount (g) of protein synthesised per day and DNA unit, and is calculated as $[(K_S/100)\times$ protein/DNA].

Table 3
Two-way ANOVA for liver protein and nucleic-acid concentration of rainbow trout fed with different dosages of maslinic acid during the experiment

	df	SS	MS	*F*-ratio	*P*-value
Liver proteins (mg g^{-1} tissue)					
Group	2	9.134	4.567	0.053	0.949
Time	3	6.689	2.230	0.026	0.994
Group–Time	6	9.153	1.526	0.018	1.000
Error	108	9367.776	86.739	–	–
Total	119	9392.753	–	–	–
Liver DNA (mg g^{-1} tissue)]					
Group	2	1.071	0.536	1.734	0.181
Time	3	17.993	5.998	19.415	0.001
Group–Time	6	3.824	0.637	2.063	0.063
Error	108	33.362	0.309	–	–
Total	119	56.250	–	–	–
Liver RNA (mg g^{-1} tissue)					
Group	2	0.657	0.329	1.806	0.169
Time	3	181.834	60.611	333.247	0.001
Group–Time	6	1.778	0.296	1.629	0.146
Error	108	19.643	0.182	–	–
Total	119	203.911	–	–	–

In each experimental group, 10 data were used. $P<0.05$ or less was considered to be statistically significant. *df*, degrees of freedom; SS, sum of squares type III; MS, mean square.

2.6. Microscopy

2.6.1. Light microscopy and picrosirium staining

After 225 days of experimental time, livers corresponding to the five trout groups were dissected out, cut into 4–5 mm blocks, and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 4 h at room temperature. Blocks of liver tissue were cryoprotected by immersion in 30% sucrose in 0.1 M phosphate buffer pH 7.4 overnight at 4 °C. Serial 40-μm-thick sections were obtained by means of a cryostat and stained with 0.2% Sirius red in picric-acid-saturated distilled water for 30 min, at room

Table 2
Liver protein and nucleic-acid concentrations in rainbow trout fed different dosages of maslinic acid over the experiment

Time (days)	0	85	150	225
Liver proteins (mg g^{-1} tissue)				
Control	$132.90\pm3.55^{x}_{a}$	$131.58\pm2.55^{x}_{a}$	$132.70\pm2.60^{x}_{a}$	$132.80\pm2.46^{x}_{a}$
25 mg kg^{-1}	$132.90\pm3.55^{x}_{a}$	$132.85\pm2.95^{x}_{a}$	$133.20\pm3.03^{x}_{a}$	$132.70\pm2.57^{x}_{a}$
250 mg kg^{-1}	$132.90\pm3.55^{x}_{a}$	$132.95\pm2.45^{x}_{a}$	$133.20\pm3.53^{x}_{a}$	$133.60\pm1.98^{x}_{a}$
Liver DNA (mg g^{-1} tissue)				
Control	$6.63\pm0.16^{x}_{a}$	$6.75\pm0.17^{x}_{ab}$	$7.15\pm0.23^{x}_{ab}$	$7.23\pm0.22^{x}_{b}$
25 mg kg^{-1}	$6.63\pm0.16^{x}_{a}$	$6.63\pm0.15^{x}_{a}$	$7.40\pm0.16^{x}_{b}$	$7.29\pm0.21^{x}_{b}$
250 mg kg^{-1}	$6.63\pm0.16^{x}_{a}$	$6.65\pm0.17^{x}_{a}$	$7.28\pm0.19^{x}_{b}$	$8.08\pm0.09^{y}_{c}$
Liver RNA (mg g^{-1} tissue)				
Control	$6.37\pm0.18^{x}_{a}$	$5.97\pm0.23^{x}_{a}$	$3.93\pm0.05^{x}_{b}$	$3.82\pm0.06^{x}_{b}$
25 mg kg^{-1}	$6.37\pm0.18^{x}_{a}$	$6.59\pm0.21^{x}_{a}$	$3.84\pm0.04^{x}_{b}$	$3.83\pm0.08^{x}_{b}$
250 mg kg^{-1}	$6.37\pm0.18^{x}_{a}$	$6.47\pm0.10^{x}_{a}$	$3.93\pm0.04^{x}_{b}$	$4.01\pm0.10^{x}_{b}$

The results are expressed as the mean±S.E.M of 10 data. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. For comparison between times, data in a row followed by different subscript letters are statistically different. For comparison between groups, data in a column followed by different superscript letters are statistically different.

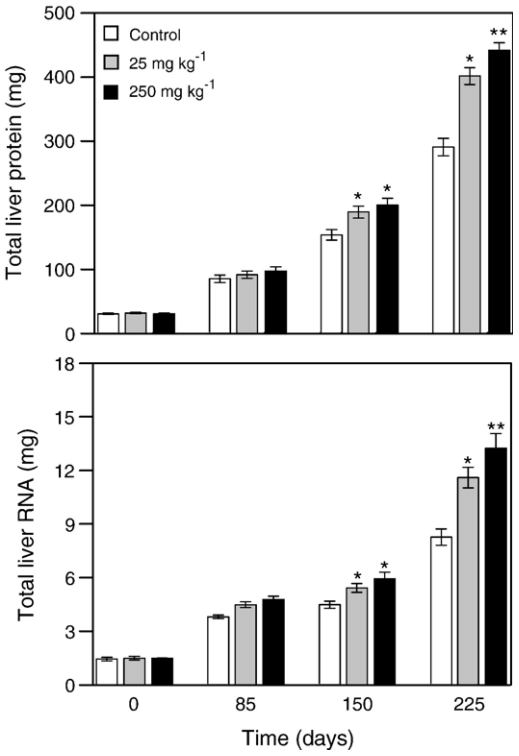

Fig. 2. Total liver protein and RNA contents in rainbow trout fed with different dosages of maslinic acid. The results are expressed as the means±S.E.M. of 10 observations. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. Asterisks above the bars indicate statistical differences ($P<0.05$) with respect to control.

temperature and in the darkness. After this, sections were washed with distilled water, dehydrated in a graded ethanol series, cleared in xylene and mounted in DPX. Sections were examined under a light microscope using polarization filters.

2.6.2. Transmission-electron microscopy

Blocks 2 mm thick of the same livers were fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 h at 4 °C. The blocks were then immersed in osmium, dehydrated in ascending ethanol series, immersed in propylene oxide, and embedded in epoxy resin. They were then sectioned with an ultramicrotome to obtain 1-μm-thick sections or ultrathin sections. Thick sections were stained with toluidin blue and examined under a conventional light microscope. Ultrathin sections were contrasted with uranyl acetate and lead citrate and viewed with an electron microscope.

2.7. Statistical analysis

All values are reported as means±S.E.M. Statistical significance was determined using a one-way or two-way ANOVA followed by Duncan's multiple-range test. Differences between means were also analysed by an unpaired Student's *t*-test. The differences were considered significant at $P<0.05$. To avoid any possible tank effect the differences were analysed by the Student's *t*-test.

3. Results

3.1. Effect of maslinic acid on whole-body and liver growth, food intake and nutritional indexes

Whole body, liver growth, relative daily intake and feed-conversion parameters were monitored for 225 days in five groups of rainbow trout fed with 0, 1, 5, 25 and 250 mg of maslinic acid per kg of diet. The results are shown in Table 1 and Fig. 1. The trout fed with maslinic acid registered significant increases in body weight and whole-body growth rate with respect to control. This effect was stronger in the groups of 25 and 250 mg kg^{-1}, where the mean final weight was 19.05% and 29.22% higher than for the control diet, respectively. Furthermore, even rates as low as 1 mg kg^{-1} and 5 mg kg^{-1} led increased body weight and G_R in relation to control. A significant increase of the whole-body growth rate, G_R, was also registered for all groups fed with maslinic acid. The highest value of G_R obtained in trout fed with 250 mg kg^{-1} was 11% higher than in control. On the other hand, the relative intake of the different nutrients and food-conversion efficiency (FER) showed no significant changes among the five groups analysed, although, in the case of FER, a non-significant tendency towards increased values appeared in the fish fed with maslinic acid. In the case of protein-conversion efficiency (PER), a similar trend is showed. The values of PER for

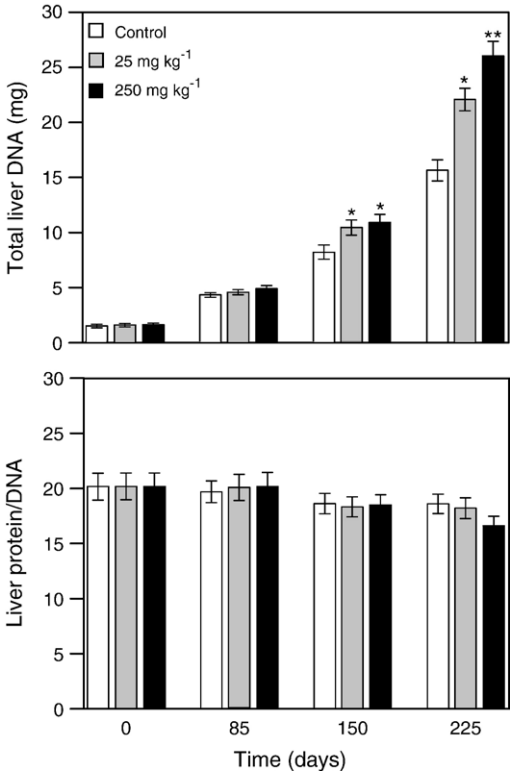

Fig. 3. Total liver DNA and protein:DNA ratio in rainbow trout fed with different dosages of maslinic acid. The results are expressed as the means±S.E.M. of 10 observations. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. Asterisks above the bars indicate statistical differences ($P<0.05$) with respect to control.

Table 4
Hepatic protein-turnover rates and protein-retention efficiency of rainbow trout fed with different dosages of maslinic acid at different experimental times

Time (days)	85	150	225
K_S[1]			
Control	$7.65\pm0.44^{x}_{a}$	$7.16\pm0.79^{x}_{ab}$	$6.47\pm0.29^{x}_{b}$
25 mg kg^{-1}	$10.57\pm0.66^{y}_{a}$	$9.20\pm0.46^{y}_{ab}$	$8.29\pm0.36^{y}_{b}$
250 mg kg^{-1}	$9.11\pm0.47^{y}_{a}$	$8.38\pm0.34^{xy}_{ab}$	$8.06\pm0.15^{y}_{b}$
K_G[2]			
Control	$1.22\pm0.06^{x}_{a}$	$1.08\pm0.05^{x}_{ab}$	$0.99\pm0.03^{x}_{b}$
25 mg kg^{-1}	$1.29\pm0.06^{x}_{a}$	$1.23\pm0.05^{y}_{a}$	$1.16\pm0.05^{y}_{a}$
250 mg kg^{-1}	$1.35\pm0.05^{x}_{a}$	$1.26\pm0.05^{y}_{ab}$	$1.19\pm0.04^{y}_{b}$
K_D[3]			
Control	$6.76\pm0.13^{x}_{a}$	$6.55\pm0.13^{x}_{a}$	$5.39\pm0.12^{x}_{b}$
25 mg kg^{-1}	$9.34\pm0.36^{y}_{a}$	$7.98\pm0.24^{y}_{b}$	$7.30\pm0.10^{y}_{c}$
250 mg kg^{-1}	$7.68\pm0.16^{z}_{a}$	$7.03\pm0.14^{z}_{b}$	$6.82\pm0.11^{z}_{b}$
PRE[4]			
Control	$16.38\pm0.18^{x}_{a}$	$13.72\pm0.30^{xy}_{b}$	$15.52\pm0.32^{x}_{c}$
25 mg kg^{-1}	$14.67\pm0.26^{y}_{a}$	$13.05\pm0.22^{x}_{b}$	$12.83\pm0.25^{y}_{b}$
250 mg kg^{-1}	$14.79\pm0.35^{y}_{a}$	$14.43\pm0.26^{y}_{a}$	$14.04\pm030^{z}_{a}$

The results are expressed as the mean±S.E.M of 10 data. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. For comparison between times, data in a row followed by different subscript letters are statistically different. For comparison between groups, data in a column followed by different superscript letters are statistically different. [1]K_S, protein-synthesis rate, is expressed as % day^{-1}; [2]K_G, protein-accumulation rate, is expresses as % day^{-1}; [3]K_D, protein-degradation rate, is expressed as % day^{-1}; [4]PRE, protein-retention efficiency, is expressed as % day^{-1}.

the group of 250 mg kg^{-1} are 13% higher than control value ($P<0.05$).

With respect to liver growth, in control trout, liver weight increased over the 225 days of experiment, following a curve of exponential regression (Fig. 1). The inclusion of maslinic acid in the diet led to significant increments in liver growth, as shown in the same figure. From 150 days of feeding with 5, 25 and 250 mg of maslinic acid per kg of diet, liver weight significantly increased with respect to control, and after 225 days of treatment the values for the groups of 25 mg kg^{-1} and 250 mg kg^{-1} were 52.1% and 39.6% higher than control, respectively (Fig. 1).

3.2. Effect of maslinic acid on liver nucleic-acid contents

The evolution of liver protein and nucleic-acid concentrations in trout fed with 0, 25 and 250 mg kg^{-1} during the experimental time is presented in Table 2. A two-way ANOVA of these data statistically analysed the effect of time and experimental group (Table 3). In control, from 0 to 225 days of the experiment, the liver-DNA concentration augmented significantly, from 6.63 to 7.23 mg g^{-1}, while RNA concentration declined, from 6.37 to 3.82 mg g^{-1}, and protein concentration did not significantly change over the experimental period. The inclusion of 25 or 250 mg maslinic acid per kg of diet prompted no significant changes in liver protein and RNA concentrations, expressed as mg g^{-1} tissue (Tables 2 and 3) with respect to control. Nevertheless, a 12% significant increase over control in the liver-DNA concentration was detected for 250 mg kg^{-1} after 225 days.

On the other hand, total liver protein, RNA and DNA in trout fed with 25 and 250 mg kg^{-1} were significantly higher than in control at 150 and 225 days (Figs. 2 and 3). Regarding the nature of growth, total DNA values or hyperplasia level for 25 and 250 mg kg^{-1} groups were 37% and 68% higher, respectively, than in control at 225 days. The magnitude of these increments was lower at 150 days. However, protein/DNA ratio, indicating cell size and thus hypertrophy, showed no significant changes (Fig. 3).

3.3. Effect of maslinic acid on liver protein-turnover rates

The effects of the different dosages of maslinic acid on protein-turnover rates in the liver of rainbow trout are summarised in Table 4. Table 5 showed a two-way variance analysis of data in Table 4. In all experimental groups, the fractional protein-synthesis rate, K_S, significantly declined over the experimental period. The highest K_S values were recorded at 85 days of feeding with 25 mg kg^{-1} and 250 mg kg^{-1} diets. At the end of the experiment, for acid groups fed high rates of maslinic acid, K_S was 32.9% and 26.3% higher than control group, respectively. The pattern of fractional protein-degradation rate, K_D, was similar to that described for K_S. The fractional protein-accumulation rate, K_G, showed the same trend, although the differences between control group and 25 mg kg^{-1}, 250 mg kg^{-1} groups were less marked (14%, 19% respectively) after 225 days. In the case of K_D and PRE, a significant interaction between the two factors analysed by the two-way ANOVA (group and time) was found (Table 5).

Fig. 4 shows the evolution of A_S, A_G and A_D values over the experimental period for different groups, the greatest differences being induced by maslinic acid in protein-turnover parameters. In

Table 5
Two-way ANOVA for live protein-turnover rates and protein-retention efficiency of rainbow trout fed with different dosages of maslinic acid during the experiment

	df	SS	MS	*F*-ratio	*P*-value
K_S					
Group	2	78.433	39.217	17.466	0.001
Time	2	33.932	16.966	7.556	0.001
Group–Time	4	5.023	1.256	0.559	0.693
Error	81	181.869	2.245	–	–
Total	89	299.258	–	–	–
K_G					
Group	2	0.422	0.211	7.902	0.001
Time	2	0.409	0.205	7.652	0.001
Group–Time	4	2.331×10^{-2}	5.826×10^{-3}	0.218	0.928
Error	81	2.139	2.673×10^{-2}	–	–
Total	89	3.005	–	–	–
K_D					
Group	2	58.606	29.303	88.298	0.001
Time	2	30.218	15.109	45.527	0.001
Group–Time	4	6.101	1.525	4.596	0.002
Error	81	26.881	0.332	–	–
Total	89	121.806	–	–	–
PRE					
Group	2	38.777	19.389	25.817	0.001
Time	2	43.170	21.585	28.741	0.001
Group–Time	4	21.144	5.286	7.038	0.001
Error	81	60.832	0.751	–	–
Total	89	163.923	–	–	–

In each experimental group, 10 data were used. $P<0.05$ or less was considered to be statistically significant. *df*, degrees of freedom; SS, sum of squares type III; MS, mean square.

the group of 25 mg kg^{-1} and 250 mg kg^{-1}, A_S and A_D values were significantly higher than in control at 85, 150 and 225 days of feeding. At 225 days, A_S of the 25 mg kg^{-1} and 250 mg kg^{-1} group was 94% and 87.5% higher, respectively, than in control. A_D values behaved similarly but at a lower magnitude than those of A_S. Thus, A_G values in trout fed with 25 and 250 mg kg^{-1} at 150 and 225 days were significantly higher than control (Tables 4 and 5).

Table 6 shows the effects of administration of maslinic acid at different dosages over C_S, K_{RNA} and K_{DNA} in the liver of rainbow trout, and Table 7 the ANOVA results. A continuous decrease in protein-synthesis capacity, C_S; over time was detected in all groups. Only slight increases, no higher than 10%, in the group of 25 and 250 mg kg^{-1} with respect to control were detected at 85 and 225 days of experimental time. When protein-synthesis efficiency, K_{RNA}, and protein-synthesis rate per DNA unit, K_{DNA}, were examined, continuous and significant increases were found. Statistical differences between the control and maslinic acid groups were detected in both parameters. After 225 days, 25 mg kg^{-1} and 250 mg kg^{-1} groups showed K_{RNA} values 31.4% and 19.7% higher than control group, respectively. In the case of C_S and K_{RNA}, a significant interaction between the two factors analysed by the two-way ANOVA (group and time) was found (Table 7). The behaviour of K_{DNA} was similar to that of K_{RNA}, with differences of 32.8% and 16.4% between control and the 25 mg kg^{-1} and 250 mg kg^{-1} groups, respectively, at the end of the experimental time.

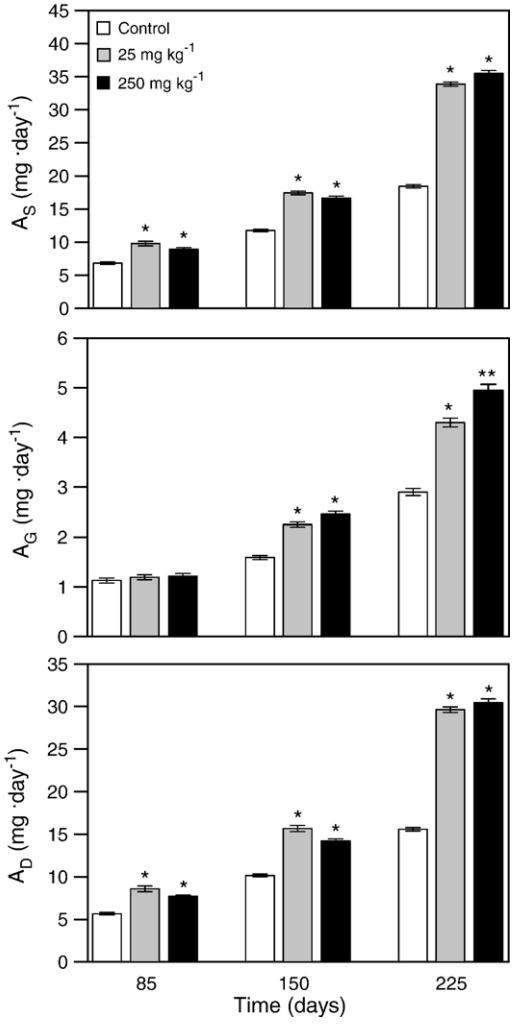

Fig. 4. Total hepatic protein-synthesis rate (A_S), total hepatic protein-accumulation rate (A_G) and total hepatic protein-degradation rate in rainbow trout fed with different dosages of maslinic acid. The results are expressed as the means±S.E.M. of 10 observations. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. Asterisks above the bars indicate statistical differences ($P<0.05$) with respect to control.

Table 6
Changes in liver parameters related with protein-turnover in rainbow trout fed with different dose of maslinic acid

Time (days)	85	150	225
C_S[1]			
Control	$44.63\pm0.56^{x}_{a}$	$29.18\pm0.38^{x}_{b}$	$28.43\pm0.39^{x}_{b}$
25 mg kg^{-1}	$49.30\pm0.42^{y}_{a}$	$28.63\pm0.21^{x}_{b}$	$28.95\pm0.28^{x}_{b}$
250 mg kg^{-1}	$48.76\pm0.56^{y}_{a}$	$29.49\pm0.37^{x}_{b}$	$30.02\pm0.35^{y}_{b}$
K_{RNA}[2]			
Control	$1.79\pm0.05^{x}_{a}$	$2.62\pm0.06^{x}_{b}$	$2.21\pm0.05^{x}_{c}$
25 mg kg^{-1}	$2.05\pm0.06^{y}_{a}$	$3.20\pm0.05^{y}_{b}$	$2.95\pm0.07^{y}_{c}$
250 mg kg^{-1}	$1.85\pm0.05^{xy}_{a}$	$3.02\pm0.09^{y}_{b}$	$2.63\pm0.09^{z}_{c}$
K_{DNA}[3]			
Control	$1.64\pm0.04^{x}_{b}$	$1.46\pm0.04^{x}_{c}$	$1.16\pm0.03^{x}_{d}$
25 mg kg^{-1}	$2.10\pm0.05^{y}_{b}$	$1.68\pm0.04^{y}_{c}$	$1.53\pm0.04^{y}_{d}$
250 mg kg^{-1}	$1.85\pm0.06^{z}_{b}$	$1.58\pm0.05^{y}_{c}$	$1.36\pm0.04^{z}_{c}$

The results are expressed as the mean±S.E.M of 10 data. Data were treated with a two-way ANOVA followed by a Student's *t*-test and Duncan's test. $P<0.05$ or less was considered to be statistically significant. For a comparison between times, data in a row followed by different subscript letters are statistically different. For comparison between groups, data in a column followed by different superscript letters are statistically different. [1]C_S, protein-synthesis capacity, is expressed as mg RNA g^{-1} protein; [2]K_{RNA}, protein-synthesis efficiency, is expressed as g protein synthesised day^{-1} g RNA^{-1}; [3]K_{DNA}, protein-synthesis rate per DNA unit, is expressed as g protein synthesized day^{-1} g DNA^{-1}.

Table 7
Two-way ANOVA for liver C_S, K_{RNA} and K_{DNA} of rainbow trout fed with different dosages of maslinic acid during the experiment

	df	SS	MS	*F*-ratio	*P*-value
C_S					
Group	2	66.258	33.129	20.304	0.001
Time	2	6 806.837	3 403.418	2 085.815	0.001
Group–Time	4	80.962	20.241	12.405	0.001
Error	81	132.167	1.632	–	–
Total	89	7 086.225	–	–	–
K_{RNA}					
Group	2	4.152	2.076	51.755	0.001
Time	2	17.159	8.580	213.883	0.001
Group–Time	4	0.709	0.177	4.419	0.003
Error	81	3.249	4.011×10^{-2}	–	–
Total	89	25.270	–	–	–
K_{DNA}					
Group	2	1.789	0.894	51.816	0.001
Time	2	3.988	1.994	115.527	0.001
Group–Time	4	0.138	3.452×10^{-2}	2.000	0.102
Error	81	1.398	1.726×10^{-2}	–	–
Total	89	7.313	–	–	–

In each experimental group, 10 data were used. $P<0.05$ or less was considered to be statistically significant. *df*, degrees of freedom; SS, sum of squares type III; MS, mean square.

3.4. Microscopy

The hepatic structure of the different experimental groups was analysed using both light microscopy and transmission-electron microscopy. Results are shown in Figs. 5 and 6. Under light microscopy a major degree of cell packaging in the liver parenchyma was detected in the liver of the 25 and 250 mg kg^{-1} groups (Fig. 5). In the liver of 250 mg kg^{-1} trout, sinusoidal space and cell size appeared smaller than in control. These results agree with the major hyperplasia index found in the groups 25 and 250 mg kg^{-1} groups. Moreover, after picrosirium staining, no differences in content and distribution of collagen in any experimental group were noted. In all cases, collagen was allocated around the major vessels.

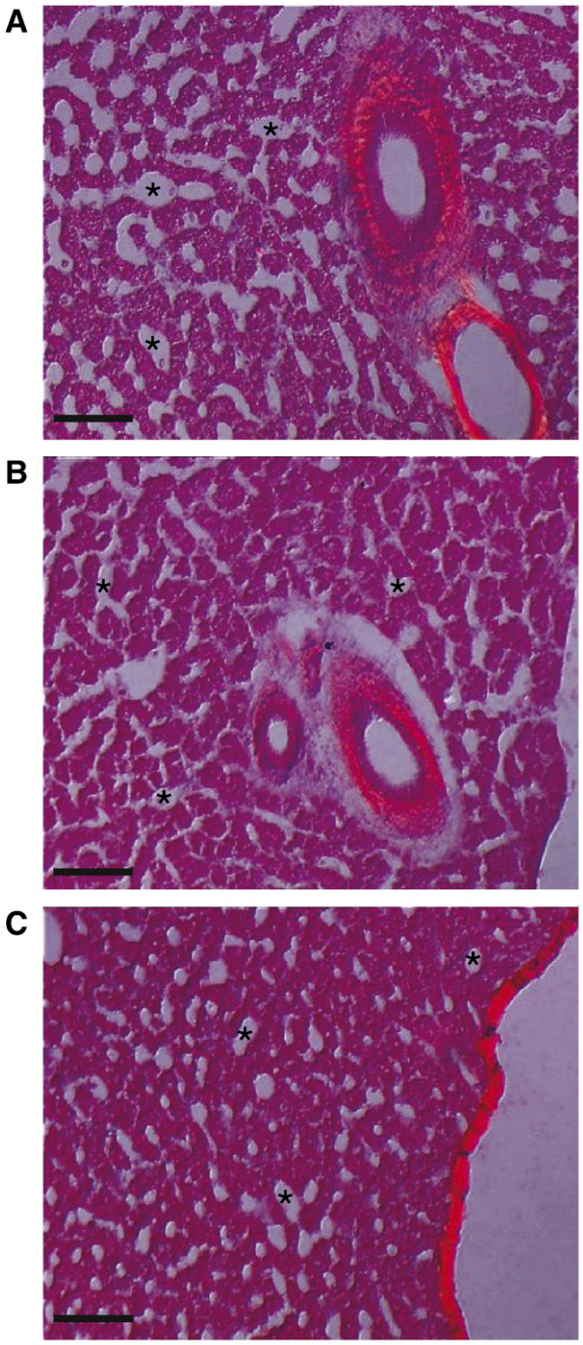

Fig. 5. Photomicrograph of liver sections from trout fed with control diet (panel A), 25 mg of maslinic acid kg^{-1} (panel B) and 250 mg of maslinic acid kg^{-1} (panel C). *=sinusoids. Barr=100 μm.

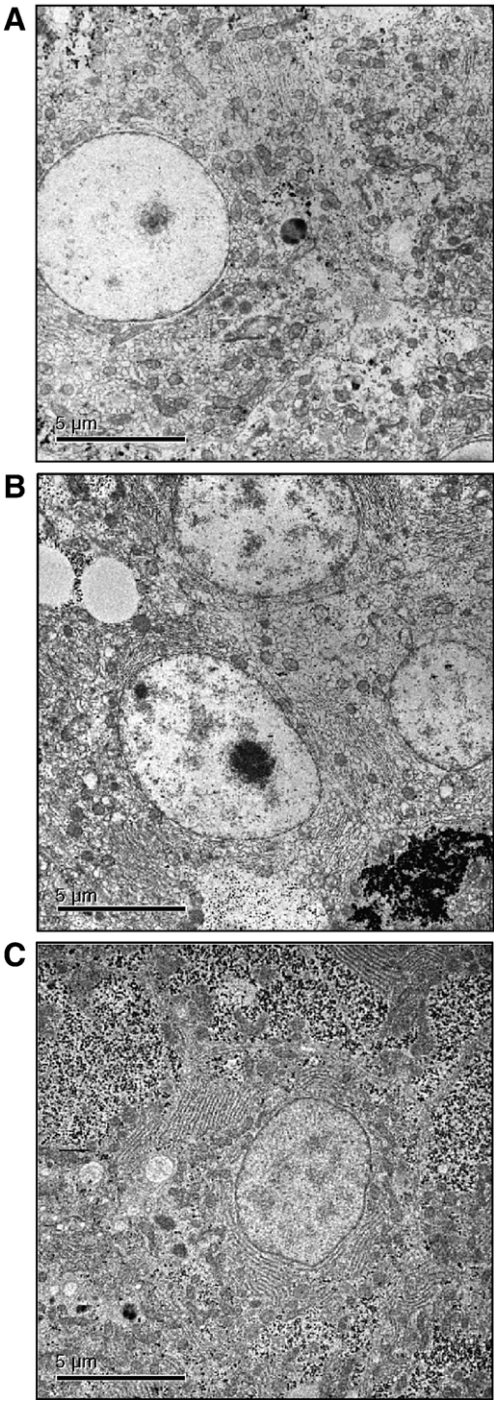

Fig. 6. Transmission-electron micrograph of liver of trout fed with control diet (panel A), 25 mg of maslinic acid kg^{-1} (panel B); 250 mg of maslinic acid kg^{-1} (panel C). Barr=5 μm.

Nevertheless, the main differences in liver structure between control and maslinic acid groups were detected with electron microscopy (Fig. 6). The hepatocytes corresponding to all experimental conditions had their characteristic pyramidal shape,

and showed a clear compartmentalization into a spherical and centrally located nucleus, an area with mitochondria, peroxisomes, smooth endoplasmic reticulum and lipid droplets, a region with rough endoplasmic reticulum cisternae and peripheral glycogen fields. However, the quantity of some of these intercellular structures varied in proportion to the dosage of maslinic acid. Thus, for the 250 mg kg^{-1} group the presence of more abundant and well-distributed rough endoplasmic reticulum was more evident than in the control group. Mitochondria were also more numerous and distributed uniformly throughout the cytoplasm. Greater quantities of peripheral glycogen granules were also detected in the 250 mg kg^{-1} group than in control group. On the other hand, the ultrastructure of the hepatocytes of the 25 mg kg^{-1} group constituted an intermediate situation between control group and 250 mg kg^{-1} group. The hepatocyte ultrastructure of the 1 mg kg^{-1} and 5 mg kg^{-1} groups was similar to that of control.

4. Discussion

The effect of nutrient availability and development stage on protein growth rate in different tissues has been the focus of our research in the past few years. The results have demonstrated that cell-growth changes in the liver and white muscle in different situations are due to differences in protein-turnover rates and nucleic-acid concentrations (Peragón et al., 1998, 2000, 2001). These works demonstrate that nutrient supply or development stage can regulate protein-turnover and growth rates. Hence, the possibility of altering protein-turnover rates (synthesis and/or degradation rates) of a certain tissue could affect its growth rate or even the rate of whole-body growth. In recent years, maslinic acid has been the goal of several studies for different reasons. This is a compound isolated from a natural product, olive waste (García-Granados, 1998a), which is currently being used as a protease inhibitor in the control of different pathologies (García-Granados, 1998b,c) as well as in other biological and therapeutical applications. The present work is based on the hypothesis that maslinic acid could regulate cell growth in different tissues and in the whole-body of trout by regulating protein-turnover rates and nucleic-acid concentrations.

This hypothesis was tested by feeding rainbow trout on diets containing maslinic acid at different concentrations: 0, 1, 5, 25 to 250 mg per kg of diet. The trout were fed at a daily ration of 1.5% body weight, an amount slightly lower than consumed when access to diets is *ad libitum*. Under these conditions, the addition of maslinic acid to the diet did not alter nutrient and energy intake of trout in the different experimental groups. The inclusion of maslinic acid in the diet increased the rate of protein use for growth in the group of 250 mg kg^{-1}. In all other groups the food- and protein-conversion efficiencies did not significantly change, although a tendency to increase was appreciable.

The results of this work demonstrate that maslinic acid fed to rainbow trout significantly increases whole-body growth rate. Whole-body weight of the trout fed 250 mg maslinic acid kg^{-1} for 225 days was 29% higher than control. That increase in growth if translated to fish farm conditions would represent an important boost in trout production and could justify its use as a feed additive.

Weight and protein-accumulation rate significantly augmented for the liver of trout fed maslinic acid. These increments were directly correlated with maslinic acid concentrations in the diet. In fish fed with maslinic acid the DNA concentration and total DNA content increased while the protein/DNA ratio did not significantly change. This indicates that greater liver-cell growth induced by maslinic acid is prime due to a higher hyperplasia level or number of cells in the tissue. Although differences in cell size can contribute to animal- or tissue-size differences, cell number usually makes a larger contribution to tissue growth (Conlon and Raff, 1999), as observed in trout liver (Peragón et al., 1998). With the exception of a few specialized cell types such as muscle and nerve cells, cell size does not vary markedly between animals of the same species, even if individuals are very different in size (Conlon and Raff, 1999). The cause of a change in tissue-cell number could be due to changes either in cell division or in cell death. Increases in cell number could be owed either to greater DNA synthesis and cell division or to less programmed cell death. Programmed cell death depends on an intracellular proteolytic cascade mediated by members of the caspase family of cysteine proteases (Nicholson and Thornberry, 1997). DNA synthesis and cell division are mediated by different growth factors or mitogens (Conlon and Raff, 1999). Further studies are necessary to elucidate the mechanisms involving maslinic acid. Maslinic acid reportedly could act as a protease inhibitor (Xu et al., 1996) and in this sense it would act as an inhibitor of caspases, lowering the level of programmed cell death in this tissue and thereby raising the number of viable cells, but this mechanism does not explain the effects detected in protein-turnover rates.

Maslinic acid significantly boosts liver protein-synthesis rates. The stimulation of liver growth is due mainly to greater protein-synthesis rates (K_S, A_S), which increase the protein-accumulation rate (K_G, A_G). The main factor responsible of the high synthesis level in maslinic-acid groups was the stimulation of the protein-synthesis efficiency rate (K_{RNA}), which is an indicator of the protein-translation rate. It could be considered as a short-term K_S regulation, possibly mediated by hormonal and metabolic signals (Sudgen and Fuller, 1991; Peragón et al., 1998). Liver is characterized by its high protein-turnover rates (Houlihan et al., 1988), a property of tissues that needs to adapt quickly to changes in nutrient conditions and plasma-protein supply. The liver synthesises structural and exportable proteins. Dietary maslinic acid induces higher protein synthesis to sustain the generation of new cells and to be exported for different purposes. At the same time, maslinic acid stimulated the protein-degradation rate, both in relative (K_D) and absolute (A_D) terms. Bearing in mind that liver K_D represents the sum of protein degradation and protein exportation rates, we propose that the increase in K_D values detected in the 25 and 250 mg kg^{-1} groups could be due to a higher exportation rate rather than to an increase in the degradation rate. Moreover, an extra energy supply would be required to maintain the high protein-synthesis rate detected in these groups, and this energy could be provided at least partly by the degradation of dispensable or damaged proteins.

The liver is a key organ in processes related to growth, detoxification, and overall metabolic homeostasis (Henderson and

Sargent, 1981). Because this organ is responsible for the degradation and excretion of metabolic products, toxics and chemicals in general, we made a microscopic analysis of trout fed on diets containing maslinic acid. In trout ingesting 25 and 250 mg kg^{-1}, a higher compaction of hepatocytes in liver parenchyma were found, although the space in the sinusoids was sufficient. This coincided with a high hyperplasia level found under these conditions. In another sense, no differences were found in the collagen content between control and groups fed with maslinic acid. Collagen is located only around larger vessels in all cases. No indications of liver injury, such as fibrosis, were detected as consequence of maslinic acid intake. By electron microscopy, the presence of abundant and well-organized rough endoplasmic reticula in the hepatocytes of trout fed 250 mg kg^{-1} maslinic acid confirms the prolific biosynthesis of exportable proteins. Furthermore, this group of fish showed abundant mitochondria to supply energy for protein biosynthesis. The larger quantities of glycogen in the liver cytoplasm of the 25 and 250 mg kg^{-1} group indicated that a great part of the available glucose is used for glycogen biosynthesis. These fish must have a high energy level by which lipids and protein but not glucose must be degraded to supply metabolic energy. In agreement with the results described in our work on glycogen, Wen et al. (2005, 2006) showed that maslinic acid and derivatives could act as glycogen phosphorylase inhibitors in mice liver.

All these results demonstrate that maslinic acid can act as an authentic growth-stimulating factor when used as a feed additive in trout diets. This factor can stimulate protein synthesis and hyperplasia processes without causing pathological hepatic alterations due to the accumulation of this compound. Nevertheless, this work marks the beginning of future studies investigating the exact molecular mechanism by which maslinic acid is absorbed, transported in blood, enters the cell and stimulates cell growth. Due to its chemical structure, comparable to the steroid hormones, we speculate that this molecule could act in a cell-signalling pathway, as do these hormones, freely crossing the plasma membrane, binding to specific soluble cytoplasm or nucleus receptors, and activating the transcription of specific genes related with protein biosynthesis and cell growth. This is the starting point for future research on this mechanism.

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