

## Effects of alanine aminotransferase inhibition on the intermediary metabolism in *Sparus aurata* through dietary amino-oxyacetate supplementation

Juan D. González<sup>1</sup>, Albert Caballero<sup>2</sup>, Ivan Viegas<sup>3</sup>, Isidoro Metón<sup>1</sup>, John G. Jones<sup>3</sup>, Joana Barra<sup>3</sup>, Felipe Fernández<sup>2</sup> and Isabel V. Baanante<sup>1\*</sup>

<sup>1</sup>Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Joan XXIII s/n, 08028 Barcelona, Spain

<sup>2</sup>Departament d'Ecologia, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

<sup>3</sup>Center for Neurosciences and Cell Biology, Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal

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### Abstract

In liver, through the reaction catalysed by alanine aminotransferase (ALT), alanine becomes an effective precursor for gluconeogenesis. In the present study amino-oxyacetate (AOA) was used to evaluate its effect on liver ALT activity of the carnivorous fish *Sparus aurata*. Moreover, the derived metabolic effects on metabolites and other key enzymes of glycolysis, gluconeogenesis and the pentose phosphate pathway were also studied. A dose-effect-dependent inhibition of AOA on hepatic cytosolic and mitochondrial ALT activity was observed *in vitro*. *In vivo*, AOA behaved as an inhibitor of hepatic cytosolic ALT activity. A long-term exposure to AOA increased pyruvate kinase activity in the liver irrespective of the composition of the diet supplied to fish. <sup>1</sup>H NMR studies showed that inclusion of AOA to the diet decreased the hepatic levels of alanine, glutamate and glycogen. Moreover, <sup>2</sup>H NMR analysis indicated a higher renewal rate for alanine in the liver of fish fed with a high-carbohydrate/low-protein diet, while AOA decreased alanine <sup>2</sup>H-enrichment irrespective of the diet. The present study indicates that AOA-dependent inhibition of the cytosolic ALT activity could help to increase the use of dietary carbohydrate nutrients.

**Key words:** Alanine aminotransferase; Amino-oxyacetate; Enzyme inhibition; *Sparus aurata*

Carnivorous fish have little capacity to utilise dietary carbohydrates; instead, they efficiently use protein amino acids for growth and to obtain energy. In fact, in these animals, amino acids appear to be a more important energy source than carbohydrates. The limited capacity to metabolise dietary carbohydrates and a metabolic profile that mimics non-insulin-dependent diabetes mellitus in mammals after a glucose load led to consider carnivorous fish as glucose intolerant<sup>(1–4)</sup>.

Alanine aminotransferase (ALT; EC 2.6.1.2) links carbohydrate and amino acid metabolism through catalysing the reversible transamination between L-alanine and 2-oxoglutarate to form pyruvate and L-glutamate. The presence of ALT isoforms in the cytosol and mitochondria has been proposed on the basis of biochemical studies<sup>(5)</sup>. In human subjects and mice, two ALT isoforms, ALT1 and ALT2, each encoded by a different gene, have been cloned<sup>(6–8)</sup>. We have previously

reported the presence of three ALT isoforms in gilthead sea bream (*Sparus aurata*): a mitochondrial enzyme (mALT) and two cytosolic isoforms that result from alternative splicing of the cytosolic ALT (cALT) gene (cALT1 and cALT2)<sup>(9,10)</sup>. In regularly fed *S. aurata*, cALT1 is expressed mainly in liver, brain, skeletal muscle, intestine and kidney, whereas cALT2 is the main cytosolic isoform expressed in heart, gill or spleen. The expression of cALT2 occurs in liver under conditions associated with enhanced gluconeogenesis, while cALT1 is predominant during postprandial utilisation of dietary nutrients. Recently, Anemaet *et al.*<sup>(11)</sup> cloned and characterised the *S. aurata* cALT promoter. This study showed that p300 and c-Myb transactivate the cALT promoter, and provided evidence about the involvement of p300 and c-Myb in the up-regulation of cALT2 in the liver of *S. aurata* under starvation.

**Abbreviations:** 6PGDH, 6-phosphogluconate dehydrogenase activity; ALT, alanine aminotransferase; AOA, amino-oxyacetate; AST, aspartate aminotransferase; cALT, cytosolic alanine aminotransferase; DMSO, dimethyl sulfoxide; FBPase, fructose-1,6-bisphosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GK, glucokinase; HC, high-carbohydrate/low-protein diet; i.p., intraperitoneal; LC, low-carbohydrate/high-protein diet; mALT, mitochondrial alanine aminotransferase; PFK-1, 6-phosphofructo 1-kinase; PK, pyruvate kinase.

\* **Corresponding author:** I. V. Baanante, fax +34 934024520, email baanantevazquez@ub.edu

Previously, our group reported that hepatic ALT activity of *S. aurata* is affected by the nutritional status and the composition of the diet<sup>(12,13)</sup> similarly described for other fish species<sup>(2,14–16)</sup>. The effect of hormones and diet composition on key hepatic enzymes was also studied in *S. aurata* and in other fish species<sup>(17–27)</sup>. These studies have shown that feeding diets with high carbohydrate content increased the expression of key glycolytic enzymes in the fish liver.

Although it has been previously found that amino-oxyacetate (AOA) behaves as an activity inhibitor of pyridoxal phosphate-dependent transaminases, no studies have been reported for the effects of ALT inhibition on the intermediary metabolism<sup>(28–32)</sup>. Since AOA was described as a non-toxic or dangerous compound for the environment, in the present study, AOA was administered to *S. aurata* with the aim to (1) analyse the effects of AOA on the hepatic cytosolic and mitochondrial ALT activity, (2) generate a picture of the effects of AOA-dependent ALT inhibition on metabolites and the hepatic activity of key enzymes involved in the use of dietary protein and carbohydrates and (3) evaluate whether ALT is a good target to optimise the use of dietary nutrients in order to spare protein.

## Materials and methods

### Experimental animals

Gilthead sea bream (*S. aurata*) obtained from Tinamenor (Cantabria, Spain) were maintained, at 20°C, in 260-litre aquaria, as described previously<sup>(13)</sup>.

To assay the effect of AOA on the hepatic ALT activity of liver crude extracts, fish of 15–25 g were daily fed at 2% body weight for 10 d on the commercial diet Microbaq (Dibaq-Diproteg S.A., Segovia, Spain; Table 1). To study the dose and short-term effects of AOA on ALT enzyme activity, fish were fed once daily at 2% body weight (BW) for 5 days with a commercial diet Dibaq Microbaq. A total of five groups of seven fish received an intraperitoneal (i.p.) injection of AOA solution ( $1.45 \times 10^{-3}$ ,  $3.625 \times 10^{-2}$ ,  $7.25 \times 10^{-2}$  and 0.145 mg AOA/kg fish) or vehicle (saline).

To study the long-term effects of AOA on metabolite levels and key enzymes involved in the intermediary metabolism in *S. aurata*, ninety-six fish were distributed in twelve aquaria and fed two diets differing in nutrient composition (low-carbohydrate/high-protein (LC) and high-carbohydrate/low-protein (HC) diets; Table 1). For each diet, three aquaria were used for fish fed in the absence of AOA or supplied with 5 or 15 mg AOA/kg fish per d. All fish were fed once daily at 2% body weight for 30 d. To analyse the <sup>2</sup>H-enrichment of alanine, thereafter, fish were placed for 48 h in 5% enriched <sup>2</sup>H-labelled water.

To obtain tissue samples, fish were anaesthetised with MS-222 (1:12500) before handling (09.30 hours). Fish were killed by cervical section, the liver was dissected out, immediately frozen in liquid N<sub>2</sub> and kept at –80°C until use. Unless stated otherwise, tissue samples were obtained 24 h after the last meal. Institutional and national guidelines for the care and use of animals were followed and all experimental

**Table 1.** Composition of the different types of diet provided to *Sparus aurata*

	LC diet	HC diet	Microbaq
<b>Formulation (%)</b>			
Mineral mixture	0.9*	0.9*	1.7
Fishmeal	84.7†	63.9†	60.5
Fish oil	5.0‡	8.4†	13.5
Starch	7.2§	24.6§	–
Wheat meal	–	–	11.4
Wheat gluten	–	–	8.3
Soluble fish extract	–	–	2.5
Yeast extract	–	–	1.0
Soya lecithin	–	–	1.0
Vitamin mixture	0.2	0.2	0.1
Carrageenan	2.0¶	2.0¶	–
<b>Chemical analysis (%)</b>			
Protein	60	45.3	51.0
Carbohydrates**	7.2	24.6	16.3
Fat	14.7	15.7	20.1
Moisture	2.3	1.7	2.1
Celluloses	1	1	0.5
Phosphate	1.3	1.3	1.2
Ash	14.6	11.2	10.5
Gross energy (kJ/g)††	21.3	21.2	23.0

LC, low-carbohydrate/high-protein diet; HC, high-carbohydrate/low-protein diet

\* Mineral mixture provided (mg/kg): CaHPO<sub>4</sub>·2H<sub>2</sub>O, 7340; MgO, 800; KCl, 750; FeSO<sub>4</sub>·7H<sub>2</sub>O, 60; ZnO, 30; MnO<sub>2</sub>, 15; CuSO<sub>4</sub>·5H<sub>2</sub>O, 1.7; CoCl<sub>2</sub>·6H<sub>2</sub>O, 1.5; KI, 1.5; Na<sub>2</sub>SeO<sub>3</sub>, 0.3.

† Corpesca S.A. Super-Prime fishmeal (Santiago de Chile, Chile).

‡ Cod-liver oil from A.F.A.M.S.A. (Vigo, Spain).

§ Pre-gelatinised maize starch from Brenntag Química S.A. (St Andreu de la Barca, Barcelona, Spain).

|| Vitamin mixture provided (mg/kg): choline chloride, 1200; *myo*-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-*rac*-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; pyridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-*trans*-retinol, 2; biotin, 0.7 cholecalciferol, 0.05; cyanocobalamin, 0.05.

¶ Iota carrageenan (Sigma-Aldrich).

\*\* Carbohydrates were calculated by difference.

†† Calculated from gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17 kJ/g).

procedures involving animals were approved by the Animal Use Committee of the Universitat de Barcelona.

### Enzyme activity assays

To assay enzyme activities, crude extracts were obtained from the powdered frozen liver (1:5, w/v) homogenised in buffer H (50 mM-Tris–HCl, pH 7.5, 4 mM-EDTA, 50 mM-NaF, 0.5 mM-phenylmethylsulfonyl flouride (PMSF), 1 mM-dithiothreitol and 250 mM-sucrose) using a PTA-7 Polytron (position 3, 30 s; Kinematica GmbH, Littau-Luzern, Switzerland). After centrifugation at 20 000 g for 30 min at 4°C, the supernatant was used to determine the activity of ALT, aspartate aminotransferase (AST; EC 2.6.1.1), 6-phosphofructo 1-kinase (PFK-1; EC 2.7.1.11), pyruvate kinase (PK; EC 2.7.1.40), fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase activity (6PGDH; EC 1.1.1.43). For the glucokinase (GK; EC 2.7.1.12) assay, 100 mM-KCl were added to the homogenisation buffer and the crude extracts were filtered through Sephadex G-25.

Mitochondrial fractions were prepared, at 4°C, from the powdered frozen liver homogenised (1/5, w/v) in buffer A (50 mM-Tris–HCl, pH 7.5, 0.5 mM-EDTA, 50 mM-NaF, 0.5 mM-phenylmethylsulfonyl flouride, 1 mM-dithiothreitol,

200 mM-mannitol and 70 mM-sucrose). To remove nuclear and cell debris, the homogenate was centrifuged at 500 **g** for 10 min. The mitochondrial fraction was pelleted by centrifugation at 9000 **g** for 20 min. The resulting supernatant contained the cytosolic fraction. The pellet was washed twice with buffer A, resuspended in the same buffer and the mitochondria were disrupted using a Dounce homogeniser.

ALT activity was determined in the direction of L-glutamate formation by monitoring the oxidation of NADH at 340 nm in a mix containing 0.1 M-phosphate buffer (pH 7.12), 0.5 mM-L-alanine, 13.7 mM-2-oxoglutarate, 0.18 mM-NADH, 1.4 U lactate dehydrogenase/ml, at 30°C in a Cobas Mira S spectrophotometric analyser (Hoffman-La Roche, Basel, Switzerland). Assays for PFK-1, PK, FBPase, G6PDH and 6PGDH and total protein were analysed as described previously<sup>(12)</sup>. GK activity was determined as described elsewhere<sup>(19)</sup>. Protein content in the extracts was determined by the Bradford method at 30°C using bovine serum albumin as a standard<sup>(33)</sup>. Glucose levels were determined using a kit (Linear Chemicals; Montgat, Barcelona, Spain).

*Western blot analysis*

Hepatic protein extract (40 µg) was loaded per lane in a 10% PAGE-SDS gel. After electrophoresis, the gel was equilibrated in transfer buffer (25 mM-Tris-HCl, 192 mM-glycine, 20% methanol, pH 8.3) and electroeluted onto NytranN nylon membranes (Whatman, Kent, UK) for 3 h at 60 V at 4°C. To detect ALT protein, the Immun-Star™ Substrate Kit (Bio-Rad, Hercules, CA, USA) procedure was used; a rabbit polyclonal antibody raised against a common domain of *S. aurata* ALT isozymes was used as the primary antibody (1:1000).

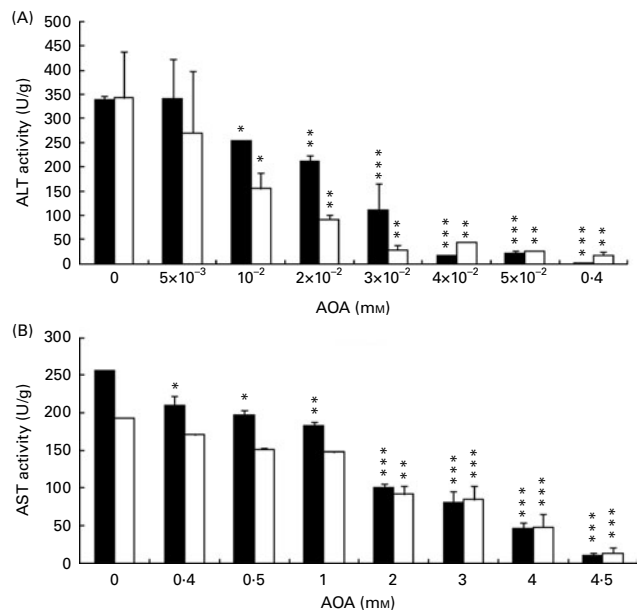
*NMR spectroscopy*

In order to isolate liver amino acids, a perchloric acid extraction was performed on pools of 700–800 mg of the powdered frozen liver from three to five different fish within the same conditions<sup>(34)</sup>. The dried extract was rinsed in 700 µl of 99%-enriched <sup>2</sup>H<sub>2</sub>O, briefly centrifuged and 30 µl of an internal standard containing 12 µmol potassium formate were added. Fully relaxed <sup>1</sup>H NMR spectra were acquired at 11.75 T with a Varian Unity 500 system equipped with a 5 mm broadband. Field-frequency lock was used and five acquisitions were performed after pre-saturation of the water signal, with a 45° pulse angle, an acquisition time of 3 s and a pulse delay of 40 s. The samples were then flushed in a column of Dowex® 50WX8 hydrogen form (Sigma-Aldrich, St Louis, MO, USA) and the amino acids eluted from the column with 2 M-NH<sub>4</sub>OH. After evaporation, the samples were rinsed in 700 µl mix of <sup>2</sup>H-depleted water (Sigma-Aldrich) and 30 µl of an internal standard containing 45 µmol dimethyl sulfoxide (DMSO) and 0.24 µmol DMSO-*d*<sub>6</sub> dissolved in <sup>2</sup>H-depleted water (Sigma-Aldrich). Proton-decoupled <sup>2</sup>H NMR spectra were acquired at 50°C without the field-frequency lock, with a 90° pulse angle, an acquisition time of 2 s and a pulse delay of 5 s. For each sample, 3500–4500 scans were acquired, corresponding to a collection time of 6–8 h. <sup>2</sup>H<sub>2</sub>O

enrichments in a water tank were determined by <sup>2</sup>H NMR as described by Jones *et al.*<sup>(35)</sup>. All NMR spectra were analysed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR Inc., Fremont, CA, USA).

Metabolites in perchloric acid extracts were quantified from the <sup>1</sup>H NMR spectrum according to the ratio of their <sup>1</sup>H signals relative to that of the formate standard multiplied by the amount of formate (12 µmol). For alanine, the methyl signal resonating at 1.47 parts per million was measured, and the area of this signal was divided by 3 to account for the three equivalent hydrogen atoms per molecule. For glutamate, the methylene pseudo-triplet signal resonating at 2.34 parts per million was measured, and the area of this signal was divided by 2 to account for the two equivalent hydrogen atoms per molecule. For glycogen, the <sup>1</sup>H signal resonating at 5.40 parts per million was measured and its ratio to that of the formate standard was directly calculated.

The mean <sup>2</sup>H enrichment of alanine methyl hydrogens in the amino acid fractions was calculated from the <sup>2</sup>H and <sup>1</sup>H NMR alanine methyl and DMSO signals as the internal standard<sup>(36)</sup>. The amount of <sup>2</sup>H (in µmol) was calculated from the ratio of the alanine methyl and DMSO signals as the internal standard in the <sup>2</sup>H NMR spectrum multiplied by the amount of <sup>2</sup>H in DMSO as the internal standard (0.24 µmol). The amount of the <sup>1</sup>H isotope (in µmol) was calculated from the ratio of the alanine methyl and DMSO signals as the internal standard in the <sup>1</sup>H NMR spectrum multiplied by the amount of <sup>1</sup>H in DMSO as the internal standard



**Fig. 1.** *In vitro* effect of amino-oxacetate (AOA) on hepatic (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) activity of *Sparus aurata*. The liver extracts were prepared to obtain cytosolic (■) and mitochondrial (□) fractions, where assayed ALT and AST activity in the absence (0) or in the presence of AOA ranged in concentration from 5 × 10<sup>-3</sup> to 4.5 mM. Values are means of duplicate determinations and corresponding to liver extracts obtained from two animals, with standard deviations represented by vertical bars. Mean values were significantly different from those of control animals: \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

(45  $\mu\text{mol}$ ). The percentage of  $^2\text{H}$  enrichment of alanine methyl hydrogen atoms was estimated as  $100 \times \mu\text{mol } ^2\text{H} / (\mu\text{mol } ^2\text{H} + \mu\text{mol } ^1\text{H})$ . There was no correction for the background  $^2\text{H}$  enrichment.

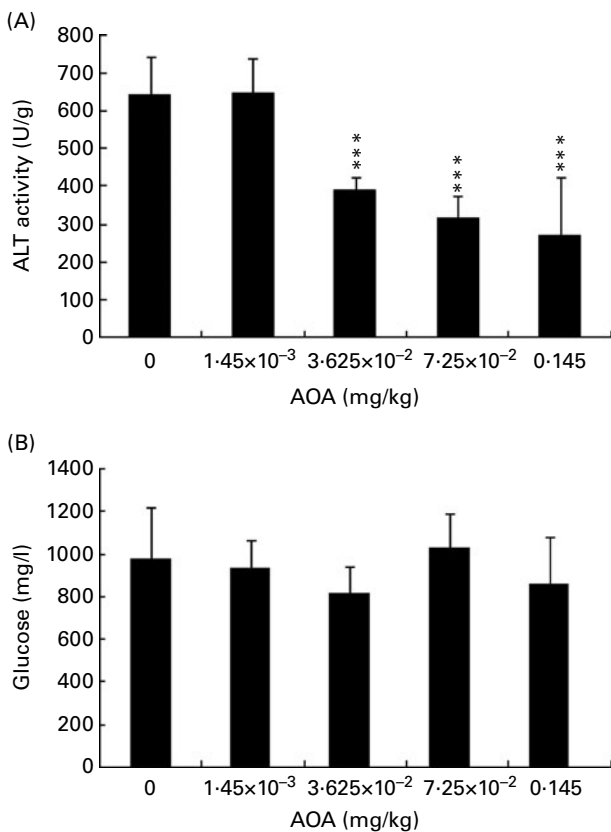
### Statistics

Data were subjected to one-way (Figs. 1–3) and two-way (Figs. 4–7) ANOVA using a computer program (StatView, Cary, NC, USA). Differences were determined by the Fisher's protected least significant difference multiple range test.

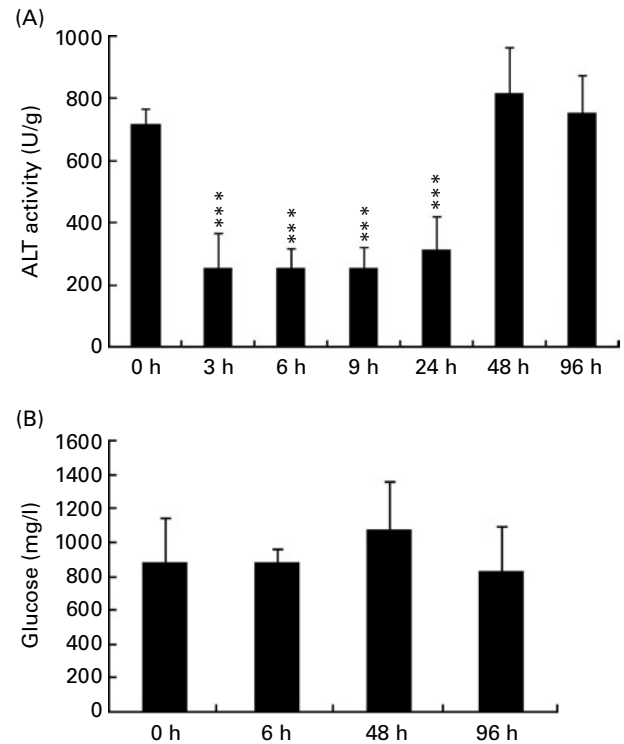
## Results

### *In vitro* effect of amino-oxyacetate on Sparus aurata hepatic alanine aminotransferase activity

To study the *in vitro* effects of AOA on the ALT activity of *S. aurata*, different amounts of AOA ranging from  $5 \times 10^{-3}$  to 4.5 mM were added to the cytosolic and mitochondrial crude extract fractions before performing the enzymatic assays. The lowest concentration of AOA required to show a



**Fig. 2.** Effect of a single intraperitoneal (i.p.) administration of amino-oxyacetate (AOA) on (A) alanine aminotransferase (ALT) activity and on the (B) glucose levels in the serum of *Sparus aurata*. Fish were injected (i.p.) with different amounts of AOA ( $1.45 \times 10^{-3}$ ,  $3.625 \times 10^{-2}$ ,  $7.25 \times 10^{-2}$  and 0.145 mg AOA/kg fish) or saline solution (control). Liver and blood samples were obtained 6 h after injection. ALT activity (U/g protein) was assayed in the liver crude extracts and the glucose blood level in serum was determined. Values are means corresponding to seven fish, with standard deviations represented by vertical bars. \*\*\* Mean values were significantly different from those of control animals ( $P < 0.001$ ).

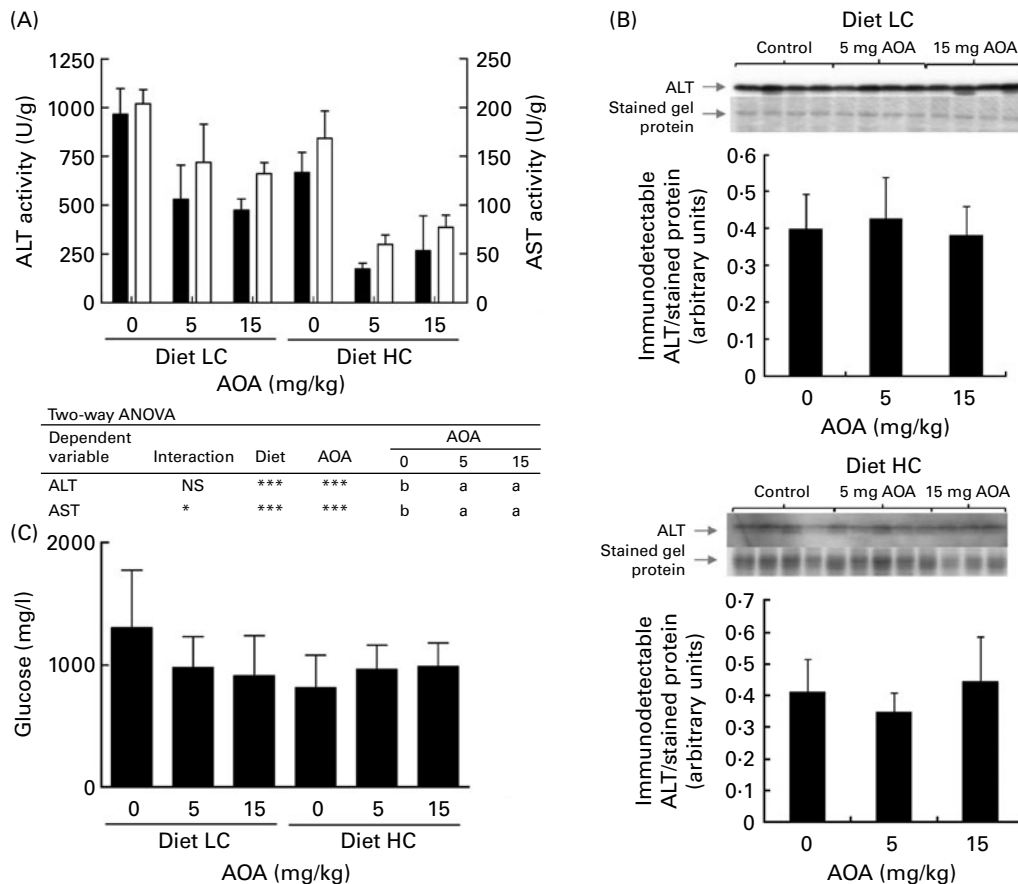


**Fig. 3.** Time course of aminotransferase (ALT) activity and glucose level in serum after the administration of a single dose of amino-oxyacetate (AOA). Fish were intraperitoneally injected with 0.145 mg AOA/kg fish or saline solution (control). Liver and blood samples were obtained at 3, 6, 9, 24, 48 and 96 h after administration. Intraperitoneal injection of AOA (time 0) was carried out 24 h after the last meal. Fish were not fed during sampling at 3, 6, 9 and 24 h after AOA administration. Thereafter, fish were fed regularly once per d and the 48 and 96 h tissue samples were obtained 24 h after the last meal. (A) ALT activity was assayed in the liver crude extracts and the (B) glucose blood level in serum was determined. Values are means corresponding to seven fish, with standard deviations represented by vertical bars. \*\*\* Mean values were significantly different from those of control animals ( $P < 0.001$ ).

significant inhibition of cytosolic ALT activity was  $10^{-2}$  mM (25% inhibition of the enzyme activity observed in the absence of AOA). The same concentration of the inhibitor caused a 55% reduction of the mitochondrial ALT activity. AST activity was measured to compare the effect of AOA on other transaminases. To attain a similar degree of inhibition on the hepatic AST activity, an AOA concentration of about 100-fold higher was needed (Fig. 1).

### Effect of a single administration of amino-oxyacetate on alanine aminotransferase activity *In vitro*

To analyse the effects of AOA *in vivo*, liver samples were obtained 6 h after the i.p. treatment with five doses of this compound to analyse ALT activity, and blood was collected to determine the glucose levels. In AOA-treated *S. aurata*, liver ALT activity significantly decreased to about 60% ( $3.625 \times 10^{-2}$  mg AOA/kg fish), 50% ( $7.25 \times 10^{-2}$  mg AOA/kg fish) and 40% (0.145 mg AOA/kg fish) of the values found in control fish (injected with saline solution). Glycaemia levels did not change significantly at any of the AOA concentrations studied (Fig. 2).



**Fig. 4.** Time course of hepatic aminotransferase (ALT) and aspartate aminotransferase (AST) activity, hepatic ALT protein level and glucose level in serum after the administration amino-oxycetate (AOA) supplied with the diet for 30 d. Fish were fed with the low-carbohydrate/high-protein (LC) and high-carbohydrate/low-protein (HC) diets with 5 and 15 mg AOA/kg fish per d or without AOA (control). (A) ALT (■) and AST (□) activity assayed in the liver crude extracts. (B) Immunodetection of ALT in the liver of fish fed the LC or HC diet in the absence or presence of 5 and 15 mg AOA/kg fish per d. (C) Blood glucose level. Values are means corresponding to six fish, with standard deviations represented by vertical bars. \* $P < 0.05$ , \*\*\* $P < 0.001$ . <sup>a,b</sup> Mean values with unlike letters within a row were significantly different between the AOA treatments ( $P < 0.05$ ).

The time-course effect of AOA on ALT activity and glycaemia was analysed in the extracted liver samples and blood collected after 3, 6, 9, 24, 48 and 96 h following a single i.p. treatment. Hepatic ALT activity was found strongly inhibited (up to 75%) between 3 and 9 h following AOA administration, whereas the enzyme activity showed a tendency to recover basal values after 24 h. Serum glucose levels did not show significant changes (Fig. 3).

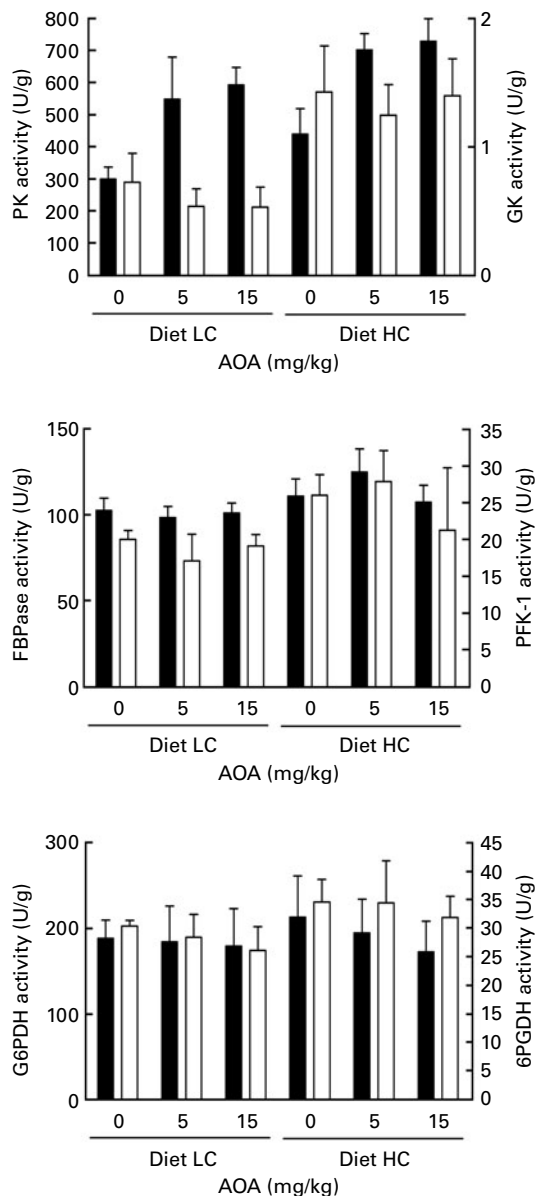
*Effect of long-term exposure to amino-oxycetate on the intermediary metabolism of Sparus aurata*

The effects of long-term exposure to AOA on *S. aurata* ALT activity in liver, metabolite levels and other key enzymes in carbohydrate and amino acid metabolism were studied in fish fed with the LC diet or the HC diet supplied in the absence or presence of AOA.

As expected, in the absence of AOA, hepatic ALT activity in fish fed a HC diet was significantly lower than the activity found in the liver extracts of fish fed a LC diet. Inclusion of 5 and 15 mg AOA/kg fish per d in the LC diet decreased ALT activity values to about 55 and 49%, respectively, of the

values found in the liver of fish fed the LC diet without AOA. A greater inhibitory effect of AOA on ALT activity was observed after the inclusion of 5 and 15 mg AOA/kg fish per d into the HC diet; ALT activity decreased to about 26 and 38%, respectively, of the values observed after feeding the HC diet in the absence of AOA. In order to determine whether AOA affects ALT activity through a mechanism involving changes in protein levels, Western blot assays were performed on liver protein extracts obtained from fish fed the LC and HC diets in the presence or absence of 5 or 15 mg AOA/kg fish per d. There were no significant changes in ALT protein content in the liver samples of fish fed the diets supplied with AOA, which suggest that the inhibitory action of AOA is due to the interaction with ALT (Fig. 4).

The long-term administration of AOA also decreased AST activity in the liver of *S. aurata*, although to a lesser extent than ALT activity. The values of AST activity in fish fed the LC diet supplied with 5 and 15 mg AOA/kg fish per d were 71 and 65%, respectively, of those found in fish fed the LC diet in the absence of AOA. Besides, the decrease observed for AST activity when fish were fed the HC diet with 5 and



Two-way ANOVA

Dependent variable	Interaction	Diet	AOA	AOA		
				0	5	15
PK	NS	***	***	a	b	b
GK	NS	**	NS	-	-	-
FBPase	*	***	NS	-	-	-
PFK-1	NS	***	NS	-	-	-
G6PDH	NS	NS	NS	-	-	-
6PGDH	NS	**	NS	-	-	-

**Fig. 5.** Effect of long-term exposure to amino-oxacetate (AOA) on pyruvate kinase (PK, ■), glucokinase (GK, □), 6-phosphofructo 1-kinase (PFK-1, □), fructose-1,6-bisphosphatase (FBPase, ■), glucose-6-phosphate dehydrogenase (G6PDH, ■) and 6-phosphogluconate dehydrogenase activity (6PGDH, □) activity in the liver of *Sparus aurata*. Enzyme activities were measured in the liver extracts of fish fed 30 d with the low-carbohydrate/high-protein (LC) or high-carbohydrate/low-protein (HC) diet supplied with 5, 15 mg AOA/kg fish per d or without AOA. Values are means corresponding to six fish, with standard deviations represented by vertical bars. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . <sup>a,b</sup> Mean values with unlike letters within a row were significantly different between the AOA treatments ( $P < 0.05$ ).

15 mg AOA/kg fish per d attained 65 and 54%, respectively, compared with fish fed the same diet without AOA (Fig. 4).

Moreover, the activity of key enzymes in glycolysis (PFK-1, PK and GK), gluconeogenesis (FBPase) and the pentose phosphate pathway (G6PDH and 6PGDH) was determined in the liver extracts of *S. aurata* fed with the two above-described diets supplied with 5 or 15 mg AOA/kg fish per d. Under the experimental conditions studied, no significant effect of AOA was observed for most of the enzymes analysed. However, in fish fed with the LC diet supplied with 5 or 15 mg AOA/kg fish per d, PK activity significantly increased 2-fold, compared with the values observed in the liver of fish fed the LC diet without AOA. Similarly, the PK values in the liver of fish fed the HC diet in the presence of 5 or 15 mg AOA/kg fish per d increased 1.6-fold in relation to their controls (Fig. 5).

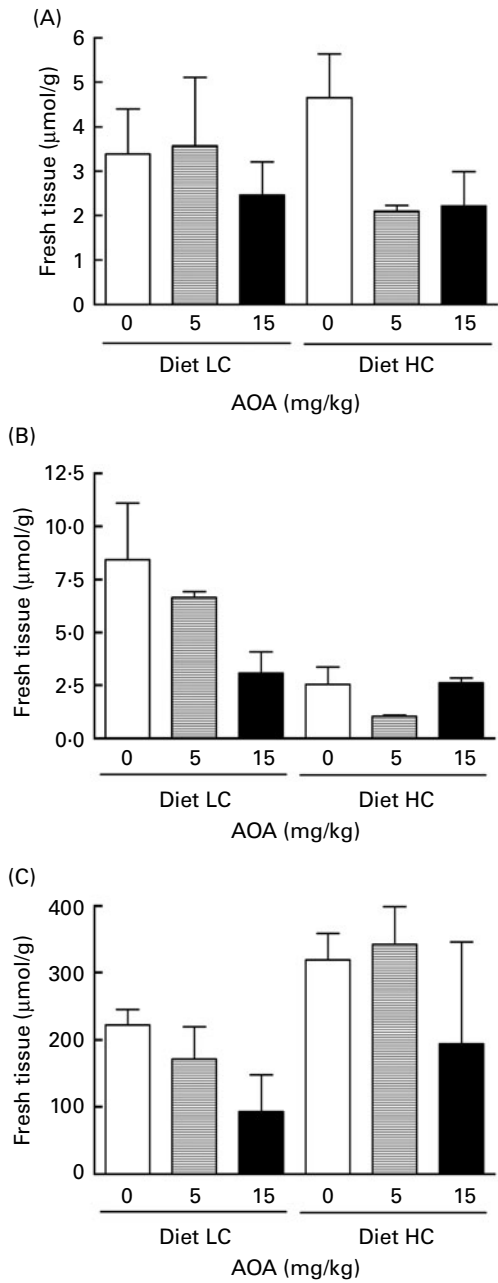
The effects of feeding the LC or HC diet with or without AOA on alanine, glutamate and glycogen hepatic levels were determined from the <sup>1</sup>H NMR spectra (Fig. 6). In the absence of AOA, alanine levels were similar when fish fed either of the diets. The glutamate values in the liver of fish fed with the HC diet were significantly lower, attaining 30% of the values in fish fed the LC diet. Consistent with the higher carbohydrate content of the HC diet, glycogen levels increased 1.5-fold in fish fed the HC diet compared with those fed the LC diet.

Irrespective of supplying the LC or HC diet, the addition of 15 mg AOA/kg fish per d significantly decreased the alanine and glutamate levels. In addition to the effect of AOA, the concentration of glutamate showed a dependence on the composition of the diet; the higher glutamate levels were found in fish fed the LC diet. On the contrary, higher levels of glycogen were found in the liver of fish fed the HC diet. Similarly as for alanine and glutamate, glycogen content decreased when the diets given to fish were supplemented with AOA.

The <sup>2</sup>H-enrichment level of alanine methyl hydrogen atoms can be directly quantified by <sup>2</sup>H NMR following partial purification of tissue amino acids by perchloric acid extraction and cation exchange. The renewal rate of alanine was determined through the NMR <sup>2</sup>H enrichment of alanine in the liver of *S. aurata* fed 30 d with the LC or HC diet in the absence or presence of AOA following a previously described procedure to quantify hepatic glutamine enrichments<sup>(36)</sup> (Fig. 7). In the absence of AOA, fish fed the HC diet exhibited a 32% increase in the *de novo* synthesis of alanine compared with the values found in fish fed the LC diet. Independently of the diet provided, *S. aurata* supplemented with 15 mg AOA/kg fish per d showed a significant decrease in alanine <sup>2</sup>H enrichment.

Discussion

Since the liver plays a central role in the intermediary metabolism, it is not surprising that hepatic cells are the main site for transamination to obtain energy either by oxidation of the carbon skeleton of the amino acids or from glucose obtained after the conversion of the carbon skeleton through gluconeogenesis.



Two-way ANOVA

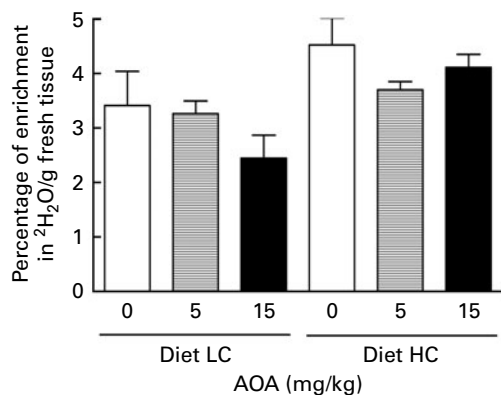
Dependent variable	Interaction	Diet	AOA	AOA		
				0	5	15
Alanine	NS	NS	*	b	a	a
Glutamate	*	***	*	b	a,b	a
Glycogen	NS	**	*	b	b	a

**Fig. 6.** Effect of long-term exposure to amino-oxyacetate (AOA) on (A) alanine, (B) glutamate and (C) glycogen levels in the liver of *Sparus aurata*. Fish fed with the low-carbohydrate/high-protein (LC) or high-carbohydrate/low-protein (HC) diet supplied with 5, 15 mg AOA/kg fish per d or without AOA were killed after 30 d of feeding. The levels of alanine, glutamate and glycogen were obtained from the <sup>1</sup>H NMR spectrum. Values are means corresponding to three to four pools of 500–800 mg of the liver from three to five different fish, with standard deviations represented by vertical bars. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. <sup>a,b</sup> Mean values with unlike letters within a row were significantly different between the AOA treatments (*P*<0.05).

The aim of the present study was to determine the effect of AOA on the ALT activity of *S. aurata* liver and whether transaminase inhibition could affect other important key enzymes and metabolites involved in intermediary metabolism. Cytosolic and mitochondrial liver fractions were used to study *in vitro* the effect of AOA on ALT isoforms. ALT activity of the cytosolic and mitochondrial fractions from the liver of *S. aurata* was strongly inhibited by AOA at concentrations higher than 10<sup>-2</sup> mM. The effect of AOA in the cytosol is essentially attributable to the inhibition of cALT1, by far the main ALT cytosolic isoform in the liver of fed *S. aurata*<sup>(10)</sup>. Nevertheless, AOA behaves as an inhibitor of cALT2, causing the same effect as for cALT1 (data not shown). It has been previously described that AOA also inhibits the hepatic AST activity of human cytosolic and mitochondrial fractions *in vitro*<sup>(37)</sup>. However, in the present study, an AOA concentration of 40-fold higher was needed to cause a similar inhibitory degree on the AST activity present in the *S. aurata* cytosolic and mitochondrial fractions.

*In vivo* analysis of ALT activity in the cytosolic and mitochondrial fractions of *S. aurata* i.p. injected with a single dose of AOA (0.145 mg AOA/kg fish) revealed that 6 h after the treatment, AOA caused a significant decrease in ALT hepatic activity, during 3 to 24 h post-injection, in the total and cytosolic fractions of the liver. ALT activity in the liver samples recovered control values 48 h after the treatment. No inhibition was observed when ALT activity was measured in the mitochondrial fractions (data not shown), which suggest that although AOA inhibits mALT *in vitro*, *in vivo*, the inhibitory compound do not enter the mitochondria and thus do not affect mALT. Consequently, the effect of AOA *in vivo* may be restricted to the cytosolic ALT activity, essentially cALT1, an enzyme isoform that we have previously shown to be mainly involved in the postprandial utilisation of dietary nutrients in fed fish<sup>(10)</sup>. Considering that mALT activity accounts for as less as about 15% of total ALT activity in the liver of fed *S. aurata*<sup>(9)</sup>, the use of AOA *in vivo* appeared to be suitable to specifically inhibit the ALT activity of the cytosolic fractions. Altogether, these findings opened the possibility of using AOA to analyse the effect of long-term cytosolic ALT inhibition on the intermediary metabolism.

In order to analyse whether the effect of AOA depends on the composition of the diet supplied to fish and whether the inhibition can be extended to a longer time period, AOA was incorporated into two diets, differing in the carbohydrate: protein ratio, that were supplied to *S. aurata* during 30 d. Irrespective of the composition of the diet, AOA caused a significant inhibition on ALT activity. Previously, we have shown that hepatic ALT activity values were low in *S. aurata* fed with HC diets<sup>(12,13)</sup>. In the present study, lower ALT activity values were found in the liver of fish fed with a HC diet. Indeed, inclusion of AOA into the diets inhibited ALT activity irrespective of the diet composition. ALT protein levels in the liver of *S. aurata*, determined through Western blot analysis, were not affected by the inclusion of AOA to the diets studied. Therefore, AOA behaved as an inhibitor of ALT activity without affecting the ALT protein levels. It has recently been reported that formation of adducts between AOA and



## Two-way ANOVA

Dependent variable	Interaction	Diet	AOA	AOA		
				0	5	15
Alanine	NS	***	*	b	a,b	a

**Fig. 7.** Effect of long-term exposure to amino-oxacetate (AOA) on the <sup>2</sup>H-enrichment of alanine in the liver of *Sparus aurata*. Fish fed with the low-carbohydrate/high-protein (LC) or high-carbohydrate/low-protein (HC) diet supplied with 5, 15 mg AOA/kg fish per d or without AOA were killed after 30 d of feeding. The <sup>2</sup>H enrichment of alanine was estimated. Values are means corresponding to three to four pools of 500–800 mg of the liver from three to five different fish, with standard deviations represented by vertical bars. \**P*<0.05, \*\*\**P*<0.001. <sup>a,b</sup>Mean values with unlike letters were significantly different between the AOA treatments (*P*<0.05).

pyruvate, α-ketoglutarate and oxaloacetate can be established<sup>(38)</sup>. Therefore, these adducts may contribute to the decrease in ALT activity without changing the ALT protein levels. In addition, AOA was described to participate in a condensation reaction with pyridoxal phosphate, a cofactor necessary for ALT activity<sup>(38)</sup>. Therefore, the higher percentage of ALT inhibition observed in the liver of fish fed with the HC diet supplied with AOA could be due to the presence of a lower number of ALT or pyridoxal phosphate–ALT molecules.

We also followed enzymatic and metabolic changes that may result from AOA-dependent ALT inhibition in the liver of *S. aurata*. In accordance with previous results<sup>(12,22)</sup>, in the present study, we observed higher levels of glycolytic enzymes (GK, PFK-1 and PK) in *S. aurata* fed with the HC diet. Interestingly, long-term exposure to AOA increased PK activity in fish fed the HC or LC diets supplied with AOA. However, no changes in GK, PFK-1, FBPase, G6PDH or 6PGDH activity values were observed by the addition of AOA to the experimental diets.

It has been reported that glutamate behaves as an inhibitor of PK activity and alanine is a strong allosteric inhibitor of hepatic PK<sup>(39)</sup>. In the absence of AOA, glutamate values were lower in the liver of fish fed the HC diet; an effect probably due to the lower supplement of dietary protein in these animals. Therefore, the elevated PK activity values found in the liver of fish fed the HC diet supplied with AOA may be a result of either the lower glutamate levels found in fish fed this diet, and the consequent loss of the glutamate-dependent PK inhibition, or the reduction of alanine levels as a consequence of the inclusion of AOA in the HC diet.

The NMR technique is a key tool for understanding metabolic processes in living systems<sup>(40)</sup>. To assess *in situ* ALT activity, we quantified the <sup>2</sup>H enrichment of hepatic alanine hydrogen atoms from <sup>2</sup>H-labelled water. Both the C3 methyl and C2 methine hydrogen atoms become enriched from <sup>2</sup>H<sub>2</sub>O via the ALT-mediated exchange of alanine and pyruvate, and enrichment of the methyl hydrogen atoms is abolished by AOA<sup>(41)</sup>. This exchange was demonstrated for catfish *in situ* by the rapid equilibration of tissue alanine and bulk water <sup>2</sup>H enrichment following placement in a tank enriched with 4% <sup>2</sup>H<sub>2</sub>O<sup>(42)</sup>. Therefore, to the extent that AOA inhibits ALT activity, enrichment of the alanine methyl hydrogen atoms from <sup>2</sup>H<sub>2</sub>O is expected to be reduced relative to that of bulk water.

In the present study, alanine levels were not significantly affected by the composition of the diet, which suggests that *de novo* synthesis of alanine in fish fed the HC diet may compensate for the lower dietary supplement of alanine. To confirm this aspect, alanine synthesised *de novo* was followed by alanine <sup>2</sup>H-enrichment analysis. Consistently, a significant increase in alanine <sup>2</sup>H enrichment was observed in fish fed the HC diet, while *de novo* synthesis of alanine decreased as a result of the inclusion of AOA in both diets.

In conclusion, since long-term *in vivo* exposure to AOA did not affect FBPase activity nor glycaemia but, indeed, increased PK activity and decreased the renewal of alanine in the liver of *S. aurata*, the present study indicates that inhibition of the cALT activity caused by the addition of this compound to the diets could help to increase the use of the dietary carbohydrate nutrients while sparing protein.

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