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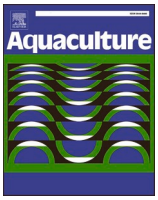

Impact of dietary glutamate and glycine on growth and nutrient utilization in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

A feeding trial was conducted to test the effect of dietary glutamate and glycine supplementation on growth potential, nutritional utilization and digestion, and energy metabolism of rainbow trout (*Oncorhynchus mykiss)*. Four isonitrogenous and isolipidic diets (2 × 2 factorial design) were formulated with glutamate (Glu) and glycine (Gly) as the main factors. The protein source consisted mainly of black soldier fly larvae meal (40% inclusion in all diets) for its low-level content of glutamate and glycine. Triplicate groups of rainbow trout (30 fish/tank with mean initial body weight of 87.5 g ± 0.9) were restrictively fed by hand twice a day for 6 weeks. Dietary Glu and Gly supplementation did not affect feed intake, specific growth rate, or the feed conversion ratio. Whole body protein, lipid, amino acid and fatty acid composition were also unaffected by the supplementation of Glu and Gly in the diets. Dietary Gly supplementation increased the apparent digestibility of amino acids. Furthermore, supplementation with Gly, increased the concentration of serine, glycine, tryptophane, tyrosine and citrulline, while supplementation with Glu in the diets increased the concentration of hydroxy-proline and β-alanine in the serum of rainbow trout. Both dietary Glu and Gly supplementation improved the digestibility of the fatty acids. The expression of genes involved in the hepatic bile acid synthesis (e.g., apical sodium dependent bile acid transporter and organic solute transporter) were affected by supplementation of Gly in the diets. In general, this study showed that dietary supplementation with Gly and Glu improved the nutrient digestibility in rainbow trout.

1. Introduction

Aquaculture is currently producing more than half of seafood destined for human consumption, and this sector is expected to continue to grow in the foreseeable future, with more pressure on the world exciting protein source in aquafeed (FAO 2020). Many strategies have been developed to reduce or to replace the level of fish meal with alternative protein sources in aquafeed, and the main alternatives come from terrestrial sources, such as soy protein concentrate or corn gluten meal (Glencross, 2020; Froehlich et al., 2018). Nevertheless, in the last few years, new sustainable alternative feedstuffs have been investigated, including marine macroalgae or insect meal (Hua et al., 2019; Van, 2020; Sahin et al., 2021; Acar et al., 2021). However, these novel feed ingredients differ in their amino acid (AA) profiles when compared to fish meal or plant-derived products (Nogales-Mérida et al., 2019; Liland et al., 2021). Therefore, it is important to establish an optimal dietary profile of AA, including essential and non-essential amino acid (EAA and NE-AA) in the novel feeds made of alternative protein sources.

Dietary AAs supply, including the EAA and the NE-AA, are the major factors influencing the productivity of farmed fish, as they play a crucial role for fish as energy substrates, for protein synthesis and to control the metabolic pathways (Belghit et al., 2014; Andersen et al., 2016; Espe et al., 2010). In fish, glutamine (Gln), glutamate (Glu) and glycine (Gly) are considered as NE-AA. Glu may be synthetized endogenously from α-ketoglutarate and branched AAs. Gln is produced from Glu by the enzyme ATP-dependent glutamine synthetase, whereas Gln is hydrolyzed to Glu by glutaminase, releasing ammonia. Gln and Glu are the most abundant AA in the body, serving as an important energy substrate in fish and are involved in purine and pyrimidine synthesis. Gly plays an important role in gluconeogenesis, sulfur AA metabolism and fat

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Table 1
Formulation, proximate composition of the four experimental diets (DM basis) fed to rainbow trout.

	Control	Gly	Glu	Gly-Glu
Ingredients (%)				
Corn starch meal	16.48	16.48	16.48	16.48
Hydrolyzed feather meal	5.0	5.0	5.0	5.0
Haemoglobin powder (spray dried)	5.0	5.0	5.0	5.0
Pea protein	10	10	10	10
Black soldier fly larvae meal[1]	40	40	40	40
Rapeseed oil + soya oil	10	10	10	10
Fish oil	6.0	6.0	6.0	6.0
Premix	1.0	1.0	1.0	1.0
$CaCO_3$	1.0	1.0	1.0	1.0
Monocalcium phosphate	2.0	2.0	2.0	2.0
DL-Methionine	0.5	0.5	0.5	0.5
Yttrium oxide	0.02	0.02	0.02	0.02
Glycine[2]	0.0	1.0	0.0	1.0
L-Glutamic acid[3]	0.0	0.0	2.0	2.0
Cellulose	3.0	2.0	1.0	0.0
Proximate analysis				
DM (%)	98	97	97	96
Crude Protein (%)	40	41	42	43
Glu (g/kg)	51	50	76	72
Gly (g/kg)	17.3	27.0	18.6	27.1
Crude Lipid (%)	23	23	23	23
Ash (%)	5.8	5.7	5.7	5.6
Gross energy (kJ/g)	23.0	23.0	23.5	23.5
Yttrium (g/kg)	0.141	0.146	0.146	0.147

Control = diet without glycine and/or glutamate supplementation; Gly = diet supplemented with glycine; Glu = diet supplemented with glutamate; Gly-Glu = diet supplemented with both glycine and glutamamte; DM = dry matter. [1] Black soldier fly larvae meal provided by Protix Biosystems BV (Dongen, The Netherlands); [2] Glycine: ReagentPlus®, ≥99%, Sigma G7126; [3] L-Glutamic acid: ReagentPlus®, ≥99%, Sigma G1251.

digestion (Li et al., 2009).

The term functional AA is used for both EAA and NE-AA, which modulate the key metabolic pathways, such as regulation of cell signaling, gene expression, energy and nutrient metabolism, reproduction, anti-oxidative response, neurotransmission and immune response (Hou et al., 2015). As the NE-AA play important roles in these metabolic pathways, it's suggested that animals, including fish have a dietary requirement for the NE-AA to obtain maximum growth and beneficial health effects. In this regard, glycine was shown to be nutritionally essential for maximal protein accretion in milk-fed piglets (Wang et al., 2014). Also, dietary supplementation with glutamate have been shown to improve the growth, digestive and absorptive function, increase the immune response and the antioxidant capacity of different fish species (Zhao et al., 2020b; Coutinho et al., 2016; Anderson et al., 2002; Hoseini et al., 2022). Thus, the objective of the current study was to test if dietary supplementation with glutamate and/or glycine increased the nutritional value of the diets by influencing the growth and nutrient utilization of rainbow trout (*Oncorhynchus mykiss*).

2. Materials and methods

This study was conducted in accordance with the Dutch/European law on the use of experimental animals (act on Animal Experiments) and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands (AVD1040020197624).

2.1. Diets preparation

The diets were produced by Research Diets Services B.V. (The Netherlands) following a 2 × 2 factorial design, with Glutamate (Glu) and Glycine (Gly) as the main factors. Four isonitrogenous (400–430 g/kg crude protein), isolipidic (230 g/kg crude lipid) and isoenergetic diets (23 kJ/g gross energy) were formulated without supplementation of Glu and/or Gly (control group, Ctl) or with supplementation of glutamate (Glu), glycine (Gly) or both glutamate and glycine (Glu-Gly). All diets were devoid of fish meal and contain the same level of black soldier fly larvae meal (40% inclusion), which was chosen for its low contents of Glu and/or Gly. Glycine and glutamate were added to the experimental diets at 1 and 2%, respectively to have a similar concentration in fishmeal-based diets (Supplementary Table 1). The proteins sources in all diets were pea proteins, hydrolyzed feather meal, haemoglobin powder and black soldier fly larvae meal, while the lipids sources were fish oil, rapeseed oil and soya oil. Diets were extruded, and 3.0 mm pellets were used. Lipids were added to the diets after extrusion by vacuum coating (Vacuüm core coater, Pegasus®-10VC, ¼ H/VV nozzle nr. 6502), at the research facilities of Wageningen University. Yttrium oxide at 0.02% was included as inert digestibility marker. Ingredients and analyzed proximate composition of the experimental diets are presented in Table 1, and the analyzed AA and the fatty acid (FA) composition of the experimental diets are presented in Supplementary Table 2.

2.2. Housing facility

Rainbow trout were purchased from Mohnen Aquaculture GmbH, Germany and were transferred to the aquatic Metabolic Research Unit (MRU) of Wageningen University in The Netherlands, where they were acclimatized for two weeks to the rearing conditions prior to the start of the feeding trial. The MRU contained twelve glass tanks (90 × 60 × 45 cm, 200 L), connected to a common water recirculation system. The water flow through each tank was kept constant at 7.0 ± 0.05 L min^{-1} using a flow meter (MAGFLOW® MAG 5000, Danfoss A/S, Denmark). To ensure that the pre-set water quality parameters remained within optimum conditions for rainbow trout, water quality parameters were measured from the common outflow. The pH was kept between 7.4 and 7.8 and the water temperature was kept between 14.4 and 14.8 °C. The concentration of dissolved oxygen in the outlet water was maintained at a level > 5.5 mg/L. The values for TAN (total ammonium nitrogen), NO_2-N, and NO_3-N did not exceed 0.5 mg/L, 0.1 mg/L, and 25 mg/L, respectively, remaining below the pre-set allowable concentrations. The oxygen consumption, TAN excretion, and NO_2-N and NO_3-N development over time were continuously measured (24 h) to determine the within-day variation using the method as described by Saravanan et al. (2012). In short, the common water inlet and outlet of each metabolic tank were connected to two separate sampling pipelines. One sampling pipeline was connected to an auto-analyzer (San^{plus} continuous flow analyzer, Skalar, Breda, The Netherlands) that measured TAN, NO_2-N, NO_3-N, urea, and CO_2 in real time. The other sampling pipeline was connected to a common measuring hub that measured dissolved oxygen continuously (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany). An electromagnetic valve (ASCO model 24/50 6 WFT, ASCO/Joucomatic, Scherpenzeel, The Netherlands) controlled the water flow through the sampling pipeline from the inlet and outlet of each metabolic tank to the common measuring hub, regulating the oxygen measurements from each tank. These electromagnetic valves were controlled by an algorithmic program through a user interface (HTBasic, Version 9.5, TransEra Corp.) and the dissolved oxygen and water flow values were automatically recorded in a personal computer. Each tank was connected to a swirl separator (AquaOptima AS, column height 44 cm; diameter 24.5 cm) to collect feces for measurement of nutrient digestibility. The feces were collected in a detachable glass bottle at the bottom of the swirl separator, in order to count uneaten feed pellets and collect feces for each tank separately. The bottles were kept under ice, to minimize the bacterial decomposition of collected feces. A photoperiod of 12 h of light and 12 h of darkness was maintained for the entire duration of the experimental trial.

2.3. Experimental trial

The four experimental diets were tested in triplicate tanks for 6

Table 2
Mean growth performance and feed utilization of rainbow trout fed a control diet or diets supplemented with glycine (Gly), glutamate (Glu) and both glycine and glutamate (Gly-Glu).

					P-value		
	Control	Gly	Glu	Gly-Glu*	Gly	Glu	Gly x Glu
IW (g)	87.0 ± 0.9	88.5 ± 1.2	86.4 ± 1.7	87.8 ± 1.0	NS	NS	NS
FW (g)	183.0 ± 1.5	187.5 ± 4.0	186.7 ± 3.8	187.0 ± 0.1	NS	NS	NS
Growth (g/d)	2.34 ± 0.01	2.42 ± 0.09	2.45 ± 0.09	2.42 ± 0.02	NS	NS	NS
Growth $_{MBW}$ (g / ($kg^{0.8} \cdot d$))	44.42 ± 2.16	45.0 ± 1.0	46.9 ± 1.9	45.5 ± 1.13	NS	NS	NS
SGR	1.81 ± 0.01	1.83 ± 0.05	1.88 ± 0.06	1.84 ± 0.03	NS	NS	NS
FI (g DM / (Fish. D))	2.17 ± 0.04	2.17 ± 0.0	2.14 ± 0.0	2.17 ± 0.0	NS	NS	NS
FI $_{MBW}$ (g DM / ($kg^{0.8} \cdot d$))	41.5 ± 0.72	40.3 ± 1.2	41.0 ± 1.3	40.8 ± 0.5	NS	NS	NS
FCR	0.94 ± 0.04	0.90 ± 0.03	0.88 ± 0.03	0.90 ± 0.01	NS	NS	NS
HSI	1.36 ± 0.22	1.23 ± 0.14	1.34 ± 0.15	1.39 ± 0.26	NS	NS	NS
VSI	11.26 ± 1.56	11.82 ± 0.83	11.23 ± 1.11	12.76 ± 2.73	NS	NS	NS
PPV	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.02	0.40 ± 0.01	NS	NS	NS
LPV	0.66^{b} ± 0.05	0.76^{a} ± 0.09	0.69^{b} ± 0.03	0.73^{a} ± 0.01	*0.05*	NS	NS
Survival rate (%)	97	100	100	100			

Control = diet without glycine and/glutamate supplementation; Gly = diet supplemented with glycine; Glu = diet supplemented with glutamate; Gly-Glu = diet supplemented with both glycine and glutamate. IW = initial weight; FW = final weight; MBW = metabolic body weight; SGR = specific growth rate; FI = feed intake; FCR = feed conversion ratio; HSI = hepatosomatic index; VSI = viscerosomatic index; PPV = protein productive value; LPV = lipid productive value. Values are presented as means ± SD with different superscripts letters next to values are significantly different. 2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$. All the growth parameters, IW, FW, SGR, FI, FCR, PPV and LPV, were studied in triplicate tanks ($n = 3$), except the dietary condition * Gly-Glu where the analyses were done in duplicate tanks ($n = 2$).

weeks. At the start of the experiment, 30 fish (mean body weight of 87.5 ± 0.9 g) were randomly stocked per tank. Since the objective of the current study was to study the impact of the NE-AA, Glu and Gly, on growth and energy metabolism of rainbow trout, fish were restrictively fed to have an equal amount of feed (on dry matter basis) per tank and per day. The feeding level was fixed at 13.5 g/kg$^{0.8}$/day, which is about 90% of the expected satiation level. Every day, the amount of feed was increased by predicting fish growth and weight, which was calculated using the average start weight of the fish and an expected feed conversion ratio of 0.9. The first day of feeding rainbow trout started at 25% of the intended feeding level, and was progressively increased to 50, 75 and then to 100% within the following 3 days. The daily feed ration was divided into equal portions fed at 8:00 and 16:00 h. The fish were hand fed, and if there were differences in feed intake between tanks, the feed list was adjusted based on the tank with the lowest feed intake during the experimental trial to maintain equal feed (nutrient) intake for all treatments.

2.4. Measurements and sampling

Oxygen consumption, and TAN measurements were done during week 5 of the experimental trial. The oxygen measurements were performed continuously for 24 h (from 08.00 to 8.00 h) in 3 subsequent runs each run of 24 h consisting of a set of 4 tanks consisting of all dietary treatments. The TAN measurements were performed continuously for 24 h in two subsequent runs each run consisting of a set of 6 tanks. The concentration of oxygen in the inlet and outlet of each tank was automatically measured using an electrode (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany) and the data were recorded in a computer. The concentration of TAN was determined with colorimetric method (San^{plus} continous flow analyzer, Skalar, Breda, The Netherlands), following the manufacturer's protocol (TAN = NH_3-N + NH_4^+-N; Skalar protocol number 155–006; Skalar, Breda, The Netherlands).

Fish were collected at the start (day 0) and at the end of the trial (day 41). At all samplings, the fish were anaesthetized with 2-phenoxyethanol (0.15 mL/L for sedation and 1 mL/L for euthanasia). At the start and the end of the sampling, all fish were batch weighted to calculate growth parameters ($n = 3$). The fish were examined externally to check for possible abnormalities. Liver and viscera were removed from individual fish and were weighed for calculation of organosomatic indices ($n = 9$). Twenty fish in total (at the start of the experimental trial) and 10 fish per tank randomly selected (n = 3, at the end of the experimental trial) were sacrificed, pooled, homogenized and stored at −20 °C for analysis of proximate composition. Feces were collected for digestibility measurement from week 3 onward, using the swirl separators for 5 days per week (not the weekends). Feces were pooled per week and stored in aluminum trays at −20 °C (n = 3). Blood was sampled 24 h postprandially and collected from the caudal vein from three fish (24 h postprandially) per tank and serum was separated from the red blood cells by centrifugation (1800 *g* for 10 min at 4 °C), and frozen in liquid N_2 ($n = 9$). Individual liver samples were taken from three fish and placed in an Eppendorf tube containing 500 uL of RNA later ® (Invitrogen, Carlsad, CA, USA) for qPCR analyses (n = 9).

2.5. Analysis of chemical composition

Proximate composition of dry matter (DM), protein, lipids, ash, energy, AA (not including cysteine and tryptophan), and FA composition (feed, whole fish, feces and liver) were analyzed as described in (Belghit et al., 2018; Belghit et al., 2019). Amino acid composition in deproteinised serum was determined on an amino acid analyzer Biochrom 20 plus Amino Acid Analyzer (Amersham Pharmacia Biotech, Sweden) equipped with a lithium column using post derivatization with ninhydrin (Espe et al., 2016). Norleucine was used as an internal standard. Serum was deproteinized by addition of sulpho salicylic acid (1:1). The amino acids were quantified using a standard containing the amino acids of interest as well as urea and ammonia (Sigma Aldrich, Germany).

2.6. Gene expression analysis: quantitative real-time PCR

Total RNA was extracted from liver samples using EZ1 RNA Universal Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and frozen at −80 °C. Quantity and quality of the RNA were assessed by spectrophotometry and the Agilent 2100 Bioanalyzer (Agilent Technologies). The samples used in this experiment had 260/280 nm absorbance ratios that varied between 2.05 and 2.12. RNA integrity number (RIN) was tested for a subset of samples that all had RIN values between 8.2 and 9.3 indicating RNA samples suitable for RT-PCR. A two-step qPCR was used to measure the mRNA levels of the target and the reference genes. RT reactions were performed on a GeneAmp PCR 9700 (Applied Biosystems) using the TaqMan® reverse transcriptase kit with oligo primers (Applied Biosystems). Real-time PCR amplification and analysis were performed on a LightCycler 480 Real-time PCR system (Roche Applied Science) with SYBR® Green I Mastermix (Roche Applied Science). Pipetting of cDNA plates was done using a Biomek® 3000 Laboratory automation workstation (Beckman Coulter, Fullerton,

Table 3
Apparent digestibility coefficients (ADC %) of crude protein, crude lipid, amino acids and fatty acids in rainbow trout fed a control diet or diets supplemented with glycine (Gly), glutamate (Glu) and both glycine and glutamate (Gly-Glu).

				P-value			
	Control	Gly	Glu	Gly-Glu*	Gly	Glu	Gly x Glu
CP	88.2^{b} ± 0.3	89.8^{ab} ± 0.3	88.7^{b} ± 1.0	90.7^{a} ± 0.3	*0.013*	NS	NS
CL	88.1^{b} ± 2.1	91.6^{a} ± 0.36	91.9^{a} ± 0.4	92.3^{a} ± 0.1	*0.02*	*0.02*	NS
Amino acid							
His	92.9^{b} ± 0.1	93.8^{a} ± 0.2	93.0^{b} ± 0.8	94.1^{a} ± 0.0	*<0.01*	NS	NS
Ser	90.3 ± 0.2	91.1 ± 0.5	90.7 ± 0.8	91.7 ± 0.4	NS	NS	NS
Arg	93.6^{b} ± 0.3	94.5^{a} ± 0.3	94.1^{b} ± 0.5	94.7^{a} ± 0.1	*0.01*	NS	NS
Gly	88.0^{b} ± 0.2	92.7^{a} ± 0.4	88.6^{b} ± 0.6	92.9^{a} ± 0.5	<0.01	NS	NS
Asp	92.8 ± 0.4	93.4 ± 0.3	93.1 ± 0.9	94.3 ± 0.2	NS	NS	NS
Glu	94.1^{b} ± 0.2	94.7^{b} ± 0.3	96.1^{a} ± 0.5	96.6^{a} ± 0.1	*0.02*	*<0.01*	NS
Thr	89.8^{b} ± 0.3	91.0^{a} ± 0.4	90.3^{b} ± 0.9	91.6^{a} ± 0.3	*<0.01*	NS	NS
Ala	93.2^{b} ± 0.2	94.0^{a} ± 0.4	93.6^{b} ± 0.5	94.4^{a} ± 0.4	*0.01*	NS	NS
Pro	91.0 ± 1.4	92.0 ± 0.6	92.1 ± 0.8	93.0 ± 0.3	NS	NS	NS
Lys	95.0 ± 0.2	95.4 ± 0.3	95.1 ± 0.6	96.0 ± 0.2	NS	NS	NS
Tyr	91.4^{b} ± 0.5	92.8^{a} ± 0.4	91.8^{b} ± 0.8	92.6^{a} ± 0.2	*<0.01*	NS	NS
Met	94.9^{b} ± 0.3	95.8^{a} ± 0.1	95.3^{b} ± 0. 6	96.1^{a} ± 0.1	*<0.01*	NS	NS
Val	91.5^{b} ± 0.3	92.6^{a} ± 0.5	92.2^{b} ± 0.6	93.0^{a} ± 0.2	*0.01*	NS	NS
Iso	91.2^{b} ± 0.4	92.3^{a} ± 0.5	91.1^{b} ± 0.7	92.8^{a} ± 0.1	*0.01*	NS	NS
Leu	92.3^{b} ± 0.2	93.4^{a} ± 0.4	93.1^{b} ± 0.64	93.8^{a} ± 0.2	*<0.01*	NS	NS
Phe	92.1^{b} ± 0.2	93.3^{a} ± 0.5	92.6^{b} ± 0.7	93.3^{a} ± 0.0	*0.01*	NS	NS
Fatty acids							
12:0	62.9^{b} ± 1.9	68.9^{a} ± 1.4	69.4^{a} ± 3.0	73.2^{a} ± 1.3	0.01	*<0.01*	NS
14:0	63.9^{b} ± 2.6	69.9^{a} ± 1.3	70.2^{a} ± 3.1	73.6^{a} ± 1.1	*0.01*	*0.01*	NS
16:0	60.2^{b} ± 3.1	65.2^{ab} ± 1.7	65.5^{ab} ± 3.8	70.6^{a} ± 0.4	*0.01*	*0.02*	NS
18:0	55.4 ± 2.9	61.3 ± 5.8	58.1 ± 4.8	65.9 ± 0.9	NS	NS	NS
18:1n-9	76.3^{b} ± 2.1	80.7^{a} ± 0.6	81.9^{a} ± 1.5	83.9^{a} ± 0.4	*0.01*	*0.01*	NS
18:1n-7	66.4^{b} ± 2.7	73.1^{a} ± 0.7	75.0^{a} ± 2.8	77.5^{a} ± 0.4	*0.01*	*0.01*	NS
18:2n-6	89.0^{ab} ± 1.5	89.7^{ab} ± 0.5	88.5^{b} ± 1.2	91.2^{a} ± 0.4	*0.04*	NS	NS
18:3n-3	90.2^{ab} ± 1,7	91.2^{ab} ± 0.4	88.1^{b} ± 1.3	92.5^{a} ± 0.6	*0.01*	NS	*0.04*
20:1n-9	63.9^{b} ± 4.4	70.7^{a} ± 0. 7	69.8^{a} ± 2.9	75.1^{a} ± 0.2	*<0.01*	*0.03*	NS
22:1n-11	63.0^{b} ± 3.6	69.2^{a} ± 1.3	70.7^{a} ± 3.0	73.3^{a} ± 0.7	*0.02*	*<0.01*	NS
Saturated FA	61.1^{b} ± 2.6	66.8^{a} ± 1.9	66.8^{a} ± 3.5	71.5^{a} ± 0.8	*0.01*	*0.01*	NS
Sum MUFA	74.9^{b} ± 2.3	79^{a}.4 ± 0.6	80.7^{a} ± 1.6	82.8^{a} ± 0.4	*<0.01*	*<0.01*	NS
Sum n-3	94.4^{abc} ± 1.0	95.5^{b} ± 0.2	92.5^{c} ± 1.0	96.1^{a} ± 0.0	NS	*0.01*	*0.03*
Sum n-6	88.7^{b} ± 1.4	89.5^{ab} ± 0.5	88.2^{b} ± 1.2	91.1^{a} ± 0.4	*0.02*	NS	NS
Sum PUFA	89.7^{b} ± 1.3	90.7^{ab} ± 0.4	88.9^{b} ± 1.2	92.1^{a} ± 0.3	*0.01*	NS	NS

Control = diet without glycine and/or glutamate supplementation; Gly = diet supplemented with glycine; Glu = diet supplemented with glutamate; Gly-Glu = diet supplemented with both glycine and glutamate. MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. Values are presented as means ± SD with different superscripts letters next to values are significantly different ± SD. 2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$. *All the analyses on digestibility were studied in triplicate tanks ($n = 3$), except the dietary condition Gly-Glu where the analyses were studied in duplicate tanks ($n = 2$).

California). Thermal cycling was done for forty-five cycles of 10 s at each at 95 °C, 60 °C and 72 °C, followed by a melting curve analysis to confirm that only one product was present. The stability of the reference genes (*β-act and elf1α*) and mean normalized expression of the target genes were calculated using CFX Maestro software (Bio-Rad CFX maestro version 1.1, Bio-Rad laboratories, Hercules, CA). The details of the qPCR primers used for amplification of the reference and target genes are provided in Supplementary Table 3.

2.7. Statistical analysis

According to the design, a two-way ANOVA followed by Tukey's post hoc test (packages *nlme*; Pinheiro et al., 2010) and *multcomp* (Hothorn et al., 2008) was used to assess differences between supplementation of Glu, Gly or Gly-Glu in the diets. All data were tested for homogeneity of variance by Levene's test. If the data were identified as having non-homogeneous variance or non-normal distribution, they were subjected to a non-parametric analysis (Kruskal–Wallis test; Giraudoux, 2011). A significance level of $p \leq 0.05$ was used. All statistical analyses were carried out using the software environment R (R Development Core Team, 2011). Figures were made using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

All the samples were analyzed in triplicates ($n = 3$, pooled samples/tank) or 9 replicates ($n = 9$, samples from individual fish/tank), except for the dietary group Gly-Glu, where one tank was not included in the analysis, due to a lower feed intake linked to the fish behavior and dominant fish. Thus, all data are presented in replicates, n = 3 or n = 9, if not otherwise stated in the footnotes.

2.8. Growth and nutritional indices were calculated as followed:

Growth (g/day) = final body weight (g)-initial body weight (g)/days.

The geometric mean body weight (GBW) is calculated as $\sqrt{}$ (final body weight x initial body weight), from which mean metabolic body weight (MBW) was calculated as $(GBW/1000)^{0.8}$.

Specific growth rate (SGR) = 100 × [ln final body weight (g) − ln initial body weight (g)]/days.

Feed intake (FI) = 100 × quantity of food taken (g DM)/[day× ((initial weight (g) + final weight(g))/2)].

Feed conversion ratio (FCR) = feed intake (g DM)/fish weight gain (g).

Hepatic Somatic Index (HSI) = liver weight (g)/body weight (g) *

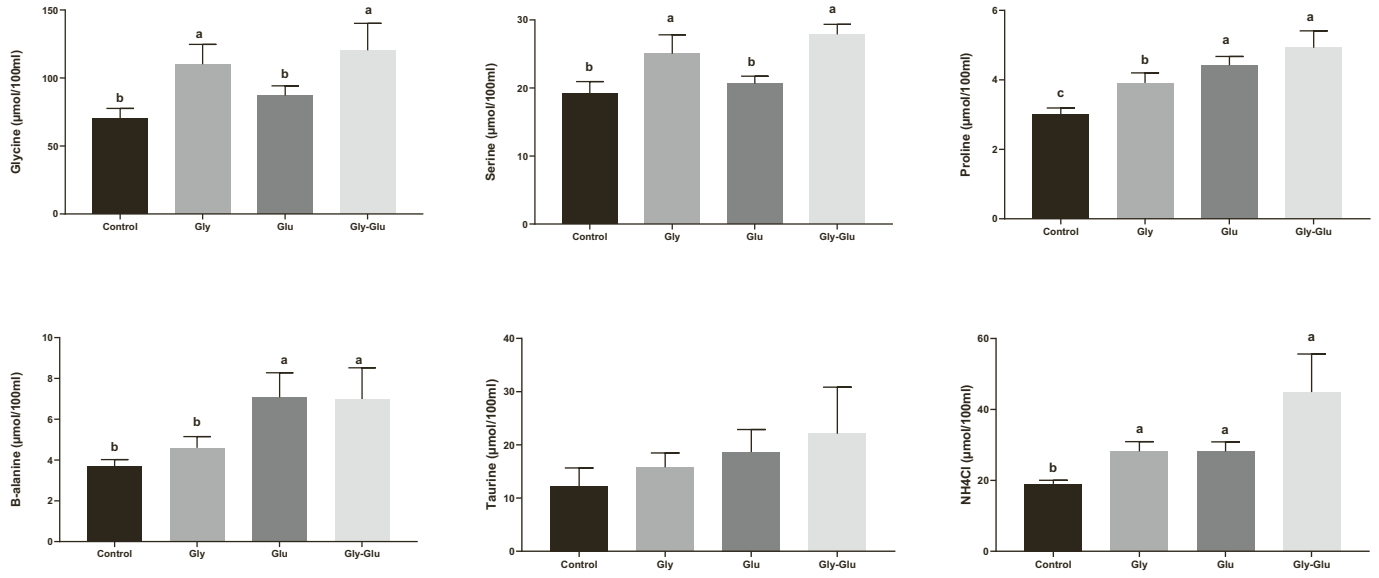

Fig. 1. Serum free amino acid and nitrogenous compounds (µmol/100 ml) composition of rainbow trout fed control diet or diets supplemented with Gly and/or Glu. Values are means of 9 values (except for the group *Gly-Glu, values are means of 6 values) per dietary treatment, with their standard deviation represented by vertical bars. Different superscript letters indicate significantly different values (2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$). Serum free amino acid and nitrogenous compounds composition for remaining amino acids are presented in Supporting Information Table S4.

100.

Visceral Somatic Index (VSI) = viscera weight (g)/body weight (g) * 100.

Protein production value (PPV) = (final protein content (g) – initial protein content (g))*protein fed $^{-1}$.

Lipid production value (LPV) = (final lipid content (g) – initial lipid content (g))*lipid fed $^{-1}$.

Apparent digestibility coefficient (ADC) = 100 – (Y_d*CX_f)*(Y_f*CX_d)$^{-1}$*100, where d is diet, f is feces, Y is yttrium concentration, and CX is nutrient concentration.

Retention of amino acids = (amino acid gained in fish)*(ingested amino acid)$^{-1}$* 100.

Fatty acid productive value (FAPV): (fatty acid per tank at end of trial (g)-fatty acid per tank at start of trial (g)) * (fatty acid eaten in total per tank during 6 weeks feeding trial (g))$^{-1}$.

3. Results

3.1. Dietary composition

Analyzed proximate compositions of the experimental diets were similar to calculated compositions. All diets were similar in dry matter, protein, lipids, ash and energy (Table 1). The diets had close to identical concentrations of FA (Supplementary Table 2). The composition of the EAA were approximatively similar in all feeds (Supplementary Table 2). Glutamate content was higher in the diets Glu and Gly-Glu, while the concentration of glycine was higher in the diets Gly and Gly-Glu (Table 1).

3.2. Growth performances

At start of the trial, the fish had a mean weight of 87.5 g ± 0.9 (mean ± SD). After 41 days, the fish had more than doubled their body weight (187 g ± 0.9, n = 3) (Table 2). Dietary supplementation with Gly had a significant effect on the lipid productive value (LPV) ($p = 0.05$); fish fed with dietary Gly or Gly-Glu had a higher LPV than the fish fed Ctl or Glu diets (Table 2). There were no significant effects of dietary Glu and/or Gly on final weight, weight gain, specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), hepatosomatic-index (HSI), visceral-somatic index (VSI) or protein productive value (PPV) (Table 2).

3.3. Apparent nutrient digestibility

The apparent digestibility of crude protein was significantly ($p = 0.013$) increased by supplementation of Gly in the diets, while the digestibility of crude lipid was significantly affected by supplementation of dietary Gly and Glu ($p = 0.02$ and 0.02, respectively); fish fed Ctl diet had a lower lipid digestibility than the fish fed dietary Glu, Gly or Gly-Glu (Table 3). The digestibility of most AAs was affected by dietary Gly supplementation ($p \leq 0.05$). Fish fed Gly or Gly-Glu diets had a higher digestibility for the following AAs, His, Arg, Ala, Gly, Thr, Glu, Tyr, Met, Val, Iso, Leu and Phe, than fish fed Glu or Ctl diets. Glutamate was the only AA that was affected by the dietary supplementation of Glu diet; fish fed Glu or Gly-Glu diets had a higher digestibility than fish fed Ctl or Gly diets ($p < 0.01$; Table 3). The apparent digestibility of most FAs were increased in an additive manner to both dietary Gly and Glu supplementation ($p \leq 0.05$, Table 3). Only for the digestibility of 18:3n-3 and total n-3 FA a significant interaction effect between Gly and Glu supplementation was observed ($p = 0.03$, Table 3). For all other nutrient digestibility, no interaction effect was present.

3.4. Whole fish amino acid and serum free amino acid composition

Dietary supplementation with Gly and/or Glu had no significant effects on whole fish dry matter (29.9% ± 0.1), crude protein (15.6% ± 0.06), crude lipid (11.4% ± 0.8), ash (1.7% ± 0.3) or energy content (7.7 ± 0.13 kJ/g) (on fresh weight basis, Supplementary Table 4). The whole-body amino acid composition was also unaffected by dietary Gly and/or Glu supplementation, except for glycine, glutamic acid and isoleucine (Supplementary Table 4). A significant interaction between Gly and Glu supplementation was observed on the composition of glycine, glutamic acid and isoleucine in the whole-body of rainbow trout ($p \leq 0.05$, Supplementary Table 4).

Dietary supplementation with Gly, increased the concentration of serine, glycine, tryptophane, tyrosine and citrulline in the serum of rainbow trout compared with the fish fed Ctl or Glu diets ($p \leq 0.05$,

Table 4
Retention of amino acids (%) in rainbow trout a control diet or diets supplemented with glycine (Gly), glutamate (Glu) and both glycine and glutamate (Gly-Glu).

					p-value		
	Control	Gly	Glu	Gly-Glu*	Gly	Glu	Gly x Glu
CP	42.3 ± 1.2	41.1 ± 1.6	41.4 ± 2.4	40.5 ± 1.4	NS	NS	NS
His	40.1 ± 3.1	37 ± 2.2	37.1 ± 3.3	39.3 ± 2.3	NS	NS	NS
Ser	33.8 ± 1.8	34 ± 1.7	33.1 ± 0.5	33.5 ± 1.4	NS	NS	NS
Arg	44.9 ± 3.1	47.5 ± 0.9	47.4 ± 1.8	47.1 ± 2.9	NS	NS	NS
Gly	58.6^{ab} ± 5.4	46.9^{b} ± 3.3	69.8^{a} ± 5.6	43.5^{b} ± 1.1	*<0.01*	NS	*0.04*
Asp	32.7 ^{ab} ± 0.9	35.4^{a} ± 1.7	32.1^{ab} ± 1.9	36.1^{a} ± 0.1	NS	*<0.01*	NS
Glu	40^{a} ± 0.9	42.5^{a} ± 1.5	26.5^{b} ± 1.5	29.1^{b} ± 0.3	*0.01*	*<0.01*	NS
Thr	43.6 ± 2.1	44.5 ± 2.0	43.7 ± 2.1	45.7 ± 2.0	NS	NS	NS
Ala	37.4 ± 1.0	40.7 ± 1.7	38.2 ± 2.0	38.8 ± 0. 2	NS	NS	NS
Pro	32.5 ± 1.3	35.7 ± 2.2	35.9 ± 0.4	32.8 ± 0.6	NS	NS	NS
Lys	45^{ab} ± 2.7	46.8^{a} ± 2.5	42.7^{ab} ± 4.1	48.7^{a} ± 0. 3	*0.04*	NS	NS
Tyr	29.5 ± 4.1	26.8 ± 0.8	27.4 ± 2.6	28 ± 2.6	NS	NS	NS
Met	47.7 ± 4.6	46.5 ± 1.6	48.3 ± 2.9	49.1 ± 2.9	NS	NS	NS
Val	31.4 ± 0.8	32 ± 0. 8	29.3 ± 3.0	31.9 ± 0. 6	NS	NS	NS
Iso	41.7 ± 1.1	43.1 ± 2.0	39.7 ± 4.7	44.1 ± 1.0	NS	NS	NS
Leu	35.1 ± 0. 9	35 ± 1.7	34.1 ± 2.8	36.2 ± 1.3	NS	NS	NS
Phe	38.8 ± 4.3	36.3 ± 1.7	37.3 ± 3.1	38.8 ± 3.7	NS	NS	NS

Control = diet without glycine and/or glutamate supplementation; Gly = diet supplemented with glycine; Glu = diet supplemented with glutamate; Gly-Glu = diet supplemented with both glycine and glutamate. Values are presented as means ± SD with different superscripts letters next to values are significantly different ± SD. 2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$. *All the analyses were studied in triplicate tanks ($n = 3$), except the dietary condition Gly-Glu where the analyses were studied in duplicate tanks ($n = 2$).

Fig. 1 and Supplementary Table 4). While supplementation with Glu in the diets increased the concentration of hydroxy-proline and β-alanine than fish fed Ctl or Gly diets ($p \leq 0.05$, Fig. 1 and Supplementary Table 4). The concentration of aspartic acid, proline and ammonium chloride in the serum was affected by Gly and Glu supplementation; fish fed Gly, Glu or Gly-Glu had a higher concentration of aspartic acid, proline and ammonium chloride than the control group ($p \leq 0.05$, Fig. 1 and Supplementary Table 4).

3.5. Amino acids retention

Dietary supplementation with Gly significantly increased the retention of Glu compared to trout fed dietary Glu, while a surplus with Glu in the diets significantly increased the retention of Gly in the whole body compared to fish fed the Gly diet ($p < 0.05$, Table 4). A significant interaction between dietary Gly and Glu was observed for glycine retention (0.04) (Table 4).

3.6. Fatty acid composition in the whole body and liver

The composition of 16:0 and 18:0 was significantly lower in the whole-body of trout fed Gly diets than fish the fed control diet (effect of Gly and interaction Gly x Glu, $p < 0.05$, Supplementary Table 5). Supplementation with dietary Glu had a significant effect on 20:5n-3 (eicosapentaenoic acid, EPA) in the whole-body; fish fed Glu or Gly-Glu diets had a lower concentration of EPA than fish fed Gly or Ctl diets ($p < 0.01$, Supplementary Table 5). Supplementation with Glu had also a significant effect on n-3/n-6 and n-6/n-3 ratio, being lower and higher, respectively, in the group of trout fed Glu or Gly-Glu than the control group ($p = 0.04$ and 0.05, respectively, Supplementary Table 5). There were no significant effects of dietary supplementation with Gly or Glu in the liver of rainbow trout. Significant interactions between dietary Gly and Glu were observed on the contents of 14:0, 18:1n-7, 18:2n-6, 18:3n-3, 20:1n-9, 20:4n-6 (arachidonic acid, ARA) and total fatty acid in the liver of rainbow trout ($p < 0.05$, Supplementary Table 5).

3.7. Fatty acid productive value

The calculated FAPV reflects the efficiency of the use of specific FA and groups of FA; values below 1 reflect a net loss of a FA during the 6 weeks of the trial, while values above 1 indicate a net production of a FA. Fig. S1 presents the FAPV for selected FA. The FAPV of most FAs was lower than 1.0, meaning that <100% of the FA eaten during the six-week trial were recuperated in the trout tissues, while the FAPV of ARA was higher than 3, reflecting a net production of this essential FA in fish. The FAPV of ARA was significantly different between the dietary groups, being higher in the fish fed Glu diet (~5.0) and lower in trout fed Gly and Gly-Glu diets (~3.0) ($p < 0.05$, Fig. S1).

3.8. Hepatic gene expression

The expression of genes involved in bile acid synthesis were significantly affected by supplementation of Gly in the diets of rainbow trout ($p < 0.05$, Fig. 2). The expression of apical sodium dependent bile acid transporter (*asbt*) and ATP-binding cassette transporter G5 (*abcg5*) were significantly influenced by supplementation of dietary Gly ($p = 0.03$ and 0.02, respectively, Figs. 2A and B). Fish fed diets supplemented with Gly-Glu and Gly had significantly increased *Asbt* and *abcg5* expression compared with control group (Figs. 2A and B). Supplementation with dietary Glu had a significant effect on expression of organic solute transporter (*osta*) ($p = 0.04$) and a significant interaction between Gly and Glu sources were also observed for the expression of *osta* ($p = 0.03$, Fig. 2C). The expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr)* significantly decreased in the group of fish fed dietary Gly compared to trout fed Ctl or Gly-Glu diets ($p < 0.01$, Fig. 2D). No effects of dietary Glu and/or Gly on the expression of ATP-binding cassette transporter G5 and G8 (*abca1* and *abca8*, respectively), bile salt export pump *(bsep)*, cholesterol 7α-hydroxylase (*cyp7a1*), sterol 12α-hydroxylase (*cyp8b1*) or sodium bile cotransporter (*ntcp*) was observed (data not shown).

Few and small dietary effects were detected on the gene expression of markers of lipid metabolism in the liver of rainbow trout. The expression of fatty acid binding protein 7 (*fabp-7)* was significantly lower in the fish fed dietary Gly compared to fish fed the control diet ($p = 0.02$, Fig. 3A). The expression of fatty acid synthase (*fas)* and carnitine palmitoyltransferase I (*cpt-1*) (Figs. 3B and C) as well as proliferator-activated receptor-α (*ppar-α*), liver X receptor (*lxr*), farnesoid X receptor (*fxr*) and pirillipin-2 (*plin-2*) (data not shown), was also measured, but no dietary effects were detected on their expression.

3.9. Metabolic rate

The oxygen consumption and TAN excretion pattern of rainbow trout over a 24-h cycle are shown in Figs. 4 B and D, respectively, and their

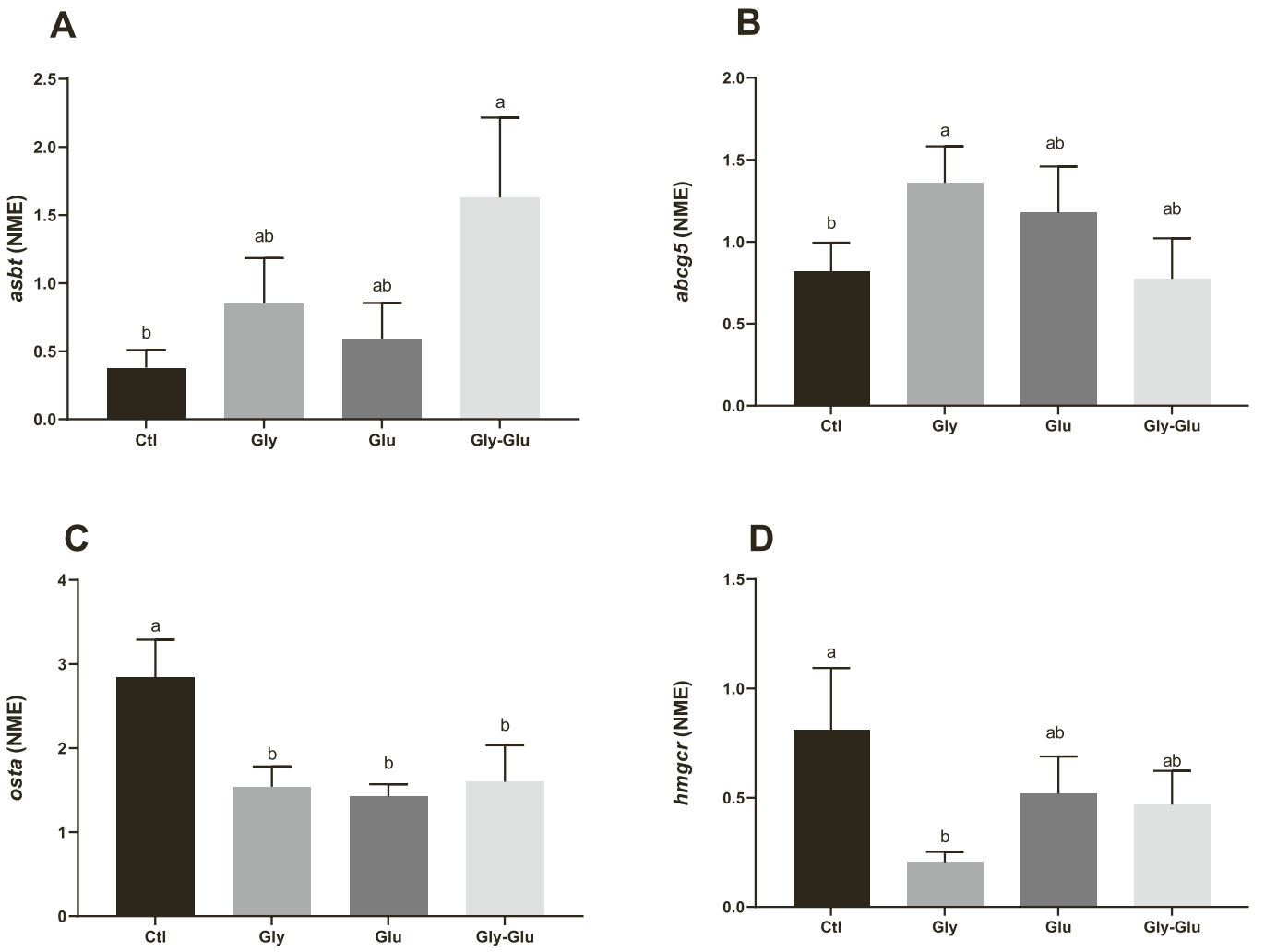

Fig. 2. Genes expression related to bile acid synthesis in the liver of rainbow trout fed control diet or diets supplemented with Gly and/or Glu. Normalized mean expression (NME) of (A) Apical Sodium Dependent Bile Acid Transporter (*asbt*); (B) ATP-binding cassette transporter G5 (*abcg5*); (C) organic solute transporter (*osta*); and (D) 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr)* . Values are means of 9 values (except for the group Gly-Glu, values are means of 6 values) per dietary treatment, with their standard deviation represented by vertical bars. Different superscripts letters indicate significantly different values (2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$).

means are presented in Figs. 4 A and C, respectively. The consumption of oxygen was quite similar between the four dietary groups and no significant differences were recorded. However, the TAN excretion was significantly affected by supplementation of Glu in the diets ($p = 0.04$) (Fig. 4 C). The value of TAN excretion was measured over an hour and is represented as the mean value in that respective hour. After feeding the fish in the morning at 08 h, the excretion of TAN increased in all the dietary groups and some divergence among the group appeared 6 h after the first meal; being high in the fish fed Glu or Gly-Glu and lower in the control group (Fig. 4C). Urea, CO_2, NO_2 and NO_3 were also measured, and no dietary effects were detected on their excretion (data not shown).

4. Discussion

Several studies have shown that dietary supplementation with Glu or Gly improves the growth of different fish and shellfish species (Zhao et al., 2020a; Caballero-Solares et al., 2015; Zhao et al., 2015; Yoshida et al., 2016a; Oehme et al., 2010; Xie et al., 2014; Xie et al., 2016; Rossi et al., 2021). In the current study, however, dietary Glu and/or Gly supplementation did not affect the growth performances of rainbow trout. There was only a small non-significant increase of 3 and 6%, on the specific growth rate of fish fed the dietary Gly and/or Glu compared to the control group, respectively. Similarly, the feed conversion ratio was slightly improved, but also not significantly so when fish were fed supplemented Gly and/or Glu. The reason for these differences in growth performance between the current study and earlier reports might be due to the feeding rate of the animals. Free amino acids are known to be attractants in fish and their supplementation in the diets might result in higher feeding rate due to olfactory stimulus (Dias et al., 1997; Oehme et al., 2010; Hughes, 1997; Li et al., 2009). In the earlier reported studies, fish were fed *ad libitium*, which could have resulted in higher feeding rate in the supplemented diets (Oehme et al., 2010; Li et al., 2009; Yoshida et al., 2016a). However, in the present study, rainbow trout were fed restrictively since the objective was to study the direct impact of Glu and/or Gly on growth performance, nutrient utilization and energy metabolism and thus eliminating any differences in feed intake. Furthermore, it should be also noted that the feeding trial in this study lasted 6 weeks and a longer evaluation could have shown some effects of dietary supplementation with Glu or Gly on growth performance of rainbow trout.

Most of the effects seen on the apparent digestibility coefficients (ADC) of AAs were observed with addition of Gly but not with Glu in the diets. Dietary Gly supplementation increased the ADC of crude protein and most of the AAs. Supplementation with Gly in the diets of rainbow

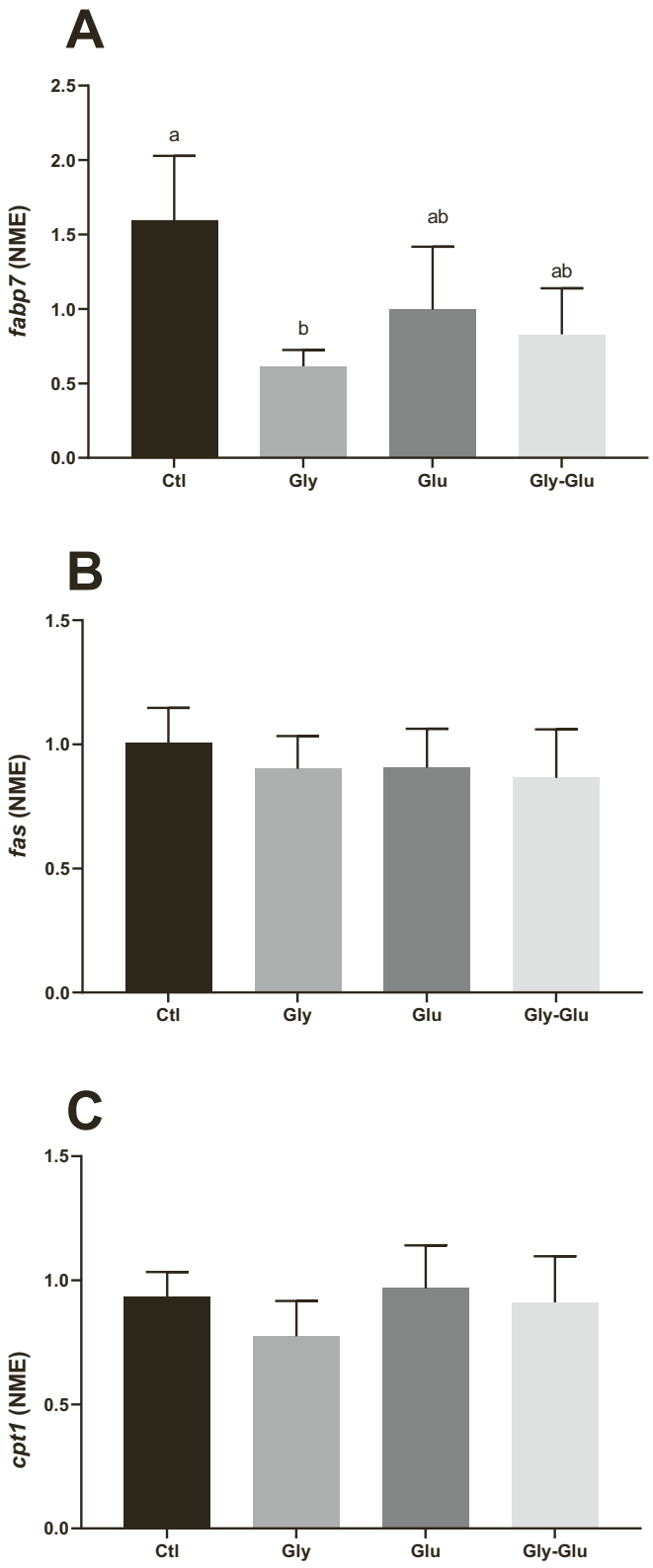

Fig. 3. Genes expression related to lipid metabolism in the liver of rainbow trout fed control diet or diets supplemented with Gly and/or Glu. Normalized mean expression (NME) of (A) fatty acid synthase (*fas*); (B) carnitine palmitoyl transterase-1 (*cpt-1*); and (C) fatty acid binding protein-7 (*fabp*-7). Values are means of 9 values (except for the group Gly-Glu, values are means of 6 values) per dietary treatment, with their standard deviation represented by vertical bars. Different superscripts letters indicate significantly different values (2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$).

trout may result in a higher absorption of AAs. These results are in agreement with previous studies, showing a better digestibility and absorption when some of the AAs (e.g., methionine) are supplemented in the diets of fish species as a soluble protein or as crystalline AAs (Espe et al., 2011; Espe et al., 2010; Riley et al., 1996). Retention of Gly and Glu in the whole body of rainbow trout reduced with supplementation of both dietary Gly and Glu. The decline in retention of individual amino acids upon increased dietary levels is in line with previous results testing diets with increased amino acid inclusions (Espe et al., 2020). However, in the current study, dietary supplementation with Gly increased the retention of Glu, while a surplus with dietary Glu increased the retention of Gly in the whole body of rainbow trout. These results might suggest that there is a mutual sparing effects between Gly and Glu in their nutrient utilization. Moreover, catabolism of glycine is important for energy and oxidative metabolism (Wang et al., 2014; Wu, 2014). Its supplementation in the diets of monogastric animals can protect against free-radical-mediated oxidative stress (ROS) (Hoseini et al., 2022; Wu, 2014). In the present study, dietary supplementation with glycine significantly increased the expression of hepatic selenoprotein P (*SePP*) compared to fish fed Glu or control diets (Supplementary Fig. 2). *SePP* is a circulating selenium carrier which functions as an antioxidant enzyme. Therefore, the oxidative status may be better in the group of fish fed with surplus of dietary glycine and thus spending less energy to prevent ROS formation. This is, however, only based on mRNA transcription of *SePP* and further investigations on the effects of glycine and the stress response would need to be done to conclude.

Furthermore, the ADC of crude lipid and most of the FAs increased with supplementation of Gly and/or Glu in the diets. These results indicated that both Gly and Glu supplementation in the diets improved the lipid digestive capacity of rainbow trout. Interestingly, some studies in monogastric animals showed that dietary supplementation with Glu or Gly can improve the proliferation, differentiation and function of enterocytes (Wang et al., 2014; Zhao et al., 2020b; Yan and Qiu-Zhou, 2006; Coutinho et al., 2016; Caballero-Solares et al., 2015). Dietary supplementation with Glu was shown to enhance the digestive capacity of Jian carp (*Cyprinus carpio* var. Jian) (Zhao et al., 2020b; Zhao et al., 2015) and promote intestinal nucleotide synthesis in rainbow trout (Yoshida et al., 2016b). These NE-AA serve as major energy substrates for the gastrointestinal tract of mammals and fish species (Li et al., 2020; Li et al., 2009; Hou et al., 2015; Hou and Wu, 2018; Jia et al., 2017). Therefore, the higher lipid digestibility observed when Gly and Glu were supplemented in trout diets may be due to the role of Gly and Glu as major fuels for trout enterocytes, as it is the case in mammals (Yang and Liao, 2019; Jia et al., 2017; Hou and Wu, 2018). However, this hypothesis could not be confirmed in the current study since the gastrointestinal tract samples collected for gene expression were degraded. Further studies are required to corroborate the metabolic role and the nutrient utilization of these NE-AA in the gastrointestinal tract of rainbow trout.

In mammals, bile acid is synthesized from cholesterol in the liver and is conjugated either with glycine or taurine to form glycocholic acid or taurocholic acid, respectively. After its conjugation, bile acid is secreted into the proximal intestine where it acts as a surfactant by emulsifying lipids into micelles, which facilitates fat hydrolysis and digestion (Chiang, 1998). In the current study, to look for effects of dietary Gly and Glu supplementation on bile acid metabolism, gene expression of markers of bile acid biosynthesis were measured in the liver. Dietary Gly and Glu supplementation did not affect the expression of cholesterol 7 alpha-hydroxylase (*cyp7a1*), the first rate-limiting enzyme for bile acid synthesis. The expression of apical sodium-dependent bile acid transporter (*Abst*) and ATP-binding cassette subfamily G5 (*abcg 5*), which play roles in the selective sterol excretion by the liver into bile, did, however, increase significantly in trout fed with surplus of Gly and/or Glu. The expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*) which is regarded as the rate-limiting step in cholesterol synthesis, significantly decreased in fish fed diet supplemented with Gly

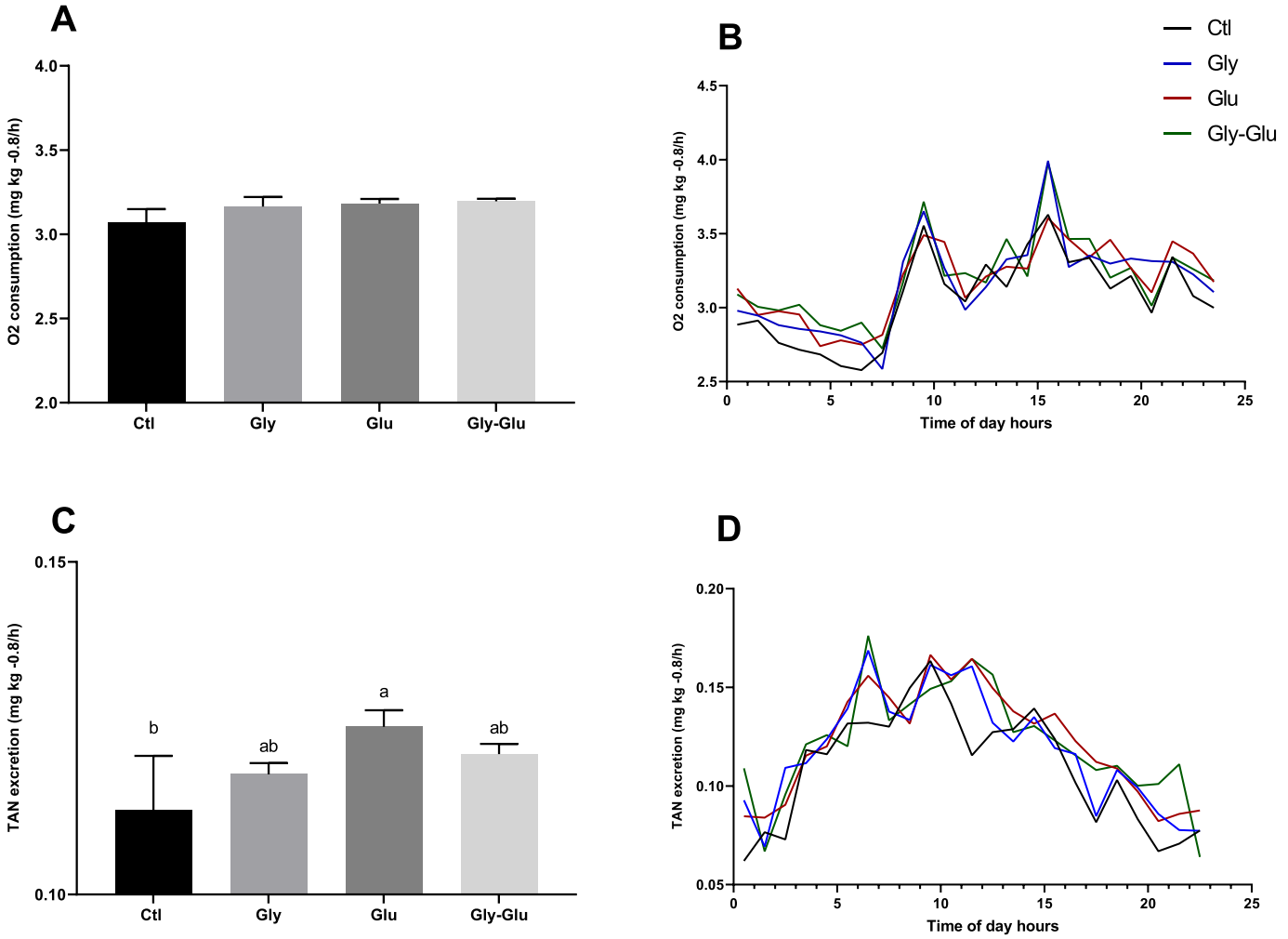

Fig. 4. Mean hourly oxygen consumption (A-B) and total ammonia nitrogen excretion (C—D) pattern over 24 h of rainbow trout fed control diet or diets supplemented with Gly and/or Glu. Values are means of 3 values (except for the group Gly-Glu, values are means of 2 values) per dietary treatment, with their standard deviation represented by vertical bars. Different superscripts letters indicate significantly different values (2 × 2-way factorial ANOVA design with Glu and Gly as varying factors and interaction between the main effects of the two factors (Glu × Gly), $p \leq 0.05$).

compared to fish fed control or dietary Glu. Similarly, organic solute transporter (*osta*) expression significantly decreased in fish fed diet supplemented with Gly. This primary solute efflux transporter is present on the enterocyte at the basolateral surface and is responsible for the secretion of bile acids from the intestine into the systemic circulation, recycled by the liver (Chiang, 1998). Bile acids are released after a contraction of the gallbladder in response to the presence of AA and fat in the proximal intestine. It has been demonstrated in mammalian studies that diet macronutrient composition can affect cycling frequency of bile acids (Chiang, 1998). Thus, the difference in the expression of genes involved in the hepatic bile acid and cholesterol transporters of trout fed with a surplus of dietary glycine might reflect the cycling frequency of bile acid within the enterohepatic circulation. Furthermore, the obtained results might suggest also that a surplus of dietary glycine may be conjugated with bile acid and stimulated the hepatic bile acid biosynthesis in rainbow trout, as shown in mammals (Chiang, 2013). In most fish species studied so far, however, bile acids are mostly conjugated with taurine and not with glycine due to a lower affinity of glycine compared to taurine for bile acid-CoA (Romano et al., 2020; Hagey et al., 2010). Accordingly, dietary taurine supplementation affected hepatic bile acid biosynthesis in different fish species (Romano et al., 2020; Kortner et al., 2013; Staessen et al., 2021). In the present study, despite taurine levels in the diet, fish and in serum being similar, significant effects were seen in key genes involved in bile acid biosynthesis and circulation, suggesting a role for dietary glu/Gly in modulating bile acid metabolism in trout. Also, it is important to note in this study that all diets were devoid of fish meal, which is a source of bile acids synthesis. The control diet might be challenging for bile acid synthesis, however dietary Gly and Glu supplementation indirectly improved the lipid digestibility, which could be either by stimulating the hepatic bile acid biosynthesis or an improve reabsorption of bile acids by the enterocytes. This is, however, only based on mRNA transcription results, but the effects of dietary supplementation with Glu and Gly on the bile acid metabolism could be an interesting path to follow further.

Total ammonia nitrogen excretion was higher in the group of fish fed with surplus of dietary Glu when compared to the control group. The level of ammonium chloride and urea were also higher in the serum of fish fed dietary Gly-Glu. Similar results were obtained in gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), where the ammonia excretion increased with an increase of NE-AA in the diets (Peres and Oliva-Teles, 2006, 2007; Gómez-Requeni et al., 2003). The increased serum ammonia and urea in fish fed with dietary Glu may be attributed to the catabolism of excessive dietary glutamate ingested by the fish. Hence, the higher ammonia and urea levels observed may be due to the excess of glutamate, resulting in the conversion of excess of glutamine to urea via the urea cycle. Studies in mammals have, showed

that the concentration of glutamate in the plasma was not affected by dietary Glu supplementation, since almost all of the dietary Glu is oxidized in the mucosal cells of the intestine as an energy source for growth and function (Tomé, 2018). On the other hand, an increase in dietary Gly led to an increase of Gly, Ser, Trp, and Tyr in the serum of rainbow trout. Dietary supplementation with Gly or Glu-Gly increased the concentrations of glycine in the serum by 45% and 70% compared to the control group, respectively. These results suggest that intestinal breakdown of the supplemented Gly was slow, thus leading to its uptake to the circulation. Similar results have been observed in other studies when some AAs (e.g., glutamate and glycine) were supplemented in the diets of animals (Oehme et al., 2010; Wang et al., 2014). Similar to the serum Gly level, the concentration of Ser in the serum of trout increased by 30% and 45% in the group of fish fed with dietary Gly or Gly-Glu compared to those fed Ctl diets, respectively. These results may indicate that surplus of Gly in the serum is converted into serine, as shown in previous studies (Wang et al., 2014). In conclusion, the results of the present study suggest that a surplus of glycine in the diets was not completely catabolized by the enterocytes due to the elevated level of glycine in the serum of trout fed dietary Gly. However, the concentration of serum glutamate was unaffected by dietary glutamate supplementation, suggesting that dietary glutamate was completely degraded in the intestine and might be used as an energy substrate by the enterocytes of rainbow trout.

5. Conclusions

The results of the present study showed that a surplus of dietary glycine and glutamate improved the digestibility of protein, lipid and most of the amino acids and fatty acids. In conclusion, given the beneficial effects of dietary glutamate and glycine supplementation on the digestive ability of rainbow trout, these NE-AA could be considered as functional amino acids in aquafeed to enhance growth in commercial cultured fish species. However, more research behind the mechanisms is needed to have a full understanding of the effects of these two AAs on digestive capacity and energy metabolism in rainbow trout.

CRediT authorship contribution statement

Ikram Belghit: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Antony Jesu Prabhu Philip:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. **Roel M. Maas:** Data curation, Formal analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing. **Erik-Jan Lock:** Investigation, Methodology, Visualization, Writing – review & editing. **Ep H. Eding:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Project administration, Visualization, Writing – review & editing. **Marit Espe:** Conceptualization, Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing. **Johan W. Schrama:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

No potential conflict of interest was reported by the author(s).

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739311.

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