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Evidence of gastrointestinal sensing and gut-brain communication in rainbow trout (*Oncorhynchus mykiss*) in response to the aqueous extract of fishmeal and its free amino acid fraction

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ABSTRACT

Using rainbow trout (*Oncorhynchus mykiss*) as a model, we aimed to obtain information about the gastrointestinal tract (GIT) amino acid sensing capacity and hormone production along regions of the GIT, in response to proline (Pro), to a solution of free amino acids (FAA) mimicking the composition of a fishmeal (FM) aqueous extract (FM-FAA), or to the whole FM aqueous extract (FM-AQE). In addition, we evaluated central responses (in hypothalamus) in mechanisms regulating food intake, 2 h following intragastric administration of these treatments. The presence of Pro in the GIT elicited changes in amino acid sensing systems and in the production of GIT hormones, especially in the more proximal regions in parallel with an anorectic response in hypothalamus. The intragastric administration of FM-AQE induced increased production of the anorectic hormones peptide tyrosine-tyrosine (PYY) and cholecystokinin (CCK) that occurred 20 min post-treatment in the proximal and middle intestine of this treatment. These changes occurred in parallel with an anorectic response in the hypothalamus 2 h post-treatment. The treatment with FM-FAA elicited a comparable anorectic response in the hypothalamus at 2 h post-treatment, which was associated however with a more complex response in the GIT. This included a comparable increased production of the anorectic hormones PYY and CCK in the proximal and middle intestine, but also a decreased production of the orexigenic hormone ghrelin (GHRL) in the stomach, 20 min after FM-FAA administration. These effects were also accompanied by some changes in parameters related to amino acid sensing systems mediated by receptors, which were not observed in the FM-AQE treatment. Overall, results indicate that all treatments elicited a response in elements of gut sensing mechanisms and gut-brain axis, despite important differences in the specific genes (likely having different substrate specificities), GIT areas and times in which responses were observed.

1. Introduction

The gastrointestinal tract (GIT) is involved in the control of food intake in mammals (Geraedts et al., 2010). The detection of nutrients (glucose, fatty acids or amino acids) by different receptors, including canonical taste receptors, and the transduction of derived signals by downstream signalling cascades occurs in the GIT, mainly in enteroendocrine cells (EECs), which are also responsible for the synthesis and release of gastrointestinal hormones. Among others, these include ghrelin (GHRL), cholecystokinin (CCK), peptide tyrosine-tyrosine (PYY), and glucagon-like peptide-1 (GLP-1), which besides having multiple paracrine and endocrine roles, bind to receptors in the vagus nerve and/or central brain areas (*i.e.* hypothalamus). In this manner, these hormones ultimately regulate food intake, digestive, absorptive and metabolic processes (Raka et al., 2019) through the so-called gut-brain axis (Goldstein et al., 2021). In fish, the GIT is also involved in the detection of different nutrients, as demonstrated for glucose (Polakof et al., 2010; Polakof and Soengas, 2013) and recently for amino acids (Calo et al., 2021), and there is also evidence for the existence of a gut-brain axis (Blanco et al., 2021). Furthermore, the co-expression of members of the taste 1 receptor (*tas1r*) gene family with mRNAs coding for the gut peptides Ghrl, Cck, Pyy and proglucagon (Pg – precursor of Glp-1) has now also been firmly established in EECs of seabream (*Sparus aurata*) (Angotzi et al., 2022). Focusing on amino acids, we recently established

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the presence of homologous genes to mammalian amino acid sensing receptors (Depoortere, 2014; Raka et al., 2019), including G protein-coupled receptor family C group 6 member A (*gprc6a*) and taste receptors type 1 members 1 and 2 (*tas1r1*, *tas1r2*) in the GIT of rainbow trout (*Oncorhynchus mykiss*) (Calo et al., 2021). We also demonstrated that the expression of these genes (together with that of molecular components of taste-related intracellular signalling) was altered 20 min after intragastric administration of L-leucine, L-valine, L-proline or L-glutamate in the stomach and proximal intestine of trout (Calo et al., 2021). Moreover, the luminal presence of these amino acids led to important changes in mRNA and/or protein levels of Ghrl, Cck, Pyy, and Glp-1 (Calo et al., 2021). However, the response of these systems in more distal regions of the intestine, during longer time periods, was not previously assessed.

Fish meal (FM) was traditionally used as the main ingredient in fish feeds due to its ideal properties for fish nutrition, including its profile of essential and non-essential amino acids, high digestibility and good palatability (Cho and Kim, 2011). However, sustainability concerns, decreasing availability and rising prices have led to the progressive reduction in FM contents and its replacement by alternative protein sources, so far mostly of vegetable origin (Jannathulla et al., 2019; Glencross et al., 2020). This replacement has been often associated with a lowered feed intake and feeding efficiency, particularly in carnivore fish species (Kissil et al., 2000; Gómez-Requeni et al., 2004; Hansen et al., 2007; Bonaldo et al., 2011), linked to potential deficiencies in amino acid composition and presence of anti-nutritional factors in vegetable sources. The high levels of soluble protein and free amino acids (FAA) in FM are believed to explain some of its well-known palatability and digestibility properties (Aksnes et al., 2006; Dong et al., 2016; Ween et al., 2017). Thus, it is reasonable to suggest that at least some of the beneficial effects related to high levels of FM in fish feeds could be associated to the activation of gut sensing mechanisms by amino acids and peptides, as well as other water soluble compounds, leaching out from the feeds. However, there is a shortage of studies evaluating the impact of FM and amino acid mixtures on putative amino acid sensing systems, on the synthesis of GIT hormones, or in the central mechanisms involved in feed intake control in fish.

Therefore, we aimed to obtain information about the amino acid sensing capacity and GIT hormone production in rainbow trout, after the intragastric administration of a single amino acid (Pro: Proline), of a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA), or of the whole FM aqueous extract (FM-AQE). The short-term (20 min) response to the intragastric presence of Pro has been assessed previously, but the present study additionally evaluated the response at a longer time period (2 h), to guarantee that the treatments also reached the distal intestine. Additionally, central responses in mechanisms regulating feed intake in hypothalamus (Soengas et al., 2018; Soengas, 2021) were also evaluated at 2 h following treatment administration.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*, body weight (bw) = 100 ± 12 g) were obtained from a local fish farm (A Estrada, Spain) and housed in 100 L tanks at the Universidade de Vigo (Spain). Fish were maintained under laboratorial conditions under a 12 L:12D photoperiod (lights on at 08:00 h, lights off at 20:00 h), in aerated and dechlorinated tap water at 15 °C. We fed fish once daily at 11:00 h with commercial dry feed (proximate analysis: 44% crude protein, 2.5% carbohydrates, 21% crude fat, and 17% ash; 20.2 MJ kg^{-1}; Biomar, Dueñas, Spain) until visual satiety. The experiments were carried out in accordance with the guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

2.2. Preparation and analysis of fishmeal aqueous extract

For the extraction of the soluble fraction of fishmeal LT70 (N.O. Hansen Agentur Aps, Nyborg, Denmark), 10 g of this ingredient were suspended in 50 mL of water and pH adjusted to 4.5 with 1 N HCl (simulating stomach pH conditions). The mixture was stirred for 3 h at room temperature, centrifuged (8000 *g* × 10 min) and manually filtered through a 0.45 μm filter provided with hydrophilic PVDF membrane (Merck Millipore, Darmstadt, Germany). The obtained solution was freeze dried to obtain 2.5 g of solid product (extraction yield = 25%), which was stored at −20 °C until use.

The dried aqueous extract of FM LT70 (FM-AQE) was subsequently analysed for proximate composition (Table 1), FAA composition (Table 2) and peptide molecular weight distribution (MWD; Table 3). Proximate analysis was performed using standard methods: dry matter after drying at 103 °C for 24 h; ash by combustion at 550 °C for 12 h; crude protein (N × 6.25) by Kjeldahl distillation; crude fat by acid hydrolysis; sugars by Luff-Schoorl; and crude fiber by Weende. Free amino acids were prepared for liquid chromatography using the Advanced Bio Amino Acid Analysis Solution provided by Agilent (Santa Clara, USA), following manufacturer's instructions with minor modifications. Samples (100 mg) were dissolved in 0.1 N HCl to proper concentration and filtered. 180 μL of the obtained solutions were mixed with 20 μL of the internal standard solution (Agilent, Santa Clara, USA) and the mixtures submitted to on-line derivatization/analysis according to manufacturer's instructions (Agilent, Santa Clara, USA). The analysis was performed on an Agilent 1290 Infinity II HPLC (Santa Clara, USA) equipped with a Kinetex EVO C18 column (100 × 4.6 mm, 100 Å, 2.6 μm particle size – Phenomenex, Torrance, USA) and detected by fluorescence on an Agilent 1260 FLD Spectra detector (Santa Clara, USA, excitation/emission wavelengths were 340/450 nm from $t = 0$ to 7.30 min and 260/325 nm from t = 730 nm to the end). Samples were eluted by linear gradient using: A) 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$, pH 8.2 and B) Acetonitrile:Methanol:Water (45:45:10, v:v:v) as mobile phases, 1 mL/min flow and the gradient described by the manufacturer. Standard solutions and calibration curves were prepared as described in the commercial methodology. Analysis of peptide MWD was performed by HPLC (1200 series HPLC, Agilent Technologies, Santa Clara, CA, USA) using size exclusion chromatography. Briefly, a Superdex Peptide 10/30, (bed dimensions 10 × 300 mm) column was used for peptide fractionation based on the differential diffusion of molecules of different molecular weight into the pores of the resin, and the UV-detector was set at 214 nm.

The concentration and profile of FAA in FM-AQE was then chemically recreated using pure amino acids (Sigma Chemical Co, St Louis, Mo, USA) to produce a FM-FAA mimic treatment.

2.3. Experimental designs

In the first experiment, fish were randomly assigned to 4 experimental tanks with 6 fish per tank, and fasted for 48 h to ensure intestinal emptying and basal levels of hormones involved in metabolic control of food intake. The day of the experiment, we lightly anesthetized fish with 2-phenoxyethanol (Sigma, 0.02% *v*/v). Then, all fish from each tank were captured, individually weighed and intragastrically administered

Table 1
Proximate analysis (% dry matter) of fishmeal aqueous extract.

Protein	69.72
Lipids	2.18
Carbohydrate	2.31
Fiber	<0.20
Ash	23.82
Humidity and volatile matter	3.12

Table 2
L-amino acid content (g 100 g^{-1} extract) and percentage of free amino acids of fishmeal aqueous extract.

L-amino acid	Content	% free amino acid
Alanine	1.02	11.47
Arginine	0.51	5.70
Asparagine	0.02	0.22
Aspartate	0.19	2.14
Glutamine	0.00	0.00
Glutamate	0.50	5.58
Glycine	0.28	3.11
Histidine	0.44	4.95
Isoleucine	0.39	4.35
Leucine	0.95	10.72
Lysine	0.63	7.08
Methionine	0.24	2.74
Phenylananine	0.51	5.73
Proline	0.03	0.37
Serine	0.16	1.76
Taurine	1.82	20.46
Threonine	0.35	3.94
Tryptophan	0.05	0.52
Tyrosine	0.33	3.75
Valine	0.48	5.40
Total	8.89	100.00

Table 3
Peptide molecular weight distribution (MWD) of aqueous extract of fishmeal LT70.

	Aqueous extract of Fishmeal LT70
Soluble Crude Protein (g/100 g sample)	71.9
Peptides, MW (Da):	% water soluble peptides
> 20,000	1.0
20,000–15,000	1.8
15,000–10,000	4.2
10,000–8000	3.0
8000–6000	4.5
6000–4000	7.1
4000–2000	12.7
2000–1000	11.3
1000–500	8.4

with 1 mL per 100 g^{-1} bw of the corresponding solution. The solution consisted in one of the following treatments (randomly administrated): water alone (control, $n = 6$ fish) or containing 4.6 mg.mL^{-1} of a single amino acid (Pro: Proline, 40 μmol.mL^{-1}, n = 6), or a mixture of FAA reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 4.6 mg.mL^{-1} or 36.5 μmol.mL^{-1}, considering an average amino acid molecular weight of 132 g/mol, Table 3, n = 6), or the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}, n = 6). Both the FM-FAA and FM-AQE test solutions were diluted to a concentration of FAA of 4.6 mg mL^{-1}, identical to that used in the Pro solution (the same dose used in the previous work Calo et al., 2021), to make treatments comparable. The FM-AQE treatment, however, additionally contained other water-soluble molecules that leached out from the fishmeal during the extraction process. Intragastrical administration was carried out using a 13 cm-long cannula attached to a blunt-tip syringe and the administrated dose was calculated from the amount of leucine ingested per day by a trout fed a standard commercial diet (Wacyk et al., 2012). We observed no regurgitation during administration of the different solutions. After treatment, fish recovered in individual tanks for 20 min, whereupon they were anesthetized as described above and sacrificed by decapitation. We collected samples from stomach, proximal, middle, and distal intestine, as shown in Fig. 1, which were immediately frozen in dry ice and stored at −80 °C until further analysis of mRNA abundance of amino acid receptors, intracellular signalling elements and gut hormones, and protein levels of gut hormones. We selected 20 min as sampling time because preliminary experiments (not shown) demonstrated this time to be the necessary for a solution containing a dye to reach the intestine when administered intragastrically.

In a second experiment, carried out during two days, we used the same experimental procedure but in this case samples were collected from 12 fish per group (6 fish each day) 2 h after treatment. We collected samples of stomach, proximal, middle, and distal intestine, as well as of the hypothalamus. Of the 12 fish sampled per treatment, we used samples from 6 fish for analysis of mRNA abundance and the remaining fish for determining protein levels of some transcriptional factors. Both experiments started at 9 a.m. each day and lasted for about 4 h.

2.4. Quantification of mRNA abundance by real-time PCR

Total RNA was extracted from tissues of 6 fish per treatment using Trizol reagent (Life Technologies, Grand Island, NY, United States). RNA purity was assessed by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland), and only samples with an OD 260 nm/280 nm ratio > 1.8 were used for analysis. After RQ1-DNAse (Promega, Madison, WI, United States) treatment, 2 μg of total RNA were reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in a reaction volume of 20 μL. Finally, gene expression levels were

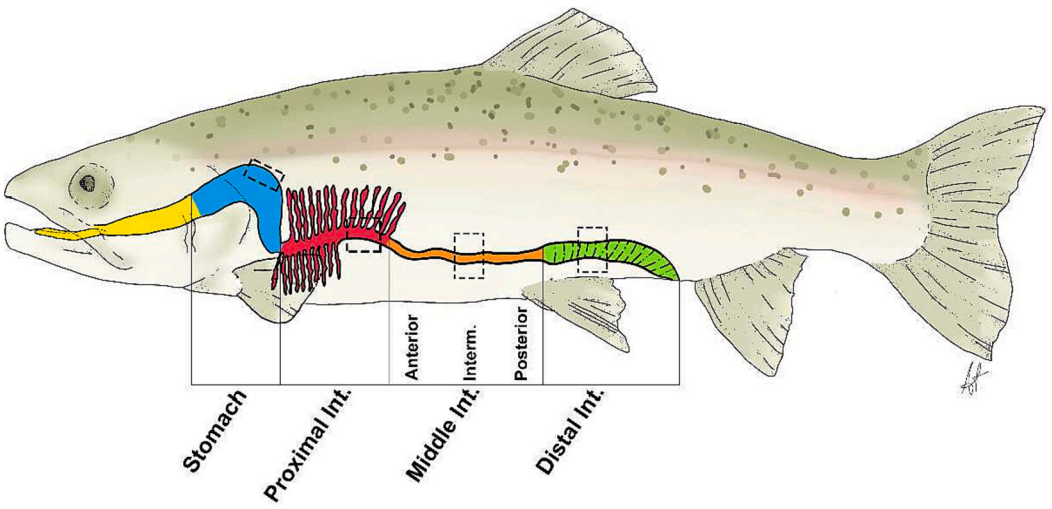

Fig. 1. Schematic representation of the rainbow trout gastrointestinal tract, showing the regions in which it was divided for the experiments carried out in this study. The areas sampled within each region are squared.

determined by real-time quantitative PCR (RT-qPCR) in an iCycler iQ (Bio-Rad, Hercules, CA, United States), using MAXIMA SYBR Green qPCR Mastermix (Life Technologies). Genes were amplified in duplicate using a 96-well plate loaded with 1 μL of cDNA and 500 nM of each forward and reverse primer (10 μM) in a final volume of 10 μL. Sequences of primers used for target and reference genes are shown in Table 4, and correspond to previously described primers in the same species (Polakof et al., 2011; Polakof and Soengas, 2013; Comesaña et al., 2018a; Conde-Sieira et al., 2018; Calo et al., 2021). Negative controls containing RNA samples and water instead of cDNA were included in every reaction. Thermal cycling was initiated with incubation at 95 °C during 10 min, followed by 40 cycles consisting of heating at 95 °C for 30 s and specific annealing and extension temperatures (Table 3) for 30 s. Following the final PCR cycle, melting curves were systematically performed and monitored (temperature gradient at 0.5 °C/5 s from 60 to 94 °C) to ensure that only one fragment was amplified. Relative expression of the target transcripts was calculated using *actb* (β-actin) and *eef1a1* (elongation factor 1α) as reference genes, both steadily expressed in this experiment, following the Pfaffl (2001). Efficiency was in the range 90–120%.

2.5. Analysis of protein levels by Western blot

Samples of stomach, proximal, middle and distal intestine, and hypothalamus ($n = 6$ fish per treatment) were homogenized in 1 mL of buffer containing 150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA (pH 7.4), 100 mM NaF, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X- 100, 0.5% NP40-IGEPAL, and 1.02 $mg.mL^{-1}$ protease inhibitor cocktail (Sigma). Homogenates were centrifuged for 15 min (1000 ×*g*; 4 °C), and supernatants were again centrifuged for 30 min (20,000 ×*g*; 4 °C). The concentration of protein in each sample was determined using the Qubit Protein Assay (Thermofisher, Waltham, MA, USA). Then, samples (each containing 50 μg protein) were mixed with 4× Laemmli buffer containing 0.2% 2-mercaptoethanol (Bio-Rad) and denatured at 95 °C for 5 min. Next, they were electrophoresed in the appropriate percentage of acrylamide gels (10% for FoxO1 and CREB, 15% for BSX and 17.5% for GHRL) or in 4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad) (for CCK and PYY) and transferred to a 0.2 μm pore-size nitrocellulose membrane (Bio-Rad) using the Trans-Blot Turbo transfer system (Bio-Rad). We blocked membranes in Pierce Protein-Free T20 (PBS) Blocking Buffer (ThermoFisher) during 60 min, and then incubated overnight with specific primary antibody. We used the following primary antibodies in GIT: anti-GHRL (1:500, ref. #H-031-31, Phoenix), anti-CCK (1:500, ref. #ab27441, Abcam, Cambridge, United Kingdom), anti-PYY (1:500, ref. #ab22663, Abcam). Primary antibodies used in hypothalamus were: anti-CREB (48H2) (1:500, ref. #9197, Cell Signalling), anti-phospho-CREB (Ser133) (1:500, ref. #9198, Cell Signalling), anti-phospho-FoxO1 (Thr24) (1:500, ref. #9464, Cell Signalling), anti-FoxO1 (L27) (1:500, ref. #9454, Cell Signalling), anti-BSX (1:500, ref. #ab236983, Abcam) and anti-β-tubulin (1:1000, ref. #ab6046, Abcam). These antibodies were validated for use in rainbow trout, as determined by band comparison between rainbow trout and rat tissues (Calo et al., 2021; Conde-Sieira et al., 2018; Velasco et al., 2016). After washing, membranes were incubated with 1:5000 goat anti-rabbit IgG (H + L) HRP conjugate (ref. #ab205718, Abcam). For protein visualization, membranes were incubated in Clarity Western ECL substrate (Bio-Rad) and imaged in a ChemiDoc Touch imaging system (Bio-Rad). Protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein (Cck, Pyy) or relative to the abundance of the housekeeping protein β-tubulin (Ghrl, Foxo1, Creb, and Bsx).

2.6. Statistical analysis

Using the statistical software SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA, USA), one-way ANOVA followed by Student-Newman-Keuls multiple comparison test were used to assess

Table 4
Primers used for determining gene expression with their accession numbers and annealing temperature (T).

Transcript	Data base	Accession	Forward primer	Reverse primer	T (°C)
actb	GenBank	NM 001124235.1	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59
agrp1	GenBank	NM_001146677	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60
bsx	GenBank	MG310161	CATCCAGAGTTACCCGGCAAG	TTTTCACCTGGGTTTCCGAGA	60
cartpt	GenBank	NM_001124627	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60
ccka	GenBank	NM_001124345.1	GGGTCCCAGCCACAAGATAA	TGGATTTAGTGGTGGTGCGT	60
cckbr2	GenBank	XM_036975022.1	GTGGCCTATGGACTCATCTCC	TCGATGATGTCAGAGTGGACA	60
creb1	GenBank	MG310160	CGGATACCAGTTGGAGGAGGA	AGCAGCAGCACTCGTTTAGGC	60
eef1a1	GenBank	AF498320	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59
foxo1	GenBank	MG310159	AACTCCCACAGCCACAGCAAT	CGATGTCCTGTTCCAGGAAGG	60
gcg	GenBank	NM_001124698.1	AGGAGTGGTGCTCCATCCAAA	TCCTGATTTGAGCCAGGAAACA	59
gcgr	GenBank	XM_021582023.1	GCCGTGATGTCAGAGGAACA	GGATGGCAACCAGTAGACCC	60
ghrl	GenBank	AB096919.1	GGTCCCCTTCACCAGGAAGAC	GGTGATGCCCATCTCAAAAGG	60
ghsr1a	GenBank	NM_001124594.1	TTCGTGCGCTCATCCTCTTT	ACTGGGTGGGTTTACACTCG	60
gnai1	Sigenae	CU073912	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAAA	60
gprc6a	GenBank	XM_021574849.1	ATGGGGATCAGCAGAATTTGG	CCGGCACCTTGTTTCTCTTTG	60
itpr1	GenBank	XM_021569164.1	AGAAGAACGCCATGAGAGTGA	ACCACTTTGTCCCCTATCACC	60
itpr3	GenBank	XM_021616029.1	GCAGGGGACCTGGACTATCCT	TCATGGGGCACACTTTGAAGA	59
npy	GenBank	NM_001124266	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58
plcb1	GenBank	XM_021611355.1	GGAGTTGAAGCAGCAGAAGG	GGTGGTGTTTCCTGACCAAC	60
plcb3	GenBank	XM_021577635.1	ATAGTGGACGGCATCGTAGC	TGTGTCAGCAGGAAGTCCAA	60
plcb4	GenBank	XM_021600840.1	ACCTCTCTGCCATGGTCAAC	CGACATGTTGTGGTGGATGT	60
pomca1	Tigr	TC86162	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60
pyy	GenBank	XM_021557532.1	GGCTCCCGAAGAGCTGGCCAAATA	CCTCCTGGGTGGACCTCTTTCCA	60
tas1r1	GenBank	XM_021614415.1	GTTGTGTTCTCCAGCAAAAGC	TCTGTCCCTATCCACACCTTG	60
tas1r2a	GenBank	MT240253	ATAGTTTTTGCCGGGCAGAGC	CCTGCAATCCACACTTTGCTG	59
tas1r2b	GenBank	XM_021625831.1	GATGAGTGGGCCAGGAATGG	CCTCCCACCGGCTGACTTTA	59

actb, beta-actin; *agrp1*, agouti-related protein 1; *bsx*, brain homeobox transcription factor; *cartpt*, cocaine- and amphetamine-related transcript; *ccka*, cholecystokinin a; *cckbr2*, cholecystokinin receptor type B-like; *creb1*, cAMP response-element-binding protein; *eefla1*, elongation factor 1α; *foxo1*, forkhead boxO1; *gcg*, proglucagon; *gcgr*, glucagon like peptide 1 receptor; *ghrl*, ghrelin; *ghsr1a*, growth hormone secretagogue receptor 1a; *gnai1*, guanine nucleotide-binding protein G subunit alpha 1; *gprc6a,* G protein-coupled receptor family C group 6 member A; *itpr1/itpr3*, inositol 1,4,5-triphosphate receptor type 1 and 3; *npy*, neuropeptide y; *plcb1/plcb3/plcb4*, genes encoding phospholipase C β1, β3, and β4; *pomca1*, pro-opio melanocortin a1; *tas1r1/tas1r2a/tas1r2b*, genes encoding taste receptor 1 family members 1, 2a, and 2b.

differences among experimental groups, which were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects after 20-min administration

Parameters assessed in stomach 20 min after treatment administration are shown in Fig. 2. The mRNA abundance of *ghrl* increased in comparison with the control group after Pro or FM-FAA treatment, and

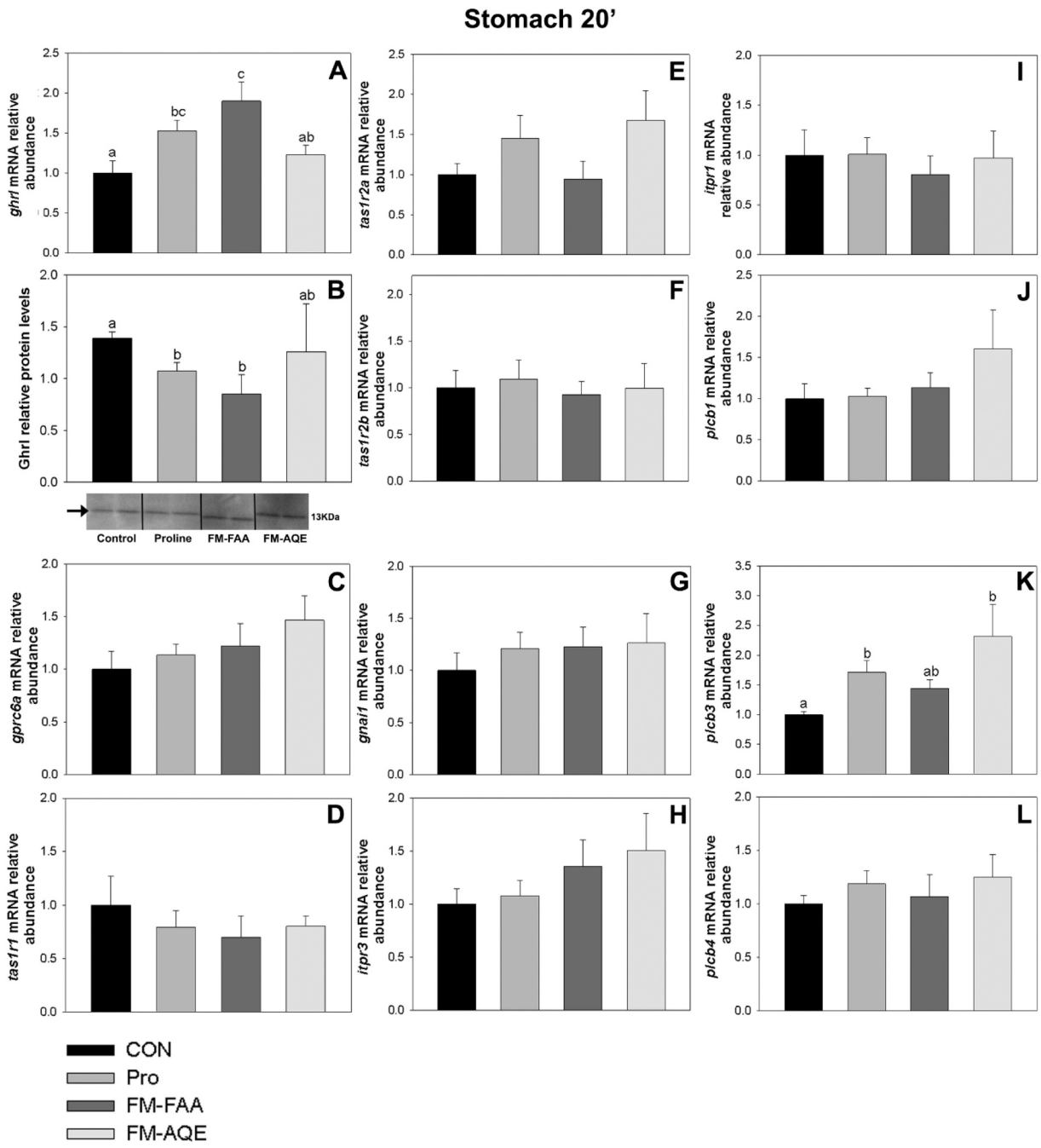

Fig. 2. mRNA abundance of *ghrelin* (A), *gprc6a* (C), *tas1r1* (D), *tas1r2a* (E), *tas1r2b* (F), *gnai1* (G), *itpr3* (H), *itpr1* (I), *plcb1* (J), *plcb3* (K), and *plcb4* (L), and Ghrelin protein levels (B) in stomach of rainbow trout 20 min after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of β-tubulin. Data are expressed as mean + SEM ($n = 6$). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

was also higher in the FM-FAA group than in FM-AQE (Fig. 2A). Ghrl protein levels (Fig. 2B), on the other hand, decreased in the Pro and FM-FAA groups compared to the control. The mRNA abundance of phospholipase C β3 (*plcb3*, Fig. 2K) increased after Pro or FM-AQE administration, compared to the control group. No significant changes occurred for mRNA abundance of *gprc6a* (Fig. 2C), *tas1r1* (Fig. 2D), *tas1r2a* (Fig. 2E), *tas1r2b* (Fig. 2F), guanine nucleotide-binding protein G subunit alpha 1 (*gnai1,* Fig. 2G), inositol 1,4,5-triphosphate receptor type 3 (*itpr3*, Fig. 2H), and 1 (*itpr1,* Fig. 2I), and phospholipase C β1 (*plcb1* (Fig. 2J), and 4 (*plcb4*,Fig. 2L)

Fig. 3 shows changes in parameters assessed in proximal intestinal 20 min after administration of test solutions. The mRNA abundance of *cck* increased in all treatments in comparison with the control (Fig. 3A), while protein levels of Cck were significantly increased only in FM-AQE relative to the control (Fig. 3B). The mRNA abundance of *pyy* in the FM-FAA group was higher than in the control group (Fig. 3C) while protein levels of Pyy increased after treatment with FM-AQE or FM-FAA (Fig. 3D). The mRNA abundance of *tas1r2b* was higher after FM-FAA treatment than in the control and Pro groups (Fig. 3I). The mRNA abundance of *itpr3* was higher after FM-FAA treatment than in the control (Fig. 3K). Levels of *plcb3* mRNA (Fig. 3N) increased after Pro and FM-AQE administration compared to the control group. The mRNA abundance of *plcb4* (Fig. 3O) was higher in the FM-FAA than in the FM-AQE group. No changes occurred in mRNA abundance of *gcg* (Fig. 3E), *gprc6a* (Fig. 3F), *tas1r1* (Fig. 3G), *tas1r2a* (Fig. 3H), *gnai1* (Fig. 3J), *itpr1* (Fig. 3L), and *plcb1* (Fig. 3M).

In middle intestine (Fig. 4), *cck* mRNA abundance increased after FM-AQE treatment compared with the control (Fig. 4A), whereas an increase in protein level was only significant in the FM-FAA group compared with the control group (Fig. 4B). In the case of PYY, its mRNA abundance was lower in the Pro and FM-FAA groups than in control, and in Pro than in the FM-AQE treatment (Fig. 4C), whereas no significant changes occurred in protein levels (Fig. 4D). The mRNA abundance of *itpr3* increased in the FM-AQE and FM-FAA groups compared with the control (Fig. 4K). The mRNA abundance of *plcb3* was higher after Pro and FM-FAA administration than in the control (Fig. 4N). No significant changes occurred in mRNA abundance of *gcg* (Fig. 4E), *gprc6a* (Fig. 4F), *tas1r1* (Fig. 4G), *tas1r2a* (Fig. 4H), *tas1r2b* (Fig. 4I), *gnai1* (Fig. 4J), *itpr1* (Fig. 4L), *plcb1* (Fig. 4M), and *plcb4* (Fig. 4O).

In distal intestine, at 20 min after treatment (Fig. 5), significant effects were only observed in the mRNA abundance of *gprc6a* (Fig. 5D) and *plcb4* (Fig. 5M), which were lower in the FM-AQE treatment compared with the control, Pro and FM-FAA groups. No significant changes occurred in CCK either for mRNA abundance (Fig. 5A) or protein levels (Fig. 5B), nor in mRNA abundance of *gcg* (Fig. 5C), *tas1r1* (Fig. 5E), *tas1r2a* (Fig. 5F), *tas1r2b* (Fig. 5G), *gnai1* (Fig. 5H), *itpr3* (Fig. 5I), *itpr1* (Fig. 5J), *plcb1* (Fig. 5K), and *plcb3* (Fig. 5L).

3.2. Changes after 2-h administration

Gene and protein expression were also assessed in stomach 2 h after administration of the test solutions (Fig. 6). As for *ghrl*, no significant differences were observed in mRNA abundance between treatments (Fig. 6A), whereas Ghrl protein levels were higher in the FM-FAA than in the Pro group (Fig. 6B). The mRNA abundance of *gprc6a* was lower in the FM-AQE group than in the control (Fig. 6C). The mRNA abundance of *tas1r2a* was lower after Pro treatment than in the control (Fig. 6E). The mRNA abundance of *tas1r2b* was higher in the FM-FAA treatment than in the Pro group (Fig. 6F). The mRNA abundance of *itpr3* was higher in the FM-FAA group than in the control (Fig. 6H). No significant changes occurred in mRNA abundance of *tas1r1* (Fig. 6D), *gnai1* (Fig. 6G), *itpr1* (Fig. 6I), *plcb1* (Fig. 6J), *plcb3* (Fig. 6K), and *plcb4* (Fig. 6L).

Fig. 7 shows changes in parameters assessed in proximal intestine 2 h after treatment. When considering CCK, no significant changes occurred in its mRNA abundance (Fig. 7A) or protein levels (Fig. 7B). As for PYY, its mRNA abundance was higher in the FM-FAA group than in the control and FM-AQE groups (Fig. 7C), while protein levels did not show significant changes (Fig. 7D). The mRNA abundance of *tas1r1* and *gnai1* increased after FM-AQE treatment, compared with the control (Fig. 7G and J). The mRNA abundance of *tas1r2b* (Fig. 7I) and *plcb4* (Fig. 7O) was higher in the FM-FAA group than in all the other treatments. No significant changes occurred in mRNA abundance of *gcg* (Fig. 7E), *gprc6a* (Fig. 7F), *tas1r2a* (Fig. 7H), *itpr3* (Fig. 7K), *itpr1* (Fig. 7L), *plcb1* (Fig. 7M), and *plcb3* (Fig. 7N).

Changes in parameters assessed in middle intestine at 2 h are shown in Fig. 8. The hormone CCK displayed no changes in its mRNA abundance (Fig. 8A), while its protein levels were lower in Pro than in all the other treatments (Fig. 8B). The gut peptide PYY had a higher mRNA abundance in the Pro and FM-FAA treatments than in the control and FM-AQE groups (Fig. 8C), whereas no changes occurred in protein levels (Fig. 8D). The mRNA abundance of *tas1r2b* was raised after FM-FAA treatment, compared with the other groups (Fig. 8I). FM-AQE treatment increased the mRNA abundance of *itpr3* (Fig. 8K) and *plcb3* (Fig. 8N) compared with the control group, and in the case of *itpr3* also compared with the FM-FAA group. No significant changes occurred in mRNA abundance of *gcg* (Fig. 8E) *gprc6a* (Fig. 8F), *tas1r1* (Fig. 8G), *tas1r2a* (Fig. 8H), *gnai1* (Fig. 8J), *itpr1* (Fig. 8L), *plcb1* (Fig. 8M), and *plcb4* (Fig. 8O).

In distal intestine (Fig. 9), CCK mRNA abundance (Fig. 9A) displayed no changes, while its protein levels were lowered in the FM-FAA group compared with the control (Fig. 9B). The mRNA abundance of *gcg* increased after FM-AQE treatment compared to the control (Fig. 9C). The FM-AQE treatment enhanced the mRNA abundance of *gprc6a* (Fig. 9D), *tas1r1* (Fig. 9E), *tas1r2a* (Fig. 9F) and *itpr3* (Fig. 9I) compared to the Pro group, and in the later the FM-FAA treatment also had a higher expression than the Pro group. On the other hand, the administration of Pro decreased mRNA abundance of *tas1r2a* (Fig. 9F) relative to the control, of *gnai1* (Fig. 9H) compared with all the other groups, of *itpr3* relative to the FM-AQE and FM-FAA treatments (Fig. 9I), of *plcb3* compared with the control and FM-AQE groups (Fig. 9L), and of *plcb4* relative to the FM-FAA group (Fig. 9M). No significant changes occurred in mRNA abundance of *tas1r2b* (Fig. 9G), *itpr1* (Fig. 9J), and *plcb1* (Fig. 9K).

Finally, we assessed the mRNA and/or protein expression of several neuropeptides, receptors, and transcription factors in hypothalamus after 2 h of treatment administration (Fig. 10). Significant differences occurred in the mRNA abundance of *agrp1*, which was lower in Pro and FM-FAA treatments compared with the control and FM-AQE groups (Fig. 10B). The mRNA abundance of ghrelin receptor 1a (*ghsr1a*) was lower in the FM-FAA group than in control and Pro groups (Fig. 10E). The mRNA abundance of *creb1* (Fig. 10K) was lower in the FM-FAA group than in the control. Foxo1 protein levels increased after FM-AQE and FM-FAA administration, relative to the control and Pro groups (Fig. 10I). Levels of phosphorylated cAMP response-element-binding protein (Creb) were higher in the FM-FAA than in the Pro group (Fig. 10M). The protein levels of brain homeobox transcription factor (Bsx) decreased in FM-AQE and FM-FAA groups compared with the control (Fig. 10O). No significant differences occurred in mRNA abundance of neuropeptide y (*npy*, Fig. 10A), cocaine- and amphetamine-related transcript (*cartpt*, Fig. 10C), pro-opio melanocortin a1 (*pomca1*, Fig. 10D), cholecystokinin B receptor 2 (*cckbr2*, Fig. 10F), glucagon receptor-like (*gcgr*, Fig. 10G), forkhead boxO1 (*foxo1*, Fig. 10H) and *bsx* (Fig. 10N), as well as in protein levels of Creb (Fig. 10L) and p-Foxo1 (Fig. 10J).

4. Discussion

The aim of this study was to assess the gastrointestinal amino acid sensing response and its signalling to the central nervous system, after gastric administration of 3 treatments: a FAA alone (Pro), a solution of FAA mimicking the composition of an aqueous extract from fishmeal, and the whole fishmeal aqueous extract. The intragastrical

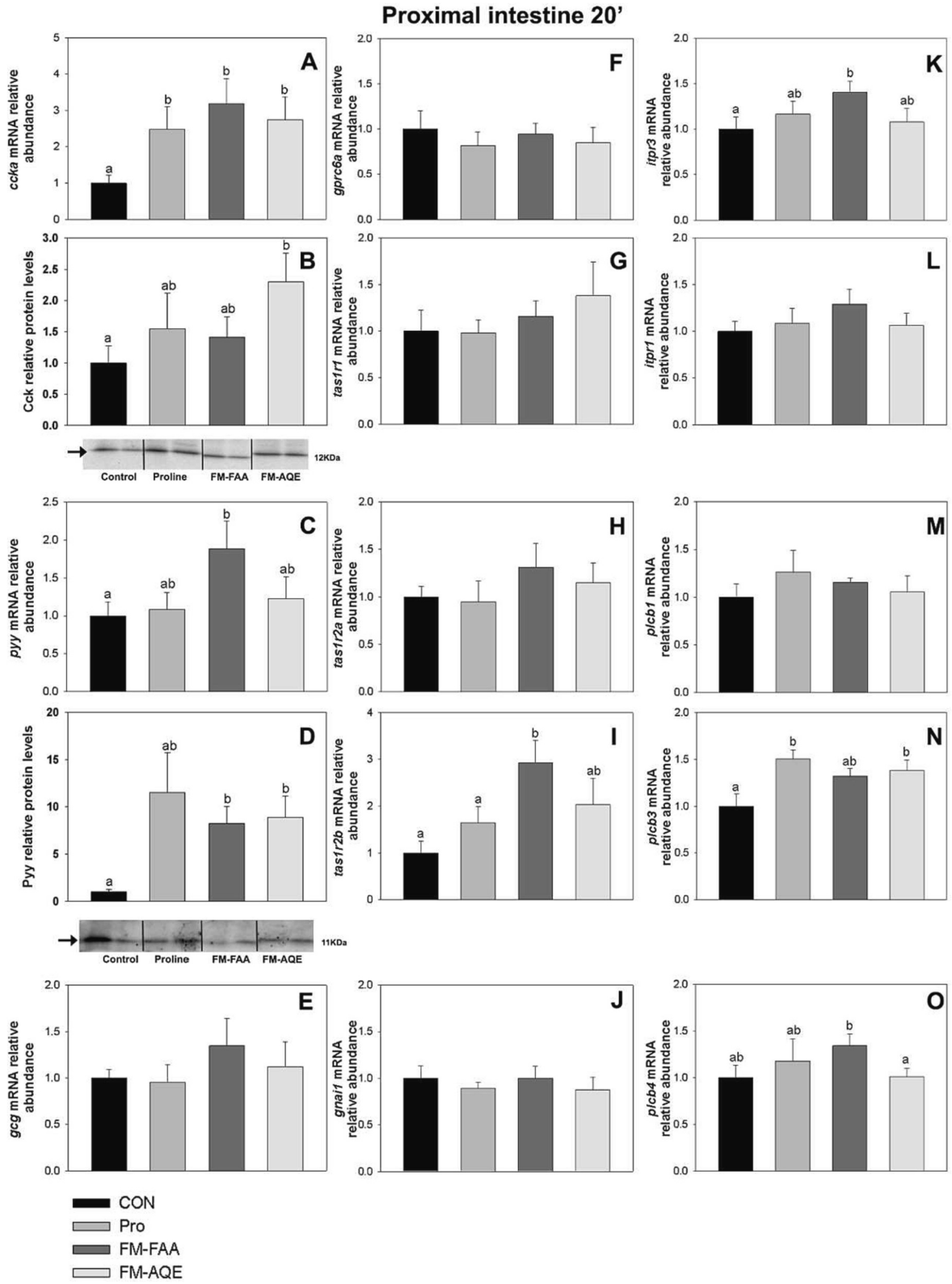

Fig. 3. mRNA abundance of *ccka* (A), *pyy* (C), *gcg* (E), *gprc6a* (F), *tas1r1* (G), *tas1r2a* (H), *tas1r2b* (I), *gnai1* (J), *itpr3* (K), *itpr1* (L), *plcb1* (M), *plcb3* (N), and *plcb4* (O), and protein levels of Cck (B) and Pyy (D) in proximal intestine of rainbow trout 20 min after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM ($n = 6$). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

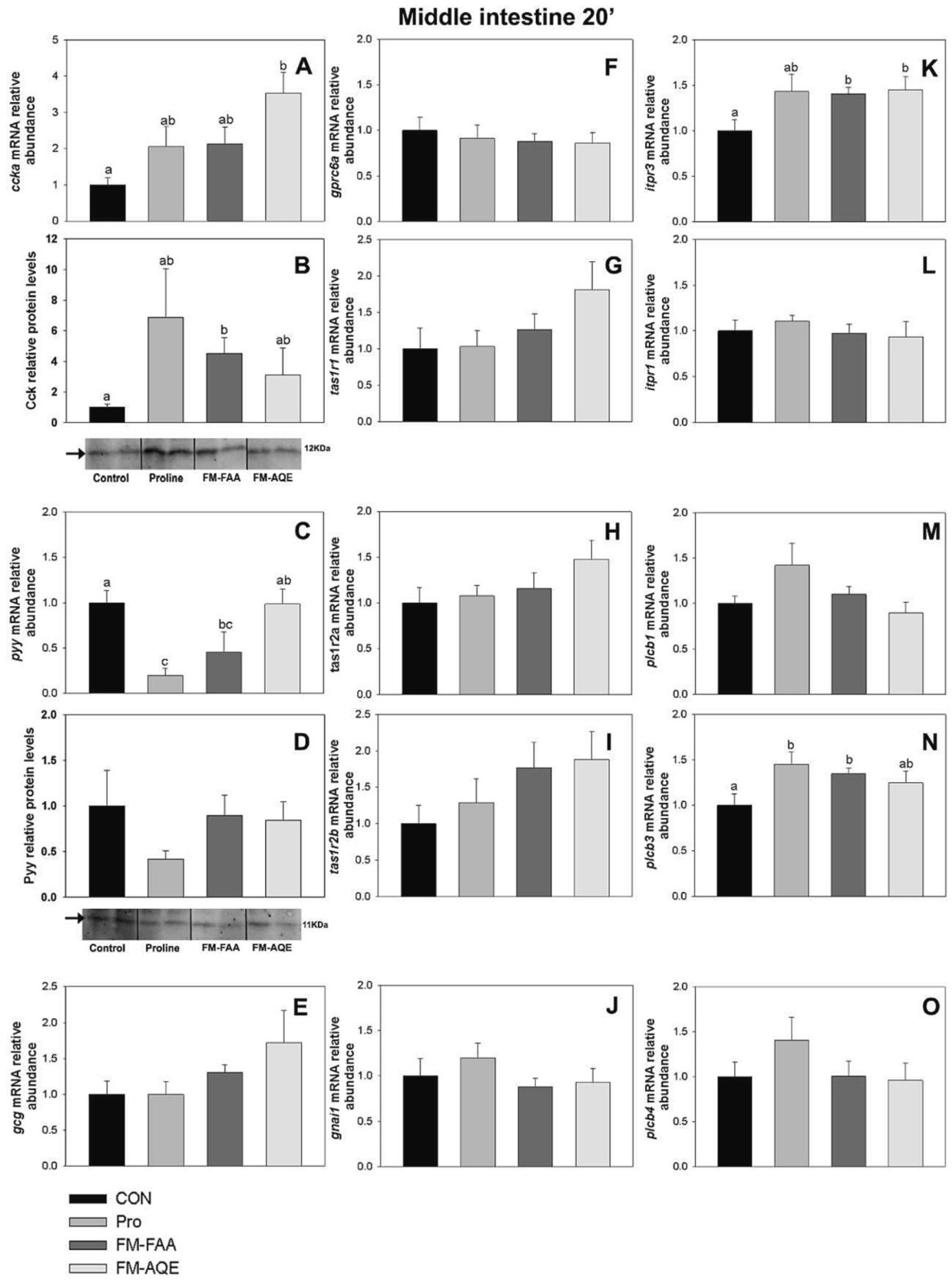

Fig. 4. mRNA abundance of *cck* (A), *pyy* (C), *gcg* (E), *gprc6a* (F), *tas1r1* (G), *tas1r2a* (H), *tas1r2b* (I), *gnai1* (J), *itpr3* (K), *itpr1* (L), *plcb1* (M), *plcb3* (N), and *plcb4* (O), and protein levels of Cck (B) and Pyy (D) in middle intestine of rainbow trout 20 min after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

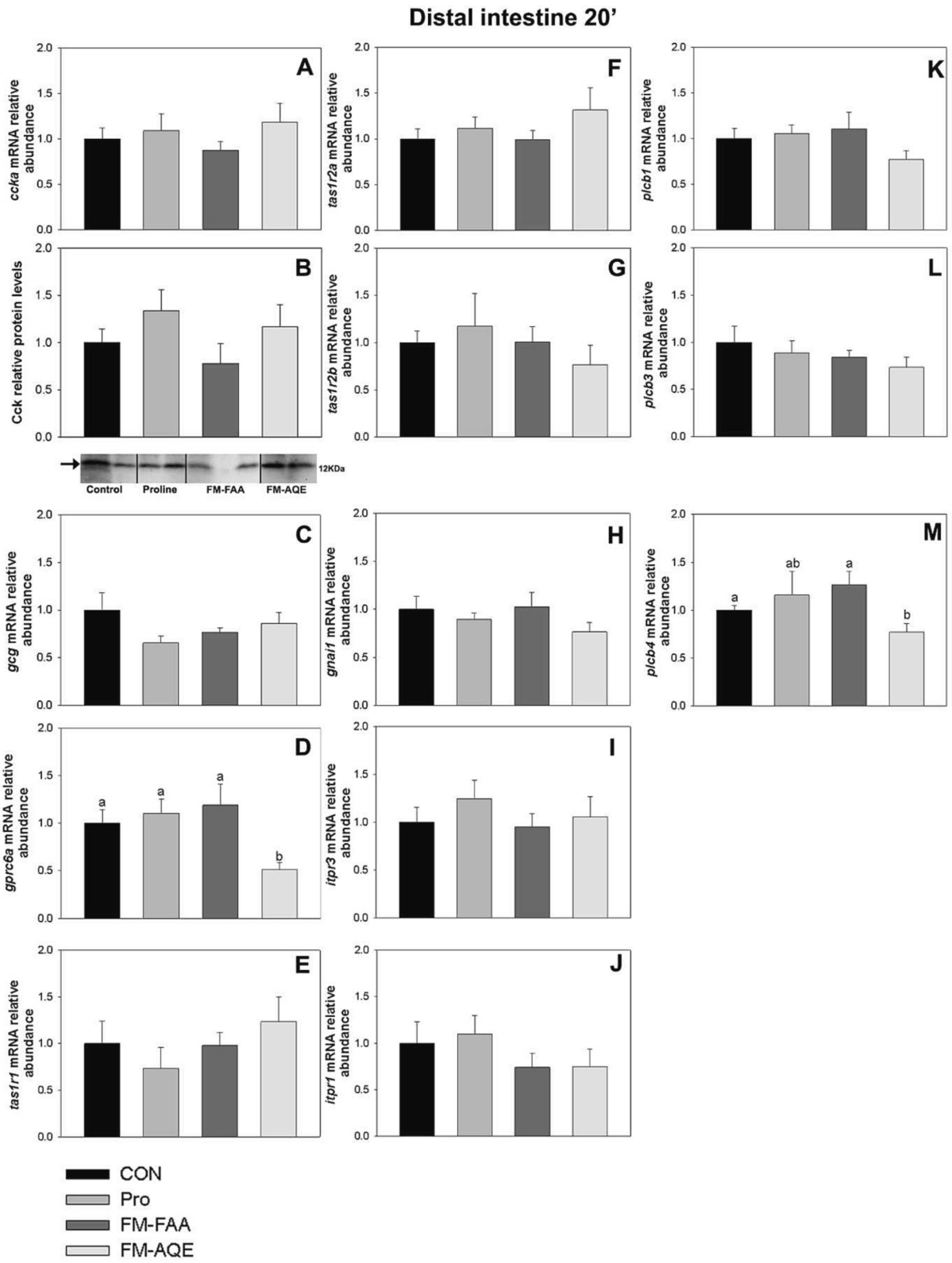

Fig. 5. mRNA abundance of *cck* (A), *gcg* (C), *gprc6a* (D), *tas1r1* (E), *tas1r2a* (F), *tas1r2b* (G), *gnai1* (H), *itpr3* (I), *itpr1* (J), *plcb1* (K), *plcb3* (L), and *plcb4* (M), and protein levels of Cck (B) in distal intestine of rainbow trout 20 min after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

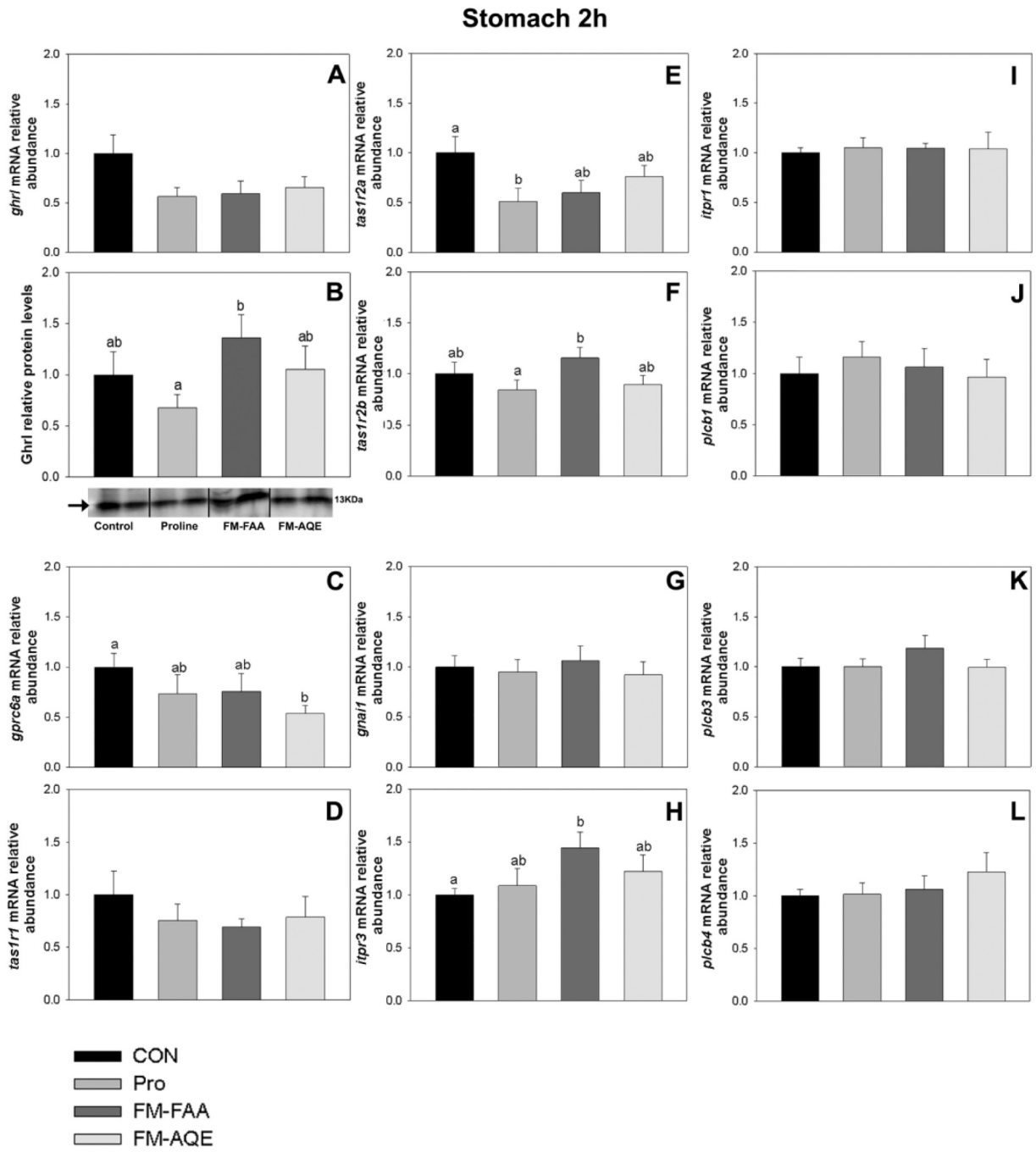

Fig. 6. mRNA abundance of *ghrelin* (A), *gprc6a* (C), *tas1r1* (D), *tas1r2a* (E), *tas1r2b* (F), *gnai1* (G), *itpr3* (H), *itpr1* (I), *plcb1* (J), *plcb3* (K), and *plcb4* (L), and protein levels of Ghrelin (B) in stomach of rainbow trout 2 h after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of β-tubulin. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

administration of Pro resulted in responses in stomach and proximal intestine at 20 min (including decreased levels of Ghrl protein expression in the stomach and increased Cck or *cck* levels in anterior intestine) generally comparable to those previously observed in Calo et al. (2021), thus supporting the reproducibility of the experimental design. In the present study, we also evaluated the impact of Pro in other areas of the GIT not assessed before, and during a longer period of time (also at 2 h). Additionally, we compared the effect of 3 treatments containing the same level of FAA but differing on: 1) the nature of the amino acids (Pro *versus* FM-FAA and FM-AQE; with the two later treatments having a mixture of FAA qualitatively and quantitatively equivalent as that found in the aqueous extract of a fishmeal FT70) or 2) presence of additional soluble components of fishmeal (FM-FAA *versus* FM-AQE). Not surprisingly, both factors had effects on the GIT response, as responses differed

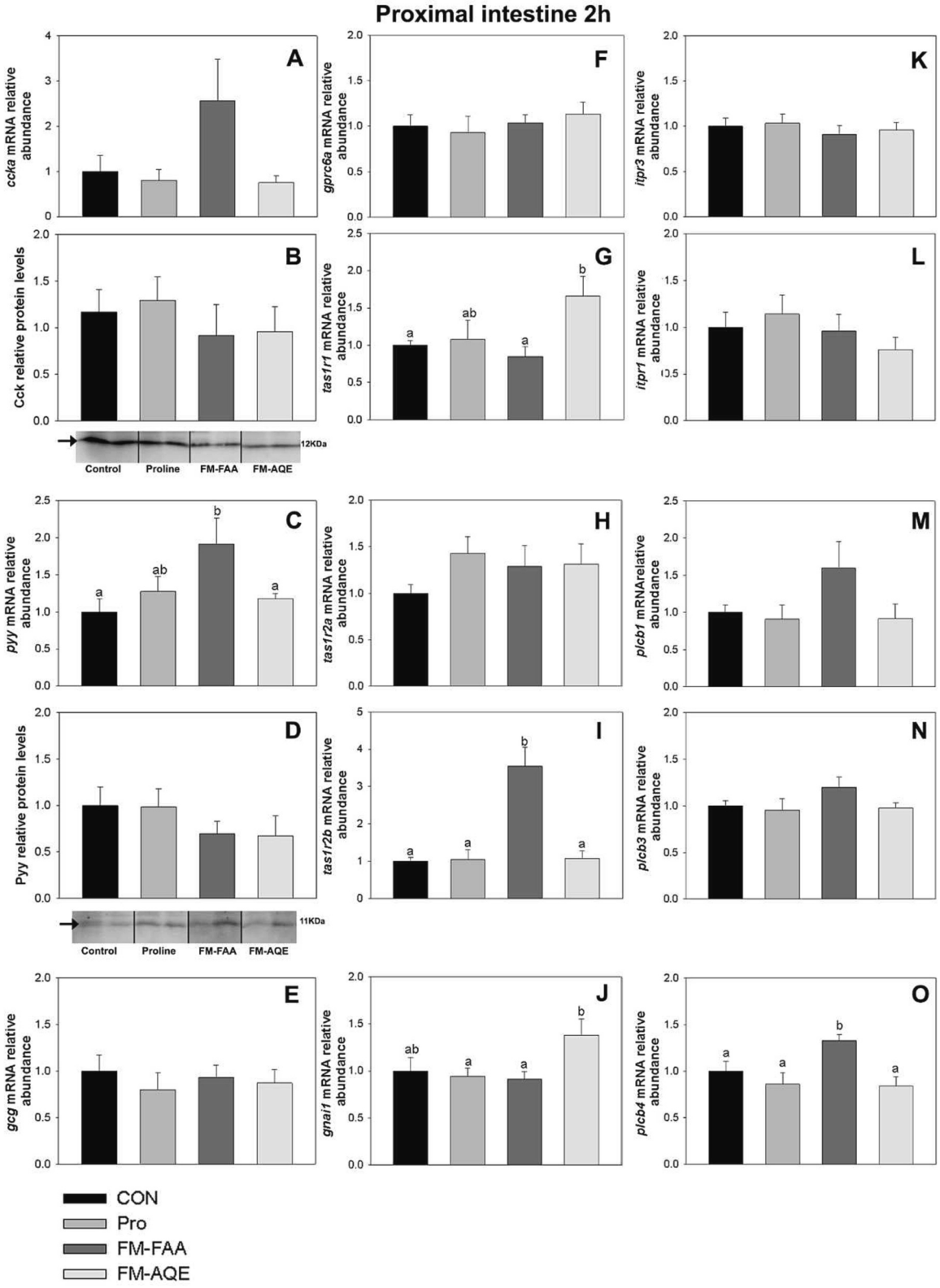

Fig. 7. mRNA abundance of *ccka* (A), *pyy* (C), *gcg* (E), *gprc6a* (F), *tas1r1* (G), *tas1r2a* (H), *tas1r2b* (I), *gnai1* (J), *itpr3* (K), *itpr1* (L), *plcb1* (M), *plcb3* (N), and *plcb4* (O), and protein levels of Cck (B) and Pyy (D) in proximal intestine of rainbow trout 2 h after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

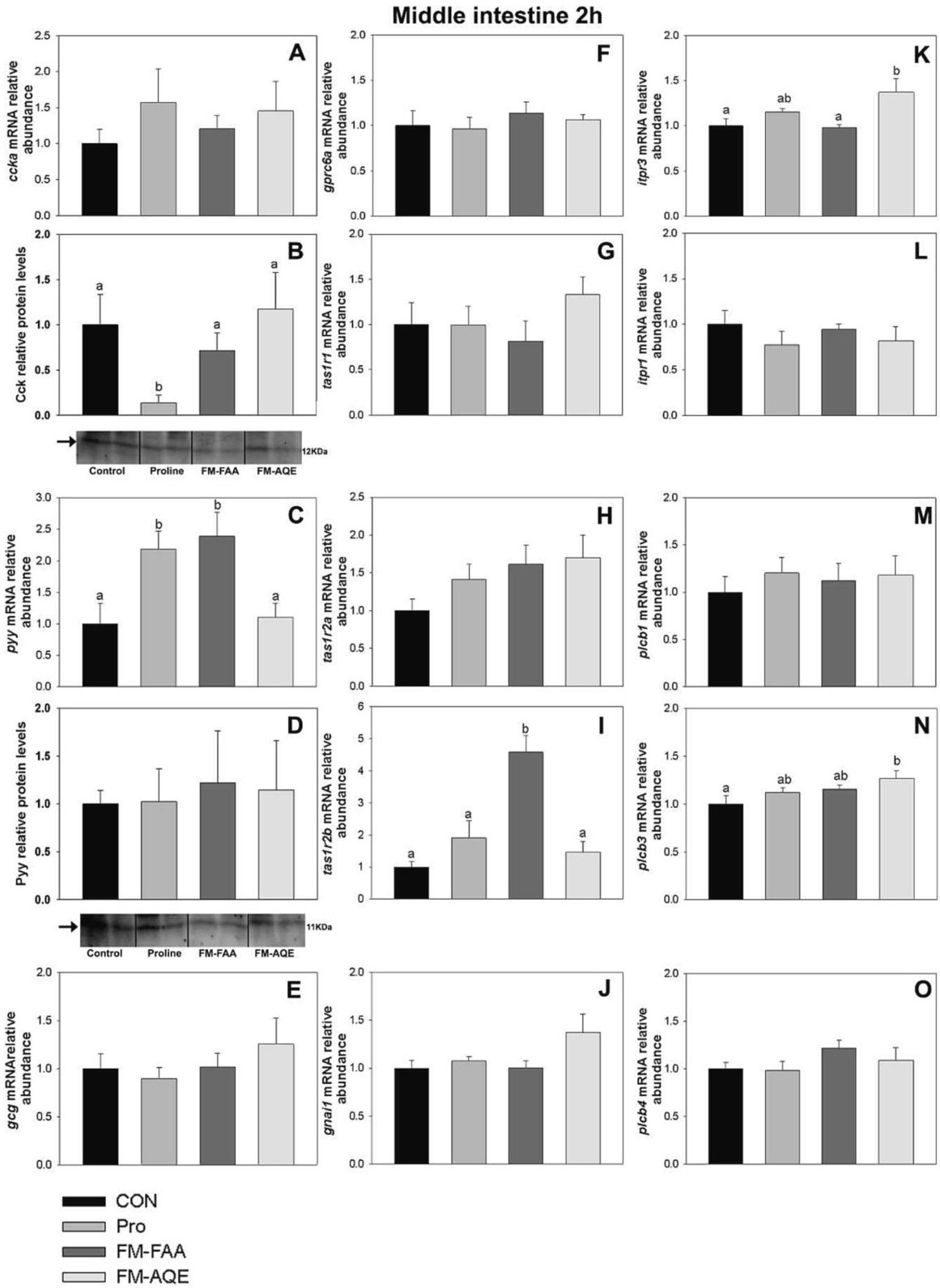

Fig. 8. mRNA abundance of *ccka* (A), *pyy* (C), *gcg* (E), *gprc6a* (F), *tas1r1* (G), *tas1r2a* (H), *tas1r2b* (I), *gnai1* (J), *itpr3* (K), *itpr1* (L), *plcb1* (M), *plcb3* (N), and *plcb4* (O), and protein levels of Cck (B) and Pyy (D) in middle intestine of rainbow trout 2 h after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

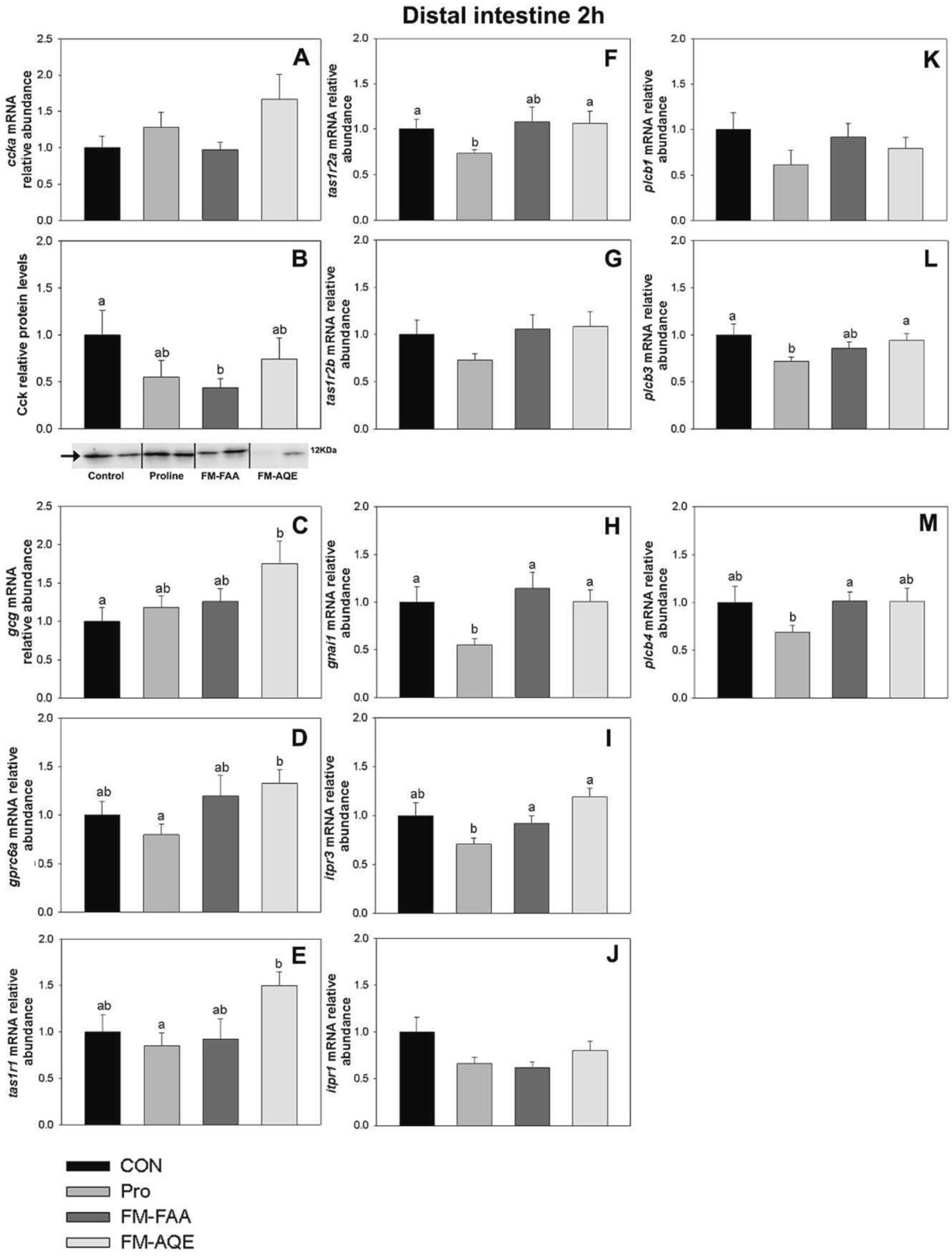

Fig. 9. mRNA abundance of *ccka* (A), *gcg* (C), *gprc6a* (D), *tas1r1* (E), *tas1r2a* (F), *tas1r2b* (G), *gnai1* (H), *itpr3* (I), *itpr1* (J), *plcb1* (K), *plcb3* (L), and *plcb4* (M), and protein levels of Cck (B) in distal intestine of rainbow trout 2 h after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of total protein. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

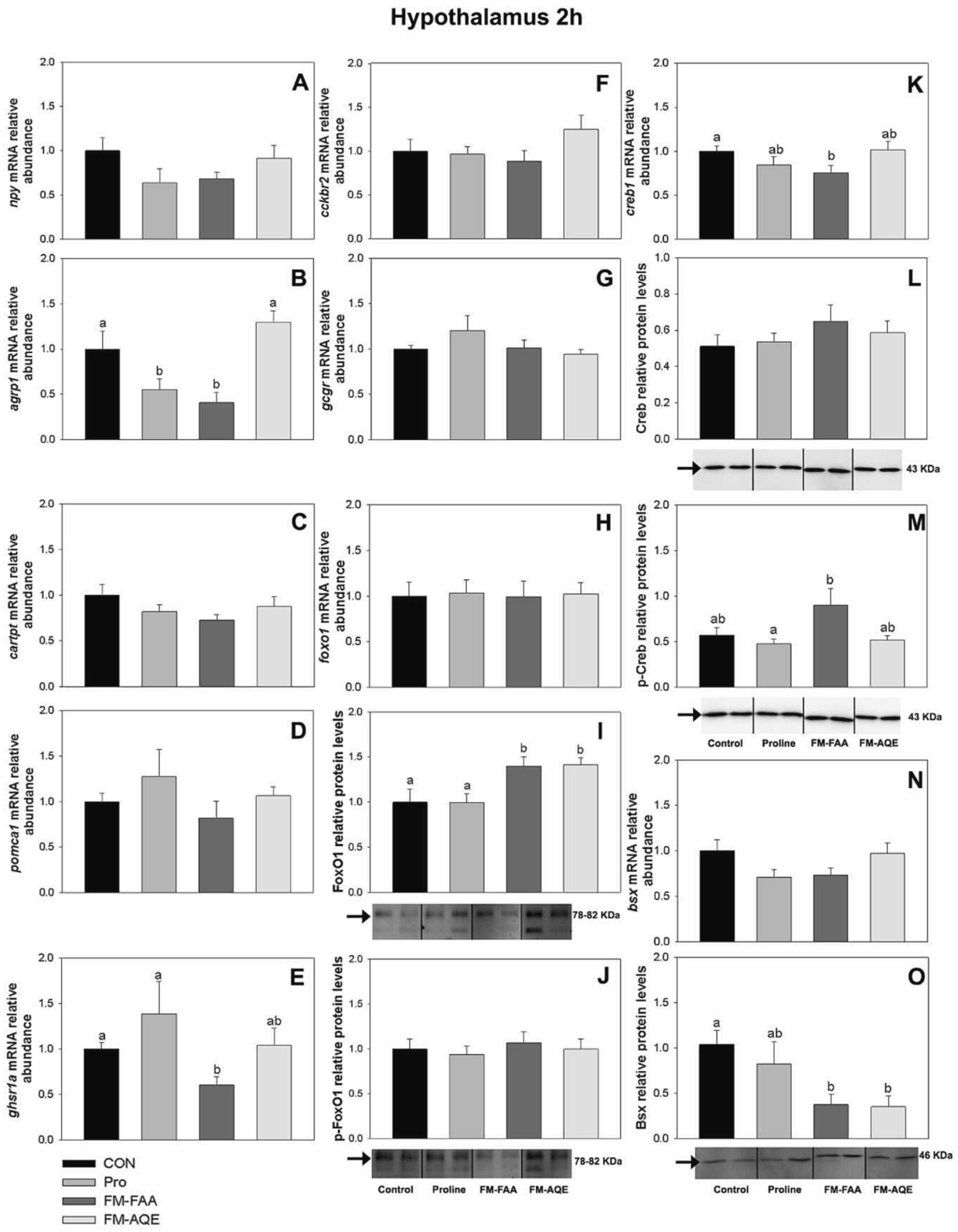

Fig. 10. mRNA abundance of *npy* (A), *agrp1* (B), *cartpt* (C), *pomca1* (D), *ghsr1a* (E), *cckbr2* (F), cggr (G), *foxo01* (H), *creb1* (K), and *bsx* (N), and protein levels of Foxo1 (I), p-Foxo1 (J9, Creb (L), p-Creb (M), and Bsx (O) in hypothalamus of rainbow trout 2 h after intragastric administration of 1 mL per 100 g^{-1} bw water alone (control, Con) or containing 4.6 $mg.mL^{-1}$ of a single amino acid (Pro: Proline, 40 $\mu mol.mL^{-1}$), or a mixture of free amino acids (FAA) reproducing the qualitative and quantitative FAA composition of an aqueous extract of FM (FM-FAA, 51.7 mg mL^{-1}), or of the whole FM aqueous extract (FM-AQE, 51.7 mg mL^{-1}). Data obtained by RT-qPCR were normalized to the expression of *actb and eef1a1* and are represented relative to the control group. From data obtained by Western blot, a representative band of each experimental group is shown for each protein; protein bands were quantified by densitometry using Image Lab software, relative to the amount of β-tubulin. Data are expressed as mean + SEM (n = 6). Statistical differences among groups were assessed by one-way ANOVA and post-hoc Student-Newman-Keuls test using SigmaPlot software version 12.0. Significant differences are denoted by the use of letters: bars sharing the same letter are not statistically significant, while if two bars have different letters they are significantly different ($p < 0.05$).

considerably between treatments. Furthermore, we observed changes in most parameters analysed, including taste and amino acid receptors and their downstream effectors. We also observed changes in gut hormones, which are part of gut sensing mechanisms in vertebrates (apparently conserved also in fish; Angotzi et al., 2022) in the different areas of the GIT, from the stomach to the distal intestine, denoting some spatial-temporal dynamics. A negligible activity was found in the distal intestine at 20 min (except for a down-regulation of *gprc6a* and *plcb4* in the FM-AQE treatment), indicating that this time period was too short for the test solutions to have reached, at least in significant amounts, the most posterior part of the intestine.

4.1. Effects of treatments in taste and amino acid receptors along the gastrointestinal tract

We analysed the expression of members of the T1R gene family, including orthologous trout genes of the mammalian umami-specific receptor subunit (*tas1r1*) and two genes coding for sweet-specific receptor subunits (*tas1r2a* and *tas1r2b*), as the *tas1r2* gene has been found duplicated in most fish species investigated so far (Hashiguchi et al., 2007; Oike et al., 2007; Morais, 2017; Angotzi et al., 2020). The receptor dimers formed by T1R members have been functionally characterized in several teleost species including medaka, zebrafish, grass carp and gilthead seabream (Oike et al., 2007; Cai et al., 2018; Angotzi et al., 2020). Studies so far have found that both the teleost umami and sweet-homologue receptors can be activated with high promiscuity by most FAA, with some species- and subunit- related specificities. They are, therefore, the perfect candidates to examine in this study. On the other hand, we also studied the response of GPRC6A, which, in mammals, has been associated with the sensing of amino acids in the intestine (Oya et al., 2013; Jørgensen and Bräuner-Osborne, 2020). Very few studies have analysed transcriptional responses of these genes to diet or amino acids, but evidence so far suggests that they might respond at a transcriptional level (Yuan et al., 2020).

In the present study we found some effects of treatment in the expression of these genes, but the results seem to indicate a higher response at 2 h than at 20 min. It is plausible that a higher individual variability might occur in early time points, as the diffusion rate across the GIT will likely be quite variable, and 20 min might be a too short period for effects to be established clearly, in each area of the GIT, with enough statistical power. Furthermore, the treatments appeared to affect different receptor genes in most areas of the intestine. An exception was possibly the distal intestine at 2 h, where the administration of Pro and FM-AQE significantly regulated *gprc6a*, *tas1r1* and *tas1r2a* expression, albeit in different directions (genes were down-regulated in Pro and up-regulated in FM-AQE treatment). It is not surprising to find regional and gene-specific differences in the response to different treatments, as fish T1R gene family members are widely and differently expressed in many body tissues, some having a broad tissue profile while others have a much more restricted expression (Yuan et al., 2020; Angotzi et al., 2022). This fact, associated with differences in AA substrate specificity (Oike et al., 2007; Cai et al., 2018; Angotzi et al., 2020), indicate that they likely have tissue-specific roles. It is however puzzling that a more homogeneous response could be observed in the distal part of the intestine at 2 h after treatment administration, and no explanation can be presently offered to explain this. It can be speculated, however, that this area potentially has an important role in the gut-brain communication in response to proteinaceous components of the feed. In the other sections of the GIT, *tas1r2b* generally responded with a different pattern of expression than the other two T1R gene members, showing an increased expression after administration of FM-FAA, in most cases significantly, compared to the other treatments at 20 min in proximal intestine, and at 2 h in stomach, proximal intestine and middle intestine. The effect of Pro administration was much less apparent and significant differences with respect to the control were only observed at 2 h in stomach and distal intestine, in the expression of *tas1r2a* (down-regulated). This could suggest a higher specificity of *tas1r2a* towards Pro than the other T1R gene members. On the other hand, the expression of *gprc6a* responded most strongly to the FM-AQE treatment, showing a down-regulation at 20 min in distal intestine and at 2 h in stomach (only significantly different from the control in the later) and, as mentioned previously, an up-regulation (compared to the Pro treatment) in distal intestine at 2 h. It could be possible that this receptor is responding to the other soluble components of the fishmeal extract, most likely to peptides (with soluble protein components being >62% of peptides, and 55% between 200 and 10,000 Da; Table 3). However, at least in mammals, there is wide consensus that GPRC6A is activated by proteinogenic L-amino acids and modulated/activated by Ca^{2+} and other cations (Oya et al., 2013), while there is conflicting evidence for the other proposed ligands (Jørgensen and Bräuner-Osborne, 2020). Considering the likely presence of minerals (including Ca^{2+}) in FM-AQE but not in FM-FAA, it might be possible that GPRC6A is also activated by Ca^{2+} in trout. Another possibility is that a more gradual and sustained amino acid release (from digestion) of these peptides in the FM-AQE treatment could be influencing the results, as this uncontrolled factor will have induced both qualitative (profile of FAA) and quantitative differences in the FAA pool over time in this treatment, compared to the FM-FAA.

4.2. Effects of treatments in putative taste signalling molecules along the gastrointestinal tract

The pathway of taste signal transduction, following G protein-coupled receptor (GPCR) activation, is generally believed to be conserved in vertebrates, with many common signalling molecules having been reported in fish (Morais, 2017). For instance, although the mammalian gustducin (Gαgust) has not been found in fish genomes so far, other Giα subunits have been proposed to be functionally homologous to it (Oka and Korsching, 2011; Ohmoto et al., 2011). This is the case of seabream genes G(i)α1 and G(i)α2, that have been functionally demonstrated to participate in taste signalling (Angotzi et al., 2020) and to co-localize with T1R3 in EECs (Angotzi et al., 2022). On the other hand, expression of the downstream signalling molecules phospholipase Cβ (PLCβ) and taste selective TRP ion channel (TRPM5) have been described in fish taste buds and gustatory tissues, co-localizing with each other in zebrafish (Yoshida et al., 2007), and PLCβ co-localizing with the T1R1/T1R3 and T1R2s/T1R3 in medaka (Yasuoka et al., 2004; Oike et al., 2007). In this study we analysed the expression of different subtypes of *itpr* and *plcb* putatively involved in taste signal transduction downstream from taste receptors activation that have been identified in rainbow trout transcriptomic databases.

The expression of *itpr3* and/or *plcb3* was significantly affected in most gut segments at both time points, except in distal intestine at 20 min, when most likely the test treatments had still not reached this area, and in proximal intestine at 2 h, when levels of FAAs at this point might also have decreased. It could be observed that although both genes were not always significantly regulated, and not always in the exact same way, the numerical trends were usually fairly well correlated, strongly suggesting that they could be acting in the same signal transduction pathway. The expression of *plcb4* was generally less responsive to the treatments than *plcb3*, and its expression was not always correlated with the other genes, except in distal intestine at 2 h post treatment administration. On the other hand, *gnai1* was generally not responsive to treatments, except in proximal intestine at 2 h (significantly elevated by FM-AQE, when neither *itpr3* nor *plcb3* were affected) and distal intestine at 2 h. Finally, neither *itpr1* or *plcb1* were significantly affected by any treatment in any of the gut segments and time points, suggesting that these might not be involved in signalling of amino acid stimuli in the GIT.

In summary, the most remarkable results were observed in distal intestine at 2 h in response to Pro. In particular, the expression pattern (across treatments) of *gnai1*, *itpr3*, *plcb3* and *plcb4* showed a high resemblance between each other, as well as with the receptors *gprc6a*,

tas1r1 and *tas1r2a*, in which the Pro treatment significantly down-regulated these genes, in comparison to the FM-AQE treatment and also the FM-FAA treatment in the case of *gnai1*, *itpr3* and *plcb4*. Therefore, these results reinforce the hypothesis that these receptors and downstream signalling molecules are involved in gut sensing pathways responding to the presence of FAA in the gut. It is challenging to speculate, however, why only the pure solution of L-Pro evoked a response.

Another general observation was that mRNA abundance of taste signalling molecules, in particular *itpr3* and *plcb3*, appeared to be more sensitive markers of gut sensing mechanisms than the taste and amino acid receptors assessed in this study, given that their transcriptional regulation by treatments was more frequently found in sections of the GIT, especially during the short-term sampling point of 20 min. However, considering the broad implication of these molecules in GPR signalling, this hypothesis must be taken with caution.

4.3. Effects of treatments in endocrine peptides along the gastrointestinal tract

Ghrelin-producing cells are found throughout the GIT but by far the highest abundance and density (correlating with mRNA and peptide levels of *ghr*/Ghr) is found in the stomach in all vertebrates, including rainbow trout (Sakata and Sakai, 2010; Calo et al., 2021). Different roles have been suggested and are still emerging for both molecular forms in which it is found - acyl-ghrelin, binding to growth hormone secretagogue receptor (GHS-R), or non-acylated ghrelin *via* other unknown specific receptor(s) - but the most well established is the stimulation of food intake and body weight gain, and modulation of energy homeostasis. Plasma Ghrl levels and hypothalamic *ghsr* are commonly elevated in a fasting state and quickly return to basal level after refeeding (Kaiya et al., 2013). Conversely, peptide content of ghrelin in the stomach has been shown to decrease after fasting, indicating that cytoplasmatic ghrelin released from gastric ghrelin cells is maintaining the high plasmatic (circulating) levels (Sakata and Sakai, 2010). In the present study, a significant reduction of Ghrl protein levels was observed 20 min after intragastric administration of Pro and FM-FAA, but not in the FM-AQE treatment. On the other hand, the inverse was observed for *ghrl* mRNA abundance, which were significantly elevated in treatments with Pro and FM-FAA, and unaffected in FM-AQE, relative to the control. These differences could indicate that Pro and FM-FAA (containing only FAA) have a quicker satiating effect (meaning that the animals would be ready to feed again earlier), and that by 20 min post-administration of treatments plasma levels of Ghrl might have been higher in these two treatments, in which mRNA synthesis rates were elevated, possibly to replenish the produced and secreted peptides. These time periods for differential responses of Ghrl in plasma are reasonable considering that levels of circulating hormone are maximal 30 min after feeding, as demonstrated in channel catfish (Peterson et al., 2012). At 2 h after treatment administration significant differences were only observed in Ghrl protein level, which was higher in the FM-FAA relative to the Pro treatment, but none of the treatments differed significantly from the control. On the other hand, Ghrl protein levels in stomach were inversely correlated to the mRNA abundance of its receptor (*ghsr1a*) in the hypothalamus at 2 h. This is as expected, considering that gene expression of *ghsr* is stimulated by ghrelin in circulation, as has also been established in non-mammalian vertebrates (Kaiya et al., 2013). Therefore, in combination, these results strongly suggest that at 2 h post treatment administration, plasma levels of Ghrl would likely be higher in the Pro group, reduced in the FM-FAA group and intermediate, and similar, in the control and FM-AQE treatments. Hence, in spite of the quick response of both treatments containing only FAA at 20 min, at 2 h the orexigenic potential related to ghrelin signalling would appear to be in the order Pro > FM-AQE > FM-FAA, suggesting a more long-lasting satiating effect of FM-FAA. However, considering that we have assessed Ghrl levels in stomach but not in plasma, further studies are required to confirm this hypothesis.

Interestingly, relatively strong responses were observed in CCK and PYY gene and protein expression in proximal intestine at 20 min, which were both increased in all treatments relative to the control (at least numerically, and in most cases significantly). However, in middle intestine, although significant responses were also observed, the response of these two peptides differed. While the treatments tended to increase *cck* mRNA (significantly for FM-AQE) and Cck protein (significantly for FM-FAA) levels, a significant reduction in *pyy* gene expression was induced by the Pro and FM-FAA treatments (no changes observed in FM-AQE), relative to the control. Among the different functions that these peptides play in mammals, they have in common a role in supressing hunger and inhibiting gastric emptying. However, CCK additionally has an important role in promoting digestion (by inducing the secretion of pancreatic enzymes and bile from the gallbladder) and promoting midgut motility (regulating peristalsis) (Cawthon and de La Serre, 2021). On the other hand, PYY, which is mostly produced and secreted in the intestine more distally than CCK (although their expression overlaps), has a key role in enhancing water and electrolyte absorption along the intestine, especially in the colon, and is involved in a negative feedback loop counteracting the secretion of CCK, to supress pancreatic secretion (Holzer et al., 2012). Therefore, differences in the results probably relate to the different roles that both peptides play in vertebrates, which, at least some of them, are believed to be conserved in fish, and their participation at different timings during the digestive process (Rønnestad et al., 2017; Assan et al., 2021). It could be speculated that the fast up-regulation of both hormones in the proximal intestine serves to inhibit gastric emptying, allowing more time for processing in the stomach and, in the case of CCK, to stimulate also the secretion of digestive enzymes into the intestine. In this context, the higher Cck protein level in anterior intestine in the FM-AQE group would be consistent with the presence of soluble proteins and peptides in this treatment. An early study performed with herring larvae showed that although both FAA and protein (bovine serum albumin; BSA) elicited a quick Cck secretion, starting at 15 min and increasing up to 120 min (first and last time points measured, respectively) after administration, whereas the treatment with BSA or a combination of BSA + FAA was responsible for a much higher response (Koven et al., 2002). In the middle intestine, on the other hand, it is challenging to speculate why the sensing of substances containing only FAA in the Pro and FM-FAA treatments led to a significant reduction of pyy expression. At 2 h post-administration some regulation of these genes in the proximal and middle intestine was still observed, but in less treatments. In particular, *pyy* mRNA abundance were significantly raised in the FM-FAA treatment, compared to the control and FM-AQE. Conversely, in distal intestine, FM-FAA treatment had a significantly lower Cck protein content than the control. On the other hand, Pro treatment significantly reduced Cck protein levels relative to all treatments, and increased *pyy* mRNA expression compared to the control and FM-AQE in middle intestine. However, no effects of the FM-AQE treatment were observed at this time. This was somewhat surprising since this treatment could have been expected to result in a longer GIT stimulation, due to the sustained release of FAA and small peptides as soluble proteins and large peptides were being digested along the GIT (causing also changes in the FAA profile).

Proglucagon is expressed in the gut, pancreas and brain of both mammals (Drucker, 2006) and fish (Lin et al., 2015). GLP-1 is cleaved from the precursor proglucagon, which also encodes multiple other peptides, including glucagon, GLP-2, glicentin, and oxyntomodulin. GLP-1 exists in two bioactive forms, GLP-$1_{7–36}$ amide and GLP-$1_{7–37}$. Both forms (at least in mammals) are rapidly inactivated in circulation, having a half-life of approximately 2 min (Orskov et al., 1993). In mammals, GPRC6A has been implicated in amino-acid induced GLP-1 secretion from intestinal L cells (Oya et al., 2013) whereas exendin-4 (GLP-1 agonist) inhibits the expression of T1R2/T1R3 in intestine (Merve et al., 2022). These EECs have a typical distal location in the mammalian GIT – found in the distal small intestine and colon (Latorre

et al., 2015) – and mRNA abundance of *gcg* was also found to be highest in distal intestine in trout, when comparing different GIT tissues (Calo et al., 2021). In the present study we observed a significantly higher expression of *gcg* in the FM-AQE treatment, compared to the control, in distal intestine at 2 h. This treatment similarly induced the highest expression (although differences were only significant with respect to Pro) of *gprc6a*, *tas1r1* and *tas1r2a* in the same tissue and time point. This result is consistent with at least one of the established roles of GLP-1 in mammals in inhibition of gastric emptying and GI motility (Holst et al., 2022), as FM-AQE is the only treatment containing molecules other than FAA, that need to undergo digestion. Hence, it makes physiological sense that *gcg* expression is elevated in distal intestine at 2 h, to slow down evacuation from the GIT. The GLP-1 is also a hunger suppression hormone, and its increase also contributes to a later initiation of feeding (or refeeding), again reducing GIT transit rate and leaving more time to process the meal. These changes agree with the anorectic effect also observed in hypothalamus (see below).

4.4. Effects of treatments on feed intake regulatory pathways in hypothalamus

The hypothalamus is the brain area involved in the integration of metabolic and endocrine information leading, through changes in the levels and phosphorylation status of transcription factors (Bsx, Creb, and Foxo1), to the production of key neuropeptides (orexigenic: *npy* and *agrp1*; or anorexigenic: *cartp* and *pomca1*) ultimately regulating feed intake in fish (Soengas, 2021). We expected that after 2 h of treatment the peripheral signals originating from different areas of the GIT should have reached the brain (Calo et al., 2021). The effect of Pro was relatively weak, although a significant down-regulation of *agrp1* was induced by this treatment, relative to the control, which could correlate to the increased expression of *pyy* in middle intestine at 2 h, suggesting an anorectic state at this time. However, as previously discussed, results point towards this treatment having slightly higher plasma Ghrl levels (lower Ghrl in stomach and higher *ghsr1a* in hypothalamus) than the control, but significantly higher than the FM-FAA treatment, and therefore the interpretation of the results is not straightforward. But the highest observed changes were induced by the FM-FAA solution, including a significant decrease in mRNA abundance of *agrp1*, reduced mRNA abundance of *creb1* and increased phosphorylation level of Creb, reduced protein level of Bsx, and increased protein level of Foxo1. Altogether, these changes (except *creb1*) are indicative of an anorectic response (Soengas, 2021), and are also consistent with the significantly reduced *ghsr1a* expression in hypothalamus and significant increase in the expression of *pyy* (hunger supressing hormone) in proximal and middle intestine induced by FM-FAA at 2 h. Similarly, an anorectic response was observed in hypothalamus of the same species after a rise in the levels of circulating nutrients (Conde-Sieira and Soengas, 2017; Soengas, 2021) including amino acids (Comesaña et al., 2018a, 2018b). Finally, treatment with FM-AQE also induced some weak responses potentially indicative of an anorectic status, such as increased protein levels of Foxo1 and decreased protein levels of Bsx, but this was not translated into changes in neuropeptides expression levels. This could also be associated with the significantly increased expression level of *gcg*, the precursor of hunger suppressing peptide GLP-1, in the distal intestine of the FM-AQE treatment at 2 h.

5. Conclusions

Results from the present study indicate that all treatments elicited a response in elements of gut sensing mechanisms along the GIT, despite important differences in the specific genes (likely having different substrate specificities), GIT areas and times in which responses were observed. This could have been expected, given the different chemical nature of the FAA stimuli (pure Pro or mixed FAAs) and the additional presence of peptides and other soluble (mainly proteinaceous) components of fishmeal. Responses were observed more clearly in putative taste or GPCR-coupled signalling molecules than in the taste and amino acid receptors assessed, especially at short time (20 min), and was also clearly seen in hormonal pathways regulating feed intake and food processing mechanisms. Changes in hormone gene and protein levels are not easy to interpret as these can have multiple physiological roles, in some cases not completely established or confirmed in fish. However, as a general observation, we could establish that administration of FAA (Pro or FM-FAA) appeared to have a stronger and/or quicker effect in ghrelin synthesis and secretion, while the presence of other molecules (mainly peptides) in the FM-AQE treatment either reduced, or substantially modified the timing of the response (not significantly different from the control at 20 min). This treatment was, however, being clearly sensed by the intestine at 20 min, where a response at least similar (possibly higher) was generated by this treatment in terms of *cck* and Cck mRNA and protein synthesis in proximal and middle intestine, and a similar regulation was observed in proximal intestine for Pyy protein levels. Other distinctive differences of the FM-AQE treatment were the up-regulation of *gcg* in the distal intestine at 2 h, and increased expression of *tas1r1* in proximal and distal intestine at 2 h. Moreover, of the three tested treatments, FM-AQE showed overall less effects, compared to the control, in hypothalamic pathways regulating feed intake. This is in contrast with the FM-FAA treatment, which had the strongest effects. It seems clear, therefore, that the additional soluble components of the fishmeal strongly modified the response of key gut-brain communication pathways, or at least the spatial-temporal dynamics of these mechanisms. However, this cannot be ascertained with certainty since only two time points were examined in this study.

On the other hand, clear differences also resulted from administrating a single FAA (Pro) or a mixture of FAA mimicking the FAA profile of a FM aqueous extract (containing 19 amino acids, including tau, Ala, Leu, Lys, Phe, Arg, Glu, Val, His, Iso, Thr, Tyr, Gly, Met, Asp, Ser, Trp, Pro, Asn, decreasing order), of which Pro represented $<0.4\%$. It is reasonable to assume that the FM-FAA treatment would be expected to result in a more potent response, as it has the potential to activate different receptors having different substrate specificity.

The current state of knowledge regarding these complex processes in fish is still at a very early stage and, therefore, does not enable predicting the final physiological and productive (*e.g.*, feed intake) outcomes of the observed changes. However, they clearly demonstrate the capacity of different systems along the rainbow trout GIT to sense amino acid levels alone or in complex mixtures simulating the FAA composition of fishmeal. The sensing of these amino acids elicits not only *in situ* and peripheral responses through changes in the secretion of GI hormones but also, through gut-brain axis, responses at central levels in hypothalamic mechanisms governing feed intake. The characterization of these mechanisms will be key to understand the importance of fishmeal components in the regulation of feed intake in aquaculture fish species.

Author statement

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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