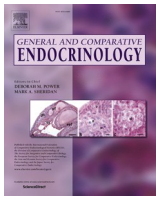
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Regulation of growth hormone and insulin-like growth factor-1 secretion in response to key amino acids in gilthead sea bream☆

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ABSTRACT

In vertebrates, growth hormone (GH) and insulin-like growth factors (IGFs) are key regulators of somatic growth. In fish, Gh and Igf1 secretion is strongly influenced by nutritional status, with dietary protein levels and, particularly, amino acids (AA) composition, being the major determinants of hormonal modulation. This study investigated the regulation of the Gh/Igf axis by selected AA in gilthead sea bream (*Sparus aurata*) using complementary experimental approaches. Intraperitoneal injection of individual AA elicited distinct endocrine responses, with lysine (Lys) being the most potent stimulator of Gh secretion, followed by arginine (Arg), whereas methionine (Met) had no significant effect. In contrast, administration of all tested AA, including Met, as well as human GH, significantly increased plasma Igf1 levels. *Ex vivo*, rainbow trout Ghrelin stimulated and human IGF1 inhibited Gh release from hemi pituitaries, validating the experimental system. Among the AA tested, Arg and leucine (Leu) did not affect hemi pituitaries Gh secretion, whereas Lys strongly stimulated it. Isolated hepatocytes gene expression analyses revealed that Arg and Met significantly increased total *igf1* mRNA levels after 24 h of incubation, while Leu and Lys had no effect. Conversely, Leu markedly upregulated *igf2* expression at 6 h. Stimulatory effects on *igf1* splice variants and related genes were mainly observed after 6 h. Overall, these results provide an integrative view of AA-dependent regulation of the Gh/Igf axis in gilthead sea bream with tissue-specific responses, highlighting the differential stimulatory effects of Lys, Arg and Met and supporting targeted AA supplementation strategies for improving aquaculture production.

1. Introduction

In fish as in all vertebrates, growth hormone (Gh) and insulin-like growth factors (Igfs) are the main regulators of somatic growth, a concept supported by comprehensive reviews addressing physiological roles and associated growth disorders (Pérez-Sánchez et al., 2018; Ranke and Wit, 2018; Vélez and Unniappan, 2021). Gh and Igf1 are pleiotropic hormones with important functions along lifespan that promote growth, and exert direct and indirect anabolic actions, including amino acids (AA) uptake, as well as RNA and protein synthesis (Fuentes et al., 2013; Vélez et al., 2017a).

The generation of the first Gh-transgenic coho salmon (*Oncorhynchus kisutch*) marked a milestone in aquaculture research (Devlin et al., 1994), and further studies using that model (Johnston et al., 2014; Panserat et al., 2014; (Raven et al., 2012)) contributed significantly to understanding the role of the Gh/Igf axis in growth regulation and metabolic adaptations in fish (Canosa and Bertucci, 2023; Pérez-Sánchez et al., 2018; Sheridan, 2021; Vélez and Unniappan, 2021).

The Gh/Igf axis regulates body growth integrating many inputs like feeding, nutrition, energy balance, reproduction, as wells as abiotic factors. Specifically, in gilthead sea bream (*Sparus aurata*), many authors have previously investigated the Gh/Igf system under different natural and experimental conditions *in vivo*, and using *in vitro* models including myocytes, adipocytes, or hepatocytes (Ballester-Lozano et al., 2015; Benedito-Palos et al., 2007; Montserrat et al., 2007; Naya-Català et al., 2021; ; Perelló-Amorós et al., 2021; Perera et al., 2021; Rius-Francino

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et al., 2011; Saera-Vila et al., 2009, (Saera-Vila et al., 2007); Vélez et al., 2018, (Vélez et al., 2016a)). In summary, the Gh/Igf system exerts in this species clear effects on growth, cell proliferation, and differentiation of different tissues in coordination with nutritional, seasonal, reproductive or health status.

Both Gh and Igf1 secretion is strongly influenced by the nutritional state in any phase of life (Ballester-Lozano et al., 2015; Benedito-Palos et al., 2007) and AA and proteins play a substantial role (Caputo et al., 2021). Thus, in mammals including humans, intravenous administration of various AA can provoke a clear rise in GH levels, with basic AA, such as arginine (Arg), histidine, and lysine (Lys), being the most potent, while the non-polar AA leucine (Leu) and valine appearing less effective (Caputo et al., 2021; Chromiak and Antonio, 2002; Knopf et al., 1965). In contrast, the information in fish about the Gh and Igf1 response to AA remains restricted. In this sense, Yang et al. (2020) and Liaqat et al. (2025) demonstrated the role of Lys regulating Gh/Igf system in largemouth bass (*Micropterus salmoides*) and basa catfish (*Pangasius bocourti*), respectively; Wang et al., 2021b investigated the effect of methionine (Met) in largemouth bass growth performance and metabolism, while Rolland et al. (2015) explored Met effects on hepatic gene expression of the Gh/Igf system and protein turnover in rainbow trout (*O. mykiss*), being the effects of Met on fish health and nutrition recently reviewed (Wang et al., 2023). Thus, a variety of physiological effects exerted by AA has been clearly demonstrated in all the fish species investigated.

Furthermore, fish are especially interesting among vertebrates due to the continuous growth that many species show, including those of commercial interest like the gilthead sea bream. This undetermined type of growth allows optimization of finfish aquaculture production by extending the hyperplasic muscle growth phase while improving flesh quality, especially muscle texture (Johnston et al., 2000). However, hyperplasic growth requires an equilibrated diet covering the adequate quantity and variety of essential AA in a species-specific manner. Hence, the role of AA in fish becomes especially relevant in many of the cultivated species due to its carnivorous nature, being significant components of the diet. Therefore, in the current context of global fisheries decline and the necessity to minimize fish meal consumption (Fernandes et al., 2024; Naylor et al., 2021; Reis et al., 2021), the research of the indispensable AA for fish feed formulation can be a useful tool for advancing aquaculture sustainability (Morin et al., 2024). Specifically, it is crucial to identify those AA that best stimulate the Gh/Igf system, guaranteeing optimal body growth and quality of the flesh, while simultaneously reducing the use of wild fish for feed production, thus preserving natural populations (Gómez-Requeni et al., 2005; Li et al., 2021).

The objective of this work was to study the regulation of Gh and Igf secretion and gene expression through different experimental approaches *in vivo*, *ex vivo* and *in vitro*, contributing to understanding which AA can be the most interesting secretagogues of these hormones in gilthead sea bream.

2. Material and methods

2.1. Animals

Gilthead sea bream (*Sparus aurata*) juveniles and adult fish were procured from the farm Piscimar (Burriana, Spain) or the Institute of Agrifood Research and Technology (IRTA, La Ràpita, Spain), respectively and transported to the animal facilities at the Faculty of Biology belonging to the Scientific and Technological Centers of the University of Barcelona (CCiTUB). Fish were acclimatized and maintained in a recirculating set-up, fed *ad libitum* twice daily with a commercial diet (Optibream, Skretting, Burgos, Spain) and fasted for 24 h before any experimental procedure. The physicochemical parameters of the water were maintained at the optimum levels (salinity: 36–38 ppt, dissolved oxygen: 7–8 ppm, temperature: 23 ± 1 °C, pH: 8.1–8.4, ammonia: <0.1 ppm, photoperiod: 12 h light: 12 h dark).

All animal handling procedures were conducted following the norms and procedures established by the European Union Council (86/609/EU) and the Spanish and Catalan Governments, with approval obtained from the Ethics and Animal Care Committee of the University of Barcelona (protocol code CEEA OB100/17).

2.2. In vivo intraperitoneal injections

Fish (60–80 g body weight; BW) were anaesthetized with a low dose (0.2 g/L) of the anesthetic (MS-222, E10521, Sigma-Aldrich, Tres-Cantos, Spain), weighted and immediately transferred to the lab bench. Fish were handled and gently immobilized using chamois cloths moistened with seawater during the injection. Sterile syringes (1 mL) and needles (23G) were used for the injection of saline buffer (NaCl solution adjusted to 380 mOsm) as a control, the different AA, either Arg, Lys, or Met (A5006, L5501, and M9500, respectively, all purchased from Sigma-Aldrich), at a dose of 6.6 μmol/g BW as previously performed in brown trout, *Salmo trutta* (Baños et al., 1999), or recombinant human GH (hGH, GenWay Biotech, Inc., CA) at 0.3 μg/g BW ($n = 8$ fish per treatment). Injections were performed in the abdominal cavity, taking care not to inject into any organs or blood vessels as performed before (Baños et al., 1999), and the entire process took no more than two min. Once the injection was completed and the area disinfected, the fish were placed in a recovery tank with adequate aeration, closely monitored during the first hour post injection, and no casualties were reported. After 6 h of the injection, fish were deeply anaesthetized with an overdose (1 g/L) of MS-222 and blood samples were taken, the plasma was separated and stored at −80 °C for posterior measurement of endogenous Gh and Igf1 levels.

2.3. Pituitaries isolation and ex vivo treatments

The current hemi pituitary isolation protocol was adapted from Cameron et al. (2005). Fish (600 to 1000 g BW) were sacrificed with an overdose of the anesthetic (1 g/L of MS-222), and immediately transferred to the dissection bench. Fish were decapitated by severing the spinal cord, and the skull was opened to expose the brain as illustrated in **Supplementary Fig. 1**. Using appropriate forceps, the brain tissue was separated, revealing the *sella turcica*, where the pituitary gland is located. The pituitary gland was extracted and correctly orientated using forceps for subsequent symmetrical fractioning following the sagittal plane with a sharp blade under a stereo microscope (Stemi SV6, Zeiss, Tres-Cantos, Spain). Each hemi fraction was collected in a microtube containing 1× ice-cold Hank's buffered salt solution (HBSS, H1641 Sigma-Aldrich), supplemented with 4.17 mM $NaHCO_3$ and 0.1% bovine serum albumin (BSA), at pH 7.5, diluted to 80%, and kept on ice until all dissections were finished. For washing, each hemi pituitary fraction was carefully transferred to 24-well plates, each well containing 1 mL of HBSS and kept on an orbital shaker placed in an incubator at 21 °C for 1 h. After washing, the HBSS was replaced by 1 mL of medium M199 (M0393, Sigma-Aldrich), supplemented with 5 mM $NaHCO_3$, 25 mM Hepes, 0.1% BSA, 0.7 mM L-glutamine, 1% of antibiotic–antimycotic solution (A5955, Sigma-Aldrich), at pH 7.5, and osmolarity adjusted to 380 mOsm with NaCl. The hemi pituitaries were then incubated in the same conditions for 4 h to establish a basal release and after that, the medium was changed to each hemi pituitary (*i.e.*, symmetric pair) to either fresh M199 for the control, or M199 supplemented with the different experimental treatments ($n = 4$ per condition). The experimental conditions were 10 nM synthetic rainbow trout acylated 24-amino-acid form ghrelin (rtghrelin, Peptide institute, Japan) as a Gh induction control, 10 nM recombinant human IGF1 (hIGF1, CU100, GroPep, Australia) as a Gh suppression control, or 2, 4, 12 or 20 mM Arg, 4 mM Leu (L8000, Sigma-Aldrich), or 4 mM Lys. After the 18 h incubations, the culture medium of each hemi pituitary was collected in a microtube and stored at −80 °C for posterior analysis of

the endogenous Gh secretion levels.

2.4. Gh and Igf1 radioimmunoassays

Gh concentration in both the plasma and hemi pituitaries incubation media were assayed by a homologous gilthead sea bream radioimmunoassay (RIA) in accordance with Martínez-Barberá et al. (1995). The sensitivity and mid-range (ED50) of the assay were 0.15 and 1.8 ng/mL, respectively. Plasma Igfs were extracted by the acid–ethanol cryoprecipitation (Shimizu et al., 2000), and Igf1 concentration measured by means of a generic fish Igf1 RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The sensitivity and mid-range of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

2.5. Hepatocytes isolation and in vitro treatments

The protocol for primary hepatocytes isolation from gilthead sea bream was adapted from Mommsen and Moon (1994) with some modifications. In brief, fish (100 to 250 g BW) were sacrificed with an overdose of MS222, bleeded, and immersed in 70% ethanol for superficial sterilization. Then, under a laminar flow cabinet the liver tissue was excised, placed in a sterile Petri dish and perfused with medium A (1× HBSS, 1 mM $MgSO_4$, 5 mM $NaHC0_3$, 10 mM Hepes, 2 mM pyruvic acid, 1% antibiotic/antimycotic solution and 0.1% gentamicin, pH 7.4, and osmolarity adjusted to 380 mOsm with NaCl) until the liver was clean of internal blood (approximately 15–20 min); followed by mechanical digestion (mincing with surgical blades) and washed twice with medium A and centrifuged at 300*g*, 21 °C for 5 min. The tissue pellet was resuspended and enzymatically digested for 30 min at room temperature and in gentle agitation with 50 mL of medium B (medium A supplemented with 0.08 mg/mL of Type II collagenase, C6885, Sigma-Aldrich). This digestion was stopped by the addition of medium C (HBSS, supplemented with 5 mM $NaHCO_3$, 10 mM Hepes, 2 mM Pyruvic acid, 2% BSA, 1% antibiotic/antimycotic solution, pH 7.4, and osmolarity adjusted to 380 mOsm with NaCl). In order to disaggregate the cells, the digested liver was filtered using 100 and 40 μm nylon cell strainers. Cells were then centrifuged (50 *g*, 21 °C for 5 min) and washed twice with medium C. Finally, cells were counted and resuspended in M199 medium (M0393, Sigma-Aldrich), supplemented with 25 mM Hepes, 5 mM $NaHCO_3$, 15% FBS, 1% antibiotic/antimycotic solution, pH 7.4, and osmolarity adjusted to 380 mOsm with NaCl and seeded in 6-well tissue culture plates (Corning® Primaria™) at a density of 1×10^6 cells/mL. The hepatocytes were let to attach for 48 h in an incubator at 23 °C and 2.5% CO_2. After this 48-h period, the hepatocyte quality was determined and confirmed by evaluating survival, attachment and morphological development based on the report of Santacroce et al. (2011). Then, 2-day hepatocytes were incubated with three concentrations (x2, x10, x20) of four AA (Arg, Leu, Lys, and Met) with respect to their corresponding content within the M199 culture medium [x1: Arg 0.33 mM, Leu 0.457 mM, Lys 0.38 mM and Met 0.1 mM]. These tested concentrations are presumed to represent low, average and high supplementation levels for cultured hepatocytes (Oh et al., 2017). The AA were dissolved in M199 culture medium supplemented with 15% FBS and 1% antibiotic/antimycotic solution, except for Leu that was first dissolved in 1 M HCl and then diluted in the M199 medium. Control conditions comprised the M199 culture medium alone or supplemented with the corresponding volume of 1 M HCl. After 6 h and 24 h of incubation, hepatocytes cell viability was analyzed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay as previously reported (Figueiredo et al., 2021). Moreover, at those same incubation times samples were also taken for RNA extraction and subsequent gene expression analysis. Four independent hepatocytes' isolations and subsequent *in vitro* experiments were performed.

2.6. RNA extraction and gene expression analysis

Total RNA was extracted from hepatocytes using Trizol as reported before (Vélez et al., 2016a), and the concentration and purity of the RNA were examined using a NanoDrop ND2000 (Thermo Scientific, Alcobendas, Spain). After genomic DNA removal by using DNAse 1 (Invitrogen, Alcobendas, Spain), RNA was reverse transcribed to cDNA using Transcriptor First Strand cDNA synthesis kit (04896866001, Roche, Sant Cugat del Vallès, Spain). Then, using Q™ SYBR® Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), reference and target genes cDNAs were evaluated using forward and reverse primers (Table 1). Elongation factor 1 alpha (*ef1α*) and ribosomal protein S18 (*rps18*) were selected as the best reference genes among the different candidates tested, and the target genes expression level was calculated relative to them as explained before (Balbuena-Pecino et al., 2022; Sánchez-Moya et al., 2023).

2.7. Statistical analysis and data availability

All data are presented as mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism version 10.4 software unless otherwise specified. For all the data, normal distribution and homoscedasticity were examined by Shapiro-Wilk and Levene's tests, respectively and normalized when necessary. For the intraperitoneal injection trial, the endogenous Gh and Igf1 levels of the treated fish were compared to those of control fish by one-way Analysis of Variance followed by Dunnett's *post-hoc* test ($p < 0.05$). In case of lack of normality or homoscedasticity, non-parametric Kruskal-Wallis followed by Dunn's *post-hoc* test was used ($p < 0.05$). For the incubated hemi pituitaries, a Gh ratio was calculated by dividing the Gh levels of the treated hemi pituitary by those of the corresponding untreated control one. To determine whether the Gh ratios of each experimental group differed from 1, inference was based on percentile bootstrap confidence intervals. In addition, a smoothed bootstrap incorporating Gaussian noise of magnitude $\sigma/\sqrt{n}$ was applied to assess the stability of the confidence intervals under additional sample variability. Ninety-five percent bootstrap confidence intervals were used to evaluate whether the mean of each group differed from the reference value. This inference based on confidence intervals was performed with R version 4.5.2 in RStudio version 1.3.1056 using the package "boot". For the primary hepatocytes, the experiments followed a Complete Randomized Design (CRD) (Casella, 2008). The significant differences in the relative expression levels between AA treatments and the corresponding control were assessed by one-way Analysis of Variance followed by Dunnett's *post-hoc* test ($p < 0.05$). In case of lack of normality or homoscedasticity, non-parametric Kruskal-Wallis followed by Dunn's *post-hoc* was used ($p < 0.05$).

The datasets generated and/or analyzed during the current study are openly available in the CORA repository (Vélez et al., 2026), at doi:10.34810/data2940.

3. Results

3.1. Intraperitoneal injection experiment

The levels of endogenous Gh and Igf1 measured by RIA in plasma samples from gilthead sea bream juveniles after the intraperitoneal injection with different AA or hGH are shown in Fig. 1. The injection with hGH did not significantly alter the endogenous plasma levels of Gh compared to the control (Fig. 1A), indicating reduced or no cross-reactivity of hGH with the primary antibody used in the RIA. In contrast, hGH was recognized by endogenous Gh receptors, as it was able to induce a significant rise of endogenous Igf1 levels (Fig. 1B), thus validating the current analysis. The tested AA had differential effects on the circulating levels of these hormones. On one hand, Arg and Lys induced approximately a 4- and 7-fold increase in Gh levels, respectively

Table 1
Primers used in the qPCR analyses.

Type	Gene	Primer sequences (5′–3′)	Ta (°C)	Accession number
Reference	*rps18*	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	60	AM490061.1
	ef1α	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	60	AF184170
	β-actin	F: TCCTGCGGAATCCATGAGA R: GACGTCGCACTTCATGATGCT	60	X89920
	rpl27a	F: AAGAGGAACACAACTCACTGCCCCAC R: GCTTGCCTTTGCCCAGAACTTTGTAG	68	AY188520
Gh-Igf axis/growth-related and associated molecules	*igf1a*	F: AGGACAGCACAGCAGCCAGACAAGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	AY996779
	igf1ab	F: AGTCATTCATCCTTCAAGGAAGTGCATCC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	EF688015
	igf1abc	F: ACAGAATGTAGGGACGGAGCGAATGGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	EF688016
	igf2	F: TGGGATCGTAGAGGAGTGTTGT R: CTGTAGAGAGGTGGCCGACA	60	AY996778
	igf1ra	F: AGCATCAAAGACGAACTGG R: CTCCTCGCTGTAGAAGAAGC	55	KT156846
	igf1rb	F: GCTAATGCGAATGTGTTGG R: CGTCCTTTATGCTGCTGATG	55	KT156847
	igfbp1a	F: AGTGCGAGTCCTCTCTGGAT R: TCTCTTTAAGGGCACTCGGC	60	KM522771
	igfbp2a	F: CGGGCTGCTGCTGACATACG R: GTCCCGTCGCACCTCATTTG	60	AF377998
	igfbp5b	F: TTTCTCTCTCGGTGTGC R: TCAAGTATCGGCTCCAG	60	AM963285
	ghr1	F: ACCTGTCAGCCACCACATGA R: TCGTGCAGATCTGGGTCGTA	60	AF438176
	ghr2	F: GAGTGAACCCGGCCTGACAG R: GCGGTGGTATCTGATTCATGGT	60	AY573601
Transduction pathways and signaling molecules	*tor*	F: CAGACTGACGAGGATGCTGA R: AGTTGAGCAGCGGGTCATAG	60	EF688015
	4ebp1	F: CCAACCTGCGACTCATCTCT R: GTTCCTCTCATCCTCCCACA	60	XM_030392259
	70s6k	F: GCACCAGAAAGGCATCATCT R: AAGGTGTGGGTCACTGTTCC	60	XM_030438737
	akt2	F: GCTCACCCCACTCTTCAGAC R: AAATTGGGAAATGTGCTTGC	60	ERA047531
	foxo3	F: CAGCAGCCTGGAGTGTGATA R: CCAGCTCTGAGAGGTCTGCT	60	XM_030405224
	erk2	F: AAAGCTCTGGACCTGTTGGA R: TCATCCAGCTCCATGTCAAA	60	ERA047531

F: forward; R: reverse; Ta: annealing temperature; *rps18*: ribosomal protein S18; *ef1α*: elongation factor 1 alpha; *β-actin*: beta-actin; *rpl27a*: large ribosomal subunit protein 27a; *igf1 1a/1ab/1abc*: insulin-like growth factor 1 splice variants; *igf2*: insulin-like growth factor 2; *igf1ra/rb*: insulin-like growth factor 1 receptor a/b; *igfbp1a/2a/5b*: insulin-like growth factor binding protein 1a/2a/5b; *ghr1/2*: growth hormone receptor 1/2; *tor:* target of rapamycin; *4ebp1*: translation initiation factor 4E-binding protein 1; *70s6k*: p70S6 kinase; *akt2:* protein kinase B; *foxo3*: forkhead box O3; *erk2*: mitogen-activated protein kinase 2.

(Fig. 1A), while Lys also induced a significant increase in Igf1 levels, and Arg tended to increase them although not significantly (Fig. 1B). On the other hand, whereas Met did not alter the levels of circulating Gh, it provoked a powerful induction of Igf1 release like the one induced by Gh, its classical stimulator.

3.2. Hemi pituitaries incubation experiment

The results of the *ex vivo* Gh secretagogue trial based on hemi pituitary incubations are displayed in Fig. 2. On the one hand, tissue incubations with the classical Gh secretagogue Ghrelin (in this case from rainbow trout, rtGhrelin), induced a significant increase in the Gh released into the culture medium compared with the secretion by the corresponding symmetric tissue portion treated with a control medium. Instead, tissue exposure to hIGF1, a well-known inhibitor of GH secretion by somatotrophs, significantly decreased the Gh secretion ratio. Together, these results validated the experimental model for the study of secretagogues in gilthead sea bream hemi pituitaries *ex vivo*. On the other hand, among the AA and concentrations tested, only Lys at 4 mM induced a potent Gh secretion (approximately a 4-fold increase compared to that caused by rtGhrelin), while a series of concentrations of Arg ranging from 2 to 20 mM, and Leu at 4 mM, did not alter the secretion of this hormone.

3.3. Primary hepatocytes experiment

Primary hepatocytes isolated from gilthead sea bream were then used to evaluate the changes in expression of several Gh/Igf1 axis-related genes after 6 and 24 h of exposure to all the previously tested AA at different concentrations (x2, x10, or x20), compared to their normal concentration in the control medium (x1). Fig. 3 shows the results concerning the expression of total *igf1* and *igf2*. While total *igf1* was not significantly modulated after 6 h of treatment (Fig. 3A), both Arg x2 and Met x10 incubations caused a significant upregulation at 24 h (Fig. 3B). In contrast, the expression of *igf2* was significantly upregulated by Leu x20 at 6 h (Fig. 3C) but did not present significant changes at 24 h post-incubation (Fig. 3D). In addition, gene expression analysis of the different *igf1* splice variants revealed significant upregulation at 6 h of *igf1a* by Leu x10, as well as of both *igf1b* and *igf1c* by Met x10 (**Supplementary Figure 2 A, 2C and 2E**), although all the differences disappeared after 24 h (**Supplementary Figure 2B, 2D and 2F**). In parallel to the stimulatory effects of Leu on *igf1a* at 6 h incubation, Leu x20 condition significantly increased the expression of the Igf1 receptor b (*igf1rb*), although not at 24 h (**Supplementary Figure 3C and 3D**),

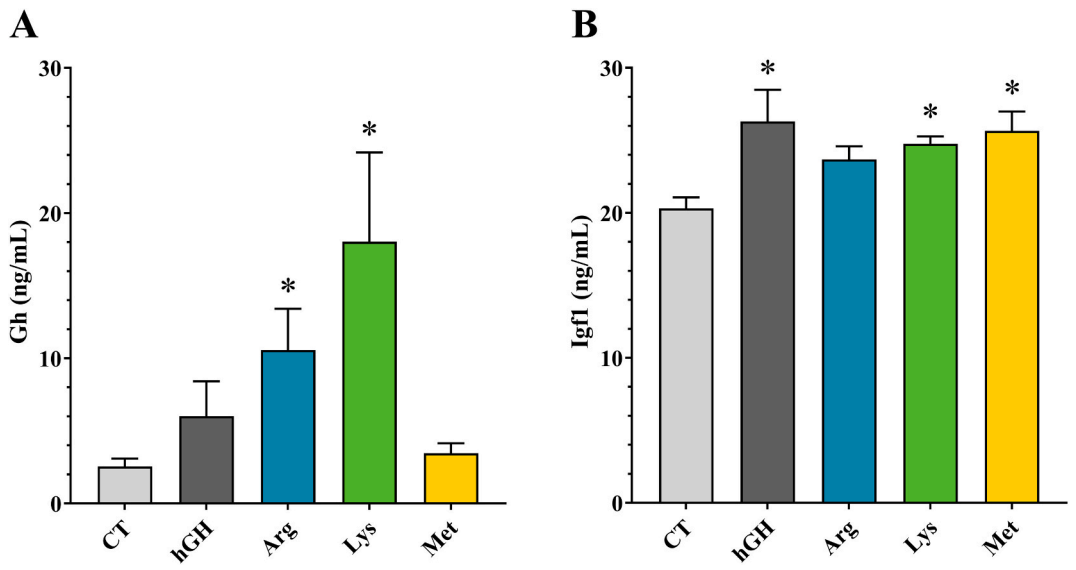


Fig. 1. Plasma levels of endogenous Gh (**A**) and Igf1 (**B**) in gilthead sea bream juveniles after 6 h of intraperitoneal injection with human GH (hGH) or different amino acids (AA); arginine (Arg), lysine (Lys) or methionine (Met). AA were dosed at 6.6 μmol/g body weight and hGH at 0.3 μg/g body weight. Data are presented as mean ± standard error of the mean ($n = 8$). Asterisks indicate significant differences compared to control (CT) fish calculated by one-way Analysis of Variance (or Kruskal-Wallis non-parametric alternative) followed by Dunnett's (or Dunn's non-parametric alternative) *post-hoc* test (*$p < 0.05$).

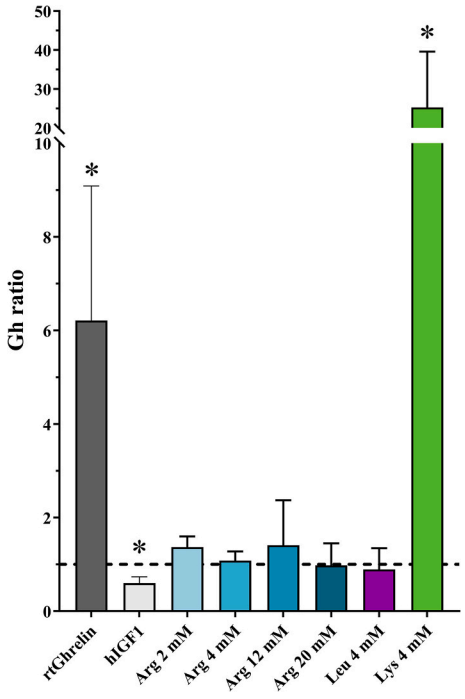


Fig. 2. Gh ratio between treated and control incubated gilthead sea bream hemi pituitaries. Treatments consisted on rainbow trout Ghrelin (rtGhrelin), human IGF1 (hIGF1), and different types and concentrations of amino acids; arginine (Arg) at 2, 4, 12 and 20 mM, and leucine (Leu) and lysine (Lys) both at 4 mM. Data are presented as mean ± standard error of the mean ($n = 4$). Asterisks indicate column means different than baseline level (1, dashed line) inferred by 95 % smoothed percentile bootstrap confidence intervals.

whereas *ig1ra* expression was not altered regardless of the AA type and incubation time (**Supplementary Figure 3A and 3B**). Furthermore, the expression of the main hepatic Igf binding proteins (*i.e.*, *igfbp1a*, *igfbp2a*, and *igfbp5b*) was analyzed (**Supplementary Figure 4**), although significant differences neither at 6 nor at 24 h post-incubation were detected respect to the corresponding control group, with the only exception of *igfbp5b* that was downregulated after 24 h of incubation with Leu x10 (**Supplementary Figure 4F**).

The hepatic gene expression results of the Gh receptors (*ghrs*) are reported in Fig. 4. Whereas *ghr1* expression was not significantly altered after 6 h of AA exposure compared to the corresponding control group (Fig. 4A), at 24 h it was significantly upregulated by Leu x2, and downregulated by Met x2 (Fig. 4B). In the case of *ghr2*, all the concentrations of Leu tested notably upregulated its expression levels after 6 h of incubation (Fig. 4C) but those returned to the control levels after 24 h (Fig. 4D).

Finally, aiming to go further into the mechanisms involved, the gene expression of key molecules of the main signaling pathways associated with the integration of signals from nutrients, energy status and growth factors, as well as with the control of the anabolic pathways and other essential cellular processes were analyzed. The results revealed that the gene expression of neither target of rapamycin (*tor*) nor its downstream effector the eukaryotic translation initiation factor 4E-binding protein 1 (*4ebp1*) were significantly altered in hepatocytes by the exposure to a single AA at either 6 or 24 h post-incubation when compared to the corresponding control medium (Fig. 5A-D). In contrast, the p70S6 kinase (*70s6k*) mRNA levels were significantly downregulated after 6 h incubation with the lowest concentration tested of Arg and Lys (x2), or by Met x10, but remained stable after incubation with Leu (Fig. 5E) as well as at longer incubation time (Fig. 5F). Of interest, all the concentrations tested of Arg, Lys and Met induced a similar significant downregulation of the protein kinase b (*akt2*) at 6 h (Fig. 6A), and a parallel strong tendency to downregulate the forkhead box protein O3 (*foxo3*) (Fig. 6C). In contrast, both *akt2* and *foxo3* remained stable after Leu exposure, and all previously observed differences disappeared after 24 h (**Fig. 6**B and D). Similarly, the results of the mitogen-activated protein kinase 2 (*erk2*) showed that the exposure to a single AA did not have any effect on the transcription of this gene (**Fig. 6**E and F).

4. Discussion

The GH/IGF1 axis is considered the main regulator of growth in vertebrates (Vélez and Unniappan, 2021). In this regard, many studies have explored different ways to potentiate the secretion of these hormones to optimizing the growth of farmed animals and increasing its production (Dohoo et al., 2003; Etherton, 1991). In aquaculture related

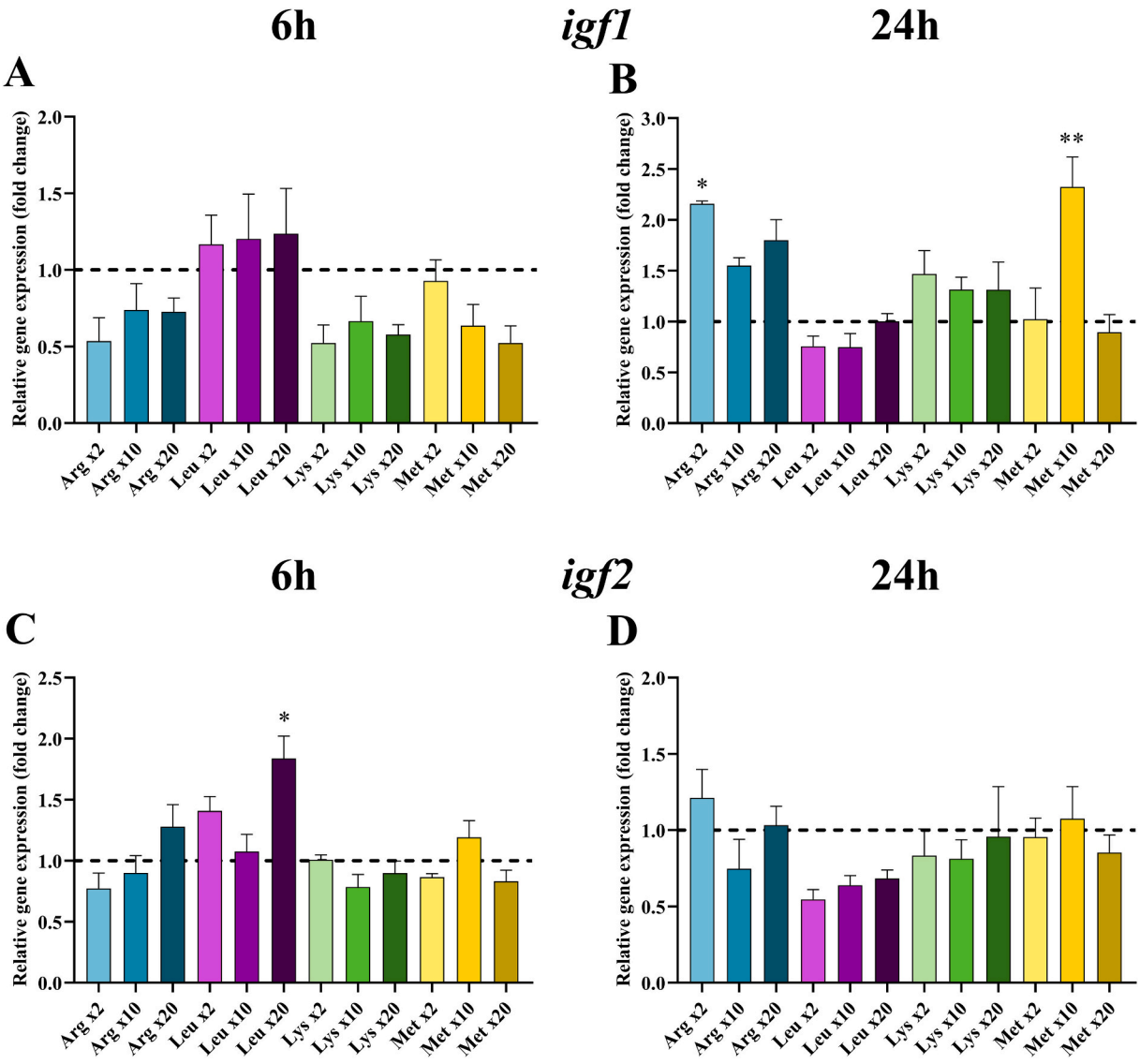


Fig. 3. Relative gene expression of *igf1* (**A** and **B**) and *igf2* (**C** and **D**) in gilthead sea bream primary hepatocytes exposed to different types and concentrations of amino acids for 6 (**A** and **C**) and 24 h (**B** and **D**). Treatments consisted on control conditions (M199 medium+15% FBS for all incubations except for leucine (Leu), whose control consisted on M199 medium+15% FBS + 1 M HCl), arginine (Arg), Leu, lysine (Lys) and methionine (Met), each at x2, x10, and x20. Data are presented as mean ± standard error of the mean ($n = 4$) normalized to the corresponding control. Significant differences were calculated by one-way Analysis of Variance (or Kruskal-Wallis non-parametric alternative) followed by Dunnett's (or Dunn's non-parametric alternative) *post-hoc* test ($p < 0.05$). Asterisks are used to highlight those significant differences between the treated and their respective control cells (*$p < 0.05$; **$p < 0.01$).

research, these strategies have spanned from the use of Gh- (Devlin et al., 2001, (Devlin et al., 1994)) or Igfbps-transgenesis (Cleveland et al., 2020) to direct hormonal treatments (Biga et al., 2005, (Biga et al., 2004a; Biga et al., 2004b); Devlin et al., 2001; Kling et al., 2012; Peterson et al., 2005, (Peterson et al., 2004); Vélez et al., 2019, (Vélez et al., 2018)), until the use of forced moderate and sustained swimming (Felip et al., 2013; Ibarz et al., 2011; Perelló-Amorós et al., 2021; Vélez et al., 2016a). Another very interesting option is to optimize diet formulation with the aim of enhancing the secretion of endogenous hormones that promote growth; that is, to potentiate the Gh/Igf1 axis actions, while ensuring a more sustainable production (Benedito-Palos et al., 2007; Perelló-Amorós et al., 2021; Pérez-Sánchez et al., 2018). In this sense, the protein content in the diet in general and its composition in AA in particular, play a fundamental role, especially in carnivorous species. With this in mind, the ultimate goal of this study combining *in vivo*, *ex vivo* and *in vitro* approaches is to better understand the role played by certain AA in the regulation of the Gh/Igf axis in gilthead sea bream.

4.1. Systemic response to AA administration: Gh and Igf1 plasma levels

There is abundant literature in vertebrates specially in mammals about the effects of AA on circulating GH levels (Caputo et al., 2021). Arg is one of the most investigated, being a classical clinical test of GH secretion. Thus, many studies have demonstrated the stimulatory effects of Arg on GH plasma levels in mammals ((Wang et al., 2021a)), and Goli et al. (2022) reviewed this topic proposing that Arg can represent a good treatment for GH deficiency in humans.

In fish, the stimulatory role of Arg on Gh secretion, as well as in the regulation of other members of the Gh/Prolactin/Somatolactin family has been demonstrated in most of the species investigated (Mao et al., 2024; Vega-Rubín de Celis et al., 2004; Wang et al., 2017; Wang et al., 2021a;). In the present study, the results clearly demonstrated that Arg injection also increases Gh plasma levels in gilthead sea bream.

Lys is also known as a good stimulator of GH secretion in vertebrates including fish (Liaqat et al., 2025; Rolland et al., 2015; Yang et al., 2020). In agreement, Lys administration provoked a significant

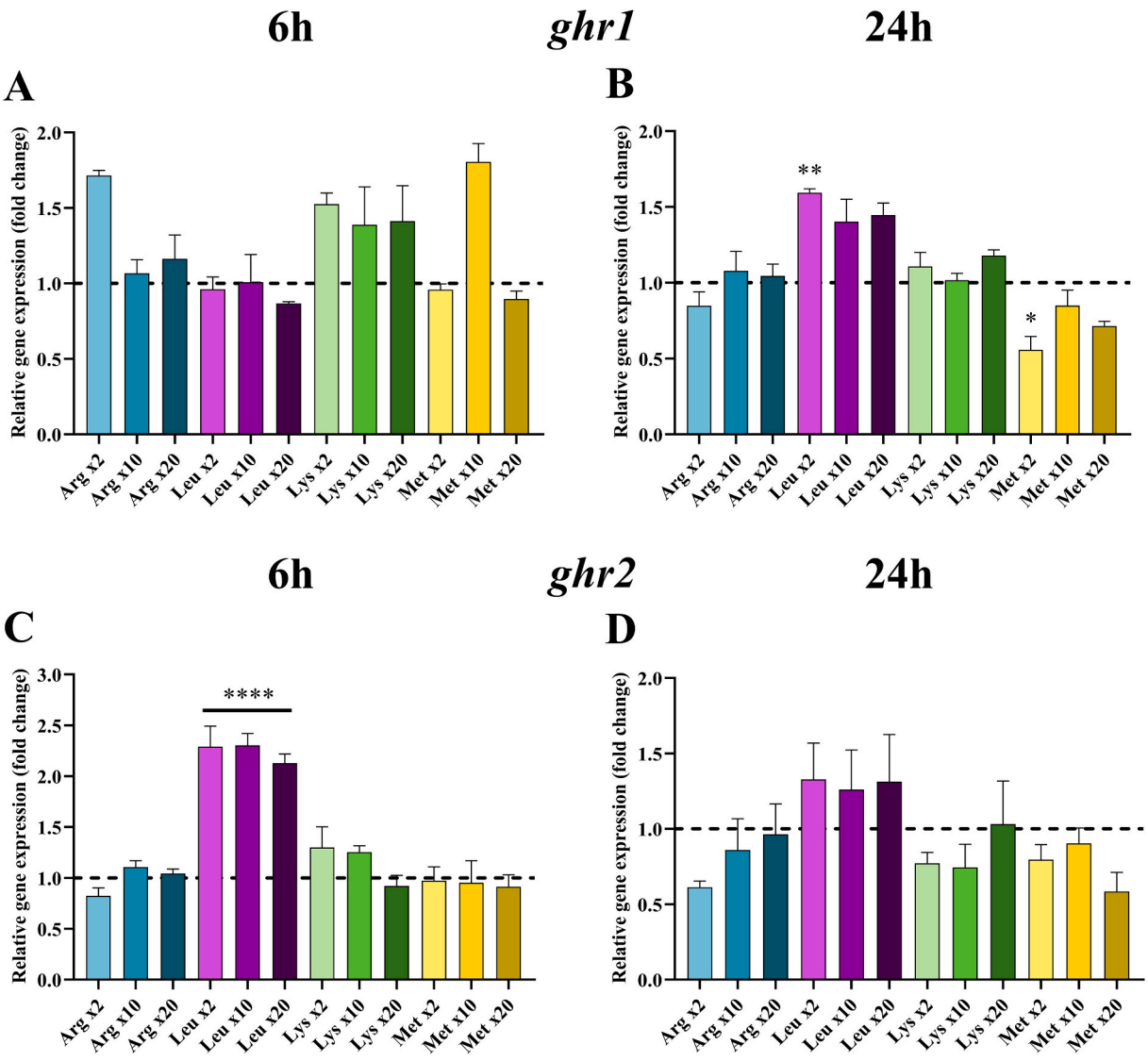


Fig. 4. Relative gene expression of *ghr1* (**A** and **B**) and *ghr2* (**C** and **D**) in gilthead sea bream primary hepatocytes exposed to different types and concentrations of amino acids for 6 (**A** and **C**) and 24 h (**B** and **D**). Treatments consisted on control conditions (M199 medium+15% FBS for all incubations except for leucine (Leu), whose control consisted on M199 medium+15% FBS + 1 M HCl), arginine (Arg), Leu, lysine (Lys) and methionine (Met), each at x2, x10, and x20 respect to the control medium. Data are presented as mean ± standard error of the mean ($n = 4$) normalized to the corresponding control. Significant differences were calculated by one-way Analysis of Variance (or Kruskal-Wallis non-parametric alternative) followed by Dunnett's (or Dunn's non-parametric alternative) *post-hoc* test ($p < 0.05$). Asterisks are used to highlight those significant differences between the treated and their respective control cells (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

increase in circulating Gh levels in gilthead sea bream. Interestingly, the results showed a stronger increase in Gh levels by Lys than those provoked by Arg administration, demonstrating the important role of Lys in the regulation of Gh secretion in this species.

Similarly, Met is also considered a growth stimulator in different fish species (Seiliez et al., 2017; Wang et al., 2023). Rolland et al. (2015) reported stimulatory effects of Met on Gh/Igf system in rainbow trout, and (Wang et al. (2021b)) growth potentiation effects in largemouth bass. However, in the current experiment with gilthead sea bream the injection of Met did not provoke a significant effect on Gh plasma levels; which could indicate that the doses utilized were not sufficient to induce secretion. In fact, Rolland et al. (2015) found a dose dependent effect of the dietary Met levels over some Gh-related molecules gene expression such as the *ghr1* and *igf1*.

Regarding the effects of the injected AA on Igf1 secretion, it is noticeable that the three AA tested presented differential effects. Whereas Lys induced the secretion of both Gh and Igf1, Arg increased only the levels of Gh, and Met enhanced only the levels of Igf1. Moreover, it is also relevant that the Igf1 plasma levels consequence of AA stimulation were comparable to those provoked by the injection of hGH, the main natural regulator of its secretion.

Among the AA tested, as previously mentioned, Met induced a clear increase of Igf1 plasma levels. Considering that Met did not stimulate Gh secretion, it can be suggested that this effect is due to a direct action on liver cells (*i.e.*, the main producers of systemic Igf1), while the stimulation of Igf1 secretion in response to Arg and Lys could be partially mediated by the effect of pituitary-derived Gh previously secreted into the plasma in response to these AA. All this emphasizes the role of Met stimulating Igf1 secretion in gilthead sea bream independently of systemic Gh. In this regard, in an *in vivo* feed trial in rainbow trout using a diet supplemented with Met, it was found a dose dependent relationship between Met levels and hepatic *igf1* expression (Rolland et al., 2015). The stimulatory effect of Met over Igf1 was also described in largemouth bass ((Wang et al., 2021b)), where higher *igf1* hepatic expression and enhanced Tor signaling were found in response to a Met supplemented diet. In the same species, higher liver *igf1* expression was also observed

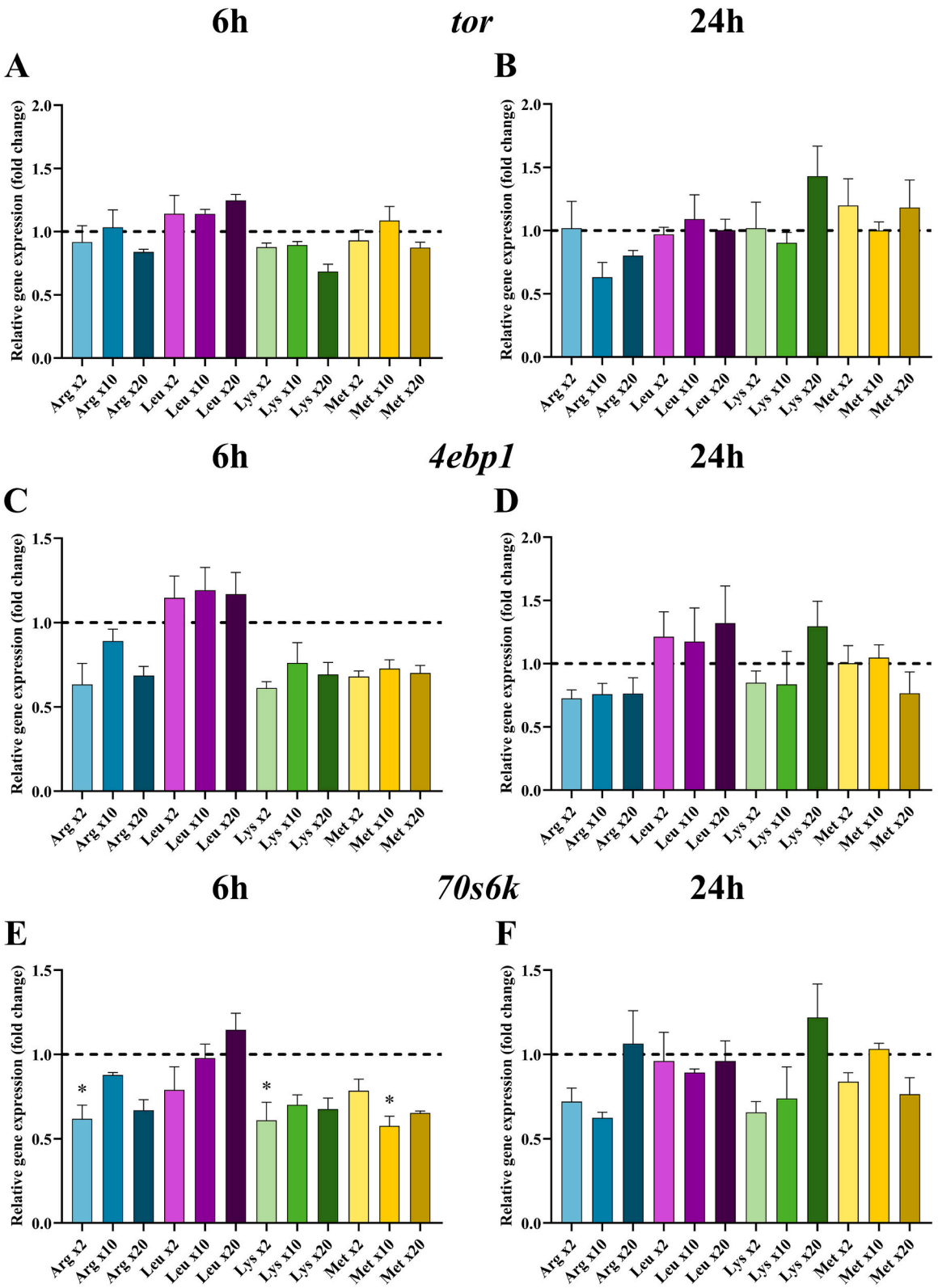


Fig. 5. Relative gene expression of *mtor* (**A** and **B**), *4ebp1* (**C** and **D**), and *70s6k* (**E** and **F**) in gilthead sea bream primary hepatocytes exposed to different types and concentrations of amino acids for 6 (**A**, **C**, and **E**) and 24 h (**B**, **D**, and **F**). Treatments consisted on control conditions (M199 medium+15% FBS for all incubations except for leucine (Leu), whose control consisted on M199 medium+15% FBS + 1 M HCl), arginine (Arg), leucine (Leu), lysine (Lys) and methionine (Met), each at x2, x10, and x20 respect to the control medium. Data are presented as mean ± standard error of the mean ($n = 4$) normalized to the corresponding control. Significant differences were calculated by one-way Analysis of Variance (or Kruskal-Wallis non-parametric alternative) followed by Dunnett's (or Dunn's non-parametric alternative) *post-hoc* test ($p < 0.05$). Asterisks are used to highlight those significant differences between the treated and their respective control cells (*$p < 0.05$).

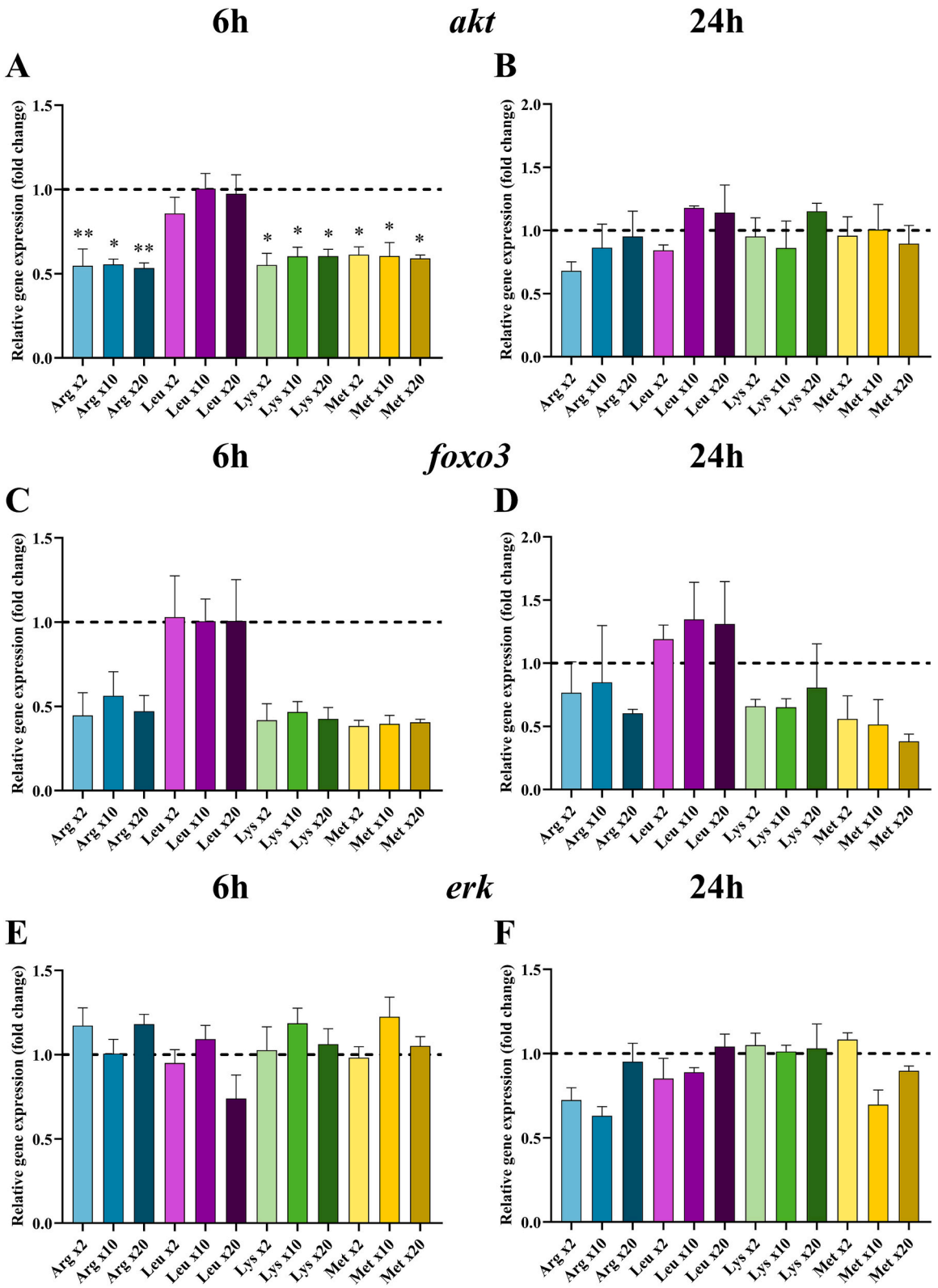


Fig. 6. Relative gene expression of *akt* (**A** and **B**), *foxo3* (**C** and **D**), and *erk* (**E** and **F**) in gilthead sea bream primary hepatocytes exposed to different types and concentrations of amino acids for 6 (**A**, **C**, and **E**) and 24 h (**B**, **D**, and **F**). Treatments consisted on control conditions (M199 medium+15% FBS for all incubations except for leucine (Leu), whose control consisted on M199 medium+15% FBS + 1 M HCl), arginine (Arg), Leu, lysine (Lys) and methionine (Met), each at x2, x10, and x20 respect to the control medium. Data are presented as mean ± standard error of the mean ($n = 4$) normalized to the corresponding control. Significant differences were calculated by one-way Analysis of Variance (or Kruskal-Wallis non-parametric alternative) followed by Dunnett's (or Dunn's non-parametric alternative) *post-hoc* test ($p < 0.05$). Asterisks are used to highlight those significant differences between the treated and their respective control cells (*$p < 0.05$; **$p < 0.01$).

in fish fed a Lys-supplemented *versus* a Lys-deficient diet (Yang et al., 2020). Moreover, a diet supplemented with a combination of Met and Lys also produced favorable results in terms of growth performance and *igf1* expression when compared to a control diet, or to diets supplemented with only Met or Lys (Liaqat et al., 2025).

On the other hand, Arg is known as a good stimulator of IGF1 secretion in mammals and Oh et al. (2017) described Arg dose dependent effects on a human hepatocyte cell line (HepG2) in terms of both IGF1 gene expression and protein synthesis. Furthermore, a significant increase in plasma Igf1 levels was observed in brown trout after 4 h of Arg injection (Baños et al., 1999). Similarly, diet supplementation with Arg stimulated growth performance in a dose dependent manner in the topmouth culter (*Culter alburnus*) fish species (Mao et al., 2024). Hence, while existing literature supports a stimulatory action of Arg on hepatic Igf1 synthesis and secretion in fish, there is no evidence demonstrating a direct action of Lys in the liver, rather pointing to an effect mediated by stimulation of the axis at the pituitary level (*i.e.*, Gh release) or a combination of both (*i.e.*, Gh and Igf1 release).

Overall, all the AA tested appear as important regulators of Igf1 plasma levels in gilthead sea bream either by the direct action on the liver or through the upstream stimulation of pituitary Gh secretion or both ways simultaneously. With the aim of understanding the regulatory mechanisms behind these systemic effects, next we studied how regulation of the Gh/Igf axis specifically occurs at both the pituitary and liver levels.

4.2. Pituitary response to AA treatments: Gh secretion

To further explore the direct effects of specific AA in the Gh/Igf axis of gilthead sea bream at the pituitary level, we analyzed the secretion of Gh *ex vivo* using symmetric portions of pituitary glands (hemi pituitaries) extracted from adult fish and incubated with a control medium supplemented with individual AA, or instead, with medium alone (control condition). First, the experimental model was validated by testing the effects of synthetic hormones respectively, one considered as a classical Gh stimulator (*i.e.*, rtGhrelin), and the other as a Gh inhibitor (*i.e.*, hIGF1) (Vélez and Unniappan, 2021), finding the expected changes.

Concerning the effects of Arg on Gh secretion from hemi pituitaries, the different concentrations (ranging from 2 to 20 mM) tested were not able to induce significant differences. In contrast, Olinto et al. (2012) demonstrated in rat hemi pituitaries that Arg stimulates *GH* expression at a dose of 71 mM (three times higher than the maximum concentration tested in the present study), while lower doses like the ones tested here did not induce significant effects. Moreover, in an *in vitro* culture model of pituitary cells from black seabream (*Acanthopagrus schlegeli*) it was found that Gh secretion is stimulated by different secretagogues in a dose dependent manner (Chan et al., 2004). Therefore, it appears that the dose of Arg used in the present study may be too low to provoke a significant effect on Gh secretion, or a dose response effect, and thus positive effects would be expected at higher doses of Arg.

A similar non-significant response to that of Arg was found for Leu 4 mM, in contrast with the strong stimulation provoked by Lys at an equal concentration, which matched the Lys-induced Gh secretion results observed in the *in vivo* injection experiment, highlighting this AA as a significant Gh secretagogue in this species. We cannot rule out that the different responses between AA observed here could be related to their nutritional requirements in this species. Of note, the importance of Lys in gilthead sea bream myogenesis has also been emphasized previously (Vélez et al., 2016b). Interestingly, the effect of Lys on Gh secretion was stronger than the effects of the hormone Ghrelin, previously identified as an important regulator of GH secretion in mammals (Khatib et al., 2014; Kojima et al., 1999). In fish, Ghrelin was first described by Unniappan et al. (2002), and its role in the regulation of energy balance reviewed (Jönsson, 2013). In gilthead sea bream, the adaptative role of Ghrelin during a fasting-refeeding experiment has been also recently characterized (Perelló-Amorós et al., 2018). However, although the function of Ghrelin in fishes is still poorly known (Lai et al., 2025), the current study strongly supports its stimulatory effect over Gh secretion in gilthead sea bream.

The Gh/Igf axis has been extensively studied in gilthead sea bream under different nutritional and environmental conditions, and the combined use of circulating Igf1 and the Igf1/Gh quotient ratio has been proposed as a reliable marker of optimal growth performance (Pérez-Sánchez et al., 2018; Simó-Mirabet et al., 2017). This concept has been reinforced by experimental evidence showing that dietary interventions modulate this Igf1/Gh ratio in parallel with metabolic adjustments that may lead to improved growth (Perelló-Amorós et al., 2018; Vélez et al., 2018, (Vélez et al., 2016a)). Thus, the inhibitory effect of Igf1 on Gh secretion observed in this model of hemi pituitary incubations, can help to further understand the regulation of the Gh/Igf system in fish.

4.3. Hepatocytes response to AA treatments: Gh/Igf axis gene expression

Aiming to further understand how AA modulate the Gh/Igf axis in fish, hepatocytes from gilthead sea bream were isolated to study *in vitro* the transcriptional effects of the exposure to increased concentrations of individual AA relative to a control medium. The results revealed that the treatments of Arg x2 and Met x10 significantly increased the expression of total *igf1* at 24 h, which is consistent with the stimulation of plasma Igf1 levels observed here after the intraperitoneal administration. Moreover, at 6 h, Leu x10 upregulated the expression of the splice variant *igf1a*, and Met x10 increased the expression of both *igf1b* and *igf1c*. Although the contribution of the different splice variants to Igf1 production in gilthead sea bream still deserves further attention (Jiménez-Amilburu et al., 2013; Tiago et al., 2008), in agreement with the Leu- and Met-induced effects observed here, increased Met dietary levels enhanced *igf1* and *igf2* mRNA levels in the liver of rainbow trout (Rolland et al., 2015), as well as a diet enriched with a mixture of branched-chain AA including Leu, valine, and isoleucine, also increased the hepatic expression of both *igf1* and *igf2* in juvenile yellowtail (*Seriola quinqueradiata*) fish (Kawanago et al., 2015). Contrarily, no effects were observed upon Lys treatment on the expression of hepatic *igfs*, suggesting that the strong stimulatory effects on Igf1 secretion induced by Lys observed in the *in vivo* experiment were indirectly mediated through its stimulation of Gh secretion, which was further supported by the hemi pituitary experiment results. Interestingly, the deficiency of Lys in the culture medium significantly downregulated the expression of both *igf1* and *igf2* in gilthead sea bream myocytes (Azizi et al., 2016), suggesting that, at least in the muscle tissue, the local Igfs production system can be modulated by this AA.

On the other hand, it was found that Leu x10 treatment upregulated the gene expression of the receptor *igf1rb* at 6 h, as previously observed with the splice variant *igf1a*, while the expression of *igf1ra, igfbp1a*, *igfbp2a* and *igfbp5b* expression, was not altered by any of the experimental treatments. Moreover, Leu x20 significantly upregulated the gene expression of *igf2*. Interestingly, this parallel regulation between the hepatic expression of *igf1a*, *igf1rb*, and *igf2*, was previously found in the liver of gilthead sea bream fingerlings injected with recombinant bovine GH (rbGH) (Vélez et al., 2018). Likely, the enhanced expression of these transcripts could represent a positive marker of anabolic situations in this species.

In line with this, the expression of *ghr1* is considered another marker of anabolic conditions in gilthead sea bream (Benedito-Palos et al., 2007; Gómez-Requeni et al., 2004; Vélez et al., 2017b), as opposed to *ghr2*; and in the present study, a clear tendency -although not statistically significant- to *ghr1* upregulation after 6 h of exposure of hepatocytes to Arg x2, Lys at different concentrations and Met x10 was observed. In contrast, at the same time, the different concentrations tested of Leu significantly upregulated the gene expression of the *ghr2*, and slightly stimulated the expression of *ghr1* at a longer incubation

time. These results therefore suggest that those AA, especially Leu, may stimulate the sensitivity of this tissue to circulating Gh to either modulate Igf1 production or to control the metabolism of carbohydrates.

Finally, the gene expression of key molecules involved in the main signaling pathways of Igf1 were analyzed. Previously, in rainbow trout hepatocytes it was reported that cell incubation with a cocktail of AA (Lansard et al., 2010), or individually with Leu, Lys, or Met (Lansard et al., 2011), was not sufficient to activate the Tor and Akt signaling pathways, respectively, unless the AA are combined with insulin. In contrast, the incubation of gilthead sea bream primary cultured muscle cells with a cocktail of AA, even without insulin, upregulated both the gene expression of *tor* and its downstream effectors *4ebp1* and *70s6k*, and increased the phosphorylation status of the Tor protein (Vélez et al., 2014). Here, while changes in the expression of *tor*, *4ebp1* or *70s6k* were not detected, −although some AA at the lowest dose reduced *70s6k* expression-, it was found that either Arg, Lys, as well as Met incubations were efficient downregulating the expression of the signaling molecule *akt2* at all the doses tested; although not significantly, *foxo3* showed the same tendency and these results would agree with the inhibition of the Foxo pathway under anabolic conditions (Senf et al., 2010; Zhang et al., 2022). Whereas we cannot rule out that changes in the Tor pathway may occur at the phosphorylation level in this situation, the results for *akt* expression after Arg, Lys, or Met incubation, which relatively mirror those observed in the case of total *igf1*, may suggest an attempt to hinder the Akt pathway and desensitize the hepatocytes to the increased Igf1 levels.

Overall, the present results have demonstrated the importance of the AA tested in modulating the Gh/Igf axis in this important marine species and Fig. 7 summarizes the main regulatory actions proposed. On the one hand, the results evidenced a role of Arg and Lys as Gh secretagogues in the gilthead sea bream pituitary, and on the other, that Arg, Met and Lys are stimulators of hepatic Igf1 secretion. Interestingly, Arg stimulates Igf1 production and secretion by acting directly on hepatocytes, but also indirectly through the stimulation of pituitary Gh secretion. In contrast, Lys appears to stimulate hepatic Igf1 indirectly by increasing systemic Gh levels, whereas Met seems to act exclusively in a direct way on hepatocytes. Furthermore, Leu administration supports the Gh/Igf axis in the liver by increasing its sensitivity to circulating Gh, as well as the production of Igf2. In conclusion, our findings confirm the anabolic role of selected AA and highlights the prominent role of Lys as a stimulator of the Gh/Igf axis in gilthead sea bream. These results provide a strong basis for developing targeted AA supplementation strategies to enhance endocrine growth regulation and improve sustainable production efficiency in aquaculture.

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Author Contribution

Conceptualization: J.B., E.C., E.J.V. and J.G.; Formal analysis: M.P-A., C.J.S., S.B.-P. and E.J.V.; Funding acquisition: I.N., J.B, E.C. and J.G. Investigation: M.P-A., C.J.S., S.B.-P., I.G.-M, M.H., A.S.-M.,J.A.C.-G. and E.J.V.; Methodology: M.P-A., C.J.S. and S.B.-P.; Project administration: I.N., J.B, E.C. and J.G.; Supervision: J.P.-S., J.B. and J.G.; Validation: J.P.-S., J.B, E.C., E.J.V. and J.G.; Visualization: M.P.-A., A.S.-M. and E.J.V.; Writing – original draft: M.P.-A. and E.J.V.; Writing – review and editing: E.C., E.J.V. and J.G.; All authors reviewed and approved the manuscript.

CRediT authorship contribution statement

M. Perelló-Amorós: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **C.J. Saromines:** Methodology, Investigation, Formal analysis. **S. Balbuena-Pecino:** Methodology, Investigation, Formal analysis. **I. García-Meilán:** Investigation. **M. Hosseinzadeh:** Investigation. **A. Sánchez-Moya:** Visualization, Investigation. **J. Pérez-Sánchez:** Validation, Supervision. **J.A. Calduch-Giner:** Investigation. **I. Navarro:** Project administration, Funding acquisition. **J. Blasco:** Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **E. Capilla:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization. **E.J. Vélez:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Conceptualization. **J. Gutiérrez:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

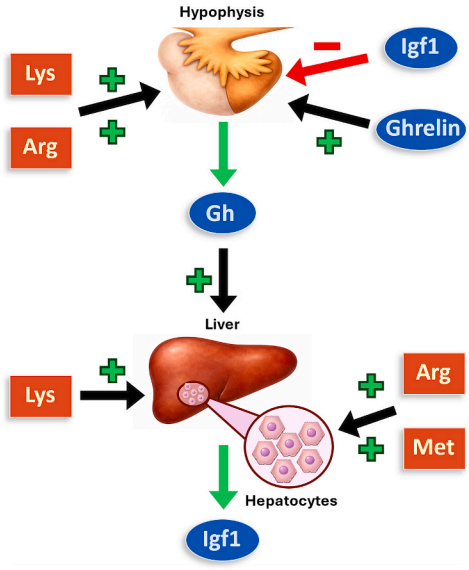


Fig. 7. Schematic view of the regulation of Gh and Igf1 secretion by amino acids in gilthead sea bream, summarizing the experiments performed in this study by combining *in vivo* (intraperitoneal injections), *ex vivo* (hemi pituitaries incubations) and *in vitro* (hepatocytes treatments) approaches. The black and red arrows symbolize the effects of the different secretagogues on hypophysis/pituitary and liver/hepatocytes, while the green arrows indicate the levels of Gh and Igf1 reached in the blood. Arginine (Arg), lysine (Lys), and rainbow trout Ghrelin (Ghrelin) stimulated, while human IGF1 (Igf1) inhibited, Gh secretion from hypophysis. In liver/hepatocytes, human GH/endogenous Gh stimulated Igf1, as well as Arg, Lys, and Met. The interaction between all these molecules contributes to regulate the circulating levels of Gh and Igf1 in gilthead sea bream. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The datasets generated and/or analyzed during the current study are openly available in the CORA repository (Vélez et al., 2026), at doi:10.34810/data2940.

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