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Research paper

Direct actions of macronutrient components on goldfish hepatopancreas *in vitro* to modulate the expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* mRNAs

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ABSTRACT

In mammals and fish, somatic growth and metabolism are coordinated by the GH-IGF axis, composed of growth hormone (GH), growth hormone receptors I and II (GHR-I and GHR-II), and the insulin-like growth factors I and II (IGF-I and IGF-II). In order to determine if dietary macronutrients regulate the hepatopancreatic expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* independently of circulating GH, organ culture experiments were conducted. Briefly, goldfish hepatopancreas sections were incubated with different doses of glucose; L-tryptophan; oleic acid; linolenic acid (LNA); eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). After two and four hours of treatment, the expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* mRNAs was quantified. We found that glucose and L-tryptophan globally upregulate the mRNA expression of *ghr-I; ghr-II; igf-I* and *igf-II*. Duration of exposure, and unsaturation level of fatty acids differentially modulate *ghr-I*, *ghr-II* and *igf-II* mRNA expression. Additionally, we found that fatty acids increase the expression of *igf-I* depending on incubation time and fatty acid class. In conclusion, here we present evidence for GH-independent, direct effects exerted by dietary macronutrients on GHR and IGF in goldfish hepatopancreas.

1. Introduction

It is widely known that somatic growth in mammals and fish is coordinated by the GH-IGF axis. Growth hormone (GH) is released from the adenohypophysis of fish in response to signals from the hypothalamus. GH functions as an endocrine regulator of many physiological processes (Björnsson, 1997; Reinecke et al., 2005), including somatic growth. The actions of GH on fish cells occur by binding to the growth hormone receptors "I" and "II" (GHR-I and GHR-II, respectively, Pérez-Sánchez et al., 2002). Initially, it was considered that GH receptors (GHRs) are expressed only in the liver of fish and mammals, mediating the release of the insulin-like growth factor-I (IGF-I) in response to GH binding. But, more recent data demonstrates that GHRs are also expressed in peripheral tissues mediating the growth-promoting effects of GH locally. Nevertheless, the major function of these receptors in the regulation of somatic growth seems to take place in the liver. It has been reported that the expression of GHRs is differentially regulated in fish liver by hormones including cortisol (Faught, 2016; Jiao et al., 2006), estradiol and testosterone (Jiao et al., 2006). These results suggest that GHR-I and GHR-II could mediate effects of GH in liver. The most studied response to the GH-GHRs binding is the release of IGF-I and IGF-II. These polypeptides promote cellular proliferation and differentiation in many vertebrates. In fish, as in mammals, IGF-I mRNA is expressed mainly in the liver, but both IGF-I and IGF-II mRNAs are also expressed in other tissues (Duan and Plisetskaya, 1993; Duguay et al., 1992; Shamblott and Chen, 1992). The biological functions of IGFs are initiated by binding to specific transmembrane receptors, which are present in both mammals and fish. IGF-I in fish has been associated not only with growth, but also with metabolism, development, reproduction, and osmoregulation in seawater (Reinecke et al., 2005). IGF-II mRNA has been detected in liver and in numerous peripheral and central organs of fish, such as brain, gills, heart, gastrointestinal tract, pancreatic islets, kidney, skeletal muscle and gonads, among others (Reinecke et al., 2005). The widespread presence of the *igf-II* gene in both juvenile and adult fish as shown by RT-PCR contrasts the situation in mammals, in which its expression is relevant only during early stages of development (Reinecke and Collet, 1998). It has been shown that IGF-II regulates metabolism in muscle cells from

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trout (Codina et al., 2004), indicating that IGF-II could act not only as a growth factor but also as a metabolic hormone in fish. There is evidence that in bony fish, the *igf-II* gene is controlled by GH (Shamblott et al., 1995; Tse et al., 2002; Vong et al., 2003) in all tissues, while in other vertebrates GH most likely regulates only the expression of the *igf-I* gene. This makes bony fish unique, and a good model to study species-specific differences in the growth axis.

Several reports have demonstrated that food composition affects the expression of GH-IGF axis components (Gómez-Requeni et al., 2012; Wood et al., 2005). Additionally, the gastric hormone ghrelin, responsible for the release of GH (Canosa et al., 2005; Grey and Chang, 2009; Unniappan and Peter, 2005), is regulated by diet composition in fish (Blanco et al., 2016a). This indicates that dietary macronutrients can modulate the release of GH. But, can these macronutrients regulate the expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* without mediating GH? To answer this, organ culture experiments were performed by exposing goldfish hepatopancreas (liver) to different doses of glucose, L-tryptophan and fatty acids. After two and four hours of treatment, the expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* mRNAs was measured.

2. Material and methods

2.1. Fish

Goldfish (*Carassius auratus*; comet variety) were acquired from Aquatic Imports (Calgary, AB, Canada). Fish with a body weight of 32 ± 8 g were keep at 20 ± 2 °C and 12 h:12 h dark-light cycle photoperiod in a 300 L tanks and were fed *ad libitum* once daily (10:00 am) on a commercial diet for goldfish (Goldfish granules, Aqueon, Franklin, WI, USA). All studies using fish strictly followed the Canadian Council of Animal Care guidelines, and protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 2012-0082).

2.2. Reagents

Glucose (Fisher Scientific, Ottawa, ON, Canada) and L-Tryptophan (Sigma-Aldrich, Oakville, ON, Canada) were reconstituted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 44 mM sodium bicarbonate, 1% penicillin-streptomycin and 0.05% gentamicin at a stock concentration of 100 mM. Oleic acid, linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), obtained from Sigma-Aldrich, were reconstituted at a stock concentration of 10 mM in ethanol. All the stock solutions were diluted in DMEM to reach the required experimental concentrations just before use. Low glucose-DMEM medium (5.6 mM) was used in order to avoid potential masking effect of high glucose levels on the macronutrients effect on *ghr-I*, *ghr-II*, *igf-I* and *igf-II* gene expression. All macronutrients concentrations were chosen following doses used *in vitro* in mouse cells by Mohan et al., 2014 and fish cells (Bertucci et al., 2017) and *in vivo* in goldfish by Blanco et al., 2016a.

2.3. Organ culture experiment

Organ culture was performed as previously described for goldfish (Sánchez-Bretaño et al., 2016) with slight modifications detailed below. Goldfish fasted for 24 h (n = 6 fish in each experiment) were sacrificed by spinal dissection and the hepatopancreas was quickly removed. Hepatopancreas was finely chopped and placed directly in culture plates, each containing a quantity of 20 mg tissue/well. Plates were pre-incubated for 2 h in 1 mL of DMEM at 23 °C under an atmosphere of 5% CO_2 and 95% O_2 for stabilization. Then, 1 mL of fresh DMEM alone, or DMEM containing glucose (25, 50 or 100 mM), L-Tryptophan (1, 10 or 50 mM), oleic acid (1, 10, 100 μM), linolenic acid (LNA; 1, 10, 100 μM), eicosapentaenoic acid (EPA; 1, 10, 100 μM) or docosahexaenoic acid (DHA; 1, 10, 100 μM) and plates were incubated for 2 or 4 h. The incubation times were chosen according to previous studies of our group (Bertucci et al., 2017; Mohan et al., 2014). Each treatment was carried out always in 6 replicates using organ samples from different fish. At the end of each incubation time, hepatopancreas samples were collected, quickly frozen in liquid nitrogen and stored at −80° C until total RNA was extracted.

2.4. Real-time quantitative PCR (qPCR)

Total RNA was isolated using Ribozol RNA Extraction Reagent (aMReSCO, Toronto, ON, Canada) and its purity was checked by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland). An aliquot of 1 μg of this RNA was reverse transcribed into cDNA in a 20 μL reaction volume using iScript Reverse Transcription Supermix for qPCR (Bio-Rad, Mississauga, ON, Canada) following the manufacturer's instructions. iQ SYBR Green Supermix (Bio-Rad) were used to perform the qPCR reactions. Primers were prepared for IDT (Toronto, ON, Canada) and sequences used for target genes *ghr-I*; *ghr-II*; *igf-I*; *igf-II* and reference gene *β-actin* are shown in Table 1. Genes were amplified as duplicates using a 96-well plate loaded with 1 μL of cDNA and 500 nM of each forward and reverse primer in a final volume of 10 μL. Each PCR run included a standard curve for the corresponding gene made of two replicates of four points of a serial dilution (1:1; 1:3; 1:9 and 1:27). Negative control was carried out adding water instead of cDNA. qPCR cycling conditions consisted of an initial step of 95 °C for 3 min, and 35 cycles of 95 °C for 10 s and 60 °C for 30 s. A melting curve was systematically monitored (temperature gradient at 0.5 °C/5 s from 65 to 95 °C) at the end of each run to confirm specificity of the amplification reaction. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression. The efficiency of the amplification for all genes studied was 100%. All runs were performed using a CFX Connect Real-Time System (Bio-Rad).

2.5. Statistical analysis

Data were analyzed by one-way ANOVA, followed by post hoc Student-Newman-Keuls test at a significance level of $P < 0.05$. Data that failed to pass homogeneity tests were log-transformed and re-tested. All tests were performed using Infostat Version 2008 (JA di Rienzo et al., 2013) software.

Table 1
Primers used for quantifying gene expression by qPCR.

Gene	GenBank accession number	Primer sequence (5′ to 3′)	Product size (bp)
ghr-I	KT985188.1	F: CGCCAATGATTCCCAGACG R: ATGGGCATGGTTGGGATTACA	200
ghr-II	KT985189.1	F: TCCACCAGTGATTCCCAGACG R: GGTAGGCATTGCTGGGAGGT	200
igf-I	AF001005.1	F: GGGGGCAGAAACTATCGCAT R: GCACGTCCCTGCAAAAATTCA	200
igf-II	FJ410929.1	F: CGTGCCGAAAAACTGTGGAA R: CTCCGCACACGAACTGAAGA	200
β-actin	AB039726.2	F: CAGGGAGTGATGGTTGGCA R: AACACGCAGCTCGTTGTAGA	168

F, Forward primer; R, Reverse primer.

3. Results

3.1. Glucose and L-tryptophan upregulate the expression of ghr-I and ghr-II mRNAs

The expression of *ghr-I* was not detectable after 2 h of organ culture neither in control conditions nor in glucose or L-tryptophan (Fig. 1A and C) treated samples. However, it was detected after 4 h of culture (Fig. 1A and C). After 4 h, all concentrations of glucose induced the expression of both *ghr-I* and *ghr-II* (Fig. 1A and B). The concentration of 10 mM of L-tryptophan caused an increase in *ghr-I* mRNA expression after 4 h of treatment (Fig. 1C), but no changes were observed in the expression of *ghr-II* after 2 or 4 h of treatment (Fig. 1D).

3.2. Length and unsaturation level of fatty acids modulated ghr-I and ghr-II mRNA expression

The expression of *ghr-I* was undetectable after 2 h in both treatment and control groups, and become detectable after 4 h of organ culture (Fig. 2). We found that the exposure of hepatopancreas to 1 μM oleic acid (18:1n-9) induced the expression of *ghr-I* at 4 h (Fig. 2A), while treatment with 10 μM during 2 h enhanced the expression of *ghr-II* (Fig. 2B). On the other hand, LNA (18:3n-3) did not cause any change in the expression of either *ghr-I* or *ghr-II* after 2 or 4 h of treatment with all the concentrations tested (Fig. 2C and D). The highest concentration of EPA (20:5n-3) tested (100 μM) upregulated both *ghr-I* and *ghr-II* mRNA expression after 4 h (Fig. 2E and F). This treatment led to a low expression of *ghr-II* compared to the control group after 2 h (Fig. 2F). The treatment of hepatopancreas with 100 μM DHA (22:6n-3) reduced the expression of *ghr-I* after 4 h (Fig. 2G). After 2 h of treatment, all DHA concentrations tested decreased *ghr-II* mRNA expression. A similar effect was detected after 4 h of treatment with 10 μM and 100 μM DHA (Fig. 2H).

3.3. Glucose and L-tryptophan globally upregulated igf-I and igf-II

The expression of *igf-I* mRNA was higher in those groups treated with 50 and 100 mM glucose compared to control groups after 2 h (Fig. 3A). All glucose treatments led to an increase in the expression of both *igf*-I and *igf*-II after 4 h (Fig. 3A and B). Treatment with 25 mM glucose downregulated the expression of *igf-II* after 2 h, while the treatment with 100 mM glucose induced *igf-II* mRNA expression compared to control group (Fig. 3B).

Exposure of hepatopancreas to 1 mM L-tryptophan led to an increase in *igf-I* mRNA expression after 2 h. All L-tryptophan concentrations induced an increase in the expression of *igf-I* and *igf-II* compared to control group after 4 h of treatment (Fig. 3C and D).

3.4. Fatty acids increased the expression of igf-I and modulated the expression of igf-II depending on time and fatty acid class

All concentrations of oleic acid increased the expression of *igf-I* after 2 and 4 h (Fig. 4A). In the case of *igf-II*, a decrease in its mRNA expression was observed after 2 h of exposure to all concentrations of oleic acid, while an increase was detected at 4 h (Fig. 4B). The concentrations of 10 μM and 100 μM of LNA increased the *igf-I* mRNA expression after 2 h, while after 4 h all treatments increased in this mRNA levels (Fig. 4C). The highest dose of LNA (100 μM) induced an increase in *igf-II* mRNA expression after 4 h (Fig. 4D). As shown in Fig. 4E, treatment with 100 μM EPA increased *igf-I* mRNA expression after 2 and 4 h. All EPA concentrations decreased *igf-II* mRNA expression after 2 and 4 h (Fig. 4F). All DHA treatments induced the expression of *igf-I* compared to control group after 2 and 4 h. The DHA treatments did not produce any changes in *igf-II* mRNA expression after 2 h of treatment (Fig. 4G), but increased its expression after 4 h (Fig. 4H).

4. Discussion

In this work, we employed a hepatopancreas organ culture technique to demonstrate that macronutrients directly alter the

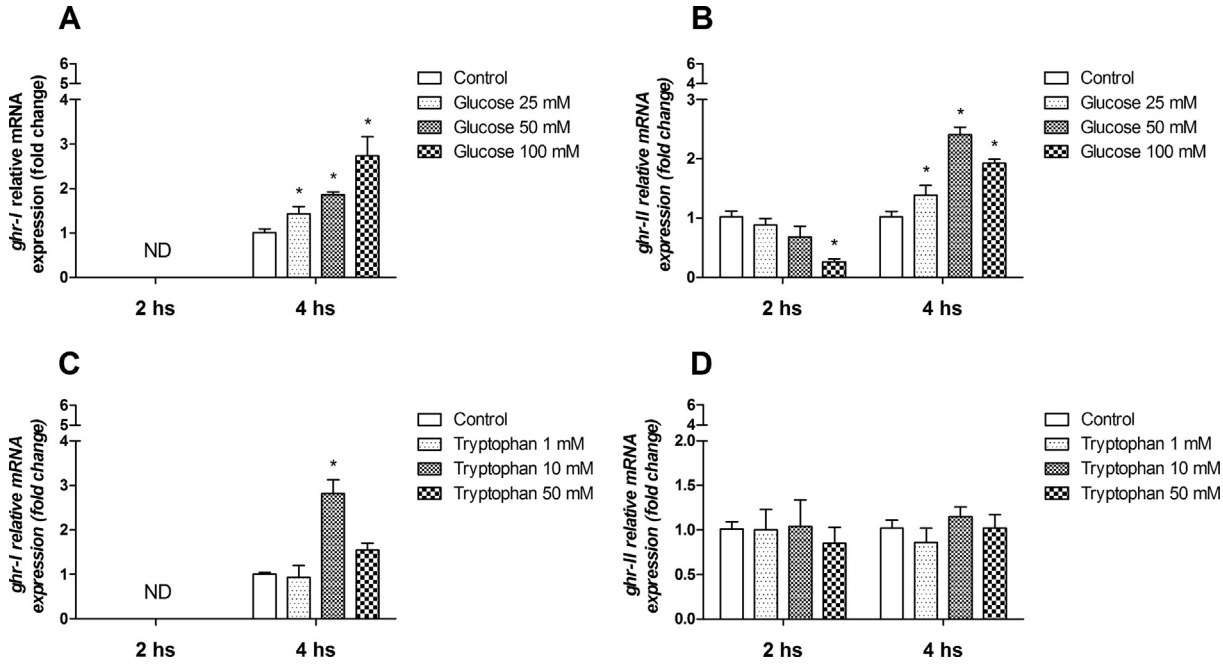

Fig. 1. *Ghr-I* and *ghr-II* mRNA expression in hepatopancreas after 2 h and 4 h of 25 mM, 50 mM and 100 mM of glucose (A, B) or 1 mM, 10 mM and 50 mM of L-tryptophan (C, D) treatments. Data obtained by qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by one way ANOVA follow by Student-Newman-Keuls post hoc test ($^{*}p < 0.05$).

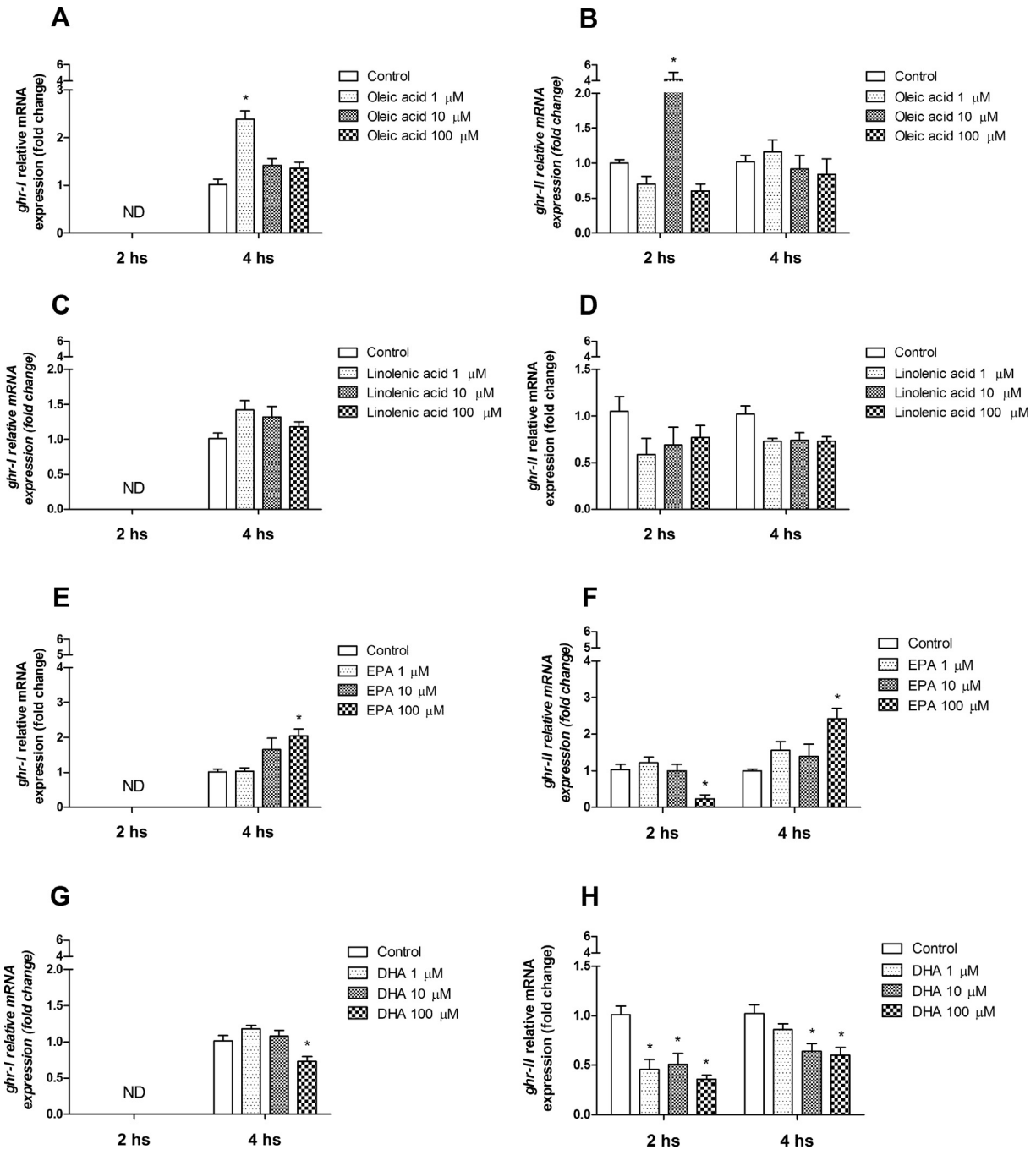

Fig. 2. *Ghr-I* and *ghr-II* mRNA expression in hepatopancreas after 2 h and 4 h of treatment with 1 mM, 10 mM and 100 mM oleic acid (A, B), linolenic acid (LNA; C, D), eicosapentanodioic acid (EPA; E, F) and docosahexanodioic acid (DHA; G, H). Data obtained by qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post hoc test ($^{*}p < 0.05$).

expression of components of the GH-IGF axis. We chose a representative components of each type of macronutrient commonly present in the diets of mammals and fish. We tested glucose to represent the effect of carbohydrates on fish metabolism. L-tryptophan was chosen to represent the effect of essential amino acids. It is also a precursor of serotonin, which regulates the secretion of the GH in pituitary, which in turn stimulates the liver cells to produce IGFs (Castrogiovanni et al., 2014). Additionally, to determine whether fatty acids influence GHRs and IGFs, and if its unsaturation level plays a role in such modulation, we tested oleic acid, linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). All macronutrient component concentrations were chosen based on the doses used *in vitro* in mouse cells by Mohan et al., 2014, in fish cells (Bertucci et al., 2017), and *in vivo* in goldfish (Blanco et al., 2016a).

Globally, the effect of different macronutrients tested (glucose; L-tryptophan; oleic acid; LNA; EPA and DHA) was an increase of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* mRNA expression, although EPA and DHA treatments produced a decrease in *igf-II* and *ghr-II* mRNA expression (respectively). The effect of macronutrients seems to be more dependent on time rather than on the concentration of the macronutrient. The expression levels of *ghr-I* fell below detection limit in all samples *in vitro* cultured for up to 4 h, in both control and treatments conditions. This situation was reverted after

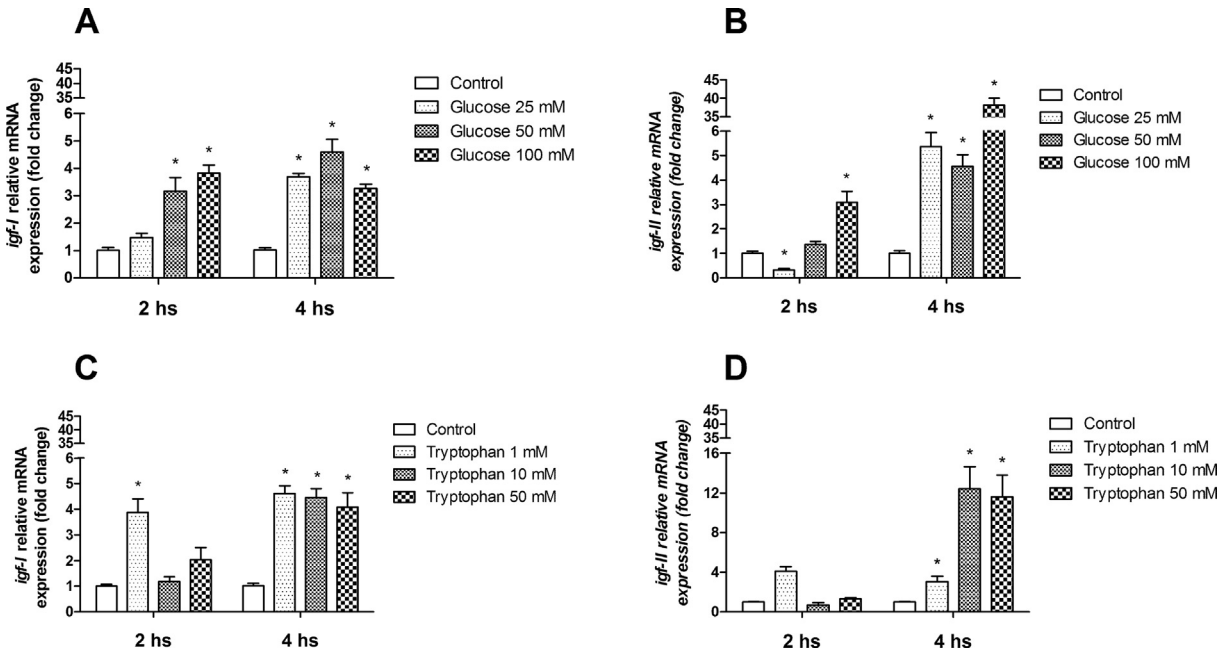

Fig. 3. *Igf-I* and *igf-II* mRNA expression in hepatopancreas of goldfish after 2 h and 4 h of 25 mM, 50 mM and 100 mM of glucose (A, B) or 1 mM, 10 mM and 50 mM of L-tryptophan (C, D) treatments. Data obtained by qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by one way ANOVA follow by Student-Newman-Keuls post hoc test ($^{*}p < 0.05$).

6 h (2 h of preincubation plus 4 h of culture time) of organ culture. Problems with the process and/or qPCR amplification of these samples can be ruled out since the same samples gave positive signals for either housekeeping or other target genes. We interpret that the expression of *ghr-I* was repressed *in vivo* in the sampled fish and this repression was removed after at least 6 h in *ex-vivo* conditions. The origin of such a repression on *ghr-I* gene expression might be related to the metabolic status of fish (fasted for 24 h) at the time of the experiment (Fukada et al., 2004; Straus and Takemoto, 1990). This starvation period was stablished to avoid the effect meals have on the expression of genes of the GH-IGF axis (Canosa et al., 2005; Peddu et al., 2009). Goldish (similar to other fish), can survive long periods of time (several weeks) without a meal (Bar, 2014; Blanco et al., 2017). We consider that 24 h was enough to avoid the previously mentioned effect, but at the same time, short enough to avoid an endocrine response to starvation (Blanco et al., 2016b) that may interfere with our objectives. Another experiment without starvation period would be necessary to clarify this point. Growth hormone receptor types I and II appear in a several teleost fish as a duplicated gene, although it is unclear if both subtypes of receptors are evolved in a new or redundant fashion (Saera-Vila et al., 2005). The differences in their mRNA expression seems to be tissue specific in fish, being highest in liver and muscle (Jiao et al., 2006; Saera-Vila et al., 2005). In general, expression of both receptor genes is modulated in liver and muscle of teleost fish by feeding, starvation and diet composition (Botta et al., 2016; Gómez-Requeni et al., 2012), but no specific biological function were attributed to each type of receptor in fish. Therefore, results presented in this work could enhance our knowledge about the regulation of these two growth hormone receptor subtypes by diet composition in fish.

All glucose treatments increased the expression of components of the GH-IGF axis after 4 h, with the major effect observed for *igf-II* expression, which was increased around 30 times after treatment with 100 mM glucose. Although the role of glucose in fish metabolism is controversial, this kind of interaction between glucose and GH-IGF axis was previously reported in fish (Riley et al., 2009). An upregulation of *ghr-I* and *ghr-II* mRNA expression in tilapia liver after an intraperitoneal injection of glucose was found. The insulinotropic effect of GH-IGF axis in mammals was demonstrated using transgenic mice by (Yakar et al., 2004). Authors found that the inactivation of GH action in liver IGF-I deficient (LID) mice led to decreased blood glucose and insulin levels, and improved peripheral insulin sensitivity. This result indicates that despite the low levels of circulating IGF-1, insulin sensitivity could be improved by inactivating GH action, suggesting that an elevation of GH levels plays a major role in insulin resistance. Also, suggests that IGF-I plays a role in maintaining the fine balance between GH and insulin to promote normal carbohydrate metabolism. Additionally, IGFs were postulated as metabolic regulators in fish, especially in the control of glucose uptake (Wood et al., 2005). This process, in mammals, has been reported to be regulated by IGF-I (Ekström et al., 2015), maybe through the modulation of GLUT-1 glucose transporters (Baumann et al., 2014). The proper processing of IGF-II was associated with hypoglycemia in a patient with a IGF-II producing tumor (Alkemade et al., 2013). These effects on glucose uptake are thought to be mediated primarily through the binding of IGF to the insulin receptor, which exhibits considerable structural similarity to IGF receptors (Wood et al., 2005). Therefore, the increased mRNA expression found *in vitro* in our study could be due to actions that are taking place *in vivo* to increase glucose uptake.

The effect of the essential amino acid L-tryptophan was highest on the levels of *igf* mRNAs, rather than that on *ghrs*. Both *igf-I* and *igf-II* mRNA expressions were modulated by L-tryptophan after 4 h of treatment, but *igf-II* levels were higher than *igf-I*. Although just IGF-I has been associated with amino acid uptake in fish cells (Castillo et al., 2004; Mommsen, 2001) through the binding of insulin receptor (Wood et al., 2005), the differences in the expression of different types of *igf* could indicate divergent actions of each insulin-like growth factors in response to dietary amino acids.

L-tryptophan is a precursor of serotonin, which increases pituitary GH secretion by inhibiting the production of hypothalamic somatostatin in mammals (Musumeci et al., 2013). It was

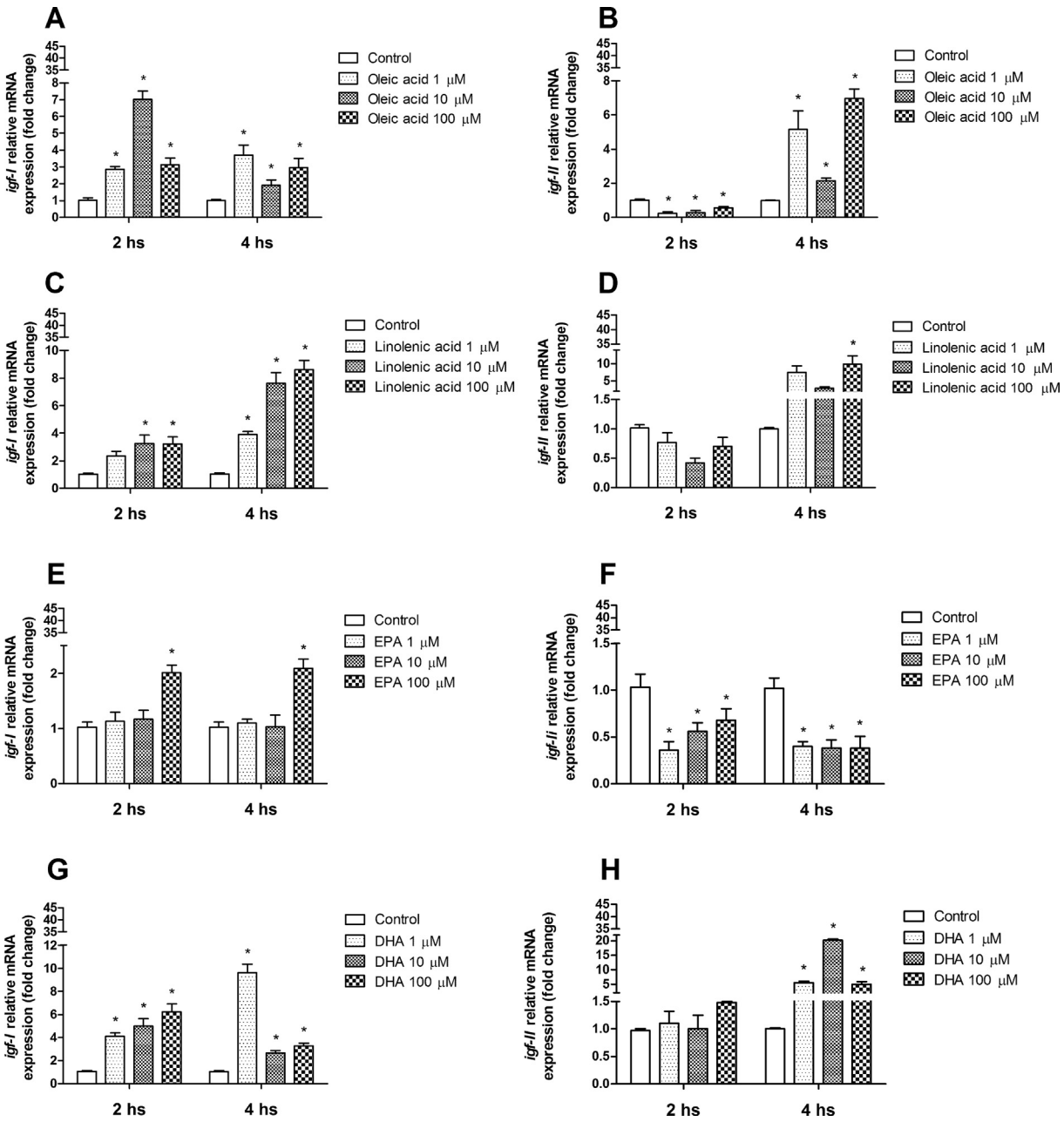

Fig. 4. *Igf-I* and *igf-II* mRNA expression in hepatopancreas after 2 h and 4 h of treatment with 1 mM, 10 mM and 100 mM oleic acid (A, B), linolenic acid (LNA; C, D), eicosapentanodioic acid (EPA; E, F) and docosahexanodioic acid (DHA; G, H). Data obtained by qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post hoc test ($^{*}p < 0.05$).

demonstrated that diet deficient in L-tryptophan generates a decrease in growth and development of muscle in rats, may be associated with an improper function of the GH-IGF axis (Musumeci et al., 2015). On the other hand, in fish, it was demonstrated that serotonin inhibits pituitary GH release *in vitro* (Canosa et al., 2007). Nevertheless, we found direct modulation of *igf* mRNAs by L-tryptophan *in vitro* in goldfish, which suggests that this essential amino acid could regulate IGF effects not mediated by GH. Porcine primary hepatocyte and HepG2 cell models also demonstrated that the mRNA and protein levels of IGF-1 increased with increasing amino acid concentration in the culture media (Wan et al., 2017). Based on these results, we hypothesize that the effects of L-tryptophan on tissue growth, could be mediated *in vivo* by serotonin and GH in pituitary, and also directly by IGFs in liver cells as reveal in our *in vitro* experiments. More studies are required to elucidate the role of L-tryptophan in the regulation of GH-IGF axis *in vivo* and growth in fish.

Fatty acids tested in this work vary in length and in number of unsaturations, as an approximation to study whether these factors induce different responses in the expression of *ghr-I*, *ghr-II*, *igf-I* and *igf-II* mRNAs. As a general observation, the effect of fatty acids seems to be time- and concentration - dependent. Comparing the effects of oleic acid with LNA (both has the same number of carbon atoms but differ in the number of unsaturations), we observed that the first one increased the expression of both *ghr-I* and *ghr-II*, while LNA did not produce any effect. EPA and DHA are poly- and highly unsaturated fatty acids, respectively, and treatments with these produce opposite effects on *ghr* mRNA expression after 4 h. These

two fatty acids are from the ω3 series and differ in length and unsaturation level, EPA being the precursor of the DHA (Los and Murata, 1998; Tocher, 2003). Given that DHA is the final product of the ω3 fatty acid synthesis pathway and inhibits the expression of both *ghr-I* and *ghr-II* mRNAs, while its precursor (EPA) increases their mRNA levels, it can be suggested that these fatty acids modulate the hepatopancreas sensitivity to the GH. In addition, GH is involved in the regulation of fatty acid metabolism in mammals (Møller et al., 2003; Vijayakumar et al., 2010) and fish (Reinecke et al., 2005; Yousefian and Shirzad, 2011). Moreover, an increase in activity of key enzyme involved in the PUFA biosynthesis, Δ6-desaturase, by GH was demonstrated in transgenic mouse overexpressing GH (Murray et al., 1994; Nakamura et al., 1996). Therefore, it is possible that DHA is inhibits the actions of GH, whereas EPA increases it, in a manner to regulate the PUFA biosynthetic pathway by its own products. In case of *igf-I*, the effect of all fatty acids regardless its length or number of unsaturations, was an increase in the mRNA expression after 2 and 4 h of treatment. But in the case of *igf-II*, the mRNA expression depends on the fatty acid class and the duration of treatment. This difference in the *igf* response could support different roles of each type of IGF in fish. *Igf-I* has been related with growth promotion and cell proliferation and *igf-II* is now associated with metabolism regulation in fish (Castillo et al., 2004; Codina et al., 2008). As fatty acids are precursors for structural components of cellular membranes (Bourre et al., 1993; Sargent et al., 1999), its availability could induce the signals to promote cell proliferation and tissue growth, and we associate this fact with the increase in *igf-I* mRNA expression found in our studies. IGF-II could mediate the process to metabolize fatty acids in order to obtain energy and precursors for cell proliferation, which might me related to the changes observed in *igf-II* mRNA expression. Although more experiments required to elucidate the role of IGFs on fatty acid metabolism in fish, our results indicate that fatty acids regulate *igf-I* and *igf-II* mRNA expression.

Since we used an *in vitro* assay and tested each macronutrient separately, the interaction between dietary macronutrients that takes place *in vivo* were not determined. The interaction between the different tissues taking part in the complex process of metabolic signaling, including pituitary, hypothalamus, and intestine was also not characetrized. Kavermann et al. (2016), reports an increase in *gh* mRNA levels in pituitary and ghr mRNA levels in liver when rats were fed with high carbohydrate-high fat diets compared to low carbohydrates-high fat diets. Additionally, it was shown in fish that a high content of lipids in fry diet cause a decrease in the ghr-I mRNA expression and no effects on the other components of the GH-IGF axis (Gómez-Requeni et al., 2012). These results, show interactions between dietary macronutrients on the regulation of the GH-IGF axis. The differences found between these studies using *in vivo* models and our *in vitro* results, could be due to the organisms' ability to sense the energy availability and metabolic precursors content of each diet and then generate a coordinated response by the GH-IGF axis.

In conclusion, we present evidence for direct effects exerted by dietary macronutrients on genes of the GH-IGF axis in goldfish hepatopancreas. An effect of dietary macronutrients on the GH-IGF axis was already reported *in vivo* in fish. This effect found *in vivo* seems to indicate a regulation of metabolism by the GH-IGF axis, which is modulated by dietary composition. But, the results obtained using *in vitro* technique that we present here suggests that the modulation could be independent of GH. That way, it is possible that macronutrients might be modulating the GH effects in hepatopancreas through the regulation of *ghr-I*, *ghr-II*, *igf-I* and *igf-II*. A limitation is that we only measured the mRNA expression. Future research should determine if these changes seen at the mRNA level are indeed translated into effects on proteins.

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