



A cross-talk between leptin and 17 β -estradiol in vitellogenin synthesis in rainbow trout *Oncorhynchus mykiss* liver

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Abstract The existence of nutritional and energy reserves is fundamental for fish female fertility, so that the existence of a correlation between metabolic reserves and reproductive capacity is suggested. Leptin regulates body weight and energy homeostasis. Estradiol induces the synthesis of vitellogenin, a phospholipoglycoprotein produced by the liver and taken up by the growing oocytes. The objective of this study was to investigate the possible existence of a crosstalk between 17 β -estradiol (E₂) and leptin in the modulation of E₂-induced vtg in the rainbow trout *Oncorhynchus mykiss*. Liver slices were incubated with recombinant trout leptin (rt-lep) at three different concentrations (1–10–100 ng/ml). rt-lep brought about the decrease of E₂-induced vtg secretion in the medium and the down-regulation of vtg mRNA expression. Moreover, rt-lep stimulated the lipase activity and diminished the liver fatty acid content. The combined employment of signal transduction inhibitors and the analysis of signal transduction phosphorylated factors revealed that rt-lep effect on E₂-induced vtg occurred through the activation of phosphodiesterase, protein kinase C, MAP kinases, and protein kinase A. In conclusion, our study suggests that leptin influences E₂-induced vtg synthesis in the rainbow trout *Oncorhynchus mykiss* by modifying both the protein and the lipid components.

Keywords Rainbow trout · Liver · Leptin · Vitellogenin · Cellular pathways

Introduction

Fish reproduction requires the central nervous system (CNS) integration of hormonal and environmental signals such as temperature, photoperiod, and food availability. Such signals influence the production of gonadotropin-releasing hormone (GnRH) in the brain, which in turn stimulates the pituitary release of gonadotropins (GtH) with downstream effects on the ovaries (Zohar et al. 2010; Degani 2016). In response to gonadotropins, the ovary produces the steroid 17 β -estradiol (E₂), which induces the liver to produce vitellogenin (Vg), the main precursor of egg yolk (Norris 1996). The hormonal control of reproduction and vitellogenin synthesis are extensively studied phenomena in many fish species due to their relevance in aquaculture (Tanaka et al. 2003; Guzmán et al., 2008; Pérez et al., 2011; Degani et al. 2017).

Leptin is a pleiotropic 16-kDa peptide hormone firstly discovered in mammals (Zhang et al. 1994), but found widely distributed phylogenetically (Prokop et al. 2012). Leptin is considered a mediator of the body metabolic status, responsible for informing the CNS about the level of energy reserves (Blundell et al. 2001). As in the case of mammals, also in teleosts, leptin appears to have an established role at the CNS level as a metabolic indicator of energy reserves and regulator of appetite and feeding behavior (Murashita et al. 2008; Li

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et al. 2010; Gorissen and Flik 2014; Chisada et al. 2014; Yan et al. 2016; Jorgensen et al. 2016).

Leptin is actively involved in the metabolic regulation of the body weight and food intake also at a peripheral level, and altered levels of leptin are associated to dysmetabolic syndromes such as obesity and diabetes (Michalakis et al. 2013). In mammals, leptin regulates peripheral lipid metabolism by reducing triacylglyceride (TAG) deposits and promoting fatty acid oxidation (FA) in both the adipose tissue and liver, sustaining its role as lipolytic hormone (Muoio and Dohm 2002). Triglycerides are transported outside the liver as lipoproteins, bound to the Apolipoprotein B (ApoB), a protein involved in lipid metabolism and the main protein constituent of very low density and low density lipoproteins (Olofsson and Boren 2005). In the mouse liver, leptin regulates lipid metabolism by promoting the incorporation of triglycerides in VLDL particles, directly affecting ApoB levels and consequently the release in the bloodstream (Huynh et al. 2013). Interestingly, it has long been proposed that vtg and human ApoB possess a common ancestor (Babin et al. 1999), sustaining the role of the liver as a crossroad of energy reserves and reproductive functions. Since vtg is a phospholipoglycoprotein that can be regarded as a lipoprotein (Nagler and Idler 1990) and the liver appears also as the major site of leptin synthesis in fish (Kurokawa et al. 2005; Londraville et al. 2017), it is possible that leptin may be involved in the regulation of vtg synthesis and somehow interact with estradiol.

In the hypothalamus of mammals, where estrogen and leptin are both crucial for the regulation of reproduction and act in overlapping neurons to regulate fertility and long-term energy balance (Xu et al. 2012), there are indications of a direct interaction between the two signals at the level of STAT3 pathway to promote gene expression (Clegg et al. 2006). The JAK/STAT pathway has been identified as the main transduction mechanism of leptin (Fruhbeck 2006; Robertson et al. 2008), although other signaling pathways, such as MAPK/ERK, can be activated (Park and Ahima 2014). Moreover, estrogen effects may include not only the activation of the canonical pathway mediated by estrogen receptors, but also the activation of non-canonical signal transduction pathways, such as PI3K, MAPK, protein kinase C (PKC), and ERK (Etgen and Acosta-Martinez 2003; Marino et al. 2002).

In this frame, we hypothesized that there may be a crosstalk between 17 β -estradiol and leptin in the

modulation of the 17 β -estradiol-induced vtg and consequently of the reproductive responses in relation to the available energy reserves. In order to test this hypothesis, we analyzed the effect of recombinant trout leptin (rt-lep) on 17 β -estradiol-induced vtg on liver slices of rainbow trout *Oncorhynchus mykiss*, one of the most economically relevant species in aquaculture (FAO report 2018) and a widely used laboratory fish model (Bailey et al. 1996; Grunow et al. 2011; Williams 2012). We measured vtg release in the incubation medium, the liver expression of vtg mRNA transcript and lipase activity, and the signal transduction pathways involved in 17 β -estradiol-induced vtg synthesis. Furthermore, we analyzed the lipid composition of rt-lep-treated liver slices by ¹H-NMR spectroscopy.

Materials and methods

Fish and sampling procedures

All the procedures involving animals were conducted as indicated in the Italian National Guidelines (D.L. No. 116 G.U., suppl. 40, 18.2.1992, circolare No. 8, G.U. July 1994) and in accordance with the European Council Directive 2010/63/EU following the rules of the D.Lgs 26/14, adhering to the Guide for the Care and Use of Laboratory Animals (United States National Research Council, 1996). The protocol was approved by the Committee on the Ethics of Animal Experiments of the same Consortium (DM n. 12/2016–UT, 29/09/2016).

Rainbow trout (*Oncorhynchus mykiss*) with an average weight of 500 g were used for this study. Fish were obtained from a local farm (Di Mella, Santa Croce del Sannio, Benevento, Italy) and acclimated for 1 week in a recirculating aquaculture system at 18 °C. Twenty trouts were employed for this study. The sacrifice took place 24 h after the last meal. Fish were euthanized by immersion in tricaine methanesulfonate (MS-222) (MP Biomedicals, LLC, Aurora, OH, USA) 80 mg/l (LC50 > 200 mg/l). Gonads were threadlike as evaluated by visual inspection.

Reagents and *Oncorhynchus mykiss* recombinant leptin (rt-lep) synthesis

17 β -estradiol, IBMX, chelerythrine, PD098059, H89, AG490, MG132, D-Mannitol, saccharose, EDTA,

Trizma-base, protease inhibitors cocktail, anti β -actin, methanol, β -mercaptaethanol, SDS, blue bromophenol, glycerol, Tween 20, nonfat dry milk, PBS, and the lipase activity assay kit were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). ATP Bioluminescence Assay Kit CLSII was from Roche Applied Science (Penzberg, Germany). Vitellogenin ELISA kit was from Biosense Laboratories AS (Bergen, Norway). SV Total RNA Isolation System was from Promega (Madison, WI, USA). DNase, SuperScript® III Reverse Transcriptase, Novex Sharp protein standard, Express SYBR®GreenER™qPCRSuperMix with Premixed ROX were purchased from Invitrogen (Carlsband, CA, USA). Bio-Rad dye protein assay was from Bio-Rad laboratories Inc. (Perth, UK). Anti-p-JNK, anti-p-ERK, and anti-p-STAT3 were bought by Santa Cruz Biotechnology (Dallas, TX, USA). Immobilon Western Chemiluminescent HRP substrate Kit was from Millipore (Billerica, MA, USA). Rainbow trout recombinant leptin (rt-lep) was produced as described in Mariano et al. (2013). Briefly, the complete trout leptin cDNA sequence was amplified from total liver cDNA by PCR. Purified leptin cDNA coding sequence was cloned into the plasmid vector and transformed into the BL21 *E. coli* strain for protein expression. Then, cells were collected and lysed by sonication for the extraction and purification by nickel affinity chromatography and stored at $-20\text{ }^{\circ}\text{C}$ until use. The protein recombinant techniques were according to Gellissen (2004).

Slice preparation, incubation, and viability

Liver slices were prepared according to Coccia et al. (2014). Briefly, livers were perfused in situ by insertion of a cannula into the portal vein followed by perfusion with sterile, ice-cold, clearing buffer (a modified Hanks' balanced salt solution, HBSS, lacking Ca^{2+} and Mg^{2+} , pH 7.2) for 10 min to remove red blood cells and prevent clotting (Shilling and Williams 2000). Livers were carefully removed, sectioned by an Oscillating Tissue Slicer EMS 5000 (Electron Microscopy Science, Hatfield, PA). Each slice was weighed and placed into a vial with 1.5 ml/100 mg of tissue of the incubation medium (HBSS). The medium was saturated with 95% O_2 to 5% CO_2 and vials were sealed. The vials were then placed on a vertically mounted wheel that was rotated at a rate of 2.3 rpm. The unit was housed within a temperature-controlled room held at $18\text{ }^{\circ}\text{C}$ at the

incubation times of 0, 24, 48, 72 h. Liver slice viability was determined by histological analysis (Imperatore et al., 2019), adenosine triphosphate (ATP) levels (ATP Colorimetric/Fluorometric Assay Kit Sigma-Aldrich, St.Louis MO USA), and genomic DNA integrity visualized by DNA laddering assay (Wyllie 1980).

Experimental design and inhibitors

In a first set of experiments, the liver slices were incubated with three concentrations of 17β -estradiol (E_2) (1–10–100 μM) to identify the most effective concentration to stimulate vitellogenin (vtg). The concentration of E_2 10 μM gave the best results and was employed in the following incubations. In a second set of experiments, we used E_2 10 μM to stimulate vtg and tested rt-lep at three different concentrations (1–10–100 ng/ml). The concentrations of rt-lep were chosen according to the circulating leptin levels in rainbow trout (Francis et al. 2014). In a third set of experiments, the liver slices were incubated for 48 h (chosen as the best compromise between the effect and the incubation time) with 10 μM E_2 , 10 μM E_2 plus 100 ng/ml rt-lep (leptin concentration was chosen as the one showing the best effect on vtg secretion, at 48 h as shown in Fig. 1) for $^1\text{H-NMR}$ assay and in presence or absence of specific cell signaling inhibitors. The inhibitors used were the following: IBMX (phosphodiesterase inhibitor that elevates cAMP), chelerythrine (protein kinase C inhibitor), PD098059 (MAP kinase inhibitor), H89 (protein kinase A inhibitor), AG490 (JAK2 tyrosine kinase inhibitor), and MG132 (NF- κB inhibitor) at the concentration of 1, 10, and 50 μM . In a fourth set of experiments, the liver slices were incubated for 30 min with 10 μM E_2 , 10 μM E_2 plus 100 ng/ml rt-lep, and 100 ng/ml rt-lep, to analyze the following phosphorylated signal transduction factors: p-JNK, p-ERK, and p-STAT3.

ELISA vtg assay

Vtg concentrations in the medium were evaluated by an enzyme-linked immunosorbent assay (ELISA) kit specific for *Oncorhynchus mykiss* (Biosense Laboratories AS, Bergen, Norway), following the manufacturer's instructions. Vtg concentration standard curve ranged from 0.39 to 200 ng/ml.

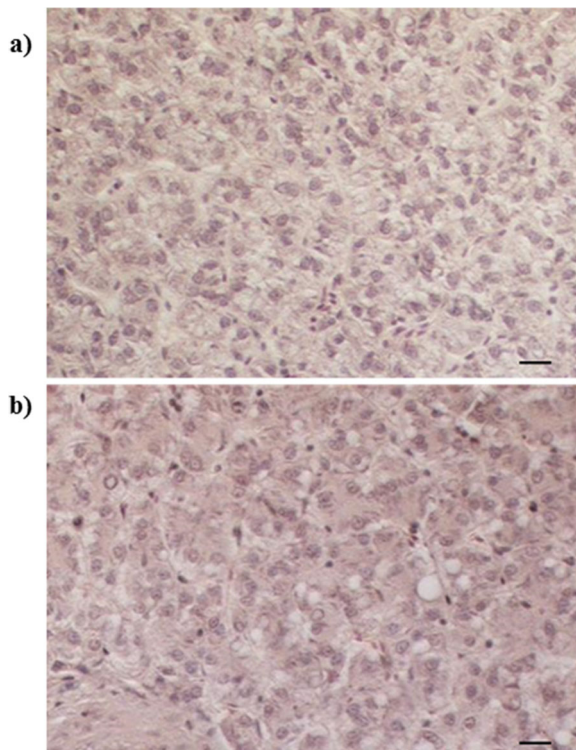


Fig. 1 Hematoxylin-eosin staining of *Oncorhynchus mykiss* trout liver slices: **a)** $T = 0$; **b)** $T = 72$ h. Scale bars = 30 μm

RNA extraction and PCR analyses

Total RNA from the liver slices was isolated and quantified with a Nanodrop 1000 Spectrophotometer (Thermo-Scientific, Waltham, Massachusetts, USA) using a kit for Total RNA Isolation (Promega Madison, WI, USA). Reverse transcription was performed using 1 μg of total RNA (previously treated with DNase), oligodT primers, and the SuperScript® III Reverse Transcriptase (Invitrogen, Carlsband, CA, USA).

cDNA preparations from liver slices were used in PCR amplifications. Vtg primers (F-5' TAGAGTGCCGGCAG ACAAAT 3', RV-5' TAAGGAGCTGAATGGGCAATC 3') were designed on the *Oncorhynchus mykiss* vtg sequence reported in GenBank (Accession n. X92804), by using the Primer3 software (Rozen and Skaletsky 2000). EF1- α primers specific for *Oncorhynchus mykiss* are reported in literature (Morash et al. 2008). All primers were designed at the exon-exon intersection.

PCR amplification of vtg was carried out in a Helix Thermal Cycler (DiaTech-Pharmacogenetics S.R.L., AN, Italy). PCR conditions consisted of denaturation at 95 °C for 5 min, followed by 35 cycles of

denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A final elongation step was performed at 72 °C for 10 min. The PCR product was separated by 2% agarose gel electrophoresis with ethidium bromide and visualized with Chemidoc UV transilluminator (BioRad, MI, Italy). The PCR fragment was purified using a QIAquick Gel Extraction (QiagenValencia, CA, USA). The PCR fragment (116 bp long) was cloned into a pGEM-T Easy Vector (Promega, WI, USA) to transform *Escherichia coli* (strain DH-5 α) using standard methods. Clones containing the PCR insert were isolated and the plasmid DNA was purified using a QIAprep Spin Miniprep Kit (QiagenValencia, CA, USA). The nucleotide sequence was carried out by PRIMM s.r.l. (NA, Italy).

Semi-quantitative PCR amplification was carried out in a Helix Thermal Cycler (DiaTech). PCR conditions consisted of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A final elongation step was performed at 72 °C for 10 min. The PCR product was separated by 2% agarose gel electrophoresis with ethidium bromide and visualized with Chemidoc UV transilluminator (BioRad). Quantification of PCR products was performed by densitometric analysis using the Quantity-One software (Bio-Rad). All samples were normalized to the housekeeping gene, EF1- α .

Real-time PCR reactions were carried out on a 7900HT Real-Time PCR System (Applied Biosystem, Foster City, CA) using Express SYBR®GreenER™qPCRSuperMix with Premixed ROX (Invitrogen, Carlsband, CA, USA) as a reference dye in a total volume of 20 μl per reaction. Each reaction contained 10 μl of SYBR Green mix, 0.4 μl each of forward and reverse primer (10 μM), 4 μl of 10 \times diluted cDNA. The thermal program included 2 min at 95 °C, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each reaction was run in duplicate. A no-template control and dissociation curve were performed to confirm the specificity of the assays. A dilution series was prepared to ensure the efficiency of the reactions. All samples were normalized to the housekeeping gene, EF1- α . Data were analyzed using the $2^{-\Delta\Delta C_t}$ method to calculate relative expression levels. All molecular techniques described were according to Farrell Jr (2009).

Lipase assay

Lipase activity in the liver slices was evaluated by a lipase activity assay kit (Sigma-Aldrich, St Louis, MO,

USA) following the manufacturer's instructions. Lipase activity was reported as nmole/min/ml = milliunit/ml.

Western blot analysis

Western blot was performed as described in Mahmood and Yang (2012). Proteins from liver slices were extracted with lysis buffer (220 mM D-mannitol, 70 mM saccharose, 1 mM EDTA, 20 mM Tris, pH 7.4) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The slices were homogenized and centrifuged at $12,000\times g$ for 30 min at 4 °C. The supernatants were collected and underwent protein determination with the Bio-Rad dye protein assay (Bio-Rad laboratories Inc., Perth, UK). Samples were boiled for 10 min in loading buffer (50 mM trisHCl pH 6.8, 100 mM β -mercaptaethanol, 2% SDS, 0.1% blue bromophenol, 10% glycerol). Then, the proteins were separated on a 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane at 100 V at 4 °C for 2 h. Membranes were blocked at 4 °C for 1 h in blocking buffer containing 0.1% Tween 20 and 5% no fat dry milk in PBS. Then, membranes were incubated overnight at 4 °C with the primary antibodies raised against Janus Kinase (p-JNK), p-ERK, p-STAT3 (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:500 and polyclonal antibodies against β -actin (Sigma-Aldrich Corp., St Louis, MO, USA) as internal marker. The incubation with the secondary anti-mouse and anti-goat IgG (1:3000) was carried out for 1 h at RT. Signals were detected by chemiluminescence with the ImmobilonWestern Chemiluminescent HRP substrate Kit (Millipore, Billerica, MA, USA) with Chemidoc (Bio-Rad). A prestained molecular weight ladder (Novex Sharp protein standard, LC5800, Invitrogen, Hilden, Germany) was used to determine the protein size. Western blotting bands were quantified by Quantity One (Bio-Rad) software version 4.6.1.

Nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy

$^1\text{H-NMR}$ spectroscopy was carried out according to Coccia et al. (2019). Briefly, liver samples were suspended in H_2O and methanol and sonicated for 30 s. Then, chloroform was added and the samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The lipophilic (apolar) phases were collected, transferred to a glass vial, and dried under nitrogen flow. Thereafter, they were dissolved in deuterated chloroform. A 600-

MHz BrukerAvance spectrometer with a TCI cryoprobe was used to acquire $^1\text{H-NMR}$ spectra at 300 K as reported in Costantini et al. (2018). A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and the gradient pulses of 1 ms each in duration adding 128 transients of 64 k complex points, with an acquisition time of 4 s/transient. Time domain data were all zero filled to 256 k complex points and an exponential amplification of 0.6 Hz prior to Fourier transformation was applied. Metabolites were assigned based on the comparison of chemical shifts and spin-spin couplings with reference spectra and tables present in the SBASE-1-1-1 database in AMIX package (Bruker, Biospin, Germany) and the biological magnetic resonance database (BMRB) (Ulrich et al. 2008). The spectral 0.50–8.60 ppm region of $^1\text{H-NMR}$ spectra was integrated in buckets of 0.04 ppm by AMIX package (Bruker, Biospin, Germany). Orthogonal Projections to Latent Structures discriminant analysis (OPLS-DA) by Metabo Analyst tool 3.0 (Xia et al. 2015) was used to analyze the different groups.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) at a significance level of 0.05, following confirmation of normality and homogeneity of variance. Where significant differences were detected by ANOVA, data were subjected to Tukey's multiple range test. All values were reported as mean \pm SEM, and all analyses were carried out with the statistical software Statistica version 7.0 (Statsoft Inc., Tulsa, OK, USA). For statistical analysis theory, we referred to the manual "An Introduction to Statistical Learning: with Applications in R" (James et al. 2013).

Results

Tissue slice viability

The histological analysis of the liver tissue slices showed normal cells with an intact nucleus and an absence of apoptotic cells with a condensed fragmented nucleus (Fig. 1). No cellular DNA fragmentation was recorded, and no statistically significant differences in the ATP levels in liver tissue slices over the entire experimental period were detected (Figs. 2 and 3). The results

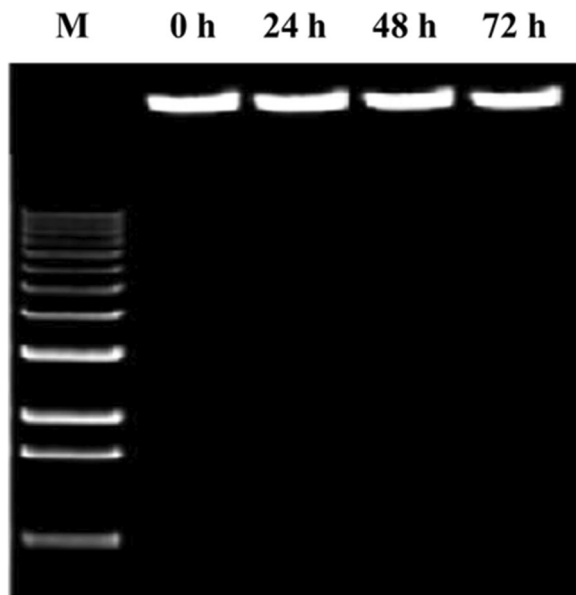


Fig. 2 Agarose electrophoresis of *Oncorhynchus mykiss* liver DNA after different times of incubation (M = marker). The gel is representative of six experiments

confirmed that the liver tissue slices used in the experimentation were viable across the experimental period.

In vitro effects of E₂ and rt-lep on vtg secretion

The in vitro effect of E₂ and E₂ plus rt-lep on vtg secretion is reported in Fig. 4. The increase of vtg concentration in the incubation medium after the treatment with E₂ was time dependent, reaching the highest concentration after 72 h of incubation. The co-incubation of E₂ plus rt-lep (1, 10, 100 ng/ml) significantly reduced the vtg concentration in the incubation medium. The decrease was both time and dose dependant.

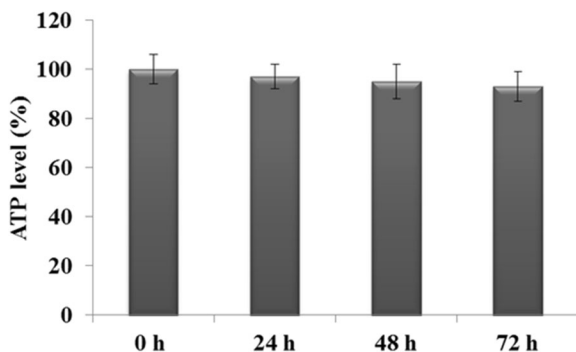


Fig. 3 ATP levels in *Oncorhynchus mykiss* liver slices after different times of incubation. The data represent the mean \pm SEM of six different trout samples

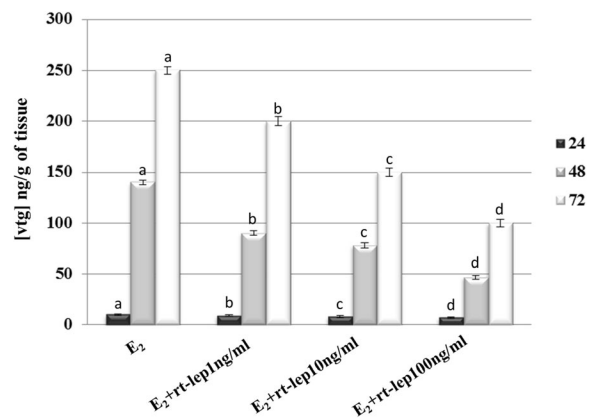


Fig. 4 Vitellogenin (vtg) concentration in the incubation medium in response to 17 β -estradiol (E₂) and recombinant trout leptin (rt-Lep) in in vitro incubation of rainbow trout (*Oncorhynchus mykiss*) liver slices at different incubation times (24, 48, and 72 h). The data represent the mean \pm SEM of different trout samples ($n = 6$). Different letters on the columns indicate statistically significant intergroup differences

In vitro effects of E₂ and rt-lep on vtg mRNA expression

In vitro effect of E₂ and rt-lep on vtg mRNA expression is shown in Fig. 5. The treatment of liver tissue slices with E₂ resulted in a significant increase in vtg mRNA expression with respect to the control. When liver slices were incubated with both E₂ and rt-lep, at any of the concentration tested (1, 10, 100 ng/ml), vtg mRNA expression was abolished. The incubation with rt-lep

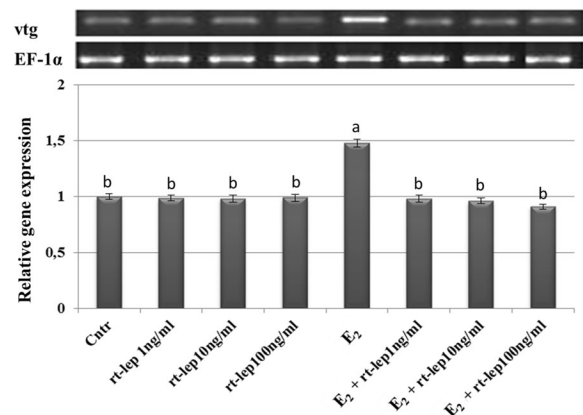


Fig. 5 Vitellogenin (vtg) mRNA expression evaluated by semi-quantitative PCR (a) and real-time PCR (b) in response to 17 β -estradiol (E₂) and recombinant trout leptin (rt-Lep) in in vitro incubations of rainbow trout (*Oncorhynchus mykiss*) liver slices after 48 h of incubation time. The data represent the mean \pm SEM of different fish samples ($n = 6$) and the different letters on the columns indicate statistically significant differences. The house-keeping gene was EF1 α

alone (1, 10, 100 ng/ml) did not cause any significant change in mRNA expression of vtg.

In vitro effects of E₂ and rt-lep on lipase activity

The administration of E₂ did not cause significant changes in the lipase activity compared to the control (2.80 ± 0.24 and 3.10 ± 0.32 mU/g of tissue for untreated and E₂ treated, respectively). The administration of rt-lep and E₂ plus rt-lep resulted in a significant increase in the lipase activity (5.36 ± 0.18 and 5.45 ± 0.16 mU/g of tissue, respectively) with respect to the control (2.80 ± 0.24) (Fig. 6).

Signal transduction pathway identification

In order to determine the signal transduction pathway activated by E₂, rt-lep, and E₂ plus rt-lep in liver, two sets of experiments were carried out. In one set, liver slices were incubated with inhibitors of specific pathways; in a second set, liver slices were incubated with E₂, rt-lep, and E₂ plus rt-lep for 30 min and antibodies against cell signaling phosphorylated molecules were employed in Western blotting analysis.

In vitro effects of signal transduction inhibitors on E₂-induced vtg secretion

As reported in Fig. 7, the co-incubation with both E₂ and rt-lep reduced significantly the vtg release in the medium at 48 h of incubation. The use of inhibitors blocking the pathway of vtg secretion influenced by leptin restores the vtg synthesis. The concentration of vtg in the medium

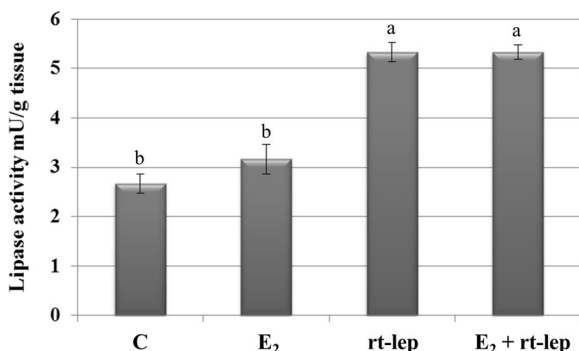


Fig. 6 Lipase activity in response to 17 β -estradiol (E₂) and recombinant trout leptin (rt-Lep) in in vitro incubations (48 h) of rainbow trout (*Oncorhynchus mykiss*) liver slices. The data represent the mean \pm SEM of different fish samples ($n = 6$) and the different letters on the columns indicate statistically significant differences

significantly increased following the co-incubation of E₂ and rt-lep together with IBMX (10 and 50 μ M), chelertine (50 μ M), PD98059 (1 and 10 μ M), H89 (10 μ M), AG490 (50 μ M), and MG132 (50 μ M).

In vitro effects of E₂ and rt-lep on signal transduction pathways

In order to determine which signal transduction pathways were activated, liver slices were incubated with E₂, rt-lep, and E₂ plus rt-lep for 30 min. MAP kinase pathway activation was evaluated by analyzing the phosphorylation of JNK and ERK. The increase in JNK phosphorylation was observed in liver slices incubated with E₂ compared with the control, while liver slices incubated with rt-lep or E₂ plus rt-lep revealed a decrease of JNK phosphorylation with respect to the control (Fig. 8a). The increase in ERK phosphorylation was observed in liver slices incubated with rt-lep compared with the control, while incubation with E₂ or E₂ plus rt-lep did not have significant effects on ERK phosphorylation (Fig. 8b). The Jak/STAT pathway activation was evaluated analyzing the phosphorylation of STAT3. The increase in STAT3 phosphorylation was observed in liver slices incubated with rt-lep compared to the control, while liver slices incubated with E₂ or E₂ plus rt-lep did not have significant effects on STAT3 phosphorylation (Fig. 8c).

¹H-NMR spectroscopy analysis

The ¹H-NMR spectroscopic analysis is reported in Fig. 9. OPLS-DA plot related to the comparison between the lipid fractions of the control liver and liver slices after incubation with E₂, rt-lep, and E₂ plus rt-lep showed that the groups clustered into four separate classes (Fig. 9a), suggesting the presence in these groups of lipids with statistically different levels (Fig. 9b). Laden plot showed that untreated liver slices had high level content of cholesterol, phospholipids, n-3 PUFAs such as DHA, and triacylglycerols (TAGs) and the levels decreased in treated liver slices, according to the following intensity: E₂ < rt-lep < E₂ plus rt-lep.

Discussion

In this study, we investigated the leptin role in the modulation of E₂-induced vtg in liver slices of rainbow trout *Oncorhynchus mykiss*.

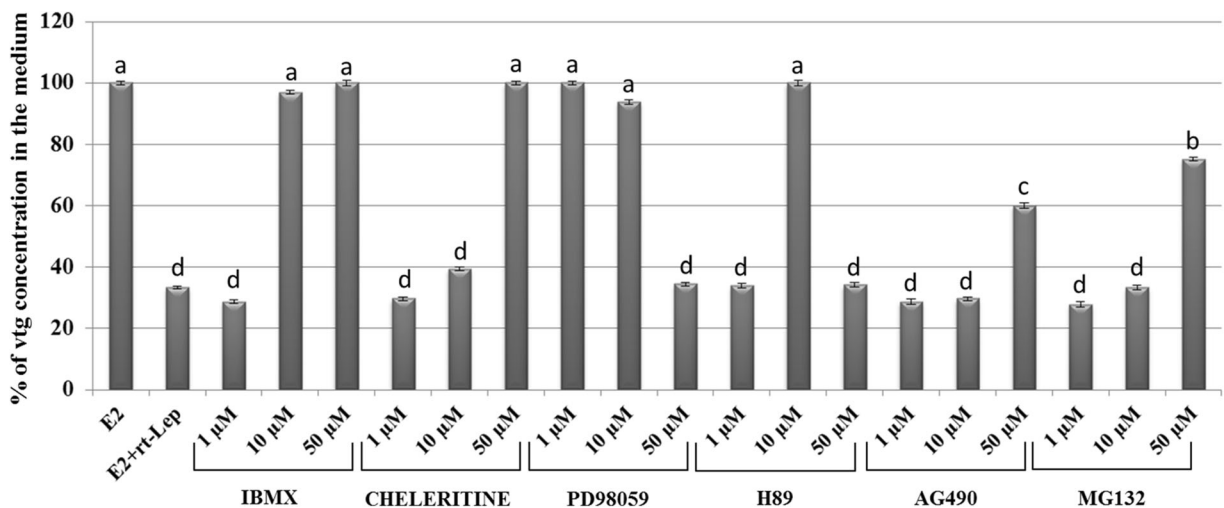


Fig. 7 Vitellogenin (Vtg) concentration in the incubation medium in response to 17 β -estradiol (E₂), E₂ + rt-Lep, E₂ + rt-Lep + inhibitor of signal transduction pathways (IBMX, cheleritine, PD98059, H89, AG490, MG132) in *in vitro* incubations of rainbow trout (*Oncorhynchus mykiss*) liver slices after 48 h. Concentrations employed were 10 μ M for E₂, 100 ng/ml for rt-Lep, and

1–10–50 μ M for each inhibitor. The data represent the mean \pm SEM of different fish samples ($n = 6$). Different letters on the columns indicate statistically significant differences. The percentage of vtg concentration in the incubation medium was calculated by setting the E₂-induced vtg value at 100

In fish, vtg represents the major egg yolk precursor, synthesized in the liver under estrogen control and transported through the bloodstream to the ovary where it is taken up by the growing oocytes (Reading and Sullivan, 2017). It is widely reported in the literature that estrogens, mainly 17 β -estradiol, have a powerful effect on the liver synthesis of vtg (Green and Tata 1976; Nagler et al. 1987). In particular, 17 β -estradiol activates the transcription of the vtg gene and increases the protein synthesis, improving the stability of the transcript (Baker and Shapiro 1977; Shapiro 1982). Accordingly, we report here the increase in the vtg concentration in the medium of liver slices of rainbow trout *Oncorhynchus mykiss*, incubated with E₂. Incubation with rt-lep at any of the employed concentrations reduced the concentration of vtg induced by E₂ in the medium, and the E₂-induced vtg mRNA expression was inhibited by rt-lep at any of the doses used. Literature studies reporting the effect of leptin on vtg transcription are lacking. Therefore, we can only hypothesize if leptin is able to physically interfere with the promoter of the vitellogenin gene. Our reasoning is based on the evidence that vtg represents an important carrier of lipids into the growing oocytes; thus, it can be regarded as a lipoprotein (Nagler and Idler 1990). The similarity between vtg and lipoproteins has led us to hypothesize that leptin could influence the synthesis of vtg, either the protein component or the lipid component, or both. Vtg is related to

other serum proteins that bind lipids in vertebrates, the so called apolipoproteins (Babin et al. 1999). Indeed, vtg and apolipoprotein B, the protein core of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (Chan 1992), are members of the same multigene superfamily (Babin et al. 1999). In this study, we report that rt-lep downregulates the E₂-induced vtg mRNA. However, leptin does not significantly influence the expressions of apoB in human HepC2 cells, while it shows a downregulation on hepatic apoM transcription and secretion *in vitro* (Luo et al. 2005). The presence of apoM in the human is associated with the production of HDL, in particular metabolism and transfer of cholesterol in the blood stream (Luo et al. 2004).

It is important to underline here that the similarities between apolipoproteins of mammals and teleosts must be considered with caution, as in teleost apolipoprotein genes have undergone a long phylogenetic differentiation, which may have led to a diversification of functions with respect to mammals. Indeed, the highest sequence similarities between zebrafish and human apolipoproteins horthologs are 54% (Otis et al. 2015).

It is worth pointing that these results are based on *in vitro* studies and their transposition to the *in vivo* level has to be carefully considered. It is interesting to note that the rt-lep minimum concentration capable of eliciting the effect of E₂-induced vtg is 1 ng/ml. The leptin circulating concentration in rainbow trout ranges

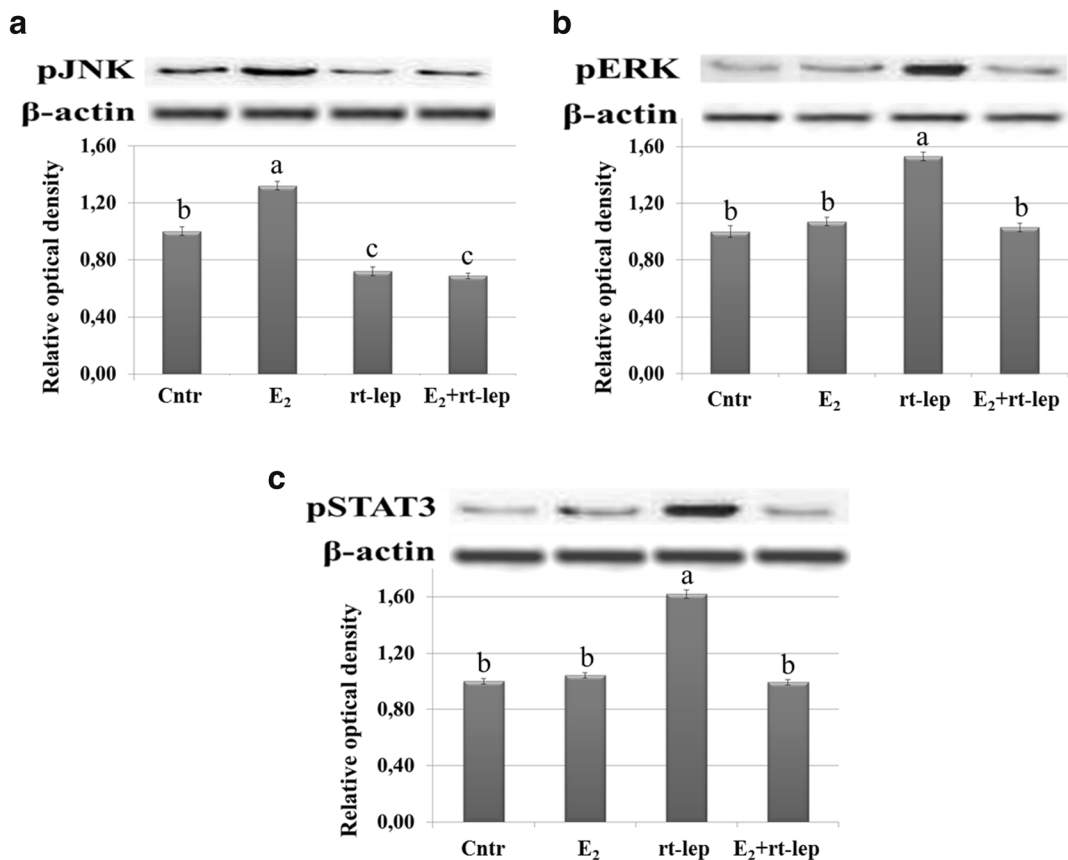


Fig. 8 Western blotting analysis of **a** pJNK, **b** pERK, and **c** pSTAT3 after 30 min of incubation of rainbow trout (*Oncorhynchus mykiss*) liver slices with 17 β -estradiol (E₂) (10 μ M), recombinant trout leptin (rt-Lep), and E₂ plus rt-Lep. The data represent

the mean \pm SEM of different fish samples ($n = 6$) and the different letters on the columns indicate statistically significant differences. β -actin was used as an internal marker

from about 1.5 ng/ml before meals to about 3.5 ng/ml after meals (Francis et al. 2014). This opens the possibility that physiologically circulating levels of leptin can affect the expression of vtg and makes possible that leptin can modulate the amount of vtg released by the liver during the reproductive cycle. Unfortunately, no reports are available about the circulating levels of leptin during the reproductive cycle in rainbow trout. However, some studies report a correlation between the levels of 17 β -estradiol and leptin, although a clear picture is not deductible. Nagasaka et al. (2006) determined leptin, 17 β -estradiol, and prolactin levels in the blood of the salmonid species ayu *Plecoglossus altivelis*, showing that leptin levels were synchronized with both 17 β -estradiol and prolactin, with plasma leptin levels low before spawning and high during and after spawning, suggesting the possibility of a regulatory link between sex hormones and leptin. Similarly, female burbot (*Lota lota*) showed low leptin levels before and during the

spawning period, compared to the after-spawning period when the leptin levels increase significantly (Mustonen et al. 2002). Yu et al. (2012) showed a relationship between overexpression of leptin gene and inhibited 17 β -estradiol synthesis in zebrafish. Choi et al. (2014) reported that leptin, 17 β -estradiol, and vtg levels increase during sexual maturation and reproduction in chum salmon *Oncorhynchus keta*. Trombley et al. (2015) indicated the ability of 17 β -estradiol to stimulate the leptin gene expression in Atlantic salmon hepatocytes, reporting that the response may depend on the developmental stage of the fish.

In addition to acting on the vtg transcript, leptin may have caused the decrease in vtg release in the medium (present study), by acting on the lipid component of the protein. To investigate this possibility, we evaluated the liver lipase activity and the lipid composition of liver slices by ¹H-NMR spectroscopy, before and after the treatments. Through ¹H-NMR spectroscopic analysis,

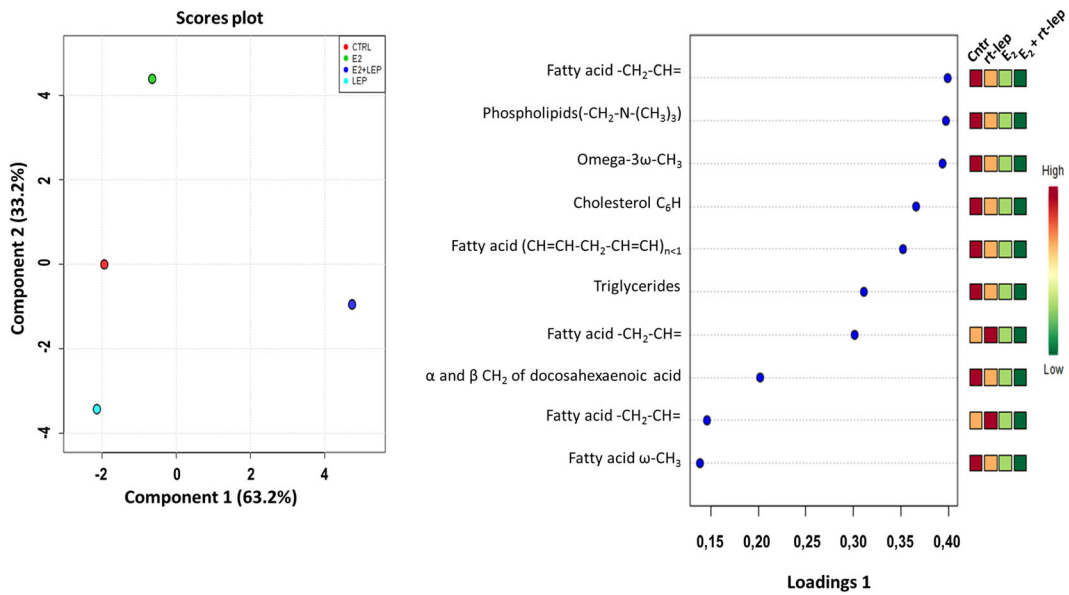
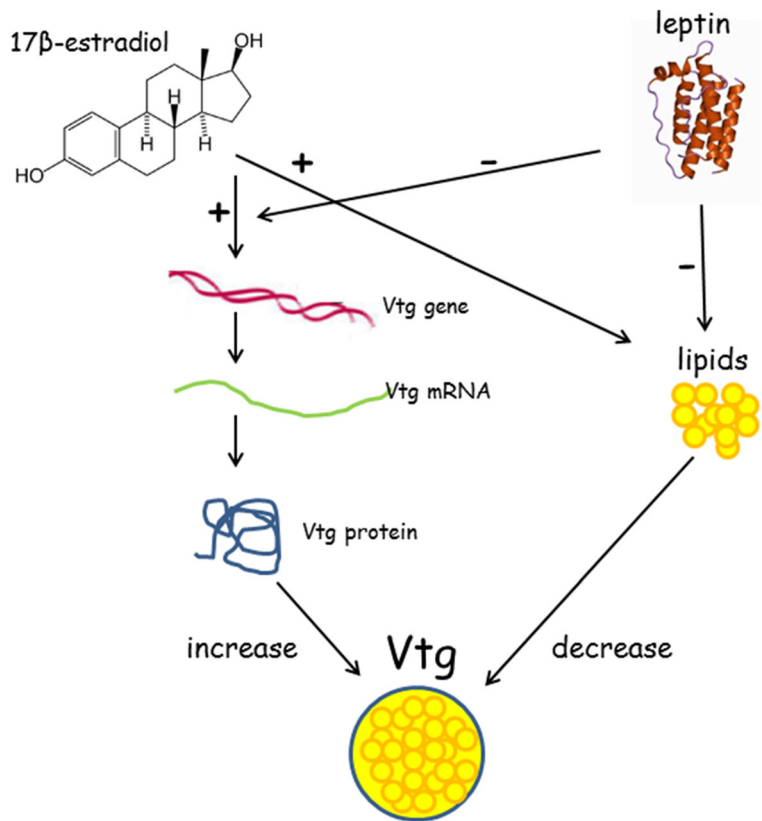


Fig. 9 ¹H-NMR spectroscopy analysis of liver slices in response to 17β-estradiol (E₂) (10 μM) and recombinant trout leptin (rt-Lep) (100 ng/ml) after an incubation time of 48 h. Orthogonal projections to latent structure discriminant analysis (OPLS-DA)

plots (a) and loading plot related to the apolar fractions (b). The Y-axis reports the metabolites while the X-axis indicates the significance values of the top ten significant metabolites

Fig. 10 Summary model reporting the effect of 17β-estradiol and leptin on vitellogenin (Vtg) synthesis in the rainbow trout *Oncorhynchus mykiss*. Vtg = vitellogenin. This model is based on literature data and present study results



we found that untreated liver slices showed the highest content of cholesterol, phospholipids, n-3 PUFAs such as DHA, and triacylglycerols (TAGs) compared to E₂, rt-lep, and E₂ plus rt-lep-treated liver slices. The liver of teleost species is the main site of lipid metabolism and it has been reported that its lipid content reflects the composition of vtg (Silversand and Haux 1995). In particular, vtg is a phospholipoglycoprotein and its total lipid content has been reported to be 16–18% in rainbow trout *Oncorhynchus mykiss*. The fatty acid (FA) composition of vtg total lipids in rainbow trout is characterized by a high content of phospholipids and a preponderance of long chain polyunsaturated fatty acids (PUFA), with considerably more n-3 than n-6 PUFAs. Precisely, the predominant n-3 PUFAs were eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Silversand and Haux 1995). Furthermore, it is known that vtg binds cholesterol and is directly involved in its transport into the oocytes (Matyash et al. 2001; Akhavan et al. 2016). We also observed a high content of TAGs in the untreated liver slices. However, this class of lipids is more likely associated with VLDL lipoprotein, a lipid transporter with a TAG rich core (Patino and Sullivan 2002; Akhavan et al. 2016).

In our study, the liver slices treated with E₂ and rt-lep showed a decrease in the levels of cholesterol, phospholipids, n-3 PUFAs such as DHA, and triacylglycerols with respect to the control. Such decrease was augmented in the liver slices treated with E₂ plus rt-lep. We believe that the greatest decrease in the case of the cotreatment with E₂ and rt-lep is the sum of two different physiological events. On one hand, the long known 17 β -estradiol lipogenic effect (Emmerson et al. 1979; Ng et al. 1984; Cleveland and Manor, 2015) may be masked by the vtg release into the medium, and on the other hand, there is the lipolytic action exerted by rt-lep. In mammals, 17 β -estradiol is a potent energy regulator and appears to interact with leptin signaling (Gao and Horvath, 2008). In teleosts, leptin induces an increase in lipolysis and a decrease in lipogenesis (Deck et al. 2017). In the liver of the grass carp *Ctenopharyngodon idellus*, leptin induced an increase in the mRNA level of hormone-sensitive lipase and a decrease in the expression of the enzyme involved in the FA synthesis (Li et al. 2010). It has been reported that in the catfish *Pelteobagrus fulvidraco* hepatocytes, leptin caused the increase in the activity and in the mRNA levels of lipolytic enzymes together with a decrease in the overall lipid content (Song et al. 2015). Furthermore, in the

hepatocytes of the goby *Synechogobius hasta*, leptin increased mRNA levels of several lipolytic genes, reduced the levels of lipogenic genes, and decreased the overall content of triglycerides (Wu et al. 2017). These data support the evaluation of enzymatic activity of lipase reported in the present study. Lipase is considered the key enzyme responsible for lipolysis. We reported that its activity was higher in the liver slices treated with rt-lep (both in the presence and absence of E₂) compared to the liver slices untreated or treated with 17 β -estradiol alone. Leptin and lipid lipase in the liver of fish increase during times of stress (Deck et al. 2017). Stress affects all aspects of vertebrate physiology, including reproduction. Our data support the hypothesis that the simultaneous presence of rt-lep and E₂ in the liver of rainbow trout *Oncorhynchus mykiss* could reflect an inconvenient condition for the establishment of reproductive responses, thus directing the metabolism towards the mobilization and use of energy during periods of stress.

In order to determine how leptin affects the E₂-induced vtg synthesis, we analyzed the effects of signal transduction inhibitors on vtg secretion and the phosphorylation of signal transduction pathway factors. We expected that blocking the pathway of vtg secretion influenced by leptin, its synthesis would be restored. Vtg secretion was re-established following the use of inhibitors of phosphodiesterase, PKC, MAPK, and PKA, suggesting that these could be the pathways through which leptin inhibits the effect of E₂ on the secretion of vtg, as also sustained by the inhibition of JNK (a MAPK), phosphorylation by rt-lep. Interestingly, the estrogen activation of MAPK and PKC pathways, among other, has been reported (Etgen and Acosta-Martinez, 2003; Marino et al. 2002), leading to the hypothesis that E₂ and leptin effects may converge on such pathways.

Our results show vtg inhibition and an increase in lipase activity after incubation with rt-lep and E₂ plus rt-lep in presence of IBMx, an inhibitor of the phosphodiesterase. Phosphodiesterase increases intracellular cAMP, which is the physiological activator of protein kinase A (PKA) which in turn stimulates lipase activity and lipolysis (Duncan et al. 2007). Thus, this pathway could be involved in the regulation of the lipid component of vtg. Moreover, Solinas and Becattini (2017) sustained that JNK acts as a negative regulator of peroxisome proliferator-activated receptor alpha (PPAR α) a gene involved in the fatty acid oxidation. Further, ERK and Jak/STAT3 are canonical pathways of leptin acting

on lipolysis and fatty acid oxidation (Procaccini et al. 2009). As we can see, rt-lep activated the phosphorylation of these proteins. However, when rt-lep was administered together with E₂, these pathways were inhibited, suggesting that a crosstalk between 17β-estradiol and leptin may occur in the liver of the rainbow trout at the level of these pathways, as it occurs in the hypothalamus (Gao and Horvath, 2008). Furthermore, phosphorylation sites recognized by MAPK and PKC have been recently identified on the estradiol receptor (ER) in the liver of a teleost, indicating that ER might activate the MAPK and PKC pathways to trigger the transcription of the vtg gene (Ding et al. 2016). Interestingly, the cAMP/PKA pathway has been recently reported as an intracellular pathway involved in vtg mRNA expression in the hepatopancreas of *Litopenaeus vannamei* (Chen et al. 2018). Although the results shown in this paper are not definitive, they represent crucial indications that the actions of E₂ and leptin can converge on the same cellular pathways. Further studies are needed to precisely define these routes.

Conclusions

In conclusion, our study shows that leptin could influence vtg synthesis by modifying both the protein and the lipid component. In particular, we propose that leptin negatively influences the E₂-induced Vtg mRNA transcript, while decreasing the vtg lipid component through its lipolytic activity. The proposed model is summarized in Fig. 10. Collectively, these data reveal an interesting role for hepatic leptin signaling in the modulation of E₂-induced vtg in teleosts.

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