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Research Article

The autophagy response during adipogenesis of primary cultured rainbow trout (*Oncorhynchus mykiss*) adipocytes

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ABSTRACT

Adipogenesis is a tightly regulated process, and the involvement of autophagy has been recently proposed in mammalian models. In rainbow trout, two well-defined phases describe the development of primary cultured adipocyte cells: proliferation and differentiation. Nevertheless, information on the transcriptional profile at the onset of differentiation and the potential role of autophagy in this process is scarce. In the present study, the cells showed an early and transient induction of several adipogenic transcription factors genes' expression (*i.e.*, *cebpa* and *cebpb*) along with the morphological changes (round shape filled with small lipid droplets) typical of the onset of adipogenesis. Then, the expression of various lipid metabolism-related genes involving the synthesis (*fas*), uptake (*fatp1* and *cd36*), accumulation (*plin2*) and mobilization (*hsl*) of lipids, characteristic of the mature adipocyte, increased. In parallel, several autophagy markers (*i.e.*, *atg4b, gabarapl1* and *lc3b*) mirrored the expression of those adipogenic-related genes, suggesting a role of autophagy during *in vitro* fish adipogenesis. In this regard, the incubation of preadipocytes with lysosomal inhibitors (Bafilomycin A1 or Chloroquine), described to prevent autophagy flux, delayed the process of adipogenesis (*i.e.*, cell remodelling), thus suggesting a possible relationship between autophagy and adipocyte differentiation in trout. Moreover, the disruption of the autophagic flux altered the expression of some key adipogenic genes such as *cebpa* and *pparg*. Overall, this study contributes to improve our knowledge on the regulation of rainbow trout adipocyte differentiation, and highlights for the first time in fish the involvement of autophagy on adipogenesis, suggesting a close-fitting connection between both processes.

1. Introduction

As in mammals, fish adipose tissue is involved in the control of energy homeostasis regulating lipid turnover and metabolism (Rutkowski et al., 2015; Todorčević and Hodson, 2015). In situations of positive energy balance, the adipose tissue grows mostly by hypertrophy, with adipocytes expanding their lipid content and size (Muir et al., 2016). Moreover, adipose tissue can also grow by hyperplasia, increasing the number of cells. This is accompanied by the formation of new adipocytes from precursor cells, also known as adipogenesis, a process that has been studied in detail in cultured cells (Hausman et al., 2001). Adipogenesis requires a complex cascade of transcriptional factors initiated by peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins alpha and beta (C/EBPα and C/EBPβ), and comprises two-step developmental stages, with the first one consisting on the commitment of multipotent cells to the adipocyte linage together with an activation of cell proliferation (Lee et al., 2019). Then, the further differentiation phase comprises the conversion of these committed cells into fully functional mature adipocytes that express lipogenic enzymes like lipoprotein lipase (*lpl*), fatty acid binding proteins (*fabp*), and high levels of the lipid-droplet-associated protein perilipin 1 (*plin*), among other genes (Ghaben and Scherer, 2019; Rosen and Spiegelman, 2000). These two well-differentiated phases have been also described in rainbow trout (*Oncorhynchus mykiss*) at a transcriptional

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level using primary adipocytes in culture (Bou et al., 2017). In that study, in accordance with mammalian studies, the proliferation phase was characterized by an upregulation of genes involved in basic cellular and metabolic processes and cellular remodelling; while in the terminal differentiation phase, genes involved in energy production, lipid and carbohydrate metabolism, as well as in lipid droplets formation were upregulated.

In the last decades, the establishment of *in vitro* preadipocytes cultures from several species has allowed further characterization of the adipogenic process in fish (Vegusdal et al., 2003; Oku et al., 2006; Bouraoui et al., 2008; Salmerón et al., 2013). Particularly, the addition of a lipid mixture into the differentiation medium is necessary to induce the complete differentiation of fish adipocytes in culture, enabling the activation of PPARγ through natural ligands, and lipid droplet formation. These *in vitro* models and the use of molecular techniques have increased the knowledge on fish adipocyte differentiation and its regulation, although transcriptional information, especially during the very early stages after its induction, is still scarce.

Macroautophagy (hereafter referred to autophagy) is a process by which intracellular material is degraded in the lysosomal compartment of eukaryotic cells. Originally described as an adaptive response to cellular stress, in recent years it has been associated with other important cellular processes, including stem cell self-renewal and differentiation (Sotthibundhu et al., 2018). During autophagy activation, the microtubule-associated protein 1A/1B-light chain 3 (LC3) is converted from LC3-I into LC3-II, being the amount of the latter a transitory marker of autophagosomes formation (Kabeya et al., 2000; Mizushima and Yoshimori, 2007). Notwithstanding, LC3-II is degraded with the progression of the process; thus, the disruption of the autophagosome-lysosomes fusion step using inhibitors like Bafilomycin A1 (BafA1) and Chloroquine (CQ) (Mauvezin and Neufeld, 2015; Klionsky et al., 2021) produces its accumulation (Sheng and Qin, 2019). In this sense, the analysis of LC3-II combined with the blocking of autophagy, turns it into a gold-standard technique to assess the cellular autophagic flux (Mizushima and Yoshimori, 2007; Klionsky et al., 2021).

Autophagy is tightly regulated by some cargo proteins like protein 62 (P62) also known as sequestosome (SQSTM1) (Levine and Kroemer, 2019), and proteins that promote autophagosome formation and maturation such as the GABA type A receptor associated protein like 1 (GABARAPL1), which also plays an important role in regulating cell metabolism and proliferation (Boyer-Guittaut et al., 2014). Moreover, it has been observed that the cellular remodelling role of autophagy could be critical for cell differentiation in mammals (Cecconi and Levine, 2008). In this regard, the knockdown of the autophagy-related gene 7 (ATG7) in mouse adipocytes caused inhibition of the autophagic function, leading to a decrease in both, adipogenic-related genes mRNA levels and lipid accumulation (Singh et al., 2009). Similarly, the same authors found that an adipocyte-specific mouse knockout of Atg7 generated lean mice with decreased white adipose mass, thus supporting that autophagy could regulate body lipid accumulation by controlling adipocyte differentiation. However, limited information is currently available on the role of autophagy in fish (Xia et al., 2019; Bolliet et al., 2017; Seiliez et al., 2012, 2016; Séité et al., 2019), particularly during the process of adipogenesis. Nonetheless, the transcriptomic analysis of rainbow trout preadipocytes during the whole adipogenic process *in vitro* (*i.e.*, proliferation and differentiation phases) revealed upregulated expression of autophagy-related genes (Bou et al., 2017), pointing a possible role of autophagy mediators in fish adipogenesis.

The present study aims to characterize, in a time-dependent manner, the transcriptional program involved in the *in vitro* differentiation of rainbow trout adipocytes using primary cultures, to obtain deeper insights into the dynamics of adipogenesis in fish. In addition, in order to explore the role of autophagy during this process, we analysed the gene and protein expression of key autophagy markers and the effects of autophagy blockage during adipocyte differentiation in the same species.

2. Material and methods

2.1. Animals and ethics statement

Adult rainbow trout (*O. mykiss*) of approximately 250 g in weight were obtained from the fishery "Troutfactory" (Lleida, Spain) and acclimated to the facilities in the Faculty of Biology at the University of Barcelona. Fish were kept in 400 L fiberglass tanks at 15 °C ± 1, under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (Optiline, Skretting, Burgos, Spain). Fish were fasted for 24 h to avoid contamination from the gastrointestinal tract during adipose tissue extraction. Before sacrifice by cranial concussion, fish were anesthetized with 0.1 g/L of ethyl 3-aminobenzoate methanesulfonate (MS222, Sigma-Aldrich, Tres Cantos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council directive (2010/63EU) and were approved by Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments (permit number CEEA OB35/17).

2.2. Primary culture of adipocytes

All cell culture reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain) and Life Technologies (Alcobendas, Spain) and all plastic items were obtained from Nunc (LabClinics, Barcelona, Spain). Cells were isolated from rainbow trout visceral adipose tissue by mechanical and enzymatic digestion and cultured according to the previously established procedure by Bouraoui et al. (2008). The cells were seeded at a density of 2×10^4 cells/cm^2 in six-well plates (9.6 cm^2/well) for Western blot and gene expression or twelve-well plates (2.55 cm^2/well), for the viability and lipid content assays, in growth medium containing Leibovitz's L15 with 0.1% fetal bovine serum and 0.01% antibiotic/antimycotic and were maintained at 18 °C. After confluence (day 7), cells were induced to differentiate by means of a growth medium supplemented with 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone and 5 μL/mL lipid mixture (4.5 mg/mL cholesterol, 10 mg/mL cod liver oil, 25 mg/mL polyoxyethylenesorbitan monooleate, and 2 mg/mL D-α-tocopherolacetate), namely differentiation medium. Media were changed every 2 days during the whole procedure.

2.3. Experimental design

Samples were collected at day 3 (preadipocytes) and every day from days 7 to 11 of culture (maturing adipocytes) to measure adipocyte- and autophagy-related genes expression. Furthermore, cells were daily assessed with a Zeiss Axiovert 40C inverted research grade microscope (Carl Zeiss Inc., Oberkochen, Germany) equipped with a Canon EOS 1000D digital camera to check the morphology and ensure that they correctly followed the already described developmental adipogenic process (Bouraoui et al., 2008).

For the autophagy disruption experiments, cells were treated with BafA1 (B1793) or CQ (C6628) from Sigma-Aldrich (Tres Cantos, Spain). Both reagents were dissolved in dimethyl sulfoxide (DMSO, D4540, Sigma-Aldrich, Tres Cantos, Spain), and control cells were treated with the same 0.2% of DMSO. In the first set of experiments, cells at day 7 were induced to differentiate by adding differentiation medium and treated at the same time with BafA1 at 20 nM or CQ at 20 μM for a period of 24 h. In the second set, cells at day 7 were treated with BafA1 at 100 nM and CQ at 40 μM prepared in differentiation medium for 8 h, and then incubated after medium renewal for further 72 h (up to day 11) in growth medium supplemented only with 5 μL/mL lipid mixture. The effects of autophagy inhibition on adipocytes gene expression were assessed at day 11, after treatment at day 7 with BafA1 at 100 nM for a period of 8 h. At this time, only one inhibitor was tested since all the previous data on adipocytes did not show significant differences

between compounds.

2.4. MTT cell assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to evaluate cell viability as previously described in Lutfi et al., (2017). Briefly, cells from 4 to 6 independent cultures were incubated the last 18 h of the treatment with a final concentration of 0.5 mg/mL of MTT. Then, cells were washed with PBS, resuspended in 250 μL of DMSO per well and absorbance was read immediately using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). Cell viability values (arbitrary units) were obtained from the absorbance measured at 570 nm, with 680 nm as the reference wavelength.

2.5. Oil red O staining

Intracellular neutral lipid accumulation was analysed by Oil Red O (ORO) staining as explained in Lutfi et al., (2017). Briefly, cells obtained from 4 to 7 independent cultures were fixed and stained with ORO and then rinsed with distilled water. Afterwards, Comassie blue staining was performed, and dye was extracted. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to the protein content (Infinite 200, Tecan, Männedorf, Switzerland). Data are presented as fold change relative to the control. The staining effectiveness was evaluated with a Zeiss Axiovert 40C (Carl Zeiss Inc., Oberkochen, Germany) inverted research grade microscope equipped with a Canon EOS 1000D digital camera (magnification 20×). To evaluate the effects of autophagy disruption on adipocyte morphology, stained cells of representative images from 4 to 6 independent cultures were classified according to round or spindle/starred shape and counted.

2.6. RNA extraction, cDNA synthesis and qPCR analyses

At different culture days (3, 7, 8, 9, 10 and 11), the cells from 4 to 8 independent cultures were lysed with a cell scraper and TRI Reagent (Applied Biosystems, Alcobendas, Spain) in a total volume of 500 μL per each of the two duplicate wells of the 6-well plates together. Total RNA

Table 1
Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Ta, annealing temperature; Acc. Num., accession number.

Gene	Primer sequence (5′ → 3′)	Ta (°C)	Acc. Num.
cebpa	F: TGTGGCGATAAAGCAAGAGC R: CTGGTGGGAATGGTGGTAGG	57	DQ423469.1
cebpb	F: CACAAAGTGCTGGAACTGGC R: TGGCACAGCGATAAATGGGT	60	FR904306.1
pparg	F: GCCAGTACTGTCGCTTTCAG R: TCCATAAACTCAGCCAGCAG	60	HM536192.1
rxr	F: AAAGAGCGCAGTGAGAACGA R: TGTAGGTCTCGGTCTTGGGT	55	AJ969439.1
lxr	F: TGCAGCAGCCGTATGTGGA R: GCGGCGGGAGCTTCTTGTC	62	NM_001159338
fas	F: GAGACCTAGTGGAGGCTGTC R: TCTTGTTGATGGTGAGCTGT	54	tcaa0001c.m.06_5.1.om.4
lpl	F: TAATTGGCTGCAGAAAACAC R: CGTCAGCAAACTCAAAGGT	59	AJ224693
hsl	F: AGGGTCATGGTCATCGTCTC R: CTTGACGGAGGGACAGCTAC	58	TC172767
cd36	F: CAAGTCAGCGACAAACCAGA R: ACTTCTGAGCCTCCACAGGA	62	AY606034
fatp1	F: AGGAGAGAACGTCTCCACCA R: CGCATCACAGTCAAATGTCC	60	CA373015
fabp11a	F: CATTTGAGGAGACCACCGCT R: ACTTGAGTTTGGTGGTACGCT	60	NM_001124713.1
plin2	F: GATGGCAATGAGGCAGAGAACA R: AGGCAGAGTGGCTAAGGGACAG	60	CB494091.p.om.8
atg4b	F: TATGCGCTTCCGAAAGTTGTC R: CAGGATCGTTGGGGTTCTGC	58	CA345181.s.om.10
atg12l	F: GATGGAGGCCAATGAACAGC R: GCGTTTGAACTGAAAAGGGCTAA	58	CB490089.s.om.10
beclin1	F: AAGGCGCTCAAGTTCATGTT R: AACATCACAGGGAAGCAAGG	60	NM_001124429
gabarapl1	F: GTGGAAAAAGCCCCCAAAGC R: CCTCTTCATGGTGCTCCTGGTA	59	CA345480
lc3b	F: GAAACAGTTTGACCTGCGTGAA R: TCTCTCAATGATGACCGGAATCT	57	CA350545
p53	F: GTGGAATTTGATCCGAGTCTGT R: AGTGTCCAGGGTAGAATGGAG	60	NM_001124692
p62	F: GGGCTAACTATGGGGATGGC R: AAAGAGAGTCCACCAGCTGC	60	NM_001124182
ef1a	F: TCCTCTTGGTCGTTTCGCTG R: ACCCGAGGGACATCCTGTG	58	AF498320
b-actin	F: ATCCTGACAGAGCGCGGTTACAGT R: TGCCCATCTCCTGCTCAAAGTCAA	61	AJ438158
ub	F: ACAACATCCAGAAAGAGTCCAC R: AGGCGAGCGTAGCACTTG	58	AB036060

Genes: CCAAT/enhancer-binding protein alpha (cebpa); CCAAT/enhancer-binding protein beta (cebpb); Peroxisome proliferator-activated receptor gamma (pparg); Retinoid X receptor (rxr); Liver X receptor (lxr); Fatty acid synthase (fas); Lipoprotein lipase (lpl); Hormone sensitive lipase (hsl); Cluster of differentiation 36 (cd36); Fatty acid transport protein 1 (fatp1); Fatty acid binding protein 11 (fabp11); Perilipin 2 (plin2); Autophagy Related 4B Cysteine Peptidase (atg4b); Autophagy related gene 12 l (atg12l); Beclin 1 (beclin1); Gamma-aminobutyric acid receptor-associated protein-like 1 (gabarapl1); Microtubule-associated protein 1A/1B-light chain 3 (lc3b); Protein 53 (p53); Sequestosome 1 (SQSTM1) or Protein 62 (p62); Elongation factor 1 alpha (ef1a): Beta-actin (b-actin); Ubiquitin (ub).

was extracted according to the manufacturer's recommendations, dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80 °C until analysis. To eliminate any residual genomic DNA, total RNA (1 μg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

The selected key genes implicated in adipogenesis and energy metabolism regulation as transcription factors or nuclear receptors, enzymes and fatty acid transporters listed in Table 1 were analysed by real-time quantitative PCR (qPCR) including the reference genes elongation factor 1 alfa (*ef1a*), *b-actin* and ubiquitin (*ub*). qPCR analyses and preliminary validation assays were performed as described in Riera-Heredia et al., (2019). The stability of the reference genes as well as the mRNA levels of expression of the genes of interest calculated relative to the most stable reference genes (geometric mean of *b-actin* and *ub*) according to the Pfaffl method (Pfaffl, 2001) were determined using the CFX Manager Software version 3.1 implemented in the CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain).

2.7. Protein extraction and Western blot analysis

Protein extraction and Western blot analyses were performed as previously described in Vélez et al. (2019) and Balbuena-Pecino et al. (2020). To perform protein extraction 50 μL of RIPA (radioimmunoprecipitation assay) buffer supplemented with proteases (#P8340 Santa Cruz Biotechnology and PMSF) and phosphatases inhibitors (NA_3VO_4) were added to each well from the 6-well plates and scrapped. The supernatants were collected after cooling the samples 20 min at 4 °C and a centrifuge of 30 min and were stored at −80 °C. Protein quantification was done by the Bradford method, and 2.4 μg of protein were subjected to electrophoresis (SDS-PAGE) on 20% polyacrylamide gels containing 6 M urea. After transfer, membranes were washed and blocked with Odyssey® Blocking Buffer (LI-COR, Inc., Biotechnology, Massatchussets, USA) and then incubated with the antibody anti-LC3B (#2775S) from Cell Signalling Technologies (MA, USA). After washing, the membranes were incubated with an IRDye secondary antibody (LI-COR, Inc., Biotechnology). The bands were visualised by infrared fluorescence using the Odyssey Imaging System (LI-COR, Inc. Biotechnology) and quantified using the Odyssey Infrared Imaging System software version 1.2. The signal band of the protein of interest (LC3-II) was normalized using REVERT Total Protein stain (LI-COR, Inc. Biotechnology).

2.8. Statistical analyses

Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. For multiple mean comparisons among days in culture of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance of data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical significance between treated groups (BafA1 or CQ) and the control group (DMSO) was performed with Student's *t*-test. Statistical analyses were performed using SPSS Statistics version 22 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM, and $p < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

3. Results

3.1. Transcriptional characterization of early adipogenesis

Preadipocyte cells from rainbow trout were isolated and maintained in culture up to day 11, with differentiation induced at day 7 once confluence was reached. Morphology characteristic of cells at the onset of differentiation, began at day 8, switching the typical fibroblastic shaped precursor cell into a more rounded and enlarged cell filled with intracellular vesicles likely corresponding to lipid droplets (Fig. 1).

Concerning relative gene expression of the key transcription factors driving the differentiation process, *cebpa*, *cebpb*, retinoid X receptor (*rxr*) and liver X receptor (*lxr*), low transcript levels were observed during the initial phase (days 3–7), while a significant peak in expression at day 8, just 24 h after the addition of the differentiation media, was detected, with the exception of *pparg* that increased steadily thus, not showing significant differences (Fig. 2A-E). Then, a marked decrease in expression was observed at day 9, recovering the cells from that time point on, similar mRNA levels to those observed during the initial phase, before the induction of differentiation.

Regarding relative expression of genes coding for enzymes involved in lipid metabolism, contrary to the consistency that was found in the case of transcription factors, the patterns observed were slightly different depending on the enzyme. *lpl* showed low levels of expression at days 3 and 7 with a posterior significant and transient peak at day 8 after induction of differentiation, and then decreased rapidly at day 9 (Fig. 3A). On the other hand, fatty acid synthase (*fas*) and hormone sensitive lipase (*hsl*) presented elevated mRNA levels during the preadipocyte phase and after the addition of the differentiation medium the expression was quickly decreased from day 9 onwards (Fig. 3B, C).

Different patterns of expression were also observed concerning the genes involved in lipid transport. Regarding *fabp11*, significant differences were not observed along the culture, but a soft peak could be seen at day 8 to then decrease up to day 11 (Fig. 4A). Low expression levels

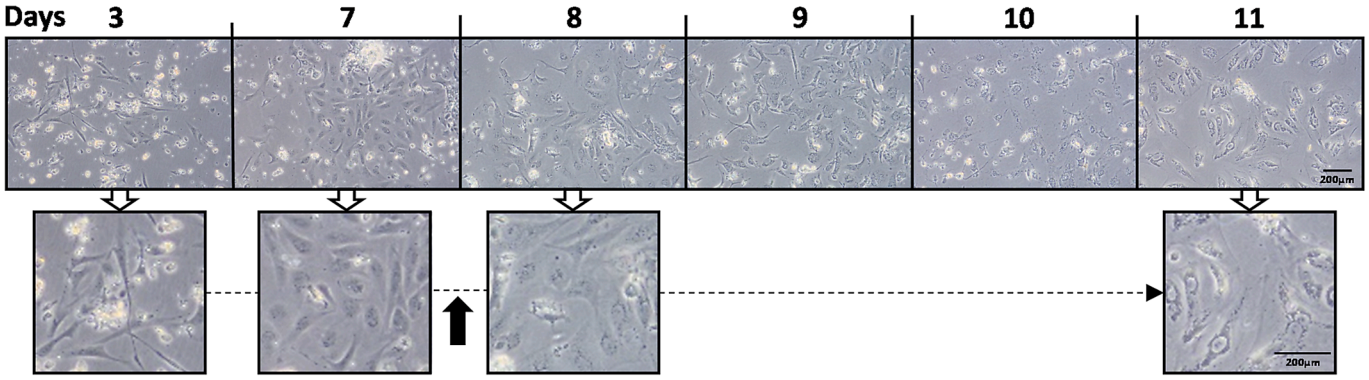

Fig. 1. Representative phase-contrast images (20× magnification) and enlarged views (50×) of rainbow trout adipocytes at different days of culture from day 3 to day 11. The moment of addition of the media to induce the differentiation of precursor cells into adipocytes is indicated by a black arrow.

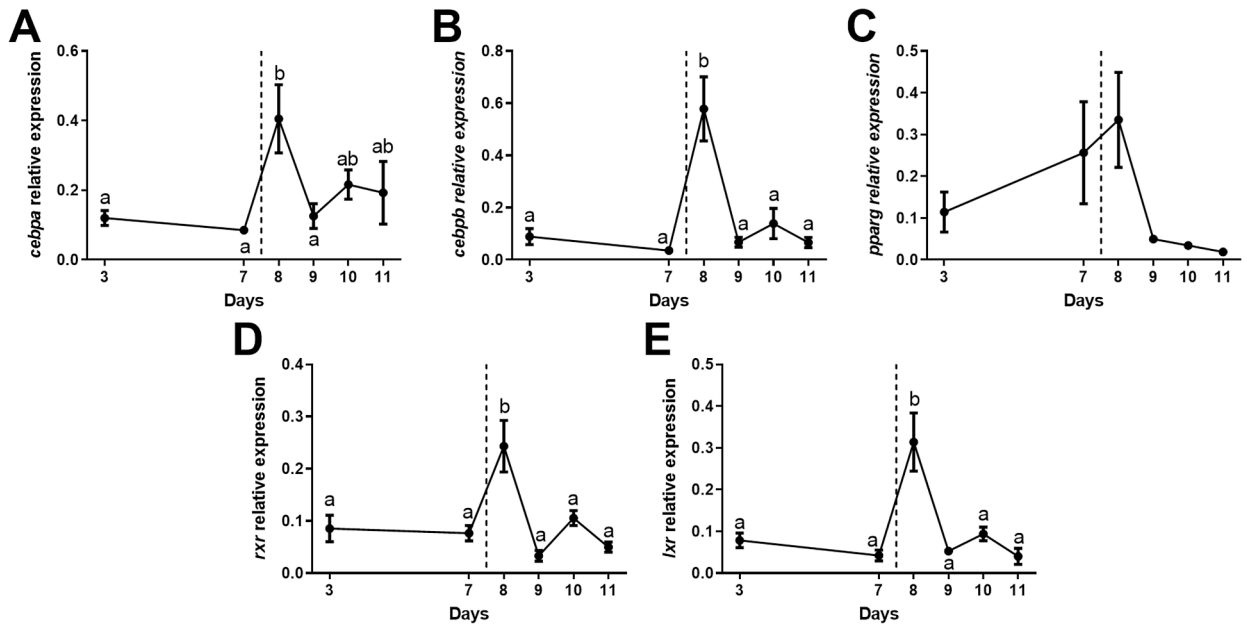

Fig. 2. Relative expression of genes coding for transcription factors (A) *cebpa*, (B) *cebpb*, (C) *pparg*, (D) *rxr*, and (E) *lxr*, in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM ($n = 4$–8). Significant differences ($p < 0.05$) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

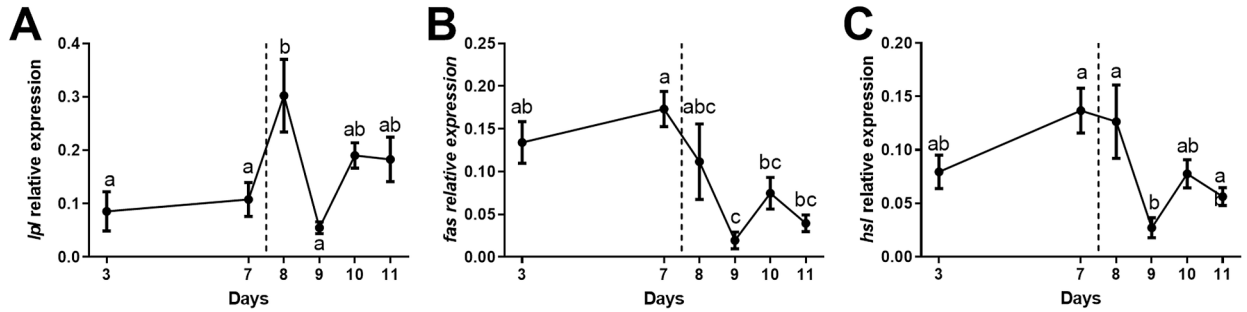

Fig. 3. Relative expression of genes coding for enzymes related to lipid metabolism (A) *lpl*, (B) *fas*, and (C) *hsl* in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM ($n = 4$–8). Significant differences ($p < 0.05$) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

between days 3 and 9 were observed for fatty acid transporter protein 1 (*fatp1*) with a subsequent significant upregulation at day 10 and a decrease at day 11 (Fig. 4B). Expression of cluster of differentiation (*cd36*) remained unaltered from days 3 to 8, to then progressively increase up to day 11 (Fig. 4C). A similar pattern of expression was observed for *plin2* mRNA levels, which remained low at the preadipocyte stage from days 3 to 7 to significantly increase at day 9 and continue to rise, up to day 11 (Fig. 4D).

3.2. Involvement of autophagy in adipocyte differentiation

Furthermore, autophagy-related genes relative expression was also analysed. Neither *p62* nor *beclin1* mRNA levels showed significant differences along the culture time (Fig. 5A, B). *atg4b, gabarapl1* and *lc3b,* showed a significant increased expression at day 8, after the addition of differentiation medium, and then were rapidly downregulated to basal levels (Fig. 5C-E). On the other hand, *atg12l* expression levels remained low prior to increase significantly at day 10 and were downregulated again at day 11 (Fig. 5F). Hence, autophagy-related genes showed specific different patterns of expression during adipocyte differentiation, although most of them presented a peak similar to the adipogenesis-related genes corresponding to the onset of differentiation.

Thereafter, to obtain more information about the possible connection between autophagy and adipocyte differentiation, the protein levels of LC3-I and LC3-II were analysed at day 7 of culture development in the absence or presence of specific inhibitors (24 h treatment). BafA1 (20 nM) incubation significantly increased the levels of LC3-II compared to the control condition (DMSO), while the induction observed with CQ (20 μM) treatment did not reach a significant value (Fig. 6A, B).

Then, the effect of both treatments in cell viability was evaluated, and although the two autophagy inhibitors seemed to slightly decrease it, BafA1 (0.094 ± 0.022) and CQ (0.074 ± 0.017) *versus* control cells (0.134 ± 0.029), the results did not show any significant differences. At the same time, lipid content was quantified and slightly lower lipid levels were observed upon either BafA1 or CQ incubations, but these differences were again not significantly different compared to the control (Fig. 6C). Pictures of cells after 24 h of DMSO treatment (control condition) reflected the lipid accumulation (stained in red) and rounded shape of the cells, characteristic of preadipocytes differentiation. On the other hand, after the autophagy blocking treatments, although the cells also presented intracellular lipids, they showed a more spindle morphology when compared to the control, and indeed, significant morphological differences were observed since the percentage of cells with a round shape in BafA1 and CQ-treated cultures was lower

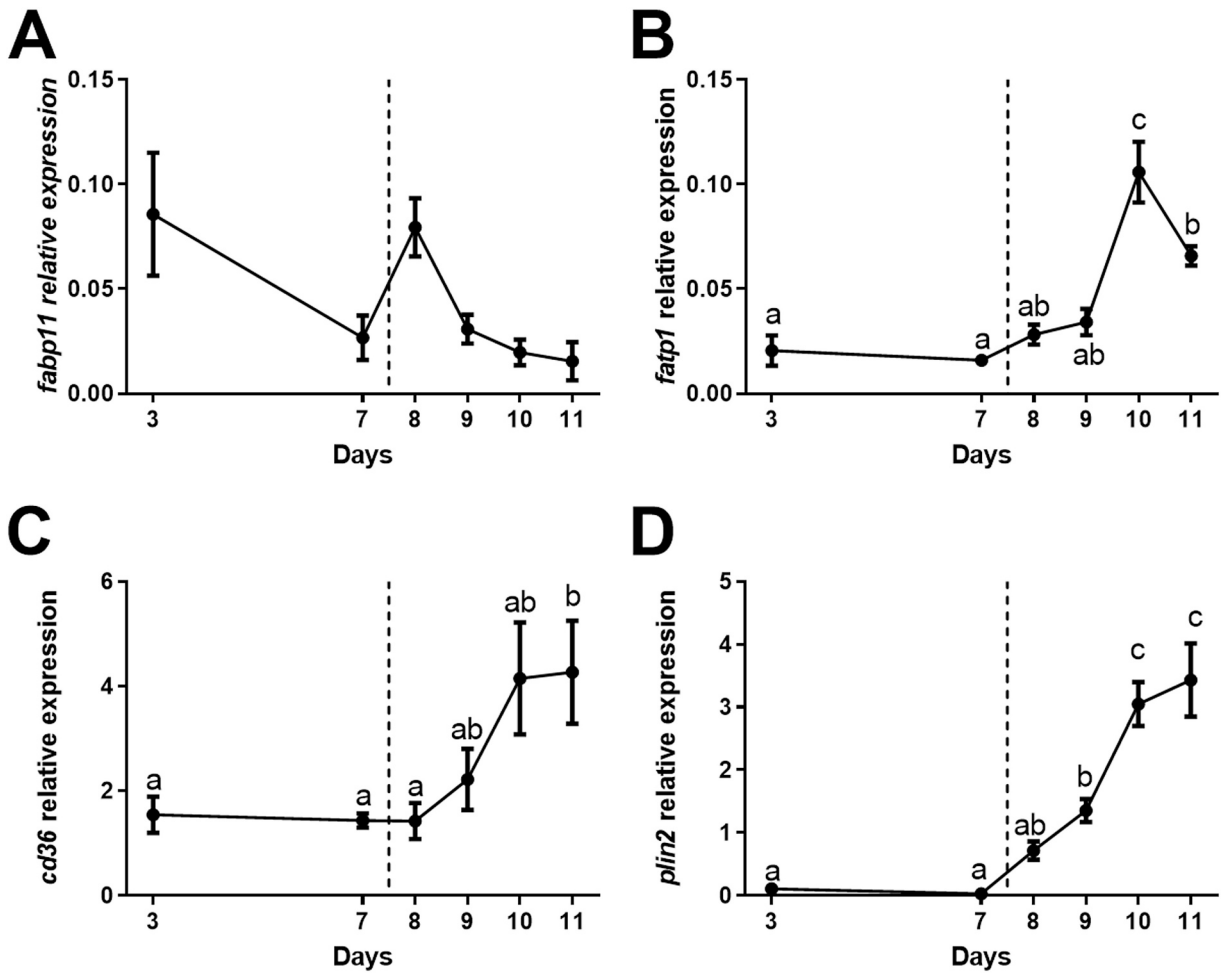

Fig. 4. Relative expression of genes coding for fatty acid transporters and proteins related to lipid binding (A) *fabp11a*, (B) *fatp1*, (C) *cd36* and (D) *plin2* in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM ($n = 4$–8). Significant differences ($p < 0.05$) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

compared to the control condition (Fig. 6D, E).

Afterwards, to further study the outcomes of autophagy inhibition during adipogenesis, cells were incubated at day 7 with BafA1 and CQ for a short period but at high doses (100 nM and 40 μM, respectively) and the posterior analyses were performed after 4 days. Regarding cell viability, significant differences were not observed between control (0.040 ± 0.011) and BafA1 (0.028 ± 0.007) or CQ (0.024 ± 0.005) treated cells. Furthermore, significant differences were neither observed after quantification of lipid content upon treatments, when compared to the control condition (Fig. 7A). Nevertheless, concerning morphology, incubation with either one of the two inhibitors showed at day 11 differences in shape, with a higher percentage of rounded adipocytes in the control, compared to cells treated with BafA1 or CQ (Fig. 7B, C). In any case, although delayed, data suggested that 72 h of recovery after 8 h blocking treatment allowed the cells to resume differentiation.

To determine the effects on gene expression following transient autophagy inhibition, adipocyte cells were again treated 8 h with BafA1 at day 7 and evaluated at day 11. The key transcription factors driving the differentiation process *cebpa* and *pparg* showed lower transcript levels compared to the control condition (Fig. 8A). On the contrary, *lxr* presented higher levels in the cells incubated with the autophagy disruptor BafA1. In the case of the genes related with lipid metabolism, while *lpl* expression was significantly downregulated after the incubation with BafA1, *fas* was upregulated when compared to the control. Moreover, significant differences were found in genes related to lipid transport, being *fatp1* and *cd36* mRNAs upregulated in the BafA1 group. Concerning genes involved in autophagy, the incubation with BafA1 significanly increased the expression of *p62*, *atg4b*, *gabarapl1* and *atg12l* compared to the control condition (Fig. 8B).

4. Discussion

Precursor cells isolated from rainbow trout adipose tissue can be differentiated into mature adipocytes when the media is supplemented with dexamethasone, IBMX, insulin and a lipid mixture (Bouraoui et al., 2008). In mammals, insulin is a powerful inductor of this process through activating *pparg* (Klemm et al., 2001; Nadeau and Draznin, 2004), while the activation of *cebpb* and *cebpd* by IBMX and dexamethasone, induces in turn, the expression of *cebpa* and *pparg* as well (Chen et al., 2016). In rainbow trout and red sea bream (*Pagrus major*), insulin favours differentiation of cultured preadipocytes, but most of the studies in fish cells have shown a high dependence on the addition of lipids (Bouraoui et al., 2008, 2012; Oku et al., 2006; Oku and Umino, 2008). In the present study, we confirm a cell morphology transition from preadipocytes into mature cells after the addition of the lipid-containing differentiation media. The cells became more rounded with an enlarged cytoplasm, and they lost the fibroblastic shape, indicating the start of visible adipogenic features with the subsequent lipid accumulation as previously observed in murine cells, and other fish species like Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*) (Mor-Yossef Moldovan et al., 2018; Vegusdal et al., 2003; Salmerón et al., 2013).

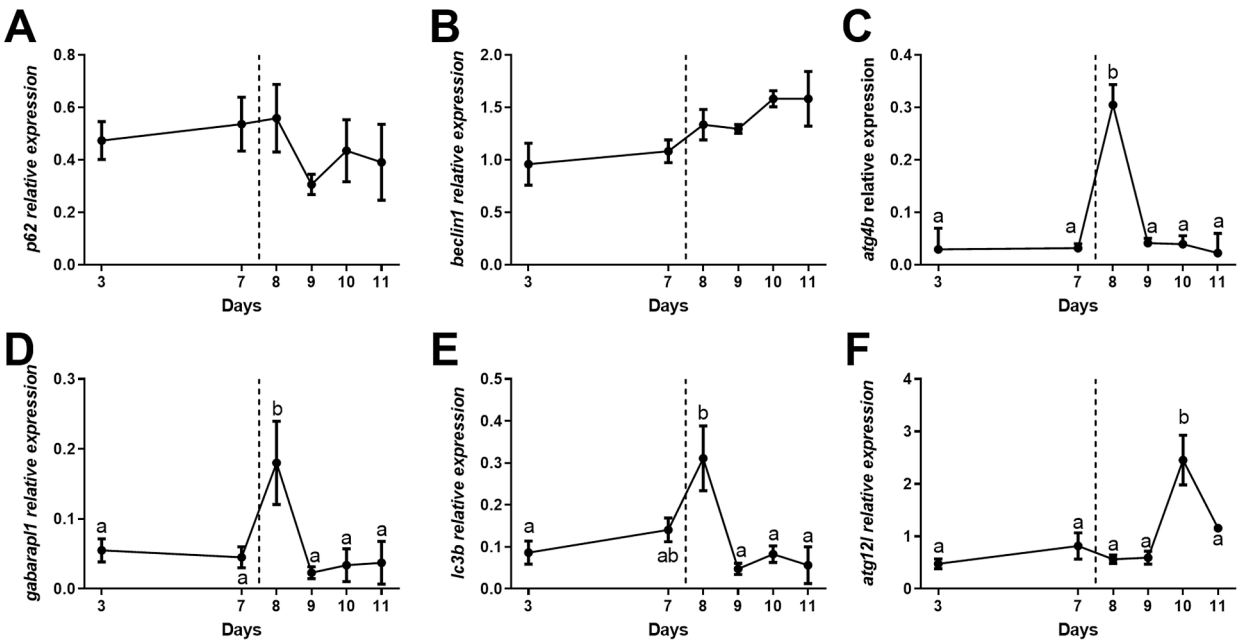

Fig. 5. Relative expression of genes related to the process of autophagy (A) *p62*, (B) *beclin1*, (C) *atg4b*, (D) *gabarapl1*, (E) *lc3b*, and (F) *atg12l* in rainbow trout adipocytes at different days of culture. Data are shown as mean ± SEM ($n = 4$–8). Significant differences ($p < 0.05$) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

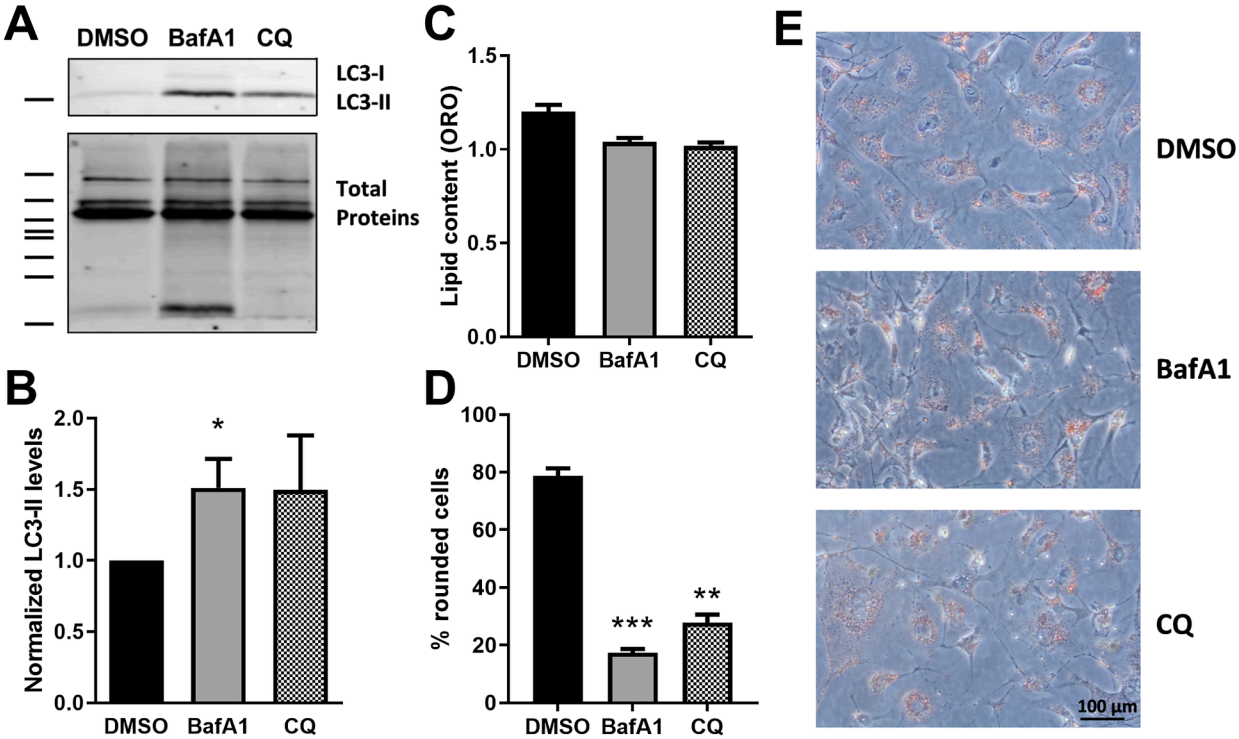

Fig. 6. (A) Representative Western blot (WB) bands of LC3-I, LC3-II, and total proteins and, (B) quantification of LC3-II protein levels in relation to total proteins, (C) quantification of lipid content after Oil Red O (ORO) staining, (D) percentage of rounded cells and, (E) representative phase-contrast images of rainbow trout adipocytes at day 7 (stained with ORO showing lipid droplets in red) after being treated during 24 h with bafilomycin (BafA1) 20 nM, chloroquine (CQ) 20 μM or dimethilsulfoxide (DMSO) 0.2% as control. Data are shown as mean + SEM (WB $n = 6$; ORO n = 6; rounded cells n = 4). Significant differences are considered at p < 0.05*; $p < 0.01$**; $p < 0.001$***. Magnification 20 × . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Adipogenesis is known to be a complex process that concerns the integration of many different signalling pathways and transcription factors (Rosen and Macdougald, 2006). Previously, the transcriptomic profile of cultured rainbow trout preadipocytes by microarray was performed (Bou et al., 2017), pointing out the relevance of PPARγ, and abundance of PLIN2, during the differentiation phase; however, a low number of culture days were analysed. In the present research, the time window in which the induction media is added to the cells was assessed

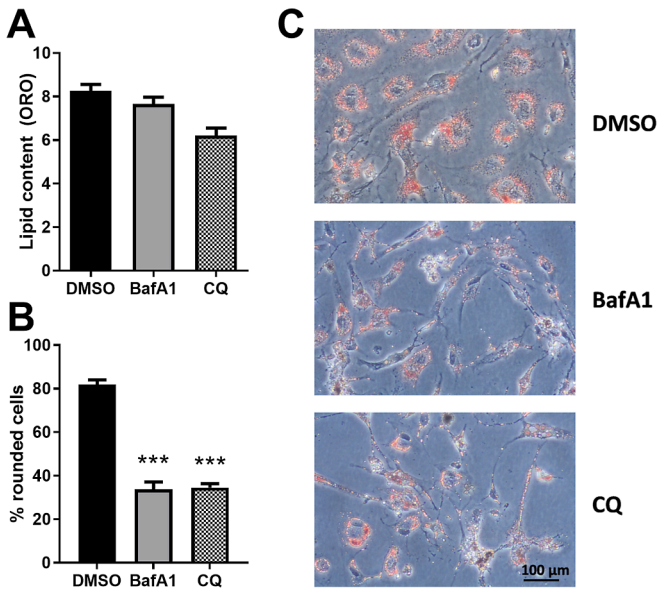

Fig. 7. (A) Quantification of lipid content after Oil Red O (ORO) staining, (B) percentage of rounded cells and (C) representative phase-contrast images of rainbow trout adipocytes at day 11 (stained with ORO showing lipid droplets in red) after being treated at day 7 during 8 h with bafilomycin (BafA1) 100 nM, chloroquine (CQ) 40 µM or dimethilsulfoxide (DMSO) 0.2% (as control) and incubated with new media for further 72 h. Data are shown as mean + SEM (ORO $n = 4–7$; rounded cells n = 4–6). Significant differences are considered at $p < 0.05$; $p < 0.001$***. Magnification 20 × . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

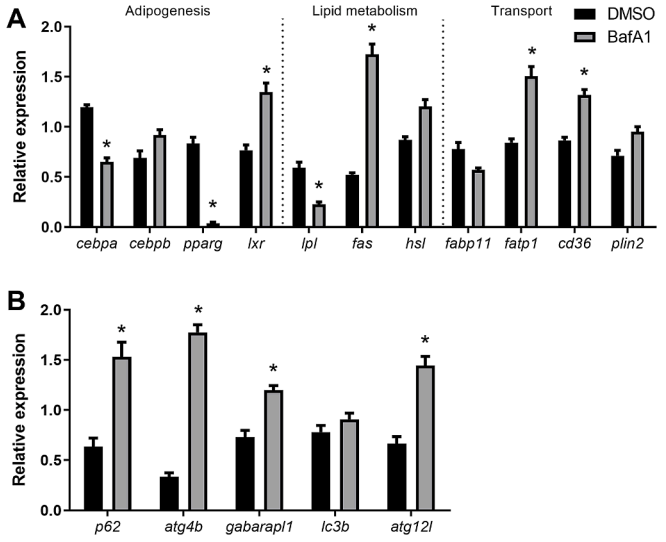

Fig. 8. Relative expression of genes related to (A) adipogenesis, lipid metabolism and transport, and (B) autophagy in rainbow trout adipocytes at day 11 after being treated at day 7 during 8 h with bafilomycin (BafA1) 100 nM or dimethilsulfoxide (DMSO) 0.2% as control and incubated with new media for further 72 h. Data are shown as mean + SEM (n = 4–6). Significant differences are considered at $p < 0.05$. Non-detectable expression was found for rxr.

to better characterize the onset of differentiation. The expression of the key transcription factors, *cebpa, cebpb,* and *rxr*, increased transiently just 24 h later, consequently activating adipocytes differentiation, in agreement with data reported from mammalian models (Cao et al., 1991), and other salmonids (Huang et al., 2010; Todorčević et al., 2010; Vegusdal et al., 2003; Bouraoui et al., 2008). Otherwise, *pparg* showed a slightly different profile of expression, mirroring that in Atlantic salmon preadipocytes, since the cells already expressed *pparg* before the induction of differentiation, possibly indicating that are fated to be adipocytes (Todorčević et al., 2010). In contrast, the expression of *pparg* remained unaffected during the adipogenic process in red sea bream (Oku and Umino, 2008), suggesting transcriptional regulation of *pparg* seems to be very sensitive to the concrete adipogenic cell conditions with species-specific dynamics.

Next, once differentiation is committed, the expression of genes characteristic of the mature adipocyte phenotype is modulated. In the present study, *lpl* showed an increased expression after adipocyte differentiation, with a subsequent decrease for the rest of the process, as occurred with the main adipogenic transcription factors, whereas *fas* and *hsl* presented a similar pattern post-differentiation, although conversely their levels of expression were elevated during the proliferation phase. These results are supported by some studies (Salmerón et al., 2016), but no others (Todorčević et al., 2010; Oku and Umino, 2008), but among studies the evaluated times were not always the same, which may explain differences in gene expression. Another possibility is that the expression of these genes may be modulated by the differentiation media composition, in which the presence of lipids makes fatty acids directly available to the cells.

Concerning lipid transport-related genes, a peak of increased expression of *fatp1* was found at day 10, while *cd36* increased steadily up to day 11, indicating that the cell is properly maturing and prepared to uptake and store lipids as previously shown (Todorčević et al., 2008; Huang et al., 2010; Sánchez-Gurmaches et al., 2012). In fact, *plin2* was expressed at highest levels also at days 10 and 11, in agreement with its essential role for lipid accumulation in 3T3-L1 adipocytes due to its presence in the lipid droplet (Love et al., 2015). Thus overall, all these genes can be considered key markers of adipocytes' maturation and fundamental for the increase in intracellular lipid content that occurs during adipogenesis. Concluding, the transcriptomic analysis along cell culture during the initial days after induction of differentiation has permitted us to characterize more in detail the transitory changes in gene expression that occur at the onset of the adipogenic process in rainbow trout.

Altogether, adipogenesis can be recognized as a complex process that is accompanied by dramatic changes in cell morphology (Rosen and MacDougald, 2006; Rutkowski et al., 2015). Moreover, the transient activation of various transcription factors seem to be crucial to trigger adipogenesis, although in mammals, it is known that other factors, such as those associated with oxidative stress and the production of reactive oxygen species (ROS), inflammation, or autophagic processes, can modulate adipogenesis (Ghaben and Scherer, 2019). In this regard, the transcriptomic analysis during *in vitro* differentiation of adipocytes from rainbow trout reported the expression of some key autophagy genes (Bou et al., 2017); however, whether those molecules have a role in fish adipogenesis is totally unknown.

In the present study, we have observed a parallel pattern between the increased gene expression of some autophagy-related genes and the induction of adipogenesis. Indeed, genes coding for proteins that are involved in the autophagosome formation (*atg4b, gabarapl1* and *lc3b*) were upregulated after induction of differentiation, in parallel with adipogenic transcription factors (*cebpa, cebpb, rxr* and *lxr*). These results are in concordance with those found in mammals (Zhang et al., 2012; Clemente-Postigo et al., 2020), suggesting that adipocyte differentiation is highly dependent on autophagy. In fact, it has been observed that the depletion of autophagy genes (*i.e.*, *atg7* or *beclin1*) and the inhibition of autophagy reduce adipogenesis *in vivo* and *in vitro* (Ferhat et al., 2019; Singh et al., 2009). In the present study with rainbow trout adipocytes, the inhibition of the autophagic process through the incubation with BafA1 or CQ slightly affected cell viability and lipid accumulation when compared to the control condition, supporting a moderate impairment over lipid accumulation and thus, adipocyte maturation. In agreement to that, when autophagy was blocked, a delay in the adipogenic process

could be inferred when looking at the cells, as they presented a significant lower percentage of rounded shape cells after the inhibitors' treatments than in the control cells. Similarly, in 3T3-L1 adipocytes, autophagy inhibition during the first two days of differentiation blocked both, phenotype changes and gene expression (*i.e.*, *pparg*), whereas inhibition at later days had only a marginal effect (Skop et al., 2014). Indeed, the loss of autophagy in an ATG4 knockout mouse model clearly led to a strong reduction in adipocyte size, probably associated with decreased lipid store in these cells (Singh et al., 2009). Even in some cases, it has been reported that the absence of autophagy in mice could led to animals that cannot produce fully differentiated adipocytes and fail to form normal depots of white adipose tissue (Cai et al., 2018). In this line, it is considered that autophagy participates in cellular remodelling as part of the dramatic cytoplasmic reorganization that occurs during differentiation (Zhang et al., 2012). It has been suggested that the clearance of the cytosolic components, such as organelles, to obtain space for the growing lipid droplets could be one of the possible roles of autophagy in adipocytes (Ferhat et al., 2019). According to this, autophagy inhibition reduced mitochondrial remodelling in 3T3-L1 adipocytes (Skop et al., 2014), and a higher number of mitochondria was observed in ATG7 deficient adipocytes when compared to control cells (Zhang et al., 2009). However, the autophagy blockages in these knockout models and cell culture systems are substantially different, which would explain more drastic effects. Impaired autophagy in the ATG4 and ATG7 knockout mice is on a permanent basis, disrupting autophagy and autophagosome cycling in fat constantly, while the present results from a transient blockade of autophagy appear to differ from the effects of a long-term autophagy disruption. With a transient inhibition with BafA1, during the initiation of adipogenesis, the balance between accumulation and use of autophagosomes is altered, and it could even lead to the contradictory situation of a more efficient cell differentiation (Nuschke et al., 2014).

Furthermore, different studies in mammals have developed the idea that autophagy may be required for the progression of adipogenesis (Zhang et al., 2012, 2013), but also, that adipogenic factors can regulate autophagy genes either directly or indirectly during adipogenesis (Ahmed et al., 2019). Some authors suggested a feed-forward loop between activation of autophagy and PPARγ2, as the activation of the latter has been shown to stimulate autophagy (Zhou et al., 2009). They found that an inhibition of autophagy potentially reduces PPARγ2 protein expression and stability thus, blocking adipogenic differentiation. It has been proposed that ahead of a differentiation signal, the autophagy-lysosome-dependent system is activated, and this interferes with the proteasome-dependent PPARγ2 degradation, which then stabilizes PPARγ2 protein and induces adipogenesis (Zhang et al., 2012, 2013). This described mechanism could explain why in our study, the gene expression level of *pparg* was downregulated when autophagy was disrupted with BafA1. Similar results were observed in 3T3-L1 adipocytes after 2 days of autophagy inhibition at the onset of differentiation (Skop et al., 2014).

Moreover, the transcriptional up-regulation of *atg4b* by C/EBPβ is another mechanism recently described involving the induction of autophagy during adipogenesis (Guo et al., 2013; Ferhat et al., 2019). Those studies showed that autophagy is necessary for the degradation of C/EBPβ-inhibitors (through their ubiquitination by P62), which directly acts through C/EBPβ-mediated induction of *atg4b* expression. In this sense, the gene expression pattern observed during rainbow trout adipocyte differentiation in the present study could support this theory. We found an upregulation of *atg4b* after the induction of adipogenesis while *cebpb* was also upregulated. Interestingly, *p62* gene expression levels remained high at the onset of differentiation but slightly decreased over time. In contrast, after incubation of the cells with BafA1, most of the autophagy-related genes expression levels were upregulated without parallelism with adipogenic transcription factors' gene expression. These differences may be explained as compensatory mechanisms induced by the inhibition of autophagy flux by BafA1. Although it is known that BafA1 produces a strong disruption of autophagy, we do not know about the persistence over time of this blockage in our system. On the other hand, it seems that the initial disruption of the autophagic flux by BafA1 still maintains a decrease in the expression of some key adipogenic genes such as *cebpa* and *pparg*. Observing the increased levels of *fas* and the fatty acid transporters (*i.e.*, *cd36* and *fatp1*) in BafA1 treated cells, we could hypothesize that these adipocytes have a delay in differentiation when compared to control cells, as they seem to be in the lipid accumulation stage, whereas the cellular remodelling to accommodate more fat (*i.e.*, rounding of the cells) is behind. Thus, one of our suggestions is that the media renewal after the 8 h treatment indicates to the cells that adipogenesis is again on, and consequently, the cells resume the normal process of differentiation after the suspension, although with a certain delay. We cannot discard either a direct effect of BafA1 on lipid metabolism, independently of the process of adipocyte differentiation, as this inhibitor increased mRNA levels of genes involved in regulating intracellular lipid stores, such as *fas* and *plin2* in rainbow trout hepatocytes (Séité et al., 2019). In fact, it is considered that inhibition of autophagy flux can induce changes in cell lipid metabolism directly or through induction of endoplasmic reticulum stress (Rutkowski et al., 2008).

In summary, specific rainbow trout adipocyte markers have been identified both, at the onset of differentiation and later at the maturation phase. Such transient increase of adipogenic-related genes expression occurred in parallel with autophagic genes in the early differentiation phase, suggesting a simultaneous activation of both processes upon addition of the induction media. Moreover, the results support a connection between adipogenesis and autophagy in preadipocytes since the blocking of autophagy seems to affect the progression of the differentiation process (*i.e.*, cell remodelling). Overall, this study highlights for the first time in fish the co-regulation of autophagy and adipogenesis and contributes to improve our knowledge on both processes. According to the literature from different mammalian species, these results support the hypothesis that autophagy is involved in adipogenesis, and that such relationship might also occur in fish. Nonetheless, further studies are required to confirm these pioneering observations in rainbow trout adipocytes.

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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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