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RESEARCH ARTICLE

Expression and distribution of leptin and its receptors in the digestive tract of DIO (diet-induced obese) zebrafish

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ABSTRACT

The expression and localization of leptin (A and B) and its receptor family in control and diet-induced obese (DIO) adult male zebrafish gut, after 5-weeks overfeeding, administering Artemia nauplii, as fat-rich food, were investigated. Recently, the obese adult zebrafish was considered an experimental model with pathophysiological pathways similar to mammalian obesity. Currently, there are no reports about leptin in fish obesity, or in a state of altered energy balance. By qRT-PCR, leptin A and leptin B expression levels were significantly higher in DIO zebrafish gut than in the control group (CTRL), and the lowest levels of leptin receptor mRNA appeared in DIO zebrafish gut. The presence of leptin and its receptor proteins in the intestinal tract was detected by western blot analysis in both control and DIO zebrafish. By single immunohistochemical staining, leptin and leptin receptor immunoreactive endocrine cells were identified in the intestinal tract either in DIO or control zebrafish. Moreover, leptin immunopositive enteric nervous system elements were observed in both groups. By double immunohistochemical staining, leptin and its receptor were colocalized especially in DIO zebrafish. Thus, our study represents a starting point in the investigation of a possible involvement of leptin in control of energy homeostasis in control and DIO zebrafish.

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1. Introduction

In recent years, obesity has become a social disease, not only due to defective genes, but also to an unhealthy lifestyle, characterized by an excessive calorie intake lacking physical activity. This condition leads to an increase in adipose tissue and correspondingly increased levels of circulating leptin in humans (Al-Hamodi et al., 2014). Leptin, a cytokine-like peptide, was first cloned in *ob/ob* mice in 1994 (Zhang et al., 1994), as the factor responsible for the morbid obesity of *ob/ob* mutant mice. In fish, leptin has been cloned from several species: pupperfish (*Takifugu rubripes*) (Kurokawa et al., 2005), zebrafish (*Danio rerio*) (Huising et al., 2006), atlantic salmon (*Salmo salar*) (Rønnestad et al., 2010), marine medaka (*Oryzias latipes*) (Wong et al., 2007), tilapia (*Oreochromis niloticus*) (Shpilman et al., 2014), striped bass (*Morone saxatilis*) (Won et al., 2012), Ya-fish (*Schizothorax prenanti*) (Yuan et al., 2014) and red-bellied piranha (*Pygocentrus nattereri*) (Volkoff, 2015), among others. In particular, two leptin paralogues, LepA and LepB, have been found in some fish lineages, such as zebrafish and pufferfish, demonstrating that they originated early in teleost, an evolution following a first duplication event of the whole genome (Gorissen et al., 2009; Prokop et al., 2012). In mammals, leptin is synthetized not only by adipose tissue but also by the digestive tract (Zhang et al., 1994; Bado et al., 1998; Sobhani et al., 2000; Cinti et al., 2000; Cammisotto et al., 2005; Cammisotto and Bendayan, 2012; Russo et al., 2011; Park and Ahima, 2014) among other tissues. In fish, gut (Bosi et al., 2004; Ronnestad et al., 2010; Liu et al., 2010; Russo et al., 2011; Varricchio et al., 2012) and liver (Huising et al., 2006; Murashita et al., 2008; Trombley et al., 2012; Zhang et al., 2013) are an important source of leptin. Moreover, different studies reported the presence of leptin and its receptors in several peripheral organs (Kurokawa et al., 2005; Ronnestad et al., 2010; Liu et al., 2010; Copeland et al., 2011; Gong et al., 2013).

Particularly in zebrafish, leptin presents a differential expression pattern: leptin A is expressed in the liver and in the gut, while

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leptin B is more expressed in the ovary (Gorissen et al., 2009; Gorissen and Flik, 2014). In addition, although the "classical" role of leptin is conserved between both isoforms, they are also pleiotropic and act redundantly or independently in a leptin network, allowing zebrafish to maintain equilibrium in the face of challenges to homeostasis (Gorissen et al., 2009; Gorissen and Flik, 2014; Conde-Sieira and Soengas, 2017). In spite of this abundant literature, there is still much to uncover in this respect.

In mammals, leptin acts through the leptin receptors (OB-Rs proteins), located in the plasma membrane. According to the length of the intracellular domain, in mammals the OB-Rs are divided into one long isoform (OB-Rb) and four short isoforms (OB-Ra, c, d, f) (Cinti et al., 2000). There is also one soluble isoform (OB-Re), which lacks the transmembrane domain and may be involved in the blood leptin transport. Moreover, Rønnestad et al. (2010) reported that the longest isoform of leptin receptor is the only form that includes both transmembrane and intracellular segments, essential for signal transduction and conserved among vertebrate leptin receptors. It is well known that, in mammals, leptin receptor isoforms show the same extracellular domain and the same affinity for leptin (Chen et al., 1996; Lee et al., 1996). These receptor isoforms were identified both in the central nervous system and peripheral organs (Cammisotto et al., 2010). Recently, isoforms of leptin receptors were also detected in zebrafish (Liu et al., 2010) and other fish species (Rønnestad et al., 2010; Prokop et al., 2012; Tinoco et al., 2012; Gong et al., 2013).

Leptin in mammals acts on the brain to regulate food intake and metabolism (Zhang et al., 1994; Pelleymounter et al., 1995). The actions of leptin occur over both short- and long-term. In the short term, plasma leptin serves as satiety signal (Ahima, 2005) and, over long periods, daily mean plasma leptin concentration communicates long-term energy status to the brain (Chehab et al., 1997). Less is known about leptin action on energy homeostasis in fish. Certainly, in goldfish, leptin potentiates the actions of the anorexigenic factors CART and CCK and inhibits the action of the orexigenic peptides NPY and orexin A (Volkoff et al., 2003), it increases fat metabolism in green sunfish (Londraville and Duvall, 2002) and acts in dose- and time-dependent manner on glucosensing and gene expression of neuropeptides involved in food intake in rainbow trout hypothalamus and hindbrain by in vitro study (Aguilar et al., 2011). Also, zebrafish lacking a functional leptin receptor have alterations in insulin and glucose levels, suggesting a role of leptin in the control of glucose homeostasis (Michel et al., 2016).

Among studied fish, zebrafish is considered an attractive simple vertebrate model (D'Angelo et al., 2016a,b) for its well-conserved organization of common organs and tissues with mammals, including the intestinal system (Harper and Lawrence, 2010; Wang et al., 2010; Goldsmith and Jobin, 2012). For these reasons, in the last decade, numerous gastrointestinal pathologies have been modeled in zebrafish (Goldsmith and Jobin, 2012). This animal model was recently used to investigate metabolic parameters such as body weight, adiposity and energy expenditure (Farber et al., 2001; Jones et al., 2008; Flynn et al., 2009; Anderson et al., 2011; Craig and Moon, 2011; Seth et al., 2013), as well as Oka et al. (2010) who used zebrafish to create a fish model for the analysis of diet-induced obesity (DIO) under conditions of high fat intake. The aim of our study was to investigate the expression (by qRT-PCR and western blot analysis) and distribution (by single and double immunostaining) of leptin, and its family receptors in the digestive tract of control (CTRL) and diet-induced obesity (DIO) adult zebrafish, in order to extend our knowledge on leptin system in this animal model species. In fact, in fish, this peptide is also expressed in the intestinal tract, where this hormone is involved in the regulation of several physiological processes, including food intake and metabolism, among other functions (Volkoff, 2015). In detail, we evaluated the expression of leptin A and leptin B in the digestive tract of CTRL and DIO zebrafish using a protocol according to Gorissen et al. (2009) who reported an involvement of these isoforms in zebrafish food intake.

2. Material and methods

2.1. Animal husbandry and experimental design: ethics statement

The experimental protocol was in accordance with the principle outlined in the Declaration of Helsinki and with the National law regarding the care and use of laboratory animals. The Italian Ministry of Health has approved the experimental protocol.

2.2. Zebrafish breeding, tissue treatment, body mass index (BMI) and body weight measurements

Adult male zebrafish (n = 30) were obtained from the breeding colony at the C.I.S.S. (Centre of Experimental Ichthyopathology of Sicily, Department of Veterinary Science, University of Messina). Fish were maintained at 28.5 °C and fed once a day, with a 14:10 h L:D cycle. The fish were divided into two dietary groups (n = 15 fish per group). The control group was fed with the equivalent of 20 mg cysts/fish/day (once a day) of freshly hatched Artemia for 5 weeks; the DIO group was fed with the equivalent of 60 mg cysts/fish/day (3 times a day, 20 mg cysts/fish each time) of freshly hatched Artemia for the same period. In this study, zebrafish were separated in different tanks (1 zebrafish per 1-l tank), according to the protocols by Montalbano et al. (2016). During the period of treatment, in order to analyze the increase in body mass index (BMI) levels, the standard length and the body weight of every fish were measured each week. Body weight was determined by weighing fish with a precision analytical scale. Body length (as standard length) was determined from the tip of the mouth to the caudal peduncle with a digital precision caliper. BMI was calculated by dividing body weight (in g) for the body length squared (in cm^2), as previously described in Montalbano et al. (2016). After 5 weeks of treatment, the fish were fasted for 24 h and then anaesthetized with MS 222 (ethyl 3-aminobenzoate methanesulfonate, 0.2 g/l; Sigma, Saint Louis, MO, USA). Successively, the animals' heads were removed from trunks and then the trunks were fixed for 24 h in Bouin's fixative at room temperature (RT), dehydrated in ascending series of alcohol and embedded in paraffin wax. Finally, 7–10 μm-thick cross and sagittal sections were routinely processed for light microscopy (Abbate et al., 2006, 2012a,b), morphometric study and immunohistochemical staining (n = 5 fish per group) (de Girolamo and Lucini, 2011), while the intestinal tracts of other fish were quickly processed for the isolation of RNA, semi-quantitative reverse PCR, and western blot analysis (n = 10 fish per group).

2.3. qRT-PCR: leptin A, leptin B and leptin receptor

Total RNA was extracted from the whole zebrafish intestine using a commercial kit (Trizol Reagent, Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. After precipitation and cold ethanol washing, RNA was dried and dissolved in an appropriate volume of Tris-EDTA buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA-Na_2). Each sample was treated with 1U of DNase I for 1 h at 37 °C to digest genomic DNA. The RNA was precipitated, washed, and dissolved again in the same buffer. RNA solution was quantified at 260 nm (Biomate 3, Thermo Electron Corporation, Waltham, MA, USA) and its purity was assessed by the ratio at 260/280 nm reading (Magnoli et al., 2012). We used the High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) and random hexamers, and 10 μg of total RNA to make cDNA following manufacturer's instructions. A 1 μg aliquot of the cDNA was used to detect leptin and leptin receptor expression using qRT-PCR.

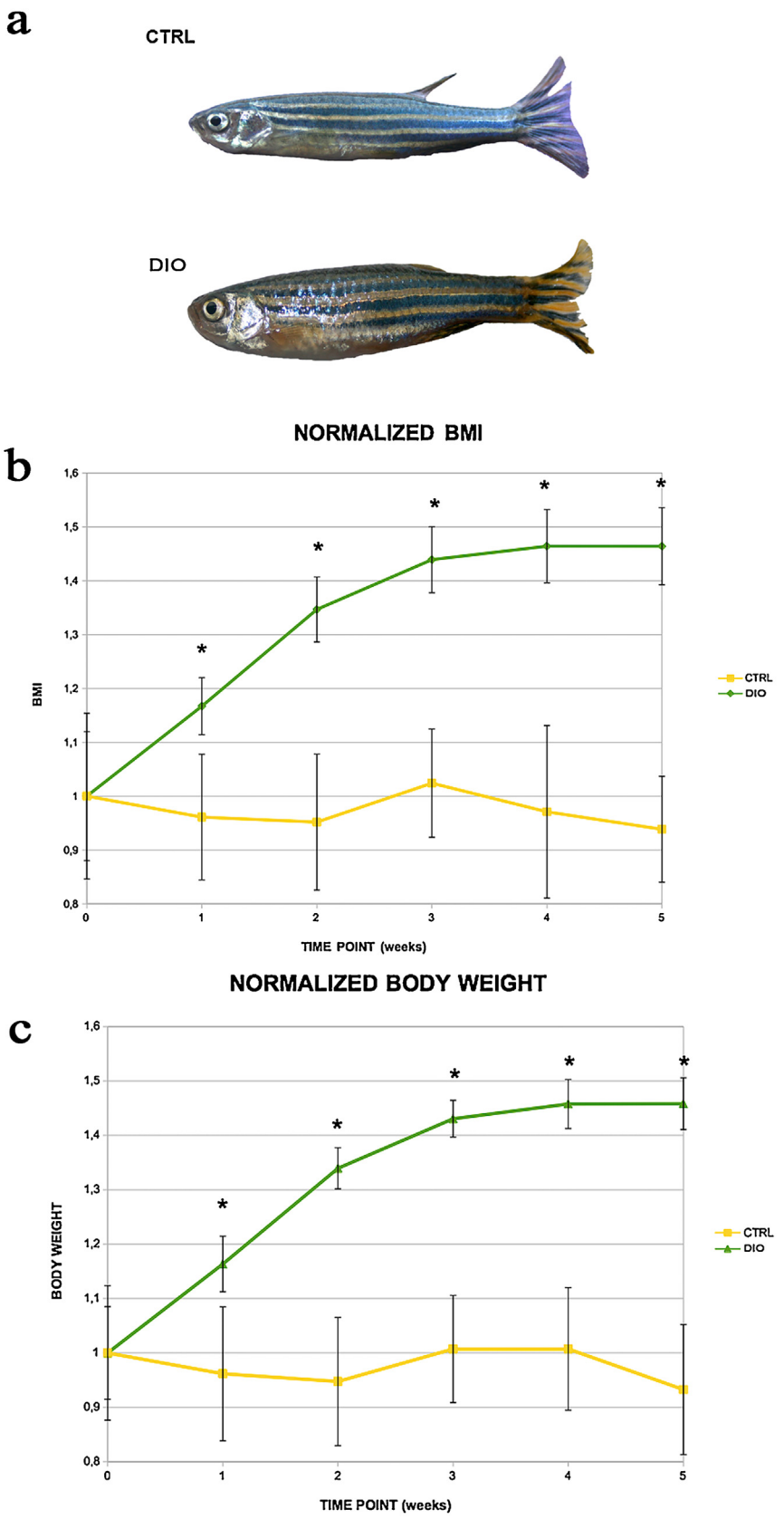

Fig. 1. (a) Control and diet-induced obese zebrafish. Pictures of a male adult control zebrafish (CTRL) and a diet-induce obese zebrafish (DIO). Scale bars = 1 cm:a–b; analysis of BMI (b) and body weight (c) in CRTL and DIO zebrafish. The results were obtained using 15 zebrafish per each experimental group and are expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) to compare the DIO and CTRL groups at each time-point. Values of $^{*}P \leq 0.05$ were considering significant.

Table 1
qRT-PCR primers. Sequences of forward and reverse primers designed for leptin A, leptin B, leptin receptor and ß-actin.

	Leptin a	Leptin b	Leptin receptor	β-Actin
FORWARD	5′-CATCATCGTCAGAATCAGGG-3′	5′-GATGAGCACTTCCAGATGTC-3′	5′-AAGTCTTCACAACGCAGGA-3′	5′-CACAGATCATGTTCGAGAGACC-3′
REVERSE	5′-ATCTCGGCGTATCTGGTCAA-3′	5′-TGTCTATGTTGAGGCAGAGC-3′	5′-ATAGGCGATGGAGCACATAG-3′	5′-GGTCAGGATCTTCATCAGGT-3′

The primers used were designed on the mRNA sequences published for leptin A, leptin B, leptin receptor and ß-actin (GenBank accession numbers: leptin A NM_001128576, leptin B NM_001030186, leptin receptor NM_001113376, β-actin NM_131031) (Table 1).

The homemade TaqMan probes were labeled at the 5′ with 6′ FAM fluorochromes for the rhodopsin, and VIC fluorochrome for β-actin, while the 3′ ends were labeled with the Minor Grow Binder (MGB) quencher. The qRT-PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using 5 pmol of each primer and 9 pmol of both target and β-actin probe. The assays were performed in triplicate using a 7500 PCR real-time System (Applied Biosystems, Foster City, CA, USA). The results were calculated through the $2^{-D\Delta Ct}$ algorithm against β-actin, and expressed as the n-fold difference compared to an arbitrary calibrator, chosen as a higher value than ΔΔCts.

2.4. Western blot analysis

Proteins from zebrafish intestinal tract and rat stomach (used as positive control) were pooled and extracted with RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) and 2 mM PMSF and protein cocktail (P8340 Sigma–Aldrich, Saint Louis, MO, USA) as protein inhibitors. Samples were homogenized with Ultra-Turrax T25 (IKA-Labortechnik, Staufer, Germany) at 13,500 rpm. Then the samples were spun at 10,000 rpm for 20 min at 4 °C and supernatants were collected. Protein concentrations were determined with the Bio-Rad dye protein assay. Samples were boiled at 98 °C for 10 min in loading buffer (50 mM Tris–HCl pH 6.8, 100 mM β-mercaptaethanol, 2% SDS, 0.1% blue bromophenol, 10% glycerol). The proteins were separated on a 15% (for leptin) and 12% (for leptin receptor) SDS-polyacrylamide gel electrophoresis with 4% and 16% stacking gel in 1% Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS [pH 8.3]) in a miniprotean cell (Bio-Rad, Laboratories In. UK) at 130 V for 2 h. The separated proteins were electrotransferred onto a nitrocellulose membrane with transfer buffer (39 mM Tris base, 0.2 M glycine, and 20% methanol [pH 8.5]) in a minitransfer cell (Bio-Rad) at 100 V for 2 h at 4 °C. Membranes were incubated at 4 °C for 1 h in blocking buffer containing 1% PBS and 0.05% Tween 20 with 5% dried non-fat milk and then were probed with polyclonal antibodies in rabbit that recognizes leptin (Ob, A-20, sc-842, Santa Cruz Biotec. Inc., Santa Cruz, CA, USA) and β-actin (A5060, Sigma) used as an internal marker and polyclonal antibody in goat that recognizes long (Ob-R, C-20, sc-1832, Santa Cruz Biotec. Inc., Santa Cruz, CA, USA) and short (Ob-R, M-18, sc-1834, Santa Cruz Biotec. Inc., Santa Cruz, CA, USA) isoforms of leptin receptor. All primary antibodies were diluted 1:500 and incubated overnight at 4 °C. This was followed by incubation with rabbit anti-goat IgG (1:5000, Millipore, Temecula, CA, USA) and goat anti-rabbit IgG (Sigma 1:5000) for 1 h at RT. Signals were detected by chemiluminescence with the Immobilon Western Chemiluminescent HRP substrate Kit (Millipore, Temecula, CA,

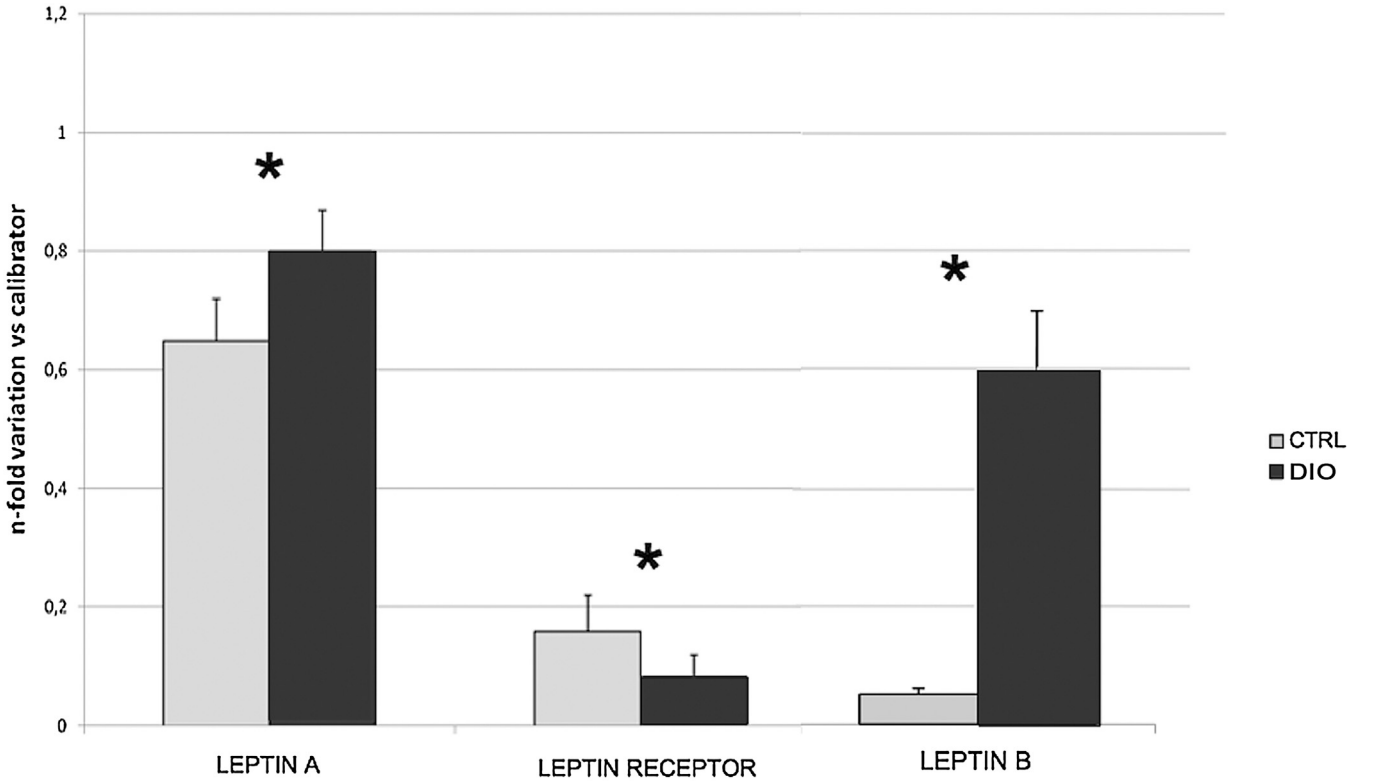

Fig. 2. qRT-PCR. The bar graph shows expression of leptin A, leptin B, and its receptor mRNA in zebrafish gut of two experimental groups. The results were obtained using 5 zebrafish per each experimental group and are expressed as mean ± SEM. Statistical analyses were done by Student's t test. Values of $^*P \leq 0.05$ were considered significant.

USA). A pre-stained molecular-weight ladder (Novex Sharp protein standard, LC5800, Invitrogen, Hilden, Germany) was used to determine protein size.

Specificity was determined by pre-absorption of primary antibodies with their relative control peptides before western blotting.

2.5. Single immunohistochemistry

Cross and sagittal sections were stained by the avidin-biotin immunohistochemical technique. In the specific step, polyclonal antibodies raised in rabbit against leptin, and polyclonal antibodies raised in goat against long and short isoform of leptin receptor were used. The antibodies were diluted 1:300 and applied on sections overnight at 4 °C. The other components of the immunological reaction were contained in the Vectastain Elite ABC kit from Vector Laboratories Inc (PK 6101 for rabbit primary antibody and PK6105 for goat primary antibodies, Burlingame, CA, USA). The final staining was performed using a solution of 10 mg of 3-3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.) in 15 ml of a 0.5M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H_2O_2. Controls were obtained by substituting the primary antiserum with PBS or normal serum in the specific step, or alternatively, by absorbing each primary antiserum with an excess of the relative blocking peptide (Ob, A-20P, sc-842P; Ob-R, C-20, sc1832P; Ob-R, M-18, sc-1834P Santa Cruz, Biotec. Inc., Santa Cruz, CA, USA).

2.6. Immunopositive cells count

In order to obtain quantitative data, 20 consecutive sections were obtained from the proximal and distal intestine of DIO and CTRL zebrafish. Five non-adjacent sections from each series of sections were immunolabelled as described above to detect leptin immunopositive cells. Slides were observed using a microscope Leica DMRA2 (Leica, Wetzlar, Germany) equipped with a DC300F digital camera and the number of leptin immunopositive cells was calculated using ImageJ program.

2.7. Double immunostaining

Double immunohistochemical staining using two primary antisera raised in different species was performed (Gatta et al., 2014). Sagittal sections were dewaxed, rehydrated, rinsed in PBS and incubated for 30 min at RT with normal rabbit serum (S-5000 Vector) 1:5. Successively, blocking of endogenous biotin was performed (Avidin-Biotin blocking kit, SP-2001, Vector). Then, the sections were incubated with the first primary antibody raised against long-or short isoform of leptin receptor (1:50) overnight at 4 °C. Successively, the sections were washed with PBS and incubated with biotinylated secondary rabbit anti-goat antibody (1:20 BA-5000 Vector) for 2 h at RT. After rinsing in PBS, the sections were incubated with Streptavidin Texas Red® conjugate (1:50; S-872, Life Technologies Europe, Monza, Italy) for 2 h at RT in dark humid chamber. After rinsing in PBS, the sections were incubated with normal donkey serum (1:5; 017-000-121, Jackson Immunoresearch Laboratories Inc., Suffolk, UK) for 2 h at RT in dark humid chamber. Afterwards, the sections were incubated with the second primary antibody in rabbit against leptin (1:50) over night at 4 °C in dark humid chamber. After rinsing in PBS, the sections were incubated Alexa Fluor® 488-conjugated affinipure donkey anti-rabbit (1:50, 711-545-152, Jackson) for 2 h at RT in dark humid chamber.

Finally, the sections were washed with PBS, mounted with glycerine diluted with PBS 1:1 and observed on a confocal microscope (Leica DM6000B by Leica, Wetzlar, Germany) equipped with a Leica DFC450C digital camera (Leica, Wetzlar, Germany). Pictures were acquired through Leica LAS AF software powered by Metamorph®.

2.8. Statistical analysis

The assays were carried out in triplicate. All experimental data are reported as mean ± SEM. Statistical analyses on BMI and body weight data were performed by one-way analysis of variance (ANOVA) and any significant difference was determined at a significance level of 0.05 via the application of a Tukey's test. Statistical analyses on qRT-PCR data were performed by the unpaired Student's t test. P values lower than 0.05 were considered significant (see Montalbano et al., 2016).

Immunopositive cell count data were analyzed by one-way analysis of variance (ANOVA) and any significant difference was determined at a significance level of 0.05 via the application of a Tukey's test. The analyses were carried out using Statistica version 7.0 (Statsoft Inc., Tulsa, OK).

3. Results

This study reports the presence and expression of leptin and its receptors family in the digestive tract of DIO zebrafish compared with the same parameters obtained in CTRL zebrafish. The pictures of a male adult CTRL zebrafish (Fig. 1a) and of DIO zebrafish (Fig. 1a) demonstrated that, under a macroscopic point of view, the abdomen width of the DIO zebrafish is due to a larger visceral

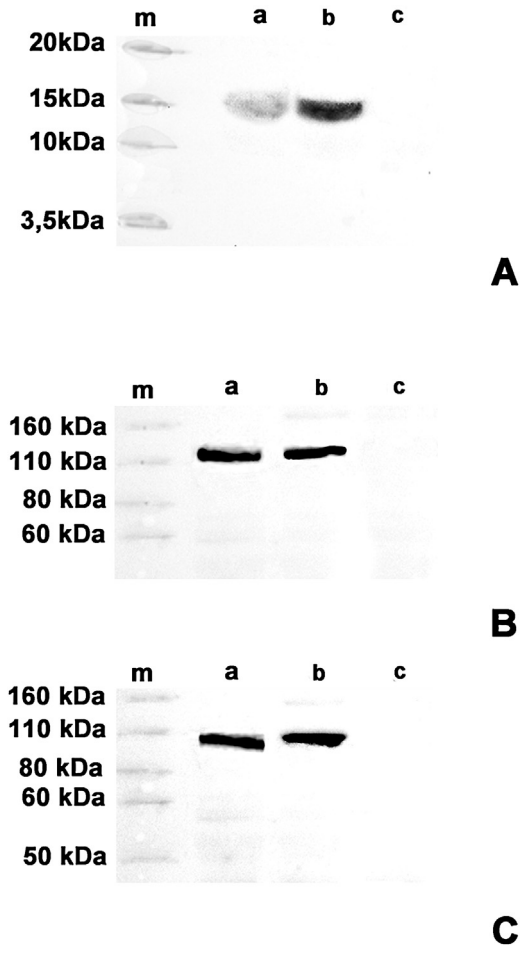

Fig. 3. Western blotting analysis. Blot A: leptin immunopositive bands of ~16 kDa in zebrafish intestine (a), rat stomach (b); blot B: Ob-R long isoform immunopositive bands of ~120 kDa in zebrafish intestine (a), rat stomach (b); blot C: Ob-R short isoform immunopositive bands of ~100 kDa in zebrafish intestine (a), rat stomach (b). (m): Marker; (a) zebrafish intestine; (b) rat stomach; (c) pre-absorption. The results were obtained using 5 zebrafish per each experimental group.

fat depot compared to CTRL group, consistently to previous results (Montalbano et al., 2016).

3.1. BMI and body weight measurements

Normalized BMI (Fig. 1b) and normalized body weight (Fig. 1c) showed a statistically significant ($^*P \leq 0.05$) increase in DIO group at every time point analyzed. At the end of the experiment, the increase in BMI was greater than 45% in DIO zebrafis (1.47 ± 0.07), compared to the starting conditions. An initial substantial increase in BMI in the first 2–3 weeks is followed by a minor increase in fat accumulation and the achievement of a "plateau", characterized by no further BMI increase. Conversely, controls show a modest decrease in BMI (approximately −10%), with respect to the starting conditions (final value 0.94 ± 0.10), thus maintaining their BMI quite constant through the whole experimental period. Similarly to what happens for BMI, weight gain in DIO zebrafish is more than 40% (1.46 ± 0.05), while in CTRL zebrafish there is a modest decrease in weight gain (0.93 ± 0.12; approximately −10%). In DIO zebrafish, an initial substantial increase in body weight in the first 2–3 weeks is followed by a minor increase in fat accumulation and the achievement of a "plateau", characterized by no further weight increase.

3.2. qRT-PCR: leptin A, leptin B and leptin receptor

In the intestinal tract, the expression levels of leptin A and leptin B (Fig. 2) were significantly ($^*P \leq 0.05$) higher in DIO zebrafish (leptin A 0.80 ± 0.07; leptin B, 60 ± 0.11) than in the CTRL group (leptin A 0.62 ± 0.08; leptin B 0.50 ± 0.01). On the contrary, leptin receptor mRNA was downregulated in the intestinal tract of DIO group (0.08 ± 0.04) compared to CTRL group (0.17 ± 0.07) (Fig. 2).

3.3. Protein expression

Western blot analysis was carried out on homogenates of intestinal tract of DIO and CTRL zebrafish. The results of western blot analysis showed the presence of leptin (~16 kDa band) in the intestinal tract of both experimental groups (Fig. 3A). The presence of Ob-R long isoform immunoreactive band of about 120 kDa (Fig. 3B) and Ob-R short isoform immunoreactive band of about 100 kDa (Fig. 3C) in zebrafish intestinal tract of both experimental groups were detected.

The specificity of the response was confirmed by pre-incubation of leptin, Ob-R long isoform and OB-short isoform antibodies with their respective blocking peptide. There was no expression of leptin, Ob-R long and Ob-R short isoform proteins in these preparations, whereas the presence of these proteins was detected in rat stomach homogenate that was used as positive control (Fig. 3).

3.4. Single immunohistochemistry

No reaction was observed in controls performed by substituting the primary antibodies with PBS or normal serum, or by adsorbing each primary antiserum with an excess their homologues antigens.

Leptin and leptin receptors immunoreactivity (IR) was observed in epithelial cells of intestinal mucosal folds (Figs. 4–6) of both experimental groups. Considering the particular morphology of the

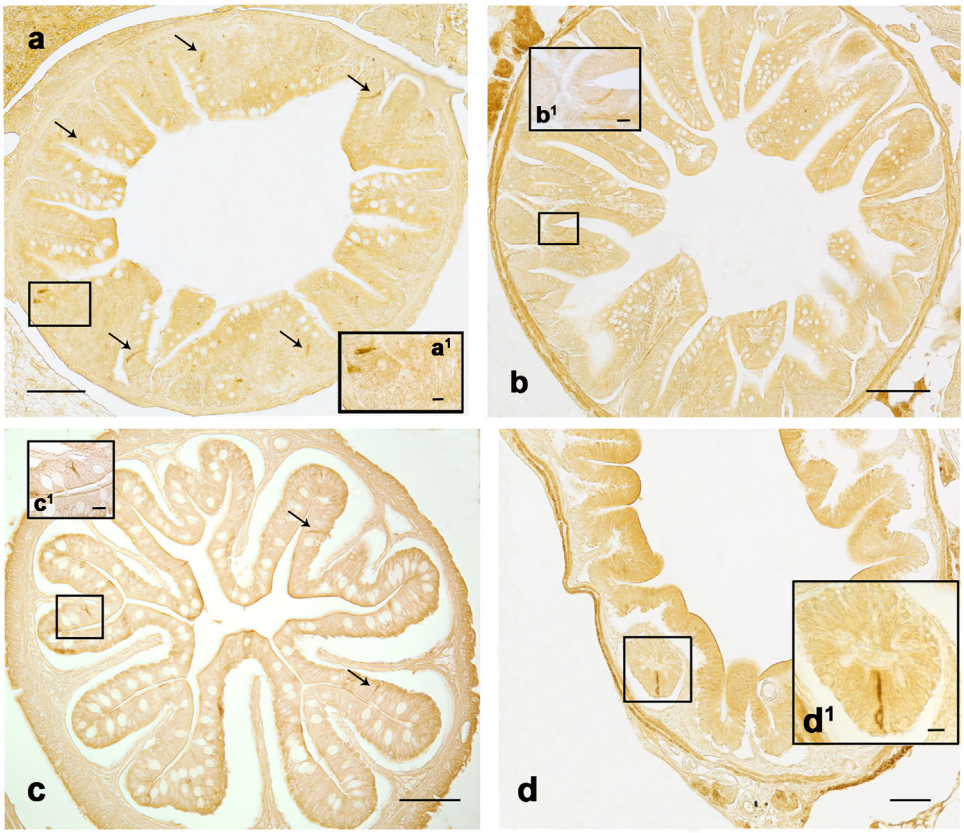

Fig. 4. Single immunohistochemistry. Cross section of DIO and CTRL zebrafish intestinal tract: numerous leptin immunoreactive endocrine cells (arrows) in epithelial mucosa of proximal intestinal tract of DIO zebrafish (a, a^1 detail of a); rare leptin immunoreactive endocrine cells in epithelial mucosa of proximal intestinal tract of CTRL zebrafish (b, b^1 detail of b); some leptin immunoreactive endocrine cells (arrows) in epithelial mucosa of distal intestinal tract of DIO zebrafish (c, c^1 detail of c); a single leptin immunopositive cell in the distal intestinal tract of CTRL zebrafish (d, d^1 detail of d). The results were obtained using 5 zebrafish per each experimental group.
Scale bars = 100 μm:a; 10 μm:a^1; 100 μm:b; 10 μm: b^1; 100 μm:c; 10 μm:c^1; 100 μm:d; 10 μm:d^1.

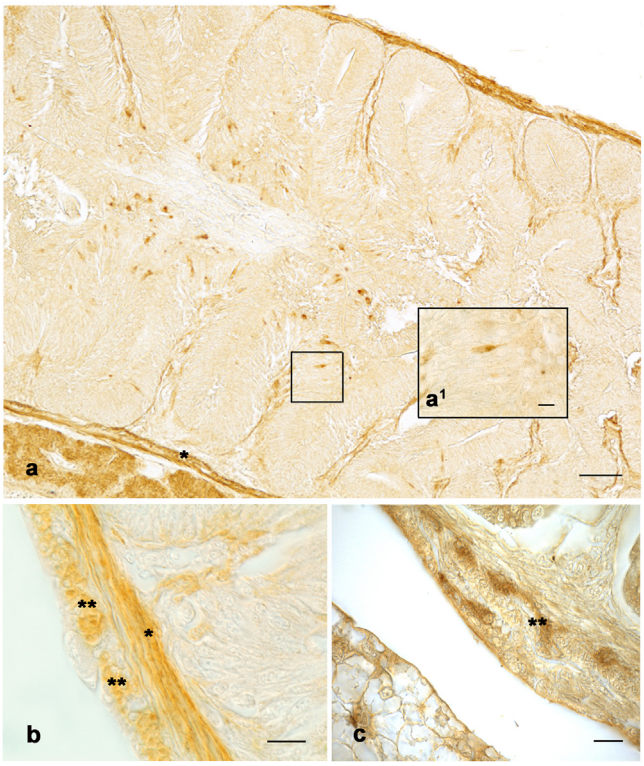

Fig. 5. Single immunohistochemistry. Sagittal and cross sections of DIO and CTRL zebrafish intestinal tract: numerous leptin immunoreactive endocrine cells in epithelial mucosa of proximal intestinal tract of DIO zebrafish (a, a^1 detail of a); leptin immunopositive fibers in circular muscle layer (*) and neurons (**) in myenteric ganglia (b,c). The results were obtained using 5 zebrafish per each experimental group.
Scale bars = 50 μm:a; 10 μm:a^1; 10 μm:b; 10 μm:c.

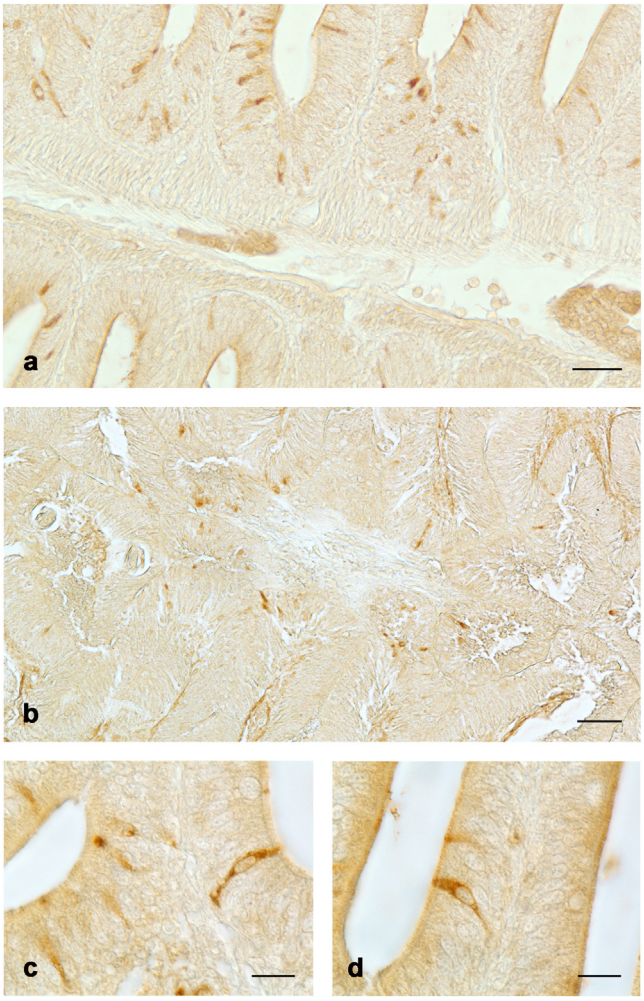

Fig. 6. Single immunohistochemistry. Sagittal and cross sections of DIO and CTRL zebrafish intestinal tract: numerous leptin receptor long (b,c) and short (a,d) isoform immunopositive cells in the epithelial lining of DIO zebrafish (a,c,d) and CTRL (b) intestinal tract. The results were obtained using 5 zebrafish per each experimental group.
Scale bars = 30 μm:a; 50 μm:b; 10 μm:c; 10 μm:d.

immunopositive cells (elongated in shape and generally bipolar), we herein refer to them as endocrine cells.

Leptin immunoreactive endocrine cells were frequently observed in the mucosal folds of proximal intestinal tract (Figs. 4a,b; 5a) and more rarely in distal intestinal tract (Fig. 4c,d) both in DIO (Fig. 4a,c) and CTRL zebrafish (Fig. 4b,d). In addition, leptin immunopositive fibers and neurons in the myenteric plexuses and fibers in circular and longitudinal muscle layers were detected in DIO and CTRL zebrafish intestine (Fig. 4).

Ob-R long isoform (Fig. 6b,c) and Ob-R short isoform (Fig. 6a,d) immunopositive fine granules were located in endocrine cells of epithelial mucosa of intestinal tract both in DIO (Fig. 6a,c,d) than in CTRL zebrafish (Fig. 6b).

3.5. Immunopositive cells count

The number of leptin immunopositive cells was increased in a statistically significant manner (*$P \leq 0.05$) in DIO zebrafish intestine (51.0 ± 1.7) compared to that of CTRL zebrafish (7.0 ± 1.5) (Fig. 7).

3.6. Double immunostaining

In DIO zebrafish intestinal tract, double immunostaining showed leptin immunoreactivity coexisted with OB-R long (Fig. 8a–c) and -short isoform receptors (Fig. 9a,c) especially in endocrine cells of epithelial mucosal folds. In CTRL zebrafish, leptin immunopositive epithelial cells did not co-localize with OB-R long (Fig. 8d–f) and -short (Fig. 9e,f) isoform receptors.

4. Discussion

In this study, we demonstrated the expression and localization of leptin and its receptor in the gut of an obese zebrafish model in response to a high-fat diet. Recently it has been demonstrated that in this experimental model many pathophysiological conditions are shared with mammalian obesity (Oka et al., 2010; Montalbano et al., 2016). It is well known that leptin plays an essential role in the regulation of food intake and control of body weight, both in fish and in mammals (Volkoff et al., 2005; Klok et al., 2007; Volkoff, 2015); however, how leptin expression in the digestive tract is regulated by nutritional status in zebrafish has not been demonstrated so far. For these reasons, this study was undertaken in order to better elucidate the involvement of leptin in the regulation of food intake in zebrafish under pathophysiological conditions. Recent studies on stomach-less and stomach-containing fish have reported the expression and localization of several neuropeptides (Gambardella et al., 2010; Micale et al., 2010, 2012; Russo et al., 2011; Varricchio et al., 2012; D'Angelo et al., 2016a,b). In agreement with those studies, our results described the majority of leptin immunoreactive endocrine cells in the proximal portion of the intestinal tract. The presence of leptin immunoreactive cells gradually decreases from the proximal to the distal portion of zebrafish intestinal tract, which

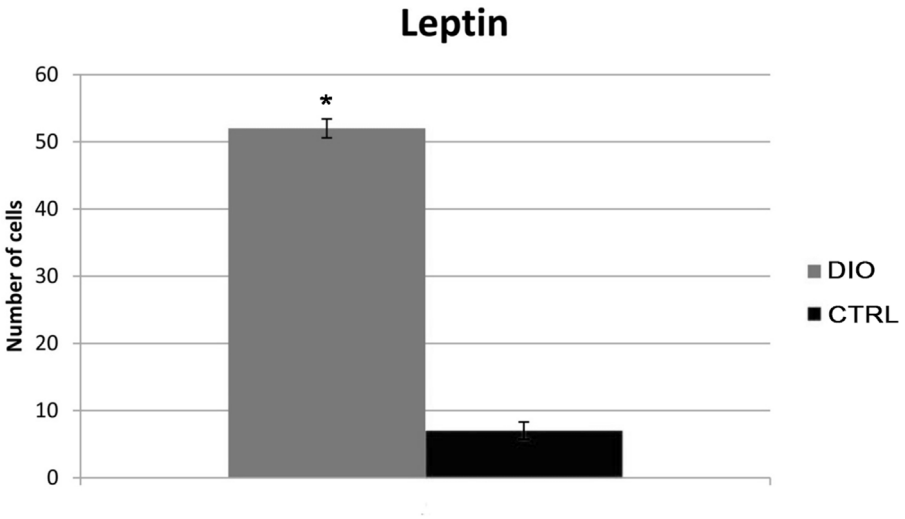

Fig. 7. Cell count graph. Bar graph showing leptin immunopositive cell counts in DIO and CTRL zebrafish intestinal tract. Immunopositive cell count data were analyzed by one-way analysis of variance (ANOVA) and any significant difference was determined at a significance level of 0.05 via the application of a Tukey's test. The analyses were carried out using Statistica version 7.0 (Statsoft inc., Tulsa, OK).

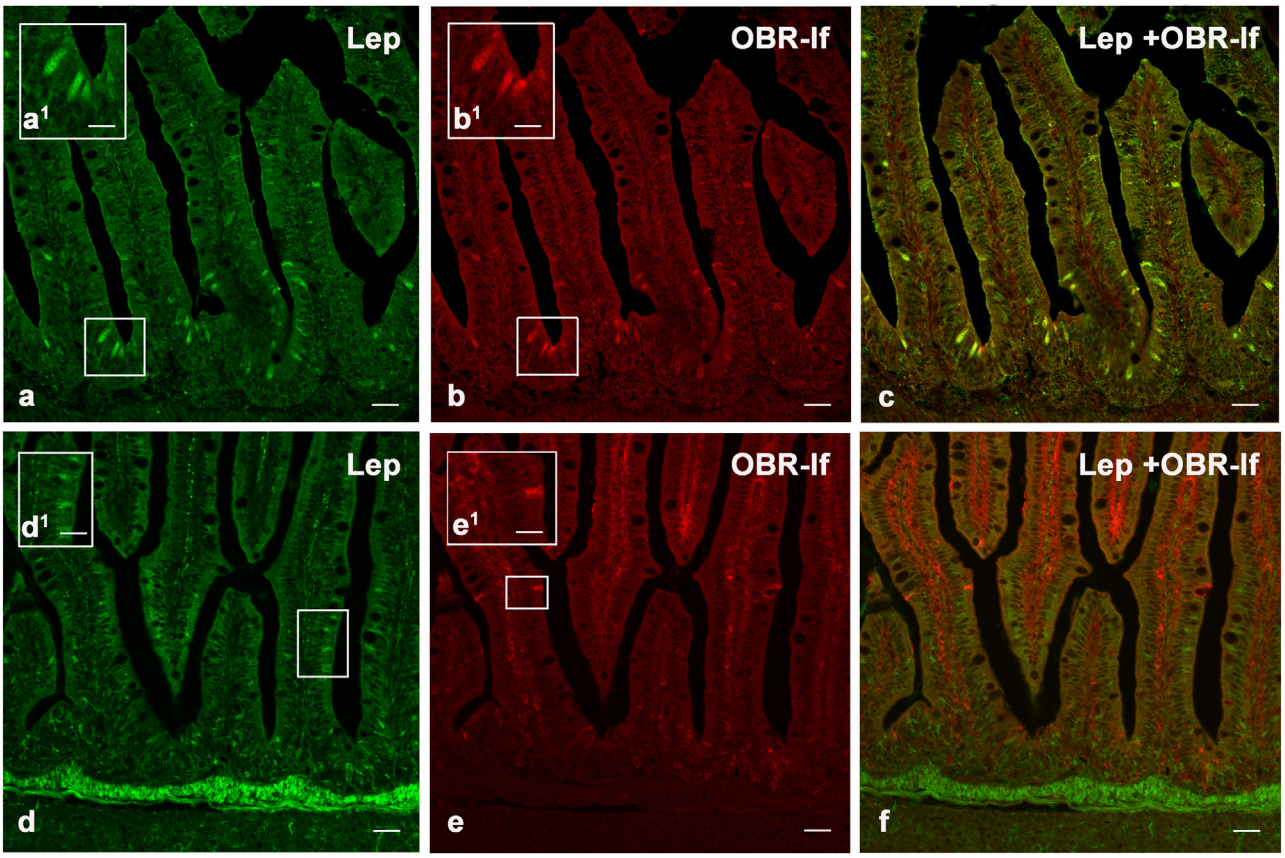

Fig. 8. Double immunostaining. Sagittal section of DIO and CTRL zebrafish intestinal tract: co-localization of leptin (Lep) (green) and leptin receptor long isoform (OB-R-lf) (red) immunopositive endocrine cells in intestinal tract epithelium of DIO zebrafish (a–c), and CTRL zebrafish (d–f). The results were obtained using 5 zebrafish per each experimental group.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Scale bars = 40 μm:a–f; 20 μm:a^1, b^1 d^1, e^1.

is consistent with results reported in goldfish and rainbow trout gut (Russo et al., 2011; Varricchio et al., 2012). Moreover, for the first time, we have demonstrated the colocalization of leptin and leptin receptor isoforms (long and short) using confocal laser microscopy in a subpopulation of enteroendocrine cells in DIO zebrafish. These findings in this teleost substantiate the previous results obtained in DIO rat experimental model (Zhang and Scarpace, 2006) and allow us to speculate that a leptin signaling disruption in a zebrafish model of diet-induced obesity is similar to the disruption causing hyperleptinemia and leptin resistance in mammals, including humans (Londraville et al., 2014; Park and Ahima, 2015; Ritze et al., 2016).

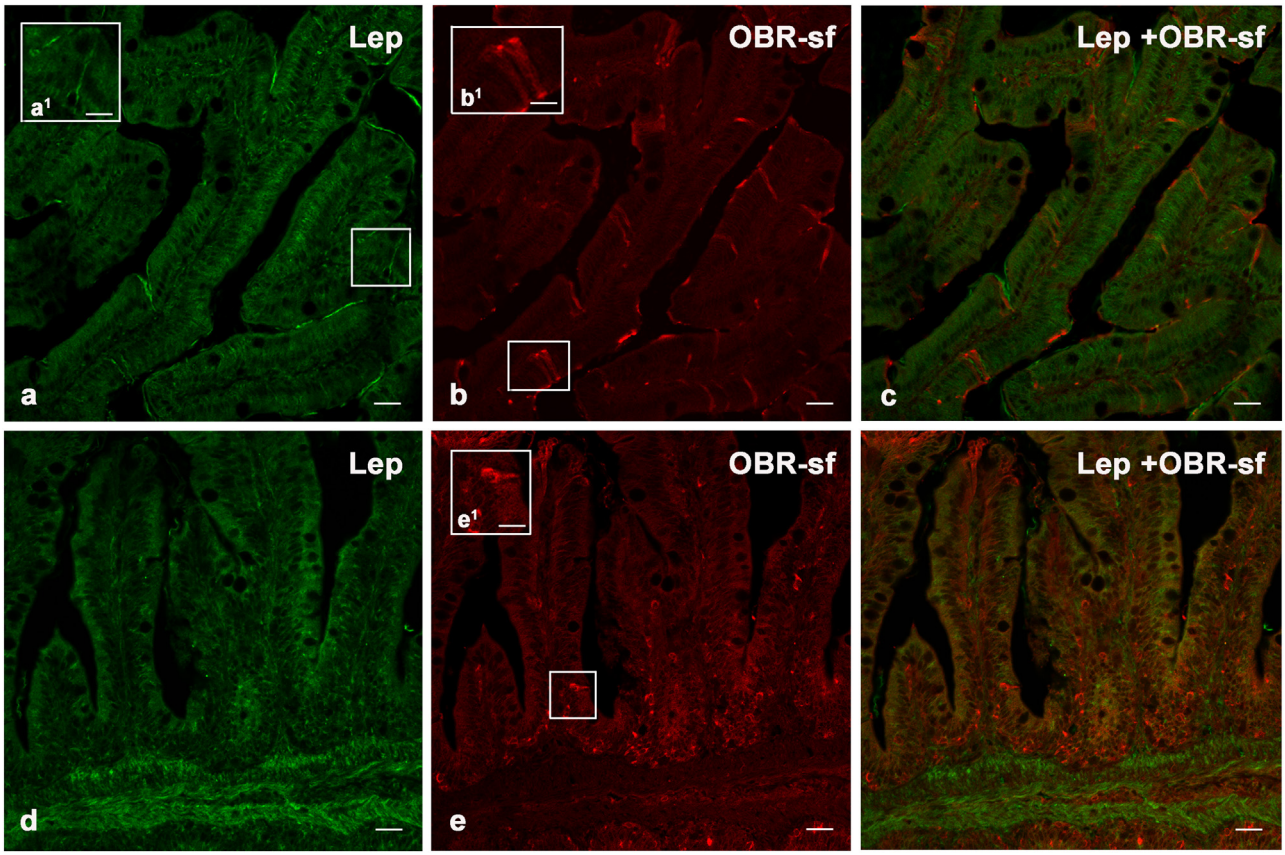

Fig. 9. Double immunostaining. Sagittal section of DIO and CTRL zebrafish intestine: co-localization of leptin (Lep) (green) and leptin receptor short isoform (OB-R-sf) (red) immunopositive endocrine cells in intestinal tract epithelium of DIO zebrafish (a–c), and CTRL zebrafish (d–f). The results were obtained using 5 zebrafish per each experimental group.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Scale bars = 40 μm:a–f; 20 μm:a¹, b¹ d¹, e¹.

The higher number of leptin immunoreactive cells observed in the proximal intestine and the increase in cells expressing leptin protein in DIO zebrafish gut could be related to an high-fat diet.

In addition, the leptin distribution in the enteric nervous system along the intestinal tract of DIO and CTRL zebrafish could suggest an involvement of this protein in the regulation of motility and absorption of nutrients as previously described in mammals (Guilmeau et al., 2004; Yarandi et al., 2011) acting through the vagal afferent pathway in response to excessive food intake (Attele et al., 2002). The action of neuropeptides on the motility and absorption has been also reported in other vertebrate species (Arcamone et al., 2014; Varricchio et al., 2014).

The presence of leptin short and long isoform receptors in zebrafish intestinal tract could suggest a possible local action of this peptide, in agreement with previous data (Attele et al., 2002; Pinto et al., 2004; Cammisotto et al., 2010). Moreover our findings could support a possible role of leptin in regulating zebrafish digestive process and informing the brain about energy and nutrient input, through signaling via the enteroendocrine system (Gambardella et al., 2010; Varricchio et al., 2012). Our results obtained by qRT-PCR report an overexpression of leptins (A and B) mRNA in the intestinal tract of DIO group compared to control group. In DIO zebrafish leptin A levels were higher than leptin B ones according to Gorissen et al. (2009). The overexpression of leptins mRNA could be related to the increased number of cells expressing leptin protein after a high-fat diet. A downregulation of leptin receptor mRNA in intestinal tract of DIO zebrafish was demonstrated, suggesting that leptin could be involved in the alteration of appetite regulation in obesity (Copeland et al., 2011). Moreover, all these data add further evidence to the hypothesis that leptin could be an upstream factor in the mechanism underlying changes in feeding and exploratory behavior associated with food intake (Attele et al., 2002). In conclusion, a variation of leptin system expression levels and immunohistochemical localization in the gut in response to a high-fat diet in DIO zebrafish was reported for the first time. Therefore, this study could be considered as a starting point to add further knowledge on the pleiotropic role of this hormone in fish. Although leptin is involved in food intake or obesity in fish (Huising et al., 2006; Oka et al., 2010; Volkoff, 2015), among other functions, Michel et al. (2016) showed that zebrafish leptin is not only required for food intake but also in glucose homeostasis, as in mammals, suggesting as glucose homeostasis appears to be conserved across vertebrates. Nevertheless, further studies are needed in order to better clarify the plethora of functions carried out by leptin system in both larval and adult life stages in fish, considering that, although the tertiary structure is highly conserved among vertebrates, the genome duplication event might have added multiple regulation roles to leptin system compared to other vertebrates (Gorissen et al., 2009; Copeland et al., 2011; Conde-Sieira and Soengas, 2017).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aanat.2017.03.005.

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