The cellular basis of compensatory muscle growth in the teleost *Odontesthes bonariensis*

Ignacio Simó[1], Mariano Faggiani[1], Daniel A. Fernandez[2,3], Andrés A. Sciara[1], Silvia E. Arranz[1]

[1]Laboratorio Mixto de Biotecnología Acuática, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), Centro Científico, Tecnológico y Educativo Acuario del Río Paraná, Av. Eduardo Carrasco y Cordiviola s/n, Rosario, 2000, Argentina
[2]Instituto de Ciencias Polares, Ambiente y Recursos Naturales (ICPA), Universidad Nacional de Tierra del Fuego (UNTDF), Fuegiabasket 251, V9410BXE Ushuaia, Argentina
[3]Centro Austral de Investigaciones Científicas (CADIC-CONICET), Bernardo A. Houssay 200, V9410BXE Ushuaia, Argentina

*Author for correspondence (sarranz@fbioyf.unr.edu.ar)
ORCID iD:0000-0002-9076-4746

KEYWORDS: Fasting, Compensatory growth, Cell proliferation, Muscle, Hyperplasia, Hypertrophy, Myogenesis, Pejerrey, fish

SUMMARY STATEMENT
Using *in vivo*labelling experiments, we demonstrate that a population of myoblasts continues proliferating, and muscle hypertrophy but not hyperplasia is inhibited under growth adverse conditions in the teleost *Odontesthesbonariensis*.

ABBREVIATIONS:
MPC: *muscle progenitor/precursor cell*
MRF: myogenic regulatory factors
EdU: 5-ethynyl-2'-deoxyuridine
Myog: Myogenin

ABSTRACT

This study evaluates white muscle growth and *in vivo* cell proliferation during a fasting and refeeding trial, using pejerrey *Odontesthes bonariensis* as animal model, in order to better understand the cellular basis governing catch-up growth. Experiments consisted in two groups of fish, a control one continuously fed *ad libitum*, and a group fasted for 2 weeks and then fed for another 2 weeks. We examined how the formation of new muscle fibers and their increase in size were related to muscle precursor cell (MPC) proliferation under both experimental conditions. During fasting, the number of 5-ethynyl-2'-deoxyuridinepositive (EdU^+) cells decreased along with myogenic regulatory factors (MRF) mRNA levels related to myoblast proliferation and

differentiation, and the muscle stem cell-markerPax7 mRNA level increased. Analysis of myomere cross-sectional area, distribution of muscle fiber sizes and number of fibers per myomere showed that muscle hypertrophy but not hyperplasia was inhibited during fasting. Both higher igf2 mRNA level and the persistence of cell proliferation could be supporting new myofibre formation. On the other hand, an exacerbated MPC proliferation occurred during catch-up growth, and this increase in cell number could be contributing to the growth of both pre-existing and newly form small fibers. The finding that some MPCs proliferate during fasting and that muscle growth mechanisms, hyperplasia and hypertrophy, are differentially regulated could help to explain why re-fed fish could growth at higher rates, and why they return to the lost growth trajectory.

INTRODUCTION

Each organism has a growth trajectory and a final body size determined mainly by genetics and epigenetics components (Nilsson, 2018; Sebens, 1987)with growth rates influenced by external factors (Alami-Durante et al., 2007; Johnston et al., 2011; Macqueen et al., 2008).One of the unsolved questions related to body growth rates is how organisms can growth at faster rate than controls after a period of delayed growth, and how deceleration occurs once the lost growth trajectory is attained. In fish, "catch-up" growth (Boersma and Wit 1997)that occurs after fasting has been related to an increase in food intake (hyperphagia) and efficiency in food utilization (Ali et al., 2003; Picha et al., 2014; Won and Borski, 2013). However, locally regulation of cell proliferation and cell growth in this situation has been poorly described.

On average, if growth trajectories were compared between different taxa, endothermic mammals and birds have higher maximum growth rates than similar sized ectothermic fishes and reptiles (Werner and Griebeler 2014). On the other hand, unlike mammals, the window of body growth covers nearly the entire life cycle in most teleost species and are considered to have indeterminate growth because body size never fixed (Froehlich et al., 2013; Mommsen, 2001; Sebens, 1987).

In almost all animal species studied, the fastest growing period is associated to an abrupt increase in muscle mass that corresponds to the period of fastest production of new muscle fibers (Alami-Durante, 1997; Koumans et al., 1993; Leitão et al., 2011; Veggetti et al., 1990), suggesting that growth rates are highly dependent on the rate of muscle hyperplasia (increase in myofibre number). Each new muscle fiber is primarily formed by the fusion of differentiated myoblasts or muscle progenitor/precursor cells (MPCs) that once committed to terminal differentiation, became mitotically inactive. Further muscle fiber growth in length and diameter (hypertrophy) depends on protein synthesis and/or new absorbed differentiated MPCs (Johnston et al., 2011). Both muscle

hyperplasia and hypertrophy depend on myoblasts proliferation. Final body size attained by a species strongly correlates with *maximum muscle fiber number*, and clear evidence exists that both parameters are mainly genetically determined (Johnston et al., 2004; Rehfeldt et al., 2011). In teleost fish, post-larval muscle growth occurred by both hyperplasia and hypertrophy throughout most of their lives (Rowlerson et al. 1995; Rowlerson et al., 1997), while in mammals, the contribution of hyperplasia to muscle growth is restricted to prenatal or a quite small postnatal period and further muscle growth primarily depends on hypertrophy (Rehfeldt et al., 2011; Rowe and Goldspink, 1969). Very little is known about the mechanisms regulating the formation of myotubes in fast muscle (Johnston, 2006), and the regulation of muscle fiber number (Johnston et al., 2009).

In mammals, determination of the total number of fibers within a muscle is technically difficult, particularly in muscles of irregular shape, and because new fiber formation takes place during intrauterine growth (Brameld and Daniel, 2008). In intrauterine growth retardation syndrome (IUGR), the results are controversial since reports used a variety of methods for the estimation of hyperplasia process (Fahey et al., 2005; Greenwood et al., 1999; Nissen et al., 2003; Yates et al., 2014). In juvenile and adult fish, determination of the total number of fibers within a muscle is also technically difficult.Recent research to elucidate the role of hypertrophy and hyperplasia during muscle compensatory growth was achieved by transcriptomic analysis of muscle of adult fish during accelerated growth and hyperplastic growth zones of the post-embryonic trout myotome, and suggest that accelerated muscle growth occurs by muscle hypertrophy (Rescan et al., 2017). However, the role of myoblast proliferation and the rate of myofibre generation in catch-up growth are still poorly described.

Increase in cell proliferation have been described in various mammals tissues after a short period of calorie restriction (Cerletti et al., 2012; Finkielstain et al., 2013), and deceleration of growth upon reaching final body size with age seems to be regulated not systemically but locally (Finkielstain et al., 2009; Finkielstain et al., 2013; Roselló-Díez and Joyner, 2015), and it was associated with the down regulation of insulin-like growth factors and cell proliferation (Yakar et al., 2018). Paracrine/autocrine signals regulate cell proliferation and fiber growth in skeletal muscle. The insulin-like growth factors type 1 and 2 (Igf1 and Igf2), and myostatin are key regulators for the formation and maintenance of the skeletal muscle (Yakar et al., 2018). Igf2 is well established as a critical factor regulating cell proliferation, growth, differentiation and survival restricted to intrauterine growth or early postnatal development in humans and mouse (Nielsen, 1992). Igf2 concentration diminishes postnatally when growth-hormone dependent Igf1 signalling dominates the signalling of these processes (Nielsen, 1992). In humans, a nonsense mutation in the *IGF2* gene, reported in a family of four members, was associated with prenatal and postnatal growth

restriction (Begemann et al., 2015). Interestingly, unlike in mammals, *igf2* muscle expression does not cease in adults fish (Duan, 1998), suggesting that the expression of *igf-2* could be associated with indeterminate growth in teleost, and possibly with muscle hyperplasia.

Pejerrey *Odontesthes bonariensis* (Atheriniformes, Atherininopsidae), a well-studied South American freshwater silverside, exhibit a broad window of growth and a relatively small size if compared with other commercial fish which show indeterminate body growth. Moreover, cross sections of juvenile specimens are small enough to allow us the examination of the whole musculature of juvenile fish under microscope. In this context, the aim of the present work was to get inside into the cellular basis of compensatory growth using *O. bonariensis* as teleost model, by analysing muscle cell proliferation using EdU *in vivo* labelling assay, muscle hyperplasia and hypertrophy. We also studied the expression of muscular proliferation and differentiation molecular markers and paracrine/autocrine factors during both the non-growing phase and during the phase of exacerbated growth.

MATERIALS AND METHODS

Fish husbandry, experimental design and sampling

Juvenile pejerrey (*Odontesthesbonariensis*) were obtained by natural reproduction of brood stock of *O. bonariensis* belonging to the Laboratory of Aquaculture Biotechnology (LBA) at Centro Científico, Tecnológico y Educativo "Acuario del río Paraná" (Rosario, Argentina) and kept at the facilities of the LBA for the trials. Juvenile pejerrey were maintained on a 14-h light/10-h dark schedule at 21 ± 1°C on a recirculating system containing 12 x 100L-tanks with a mechanical and biological filter. In all cases, the fishes were randomly divided into the experimental tanks, fed to satiation with a commercial diet (Shulet S.A., Argentina) and acclimatized for two weeks before the start of the trial. As a first step in studying compensatory growth in this fish species, a fasting and refeeding experiment was carried out in order to gain basic knowledge on its somatic growth response. Based in this result, a second fasting-refeeding experiment was designed to analyze *in vivo* MPC proliferation. In all cases, fish were anesthetized with Eugenol 10mg/L or euthanized with Eugenol at 50 mg/ml. The first two experiments presented in this work were conducted assigning a group of fish per tank (3 controls and 3 treated tanks). This approach was elected over the classical many fish in a single tank in order to minimize unpredictable effects of intra-tank interaction between individuals and growth, food ingestion and related variables are under study in this work. Experiments consisted in two experimental groups, a control one which was continuously fed *ad libitum* and a treated group, which consisted in 2 weeks of fasting followed by 2 weeks of refeeding. On first experiment (Fig. 1A), 120 fish (1.02 ± 0.12 g body weight; 52.8 ± 2.12 mm standard length)

were distributed on 6 tanks (3 control tanks and another 3 treated tanks) and were allowed to acclimatize for two weeks to the new conditions. Body weight and standard length of 6 fish per tank were registered after acclimatization (time 0), fasting (2 weeks) and refeeding (4 weeks). At each time, 3 fish per tank were killed and muscle samples were taken to RNA extraction and histological analysis (see histological analysis). A second experiment was designed in order to quantify *in vivo* cell proliferation in white muscle (Fig. 2A). Fish (72 individuals) were distributed among 12 tanks. Fish on tanks 1, 2, 3, 7, 8 and 9 were continuously fed *ad libitum*, fishes on tanks 4, 5 and 6 were starved for 2 weeks and fishes on tanks 10, 11 and 12 were starved for 2 weeks followed by 2 weeks of refeeding. At days 9 and 23 after the start of the experiment fishes on tanks 1-6 and 7-12 respectively received an intra peritoneal injection of 10mM 5-ethynyl-2'-deoxyuridine (EdU) in PBS in order to track and label MPCs. The volume of the injection was adjusted so that each fish receives a dose of 50 mmol of EdU per Kg of body weight. Fish (4 fishes per tank) were euthanized after 2 weeks of treatment (tanks 1-6), and after refeeding period (tanks 7-12). Then, a slice of 5mm thickness was obtained from a distance of ¾ of the total length from the head to the tail and frozen in isopentane which had been cooled (-160°C) in liquid nitrogen for 30 sec. These samples were sectioned at 14 µm in thickness by cryostat and were stored at - 80°C until histology and other analyses could be performed. To determine if EdU^+ cells belong to muscle lineage a third experiment were conducted. Thirty *Odontesthes bonariensis* juveniles of 4,06 ± 1,14 g of body weight and 8,05 ± 0,93 cm of total length were distributed in 6 tanks (5 fish per tank). Fish from three of the tanks were subjected to fasting conditions for 10 days, while fish from the remaining three tanks were fed 4 times a day until satiety. On the tenth day of the experiment, the fish in each tank were euthanized. Following previously published methods (Froehlich et al., 2014), for the fish pool of each tank, cells were extracted from the white muscle and cultured *in vitro* in laminin-coated glass coverslips. The cells extracted from each tank were seeded in 3 culture coverslips to be counted and analyzed by immunofluorescence.

For all the experiments the specific protocols were approved by the Animal Use Ethics Committee of Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina (permit nº 361/2016).

Immunohistochemistry

Muscle slices

Muscle slices were thawed for 15 minutes at RT and then incubated and fixed with 3.7% formaldehyde solution in PBS for 15 min at RT. To remove the excess of fixative, 2 washes of 30 s were performed with 100 µl of blocking solution (PBS 1X pH 7.6 BSA 3%, BLOTO). All the washing

steps mentioned in this section were carried out under the same conditions. Subsequently, it was incubated with 0.5% Triton X 100 solution in PBS pH 7.6 for 20 min for tissue permeabilization and then 3 washes with BLOTO were performed. It was incubated with the built-in EdU development solution (Click-iT® EdU Imaging Kits, Thermo Fisher Scientific) for 30 min in the dark and then two washes were performed. Finally, it was incubated with anti-laminin antibody (Sigma L-9393) in a 1/40 dilution in BLOTO overnight at 4 ° C. The next day, 3 washes were performed and then incubated with anti-mouse IgG antibody conjugated to the Cy3 flourophore (Jackson ImmunoResearch, West Grove USA, NB 120-6939) in BLOTO diluted 1/200 and a Hoechst 1x solution (Click-iT® EdU Imaging Kits, Thermo Fisher Scientific) for 1 hour at room temperature. After the incubation, two washes were made with 0.05% Tween 20-PBS and finally the preparations were mounted with a cover-slices using the DABCO assembly solution (Sigma Aldrich Co.). The slices were stored at -20 ° C overnight and the next day were observed with a fluorescence microscope (Nikon Eclipse 800). To evaluate cell proliferation, all the EdU^+ cells present on half epaxial cross section of each fish were counted.

Isolated cells

A fraction of isolated cells from white muscle from control and fasted fish were incubated on laminin-coated glass coverslips (200.000 cells per cm^2) for 40 min and then washed twice with culture medium to remove any loosely attached and unattached cells. The coverslips were washed twice in a 3% BSA in PBS (3% BSA solution) and the cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT and washed twice 5 min each with 3% BSA solution. Then, cells were permeabilizated with 0.5% Triton X-100 in PBS for 20 min, washed 2 times 5 min each with 3% BSA solution. For the development of EdU, each coverslip was incubated in the dark for 30 min with the Click-iT® reaction cocktail (Click-iT® EdU Imaging Kit) at room temperature followed by two washes with BSA solution. Then, immunofluorescence was performed with Anti-Myod antibody (Jackson ImmunoResearch, West Grove USA, NBP1-54153) to detect Myod. Coverslips were blocked for 1 hour on BSA solution and then incubated in Anti-Myod antibody 1/100 in 1% BSA in PBS ON at 4° C, followed by two washes with 3% BSA solution and 1 hour incubation with secondary anti-rabbit IgG antibody Cy3 1/1000 in 1% BSA and Hoechst 33342. Coverslips were washed two times in 3% BSA solution and mounted using an anti-quencher solution. Using fluorescence microscopy, the EdU^+/$Myod^+$/$Hoechst^+$ cells were counted from three representative areas of each coverslip (more than 100 nuclei in each area) and these values were relativized to the total number of counted nuclei ($Hoechst^+$) in the three areas. Finally, the results obtained from the 3 pools of each condition were averaged to obtain the percentage of cells with different markers on each one.

The cells that did not adhere to the glass coverslips and another fraction of the total isolated cells from control and fasted fish were fixed with 4% (m/v) paraformaldehyde for 15 min and rinsed with PBS. Then, EdU^+ cells were revealed as mentioned earlier and the total nuclei were marked with Hoechst 33342. The concentration of EdU^+ cells in the suspensions was determined with a hemocytometer using a fluorescence microscope (Nikon Eclipse 800) and the percentage of EdU^+ cells adhered to laminin was calculated based on these values.

Digitization of images

The images were digitized using the program EZC1 3.9 Free Viewer (http://nikon-ez-c1-freeviewer.software.informer.com/3.9/). Then, using each obtained image, the complete cross section was reconstructed using the Image Composite Analyzer image overlay program (https://www.microsoft.com/en-us/research/product/computational-photography-applications/image-composite -editor/). Once digitized, the diagrams of the fibers and the calculations in the distribution, size and quantity of them were made through the software Image Pro Plus Analysis (http://www.mediacy.com/imageproplus).

Histological and morphometric studies

Morphometric analysis was performed on digital muscle cross sections, stained with hematoxylin and eosin, andphotographed using an Andor Clara digital camera coupled to a Nikon Eclipse 800 fluorescent microscope. Analysis of the muscle myomere area, fiber diameter, number of fibers per myomere was carried out in a specific myomere. Each white fiber of the myomere was manually diagrammed on the scanned image (Fig. S1). All the values of number of fibers and area mentioned in the present work were obtained from the analysis of the fibers of the same type of myomere for all the fish analysed (fig S1). The selected myomere was previously analysed on three different juveniles and did not show significant differences in the distribution of mean diameters of the fibers of different size-ranges compared with the diameter distribution of the total fibers in the half cross section. Since the fibers cross sections are not perfect circles, the mean diameter of each one is automatically calculated by the Image Pro Plus software as the average of the diameters measured at 2 degrees intervals passing through the center of the object.

pax7, myod, myf5, myog, igf1 and igf2 mRNA expression

Gene expression on muscle was analyzed by RT-qPCR. The specific qPCR primers for ef1a and igf1 have been previously validated (Botta et al., 2016; Sciara et al., 2011). Primers for igf2, pax7, myod, myf5 and myog were designed using the primer3web vs 4.1.0 (https://primer3.ut.ee) based on sequences obtained from *Odontesthes bonariensis* (pejerrey) genome (NCBI Genome txid219752).

Amplified PCR sequences of igf2, pax7, myod, myf5 and myog were sent to Macrogen sequencing service (https://dna.macrogen.com/#) to confirm their identity. Muscle tissue (50 mg) were immediately homogenized in 500 µl of TRIzol REAGENT (Invitrogen™) for total RNA extraction following manufacturer's instructions. RNA quantification and purity were determined by using NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). To eliminate possible genomic DNA contamination, all samples were DNase I treated (Promega, Madison WI, USA) starting from 2µg of total RNA according to manufacturer's instructions. Then, first strand cDNA synthesis was performed with M-MLV enzyme (Promega, Madison WI, USA) for 50min at 37°C followed by 10min at 70°C using Oligo dT as primer in a 10-µl final volume. RT-qPCR reactions were conducted on Step One Plus PCR System (Thermo Fisher Scientific) in a 10-µL final volume with 5µL of 5X PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific), 1 µL of forward/reverse primer mix (Table S1; 500 nM), 2 µL of cDNA template and 2 µL of water. Samples were run in duplicate and no template controls were performed in every run for each set of primers. Primer efficiencies were calculated on every run for each tissue by using LinRegPCR software (Ruijter et al., 2009). Quantification of cDNA was carried out by calculating the initial fluorescence (N0) per sample, which is representative of the initial amount of cDNA(Ruijter et al., 2009)and obtaining EGOI=N0GOI/N0RG, where EGOI is the expression of a gen of interest (GOI) normalized against a reference gen (RG), N0GOI is the initial fluorescence of a GOI and N0RG the initial fluorescence of RG.

Statistical analysis

Results are presented as mean ± standard error of the mean (s.e.m.). By means of nested ANOVA it was confirmed that in the observed effect it is not dependent on the tank but on the treatment. One-way nested ANOVAs were run whenever two means were to be compared. All assumptions were tested for each test and if not met, a Ln(x) transformation was carried out. For weight, length, number and diameter of fibers and area of the myomere the data were analyzed applying two-way nested ANOVAs and subsequent multiple comparisons according to Tukey in case of detecting significant differences. Compliance with the assumptions was verified through waste analysis (normality and equality of variances). In all cases it was considered significant when $p < 0.05$.

RESULTS

Effects of fasting and re-feeding on growth performance, and the number and size of skeletal muscle fibers in juveniles pejerrey

To delay growth trajectory, juvenile pejerrey individuals (53 mm mean body length) in the fast-growing phase were subjected to 2 weeks of fasting, and then to 2 weeks of re-feeding (treated group) to test for compensatory growth, or were subjected to a daily feeding protocol (control group) (Fig. 1A). Fish (3 tanks per treatment, 6 fishes per tank) were weighed and measured in standard length at time 0, 2 and 4 weeks. The weights and lengths of treated fish were constant after 2 weeks of fasting, but differed ($p<0.05$) from control (Fig. 1B, C). After 2 weeks of refeeding, no significant differences were found in the body size (weight and length) between groups (Fig. 1B, C), showing that fasted *O. bonariensis* undergo complete compensatory growth. The mean area of fasted fish myomere was significantly lower than the one corresponding to control fish (Fig. 1D, $P<0.05$) and did not differ from the mean area of the myomere at the beginning of the experiment ($P>0.05$). After the compensatory growth period, not significant differences between myomeres areas in each group were found (Fig. 1D).

Histological analysis of white muscle fibers cross-section showed that the mean number of fibers increased significantly between weeks 0-2 and 2-4 ($P<0.05$) for both groups, control and fasted-refed (Fig. 1E). The mean number of fibers did not differ significantly between treatments (Fig. 1E, *P>0.05*). Instead, the proportion of small fibers (mean diameter <20 µm) was significantly higher in the fasted group than in controls (Fig. 1F, H, $P<0.001$). In addition, average (mean ± s.e.m.) diameter of small fibers(<20 µm) was smaller in fasted groupthan in control (14.25 ± 1.16 µm vs 17.07 ± 1.08µm, *P<0.05*).The data show that new muscle fiberswere generated during 2 weeks of fasting but did not grow. No significant association between weight and number of white fibers (r^2=0.4062, P = 0.21), nor between standard length and number of white fibers (r^2=0.5043, P = 0.11) were observed in fasted fish after two weeks. In contrast, the association was significant between body weight and cross-sectional area of the myomere (r^2=0.7657, P = 0.006), body weight and standard length (r^2 =0.9632, P = 0.0000) and between fish standard length and myomere cross-sectional area (r^2=0.8966, P = 0.0002). Neither the number, nor the distribution of myofibre sizes differed between groups after the re-feeding period (Fig 1G). The percentage increase in mean myomere area and in the number of myofibreswas165.8 %and 25.7% respectively in refed fish, suggesting that hypertrophy is the process that mainly contributes to muscle compensatory growth.

Effects of fasting and re-feeding on muscle cell proliferation in juvenile pejerrey

Considering that hypertrophy but not hyperplasia was inhibited in juveniles pejerrey during fasting under the experimental conditions, and that the formation of new myotubes requires cell proliferation, we tested if cell proliferation in white muscle took place during the non-growing period. In order to assess the occurrence of cell proliferation, an assay for *in vivo* labelling of replicating DNA with EdU was standardized for juvenile pejerrey (Fig. S2). Then, an experimental approach of fasting-refeeding experiment (2 weeks fasting + 2 weeks refeeding) was conducted in which EdU was injected to juveniles pejerrey nine days after the beginning of the fasting period, and nine days after the beginning of the re-feeding period in another two control and treated groups (Fig. 2A). EdU^+ cells were measured in a half cross section area of each fish after the completion of both periods. Cell proliferation still took place in muscle during the fasting period but we observed fewer proliferating EdU^+ cells in fasted fish (43 ± 31 EdU^+ nuclei) if compared with controls (220 ± 34 EdU^+ nuclei) (Fig. 2B, C, $P<0.05$). At the end of the re-feeding period, a significant increase in EdU^+ nuclei (354 ± 21 EdU^+ nuclei) was observed in fasted-refed fish if compared with control group (164 ± 52 EdU^+ nuclei) (Fig. 2B, C, $P<0.05$). In order to assess the identity of EdU^+ cells, mononucleated cells were isolated from white skeletal muscle of fasted and control fish. The number of EdU^+ cells from total suspension and attached to laminin-coated coverslips were estimated. The percentage of isolated EdU^+ cells that adhered to laminin was 97.5% and 98.1% from control and fasted fish respectively, without significant differences. For both treatments, the EdU^+ cells that bind to laminin also resulted in $Myod^+$ cells (Fig. S5, Table 1). Neither the percentage of $EdU^-/Myod^-$nor the $Myod^+/EdU^-$ cells showed significant differences in percentage between control and fasted fish. However, consistent with previous observations in muscle histochemistry, the percentage of EdU^+ cells obtained from control fish was significantly higher (11.45 ± 1.98 %) compared to fasted group (4.08 ± 2.03 %) (Table 1, $P<0.05$).

Effects of fasting and re-feeding on gene expression patterns in muscle

The genes involved in muscle cell determination (*myod*, *myf5*) and differentiation (*myog*) showed significantly lower expression levels ($P<0.05$) during the fasting phase if compared with control fed fish (Fig. 3). In contrast, the expression of *pax7*, a quiescent stem cell marker, was significantly higher ($P<0.05$) after two weeks of fasting, and no differences between groups were observed after the re-feeding period (Fig. 3). The analysis of growth factors transcript levels in white muscle samples showed that *igf1* and *igf2*expression are differentially regulated in muscle during fasting. After the fasted period, the level of igf2 mRNA was higher, and igf1 mRNA were significantly lower than in control fish (Fig. 3, $P<0.05$). No significant differences were found in the mRNA levels of the growth factors analysed after two weeks of re-feeding (Fig. 3).

DISCUSSION

One of the unsolved questions related to body growth rates is related to how organisms can growth at faster rate than controls after a period of delayed growth. In fish, compensatory growth that occurs after fasting has been related to an increase in food intake (hyperphagia) and efficiency of food utilization (Ali et al., 2003; Picha et al., 2014; Won and Borski, 2013).However, the cellular bases of compensatory growth are poorly understood. Recent transcriptomic analysis of the genes that compose the compensatory muscle growth signature of adult rainbow trout and the genes that were up-regulated in hyperplastic growth zones of the post-embryonic myotome suggests that accelerated muscle growth occurs mediated by myofibre hypertrophy (Rescan et al., 2017).The present work provides strong evidence that fiber hypertrophy is the main process of muscle compensatory growth, and adds a new perspective to explain catch up growth. Our findings on full compensatory muscle growth after 2 weeks of fasting followed by 2 weeks of refeeding in juvenile pejerrey lead to the following conclusions: a) muscle hypertrophy but not hyperplasia is mainly regulated by nutritional status, b) a population of MPCs continues proliferating during fasting, and could be related to the new myofibres generated during the non-growing phase, c) hypertrophy of muscle fibers, including the small muscle fibers formed during fasting, is the main mechanism that contributes to catch up growth, d) the enhancement of MPCs proliferation after food restriction could be a key cellular contributor of nucleus to the compensatory skeletal muscle hypertrophy.

Muscle hyperplasia and hypertrophy are differentially regulated.

The pattern of myomeres observed in a whole-body cross-section at a given position on the anterior-posterior axis is highly conserved between individuals of a certain teleost species. In juvenile pejerrey, there was a strong correlation between the cross-section area of a myomere, determined by the sum of the areas of the whole myofibre areas within it, and the weight and length of each fish. Determining the total number of myofibres from across section area of a defined myomere is a powerful method to measure the occurrence of hyperplasia. Under the experimental conditions of the present study, the number of muscle fibers per myomere increased at similar rates (~40 myofibres per week) both in control and fasting-refeeding groups, showing that neither fasting nor refeeding induced changes in hyperplasia rate, while hypertrophy was highly dependent on food availability during the hyperplastic growth phase. The distribution of muscle fiber diameters in the myomere cross-section after two weeks of fasting suggests that not only hyperplasia but also atrophy could contribute to the increase in small muscle fibers proportion in fasted fish, since total myomere cross-sectional area did not change from time 0 to 2 weeks of fasting, although new fibers have been formed during this period. Previous data on the effect of starvation on muscle fiber size and number in juvenile fish are scarce (Beardall and Johnston, 1983;

Johansson and Kiessling, 1991). Correlations between cross-sectional area of white muscle fibers and carcass weight have been previously observed in rainbow trout (Johansson and Kiessling, 1991; Weatherley et al., 1988). However, the studies on the effect of calorie restriction or starvation on hyperplasia are more elusive. In mammals, determination of the total number of fibers within a muscle is technically difficult, particularly in muscles of irregular shape, and because new fiber formation takes place during intrauterine growth (Brameld and Daniel, 2008). Calorie restriction during gestation seems to influence total fiber number only during primary myofibre formation in the embryo but not during the production of secondary fibers during the foetal period (Kalbe et al., 2017; Wilson et al., 1988), whereas postnatal under nutrition exclusively affects fiber hypertrophy by means of reduced nuclear and protein accumulation(Rehfeldt et al., 1999). In fish, growth retardation during fasting conditions at post larval stages has been mainly associated with muscle atrophy and hypertrophy inhibition (Beardall and Johnston, 1983; Nebo et al., 2013). Only during larval stages, both muscle hyperplasia and hypertrophy seem to be conditioned by diet quality and temperature in fish (Campos et al., 2013; Canada et al., 2018; Johnston et al., 1999; Ontell et al., 1988; Ostaszewska et al., 2008). Interestingly, Rescan et al.(Rescan et al., 2015), using juveniles from a transgenic line carrying GFP cDNA driven by the myogenin promoter, showed that the distribution of *GFP*-expressing small muscle myofibres in the muscle of fasted trout was similar to that observed in the muscle of the well-fed and suggest that myogenin-positive muscle fibers are still produced in atrophied muscle from fasted trout. Our work brings strong evidence that the formation of new fast-twitch muscle fibers during the fast-growing phase is not influenced by temporally food deprivation during post-larval growth in teleost.

Our data also suggests that complete compensatory muscle growth strongly depends on accelerated hypertrophy of the pre-existing and newly formed myofibres during the fasting period. In this way, small fibers will grow more rapidly than larger ones (Weatherley et al., 1988).The fact that nutrient assimilative capacity by fibers is a function of their surface areas and is influenced by the critical diameter of the myofibres support this observation(Johnston et al., 2011). In addition, the percentage of small fibers is an indicator of the body growth potential(Canada et al., 2018; Valente et al., 1999). This is in accordance with previous data that showed that the stage of greatest growth coincides with that of the highest rate of hyperplasia (Veggetti et al., 1990). Our data allows us to hypothesize that skeletal muscle can grow faster and completely compensate growth only if growth delay occurs in a period in which new fibers are being generated. This could partly explain why catch-up growth is observed only during early phases of animal growth, when hyperplasia is still taking place (Finkielstain et al., 2013).Moreover, the fact that the new myofibre generation rate did not change between fasted-refed and control fish could partly explain why fasted-refed fish return to the controls growth trajectory after the catch-up growth period.

Muscle progenitor cell proliferation during catabolic and anabolic stages

Muscle growth involves several processes: formation of new fibers, fusion of additional myoblasts to existing fibers and increase in cell volume per nucleus. The first two of them depends on previous myoblast proliferation. In this work, nutritional status affected muscle progenitor cell proliferation in juveniles pejerrey; while myoblast proliferation was reduced in fasted fish, it was clearly exacerbated during refeeding period showing that systemic metabolic state modulates MPC proliferation. Interestingly, a myoblast proliferation deficit was observed in sheep intrauterine growth restriction (IUGR) model (Soto et al., 2017). In rainbow trout *Oncorhynchus mykiss*, primary myogenic cell lines isolated from fed animals were found to differ extensively in terms of proliferation rate to those isolated from fasted animals (Fauconneau and Paboeuf, 2000)suggesting that nutritional status influence metabolic state of fish MPCs. Nowadays, a significant amount of evidence from mammalian model organisms demonstrate the molecular link between calorie intake and the physiology and function of muscle stem cells (Fulco et al., 2008; Purohit and Dhawan, 2019). In this work, muscle hyperplasia but not hypertrophy took place during fasting, and a smaller population of MPCs continues proliferating. This suggests that in teleost white muscle, a subpopulation of MPCs could be associated to new fiber formation and could has differential susceptibility to calorie restriction or different metabolic requirements. Interestingly, evidence that myogenic cells with distinct metabolic requirements could exist in adults and embryos was recently found in mouse (Pala et al., 2018). Because both hypertrophy and hyperplasia persist during fish post larval growth, a major uncertainty is whether foetal-like myoblasts besides the satellite-like cells coexist in different niches in teleost muscle architecture. Transcriptomic, metabolic state and niche characterization of muscle cell populations isolated from both fasted and refed fish will be the next step in order to clarify the existence of more than one muscle stem cell type/status and to develop molecular markers for further analyses. Previous analyses of larval zebrafish muscle repair (Knappe et al., 2015; Pipalia et al., 2016) revealed two Pax7-expressing myoblast subpopulations that accumulate, differentiate and fuse distinctly during regeneration (Pipalia et al., 2016). Moreover, the transcriptomic analysis and *in vitro* cell behavior of MPCs extracted from hyperplastic muscle of juvenile trout showed to be more potent to form myofibres than myogenic cells extracted from non-hyperplasic muscle (Jagot et al., 2018).Whether such myoblast diversity underlies fiber formation during development in vertebrates is still unknown(Roy et al., 2017).

MPCs proliferation was exacerbated under our experimental conditions in refed pejerrey, and these nuclei probably contribute to the accelerated muscle hypertrophy that takes place during compensatory growth. The contribution of new nuclei to fiber hypertrophy was proposed for muscle growth during early stages of muscle differentiation and during early postnatal development in mice and rats, but seems to be absent in muscle hypertrophy at postnatal stages

(Schiaffino et al., 2013). However, a similar proliferation effect was observed in the muscle of young and old mice after a short-term calorie restriction period (Cerletti et al., 2012). *In vivo* direct stimulation of myogenic cell proliferation by food intake has been previously described previously (Brodeur et al., 2003) for a notothenioid fish.

Effect of nutritional status on MRFs and insulin-like growth factors expression in relation to muscle cell proliferation, differentiation and myofibres growth

The effect of a fasting and refeeding schedule in muscle gene expression profiles has been previously characterized in fish species of different taxonomic orders, like Perciformes(Lavajoo et al., 2020; Nebo et al., 2013), Salmoniformes (Jagot et al., 2018; Rescan et al., 2007; Rescan et al., 2017; Valente et al., 2012), Characiformes (De Paula et al., 2017; He et al., 2015) and Cypriniformes (Yang et al., 2019). Decrease and increase in muscle mass are clearly associated to protein degradation (for energy purposes) and protein synthesis (mainly myosin-related proteins) respectively (De Paula et al., 2017; He et al., 2015). Muscle hypertrophy occurs when the overall rates of protein synthesis exceed the rate of protein degradation (Schiaffino et al., 2013). On the other hand, fasting induce breakdown of muscle mass and involves different proteolytic processes such as the autophagy–lysosome and the ubiquitin–proteasome systems (Bonaldo and Sandri, 2013; Schiaffino et al., 2013). Although transcriptomic analysis of white muscle from pejerrey during fasting and re-feeding will be a next experimental step in order to understand the metabolic pathways and biological processes influenced by external factors, the maintenance of the myomere area while new fiber have been generated suggests the occurrence of proteolysis and a decrease in the size of pre-existing fibers, as could be observed in the lower percentage of fibers with a mean diameter between 20 and 40 µm in fasted fish. The production of nascent myofibres in the muscle of fasted pejerrey suggests that amino acids released from protein breakdown during muscle atrophy are in part reused for building new muscle fibers from activated myogenic cells, as proposed previously in trout(Rescan et al., 2015).

Based on gene expression profiles, some authors suggest that in compensatory growth refeeding stimulates proliferation of fish myogenic cells (Lavajoo et al., 2020) and muscle fiber hypertrophy (Rescan et al., 2017). In this work, both the expression of genes and cell proliferation associated to muscle growth were studied during pejerrey compensatory growth.

The specification, determination and differentiation of the cells that will give rise to differentiated myoblasts or myocytes involves different cell populations and the expression of different genes (Comai and Tajbakhsh, 2014; Dumont et al., 2015). The transcription factors responsible for the specification of mesodermal cells into the muscle lineage (myogenic regulatory factors, MRF) are conserved in teleost and mammals (García de la serrana et al., 2014; Hinits and Hughes, 2007;

Rescan, 2001; Rossi and Messina, 2014). In mammals, Pax7 is the main marker of muscle stem cells during postnatal growth and has an anti-apoptotic effect (Buckingham and Relaix, 2015). During muscle growth or regeneration, proliferating myoblasts co-express Pax7 and Myod, the latter being the main marker of active myoblasts, along with Myf5 (Comai and Tajbakhsh, 2014). The myoblasts that enter the differentiation process express a series of transcription factors that determine the identity and function of differentiated cells, being myogenin (Myog) and Myf6/MRF-4 the most characterized in fish (Froehlich et al., 2013; Gabillard et al., 2010; Rescan et al., 2015) and mammals (Sabourin and Rudnicki, 2000). Studies conducted in *in vitro* primary cultures of myoblasts obtained from teleost fish showed that *pax7* is expressed in newly activated stem cells but not during late stages of differentiation (Froehlich et al., 2013; García de la serrana et al., 2014; Seiliez et al., 2015).

In this work, exacerbated MPC proliferation, accelerated myofibre hypertrophy and whole-body growth, correlated with the recovery of the expression levels of MRFs genes (*myod*, *myf5*, *myog* and of *igf1,* down-regulated during fasting when proliferation (and expected differentiation) of MPCs is also down-regulated. In the gilthead sea bream (*Sparus aurata*), 30 days of fasting resulted in muscle fiber atrophy, a reduction in *myod2*, *myf5* and *igf1* expression, lower number of Myod-positive cells, and decreased PCNA protein expression, whereas myogenin expression was not significantly affected(García de la serrana et al., 2014). The down regulation of myog transcript level was observed in juvenile trout after 2 weeks of fasting, although *myog* expression levels did not differ between controls and starved fish after 4 weeks (Montserrat et al., 2007a).In Atlantic salmon (*Salmo salar*), the expression of *myog* and *stac3* has been correlated with the differentiation of myogenic cells in primary cultures, and the expression of both genes has been shown to be regulated by the availability of amino acids (Valente et al., 2012).

In mammals, Pax7 is a satellite cell marker whose expression is restricted to quiescent cells and proliferating myoblast in postnatal growth and regeneration, and down regulated during terminal differentiation (Seale et al., 2000). In humans, homozygous individuals with a loss of function *Pax7*-variant showed exhaustion of the satellite cell pool and consequently decreased muscle growth and regeneration capacity (Feichtinger et al., 2019). However, the role and regulation of Pax 7 in teleost is still poorly understood (Froehlich et al., 2013). Significant expansion of the $Pax7^{+}$ MPC population was observed during embryonic myogenesis in the dermomyotome of mutant fish that lack the muscle regulatory factor Myod (Hinits et al., 2011; Roy et al., 2017). Interestingly, *pax7* expression has also been associated to quiescent MPC state in *Salmo salar*, since depriving isolated muscle progenitor cells of amino acids and serum led to a rapid increase in *pax7* and a decrease in *myod1* and *pcna* expression, indicating a transition to a quiescent state (Bower and Johnston, 2010). In rainbow trout, *pax7* along with MRFs genes seem to be overexpressed in muscle hyperplastic zones of the late embryo myotome compared to adult fast muscle samples (Rescan et al., 2013). In this

work, higher pax7 mRNA levels in fasted fish could be associated to an increase in the number of quiescent MPCs or higher expression levels related to partial inhibition of cell proliferation associated to the delay in the growth process.

Levels of circulating and locally-producedIgf1 have been extensively correlated with specific growth rate in numerous teleost species and are nutritionally regulated (Beckman, 2011; Gabillard et al., 2006; Pérez-Sánchez et al., 2018; Picha et al., 2006; Picha et al., 2014; Sciara et al., 2011). Although Igf1 and Igf2 had been both implicated in muscle cell proliferation and differentiation(Montserrat et al., 2007b), in the present study, opposite trends were observed between muscle mRNA of igf1 and igf2 after fasting and refeeding compared to controls. Strikingly, the mRNA level of igf2 significantly increased, and mRNA of igf1 significantly decreased after fasting. Igf genes appear to be differentially regulated indifferent teleost species(Jiménez-Amilburu et al., 2012; Lavajoo et al., 2020; Valente et al., 2012). The transition to a re-feeding schedule after 22 days of fasting involves upregulation of muscle Igf1 and down-regulation of ifg2 transcript levels also in *Atlantic salmon* (Bower et al., 2008), and no effect of fasting and refeeding treatment was observed on the expression of igf2 in muscle of rainbow trout (Gabillard et al., 2006; Montserrat et al., 2007a). In addition, Igf2 action seems to be mainly regulated at the muscular tissue level during growth depression (Pérez-Sánchez et al., 2018).Interestingly, fast growing families of channel catfish express hepatic and muscle *igf2* at higher levels than slow growing family (Peterson et al., 2004), suggesting same kind of relationship between genetically determined Ifg2 levels, hyperplasia rate and growth rates.

In summary, a model depicting the cellular bases of compensatory growth in pejerrey is proposed (Fig. 4) in which catch-up growth is a consequence of increased MPC proliferation that contributes new nuclei for the growth of pre-existing muscle fibers, some of which were formed, but did not grow, during the starvation period. Moreover, the combined observations of MPC proliferation, new fiber formation and the *ifg2* overexpression during fasting, together, provide us with invaluable information to untie the catch-up growth gordian knot in the future.

Acknowledgements

We are grateful to the members of Statistics Department at Facultad de Ciencias Bioquímicas y Farmacéuticas (FCByF), Universidad Nacional de Rosario (UNR), for help with statistical analysis. We would like to thank Alexis Grimberg and Sergio D'Amelio for fish care at Centro Científico, Tecnológico y Educativo "Acuario del río Paraná" facilities (Rosario, Argentina), and also Alejandra Martinez of the Area of Morphology at FCByF-UNR for her expert technical assistance in muscle histology.

Author contributions

S.A. and I.S. conceived the study; I.S. performed the sampling; I.S. and M.F. performed the laboratory analyses; S.A., D.F., I.S., M.F. and AS, analyzed and interpreted the data; S.A., I.S. and M.F. acquired funding; SA and IS wrote the manuscript with D.F., A.S., and M.F. critical inputs. All authors read and approved the final paper.

Competing interests

The authors declare no competing or financial interests.

Funding

This work was financially supported by the Agencia Nacional de Promoción de la Investigación, el Desarrollo tecnológico y la Innovación (PID 020-2013 to SA). I. Simó postdoctoral and M. Faggiani predoctoral fellowships were supported from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentine Government. The authors acknowledged the financial support of UNR, Argentina (SEA, ASS) and CONICET Argentina (ASS, DAF).

References

Alami-Durante, H. (1997). Growth and multiplication of white skeletal muscle fibres in carp larvae in relation to somatic growth rate. *J. Fish Biol.* **50**, 1285–1302.

Alami-Durante, H., Olive, N. and Rouel, M. (2007). Early thermal history significantly affects the seasonal hyperplastic process occurring in the myotomal white muscle of Dicentrarchus labrax juveniles. *Cell Tissue Res.* **327**, 553–570.

Ali, M., Nicieza, A. and Wootton, R. J. (2003). Compensatory growth in fishes: A response to growth depression. *Fish Fish.* **4**, 147–190.

Beardall, C. H. and Johnston, I. A. (1983). Muscle atrophy during starvation in a marine teleost. *Eur. J. Cell Biol.* **29**, 209–217.

Beckman, B. R. (2011). Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. *Gen. Comp. Endocrinol.* **170**, 233–252.

Begemann, M., Zirn, B., Santen, G., Wirthgen, E., Soellner, L., Büttel, H.-M., Schweizer, R., van Workum, W., Binder, G. and Eggermann, T. (2015). Paternally Inherited IGF2 Mutation and Growth Restriction . *N. Engl. J. Med.* **373**, 349–356.

Boersma, B. and Wit, J. M. (1997). Catch-up Growth. *Endocr. Rev.* **18**, 646–661.

Bonaldo, P. and Sandri, M. (2013). Cellular and molecular mechanisms of muscle atrophy. *DMM Dis. Model. Mech.* **6**, 25–39.

Botta, P. E., Simó, I., Sciara, A. A. and Arranz, S. E. (2016). Growth hormone receptors in the atherinid Odontesthes bonariensis: Characterization and expression profile after fasting-refeeding and growth hormone administration. *J. Fish Biol.* **88**, 1870–1885.

Bower, N. I. and Johnston, I. A. (2010). Discovery and characterization of nutritionally regulated genes associated with muscle growth in Atlantic salmon. *Physiol. Genomics* **42 A**, 114–130.

Bower, N. I., Li, X., Taylor, R. and Johnston, I. A. (2008). Switching to fast growth: The insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. *J. Exp. Biol.* **211**, 3859–3870.

Brameld, J. M. and Daniel, Z. C. T. R. (2008). In utero effects on livestock muscle development and body composition. *Aust. J. Exp. Agric.* **48**, 921–929.

Brodeur, J. C., Calvo, J. and Johnston, I. A. (2003). Proliferation of myogenic progenitor cells following feeding in the sub-antarctic notothenioid fish Harpagifer bispinis. *J. Exp. Biol.* **206**, 163–169.

Buckingham, M. and Relaix, F. (2015). PAX3 and PAX7 as upstream regulators of myogenesis. *Semin. Cell Dev. Biol.* **44**, 115–125.

Campos, C., Valente, L. M. P., Conceição, L. E. C., Engrola, S., Sousa, V., Rocha, E. and Fernandes, J. M. O. (2013). Incubation temperature induces changes in muscle cellularity and gene expression in Senegalese sole (Solea senegalensis). *Gene* **516**, 209–217.

Canada, P., Engrola, S., Mira, S., Teodósio, R., Yust, M. del M., Sousa, V., Pedroche, J., Fernandes, J. M. O., Conceição, L. E. C. and Valente, L. M. P. (2018). Larval dietary protein complexity affects the regulation of muscle growth and the expression of DNA methyltransferases in Senegalese sole. *Aquaculture* **491**, 28–38.

Cerletti, M., Jang, Y. C., Finley, L. W. S., Haigis, M. C. and Wagers, A. J. (2012). Short-Term Calorie Restriction Enhances Skeletal Muscle Stem Cell Function. *Cell Stem Cell* **10**, 515–519.

Comai, G. and Tajbakhsh, S. (2014). *Molecular and cellular regulation of skeletal myogenesis*. 1st ed. Elsevier Inc.

De Paula, T. G., Zanella, B. T. T., De Almeida Fantinatti, B. E., De Moraes, L. N., Da Silva Duran, B. O., De Oliveira, C. B., Salomäo, R. A. S., Da Silva, R. N., Padovani, C. R., Dos Santos, V. B., et al. (2017). Food restriction increase the expression of mTORC1 complex genes in the skeletal muscle of juvenile pacu (Piaractus mesopotamicus). *PLoS One* **12**, 1–20.

Duan, C. (1998). Nutritional and Developmental Regulation of Insulin-like Growth Factors in Fish. *J. Nutr.* **128**, 306S-314S.

Dumont, N. A., Wang, Y. X. and Rudnicki, M. A. (2015). Intrinsic and extrinsic mechanisms regulating satellite cell function. *Dev.* **142**, 1572–1581.

Fahey, A. J., Brameld, J. M., Parr, T. and Buttery, P. J. (2005). The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. *J. Anim. Sci.* **83**, 2564–2571.

Fauconneau, B. and Paboeuf, G. (2000). Effect of fasting and refeeding on in vitro muscle cell proliferation in rainbow trout (Oncorhynchus mykiss). *Cell Tissue Res.* **301**, 459–463.

Feichtinger, R. G., Mucha, B. E., Hengel, H., Orfi, Z., Makowski, C., Dort, J., D'Anjou, G., Nguyen, T. T. M., Buchert, R., Juenger, H., et al. (2019). Biallelic variants in the transcription factor PAX7 are a new genetic cause of myopathy. *Genet. Med.* **21**, 2521–2531.

Finkielstain, G. P., Forcinito, P., Lui, J. C. K., Barnes, K. M., Marino, R., Makaroun, S., Nguyen, V., Lazarus, J. E., Nilsson, O. and Baron, J. (2009). An extensive genetic program occurring during postnatal growth in multiple tissues. *Endocrinology* **150**, 1791–1800.

Finkielstain, G. P., Lui, J. C. and Baron, J. (2013). Catch-up growth: Cellular and molecular mechanisms. *World Rev. Nutr. Diet.* **106**, 100–104.

Froehlich, J. M., Fowler, Z. G., Galt, N. J., Smith, D. L. and Biga, P. R. (2013). Sarcopenia and piscines: The case for indeterminate-growing fish as unique genetic model organisms in aging and longevity research. *Front. Genet.* **4**, 1–8.

Fulco, M., Cen, Y., Zhao, P., Hoffman, E. P., Mcburney, M. W., Sauve, A. and Sartorelli, V. (2008). Dev. cell. **14**, 661–673.

Gabillard, J. C., Kamangar, B. B. and Montserrat, N. (2006). Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (Oncorhynchus mykiss). *J. Endocrinol.* **191**, 15–24.

Gabillard, J. C., Sabin, N. and Paboeuf, G. (2010). In vitro characterization of proliferation and differentiation of trout satellite cells. *Cell Tissue Res.* **342**, 471–477.

García de la serrana, D., Codina, M., Capilla, E., Jiménez-Amilburu, V., Navarro, I., Du, S. J., Johnston, I. A. and Gutiérrez, J. (2014). Characterisation and expression of myogenesis regulatory factors during in vitro myoblast development and in vivo fasting in the gilthead sea bream (sparus aurata). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **167**, 90–99.

Greenwood, paul l., Slepetis, R. M., Bell, A. W. and Hermanson, J. W. (1999). Intrauterine growth retardation is associated with reduced cell cycle activity, but not myofibre number, in ovine fetal muscle. *Reprod. Fertil. Dev.* **11**, 281.

He, L., Pei, Y., Jiang, Y., Li, Y., Liao, L., Zhu, Z. and Wang, Y. (2015). Global gene expression patterns of grass carp following compensatory growth. *BMC Genomics* **16**, 184.

Hinits, Y. and Hughes, S. M. (2007). Mef2s are required for thick filament formation in nascent muscle fibres. *Development* **134**, 2511–2519.

Hinits, Y., Williams, V. C., Sweetman, D., Donn, T. M., Ma, T. P., Moens, C. B. and Hughes, S. M. (2011). Defective cranial skeletal development, larval lethality and haploinsufficiency in Myod mutant zebrafish. *Dev. Biol.* **358**, 102–112.

Jagot, S., Sabin, N., Le Cam, A., Bugeon, J., Rescan, P. Y. and Gabillard, J. C. (2018). Histological, transcriptomic and in vitro analysis reveal an intrinsic activated state of myogenic precursors in hyperplasic muscle of trout 06 Biological Sciences 0604 Genetics. *BMC Genomics* **19**, 1–11.

Jiménez-Amilburu, V., Salmerón, C., Codina, M., Navarro, I., Capilla, E. and Gutiérrez, J. (2012). Insulin-like growth factors effects on the expression of myogenic regulatory factors in gilthead sea bream muscle cells. *Gen. Comp. Endocrinol.* **188**, 151–158.

Johansson, L. and Kiessling, A. (1991). Effects of Starvation on Rainbow Trout. *Acta Agric. Scand.* **41**, 207–216.

Johnston, I. A. (2006). Environment and plasticity of myogenesis in teleost fish. *J. Exp. Biol.* **209**, 2249–2264.

Johnston, I. A., Strugnell, G., McCracken, M. L. and Johnstone, R. (1999). Muscle growth and development in normal-sex-ratio and all-female diploid and triploid Atlantic salmon. *J. Exp. Biol.* **202**, 1991–2016.

Johnston, I. A., Abercromby, M., Vieira, V. L. A., Sigursteindóttir, R. J., Kristjánsson, B. K., Sibthorpe, D. and Skúlason, S. (2004). Rapid evolution of muscle fibre number in post-glacial populations of Arctic charr Salvelinus alpinus. *J. Exp. Biol.* **207**, 4343–4360.

Johnston, I. A., Lee, H. T., Macqueen, D. J., Paranthaman, K., Kawashima, C., Anwar, A., Kinghorn, J. R. and Dalmay, T. (2009). Embryonic temperature affects muscle fibre recruitment in adult zebrafish: Genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *J. Exp. Biol.* **212**, 1781–1793.

Johnston, I. A., Bower, N. I. and Macqueen, D. J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214**, 1617–1628.

Kalbe, C., Lösel, D., Block, J., Lefaucheur, L., Brüssow, K. P., Bellmann, O., Pfuhl, R., Puppe, B., Otten, W., Metges, C. C., et al. (2017). Moderate high or low maternal protein diets change gene expression but not the phenotype of skeletal muscle from porcine fetuses. *Domest. Anim. Endocrinol.* **58**, 63–75.

Knappe, S., Zammit, P. S. and Knight, R. D. (2015). A population of Pax7-expressing muscle progenitor cells show differential responses to muscle injury dependent on developmental stage and injury extent. *Front. Aging Neurosci.* **7**, 1–17.

Koumans, J. T. M., Akster, H. A., Booms, G. H. R. and Osse, J. W. M. (1993). Growth of carp (Cyprinus carpio) white axial muscle; hyperplasia and hypertrophy in relation to the myonucleus/sarcoplasm ratio and the occurrence of different subclasses of myogenic cells. *J. Fish Biol.* **43**, 69–80.

Lavajoo, F., Perelló-Amorós, M., Vélez, E. J., Sánchez-Moya, A., Balbuena-Pecino, S., Riera-Heredia, N., Fernández-Borràs, J., Blasco, J., Navarro, I., Capilla, E., et al. (2020). Regulatory mechanisms involved in muscle and bone remodeling during refeeding in gilthead sea bream. *Sci. Rep.* **10**, 1–14.

Leitão, N. de J., Pai-Silva, M. D., de Almeida, F. L. A. and Portella, M. C. (2011). The influence of initial feeding on muscle development and growth in pacu Piaractus mesopotamicus larvae. *Aquaculture* **315**, 78–85.

Macqueen, D. J., Robb, D. H. F., Olsen, T., Melstveit, L., Paxton, C. G. M. and Johnston, I. A. (2008). Temperature until the “eyed stage” of embryogenesis programmes the growth trajectory and muscle phenotype of adult Atlantic salmon. *Biol. Lett.* **4**, 294–298.

Mommsen, T. P. (2001). Paradigms of growth in fish. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **129**, 207–219.

Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I. and Gutiérrez, J. (2007a). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (Oncorhynchus mykiss). *Gen. Comp. Endocrinol.* **150**, 462–472.

Montserrat, N., Gómez-Requeni, P., Bellini, G., Capilla, E., Pérez-Sánchez, J., Navarro, I. and Gutiérrez, J. (2007b). Distinct role of insulin and IGF-I and its receptors in white skeletal muscle during the compensatory growth of gilthead sea bream (Sparus aurata). *Aquaculture* **267**, 188–198.

Nebo, C., Portella, M. C., Carani, F. R., de Almeida, F. L. A., Padovani, C. R., Carvalho, R. F. and Dal-Pai-Silva, M. (2013). Short periods of fasting followed by refeeding change the expression of muscle growth-related genes in juvenile Nile tilapia (Oreochromis niloticus). *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **164**, 268–274.

Nielsen, F. C. (1992). The molecular and cellular biology of insulin-like growth factor II. *Prog. Growth Factor Res.* **4**, 257–290.

Nilsson, O. (2018). Genetic and epigenetic regulation of childhood growth. *Nat. Rev. Endocrinol.* **14**, 70–72.

Nissen, P. M., Danielsen, V. O., Jorgensen, P. F. and Oksbjerg, N. (2003). Increased maternal nutrition of sows has no beneficial effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring. *J. Anim. Sci.* **81**, 3018–3027.

Ontell, M., Hughes, D. and Bourke, D. (1988). Morphometric analysis of the developing mouse soleus muscle. *Am. J. Anat.* **181**, 279–288.

Ostaszewska, T., Dabrowski, K., Wegner, A. and Krawiec, M. (2008). The effects of feeding on muscle growth dynamics and the proliferation of myogenic progenitor cells during pike perch development (Sander lucioperca). *J. World Aquac. Soc.* **39**, 184–195.

Pala, F., Di Girolamo, D., Mella, S., Yennek, S., Chatre, L., Ricchetti, M. and Tajbakhsh, S. (2018). Distinct metabolic states govern skeletal muscle stem cell fates during prenatal and postnatal myogenesis. *J. Cell Sci.* **131**,.

Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha, J. A., Perera, E., Bermejo-Nogales, A., Benedito-Palos, L. and Calduch-Giner, J. A. (2018). Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Front. Endocrinol. (Lausanne).* **9**, 1–24.

Peterson, B. C., Waldbieser, G. C. and Bilodeau, L. (2004). IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (Ictalurus punctatus). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **139**, 317–323.

Picha, M. E., Silverstein, J. T. and Borski, R. J. (2006). Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (Morone chrysops × Morone saxatilis). *Gen. Comp. Endocrinol.* **147**, 196–205.

Picha, M. E., Biga, P. R., Galt, N., McGinty, A. S., Gross, K., Hedgpeth, V. S., Siopes, T. D. and Borski, R. J. (2014). Overcompensation of circulating and local insulin-like growth factor-1 during catch-up growth in hybrid striped bass (Morone chrysops×Morone saxatilis) following temperature and feeding manipulations. *Aquaculture* **428–429**, 174–183.

Pipalia, T. G., Koth, J., Roy, S. D., Hammond, C. L., Kawakami, K. and Hughes, S. M. (2016). Cellular dynamics of regeneration reveals role of two distinct Pax7 stem cell populations in larval zebrafish muscle repair. *DMM Dis. Model. Mech.* **9**, 671–684.

Purohit, G. and Dhawan, J. (2019). Adult Muscle Stem Cells: Exploring the Links Between Systemic and Cellular Metabolism. *Front. Cell Dev. Biol.* **7**, 1–14.

Rehfeldt, C., Stickland, N., Fiedler, I. and Wegner, J. (1999). Environmental and genetic factors as sources of variation in skeletal muscle fibre number. *Bam-Padova-* **9**, 235–254.

Rehfeldt, C., Te Pas, M. F. W., Wimmers, K., Brameld, J. M., Nissen, P. M., Berri, C., Valente, L. M. P., Power, D. M., Picard, B., Stickland, N. C., et al. (2011). Advances in research on the prenatal development of skeletal muscle in animals in relation to the quality of muscle-based food. I. Regulation of myogenesis and environmental impact. *Animal* **5**, 703–717.

Rescan, P. Y. (2001). Regulation and functions of myogenic regulatory factors in lower vertebrates. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **130**, 1–12.

Rescan, P. Y., Montfort, J., Rallière, C., Le Cam, A., Esquerré, D. and Hugot, K. (2007). Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. *BMC Genomics* **8**, 1–18.

Rescan, P. Y., Montfort, J., Fautrel, A., Rallière, C. and Lebret, V. (2013). Gene expression profiling of the hyperplastic growth zones of the late trout embryo myotome using laser capture microdissection and microarray analysis. *BMC Genomics* **14**, 1–11.

Rescan, P. Y., Ralliere, C., Lebret, V. and Fretaud, M. (2015). Analysis of muscle fibre input dynamics using a myog:GFP transgenic trout model. *J. Exp. Biol.* **218**, 1137–1142.

Rescan, P. Y., Cam, A., Rallière, C. and Montfort, J. (2017). Global gene expression in muscle from fasted/refed trout reveals up-regulation of genes promoting myofibre hypertrophy but not myofibre production. *BMC Genomics* **18**, 1–12.

Roselló-Díez, A. and Joyner, A. L. (2015). Regulation of long bone growth in vertebrates; It is time to catch up. *Endocr. Rev.* **36**, 646–680.

Rossi, G. and Messina, G. (2014). Comparative myogenesis in teleosts and mammals. *Cell. Mol. Life Sci.* **71**, 3081–3099.

Rowe, R. W. and Goldspink, G. (1969). Muscle fibre growth in five different muscles in both sexes of mice. *J. Anat.* **104**, 519–30.

Rowlerson, A., Mascarello, F., Radaelli, G. and Veggetti, A. (1995). Differentiation and growth of muscle in the fish Sparus aurata (L): II. Hyperplastic and hypertrophic growth of lateral muscle from hatching to adult. *J. Muscle Res. Cell Motil.* **16**, 223–236.

Rowlerson, A., Radaelli, G., Mascarello, F. and Veggetti, A. (1997). Regeneration of skeletal muscle in two teleost fish: Sparus aurata and Brachydanio rerio. *Cell Tissue Res.* **289**, 311–322.

Roy, S. D., Williams, V. C., Pipalia, T. G., Li, K., Hammond, C. L., Knappe, S., Knight, R. D. and Hughes, S. M. (2017). Myotome adaptability confers developmental robustness to somitic myogenesis in response to fibre number alteration. *Dev. Biol.* **431**, 321–335.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. and Moorman, A. F. M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* **37**, e45–e45.

Sabourin, L. A. and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clin. Genet.* **57**, 16–25.

Schiaffino, S., Dyar, K. A., Ciciliot, S., Blaauw, B. and Sandri, M. (2013). Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* **280**, 4294–4314.

Sciara, A. A., Vigliano, F. A., Somoza, G. M. and Arranz, S. E. (2011). Muscular hypertrophy and growth-promoting effects in juvenile pejerrey (Odontesthes bonariensis) after oral administration of recombinant homologous growth hormone obtained by a highly efficient refolding process. *Aquac. Res.* **42**, 844–857.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777–786.

Sebens, K. P. (1987). The ecology of indeterminate growth in animals. *Annu. Rev. Ecol. Syst. Vol. 18* 371–407.

Seiliez, I., Froehlich, J. M., Marandel, L., Gabillard, J. C. and Biga, P. R. (2015). Evolutionary history and epigenetic regulation of the three paralogous pax7 genes in rainbow trout. *Cell Tissue Res.* **359**, 715–727.

Soto, S. M., Blake, A. C., Wesolowski, S. R., Rozance, P. J., Barthel, K. B., Gao, B., Hetrick, B., McCurdy, C. E., Garza, N. G., Hay, W. W., et al. (2017). Myoblast replication is reduced in the IUGR fetus despite maintained proliferative capacity in vitro. *J. Endocrinol.* **232**, 475–491.

Valente, L. M. P., Rocha, E., Gomes, E. F. S., Silva, M. W., Oliveira, M. H., Monteiro, R. A. F. and Fauconneau, B. (1999). Growth dynamics of white and red muscle fibres in fast- and slow-growing strains of rainbow trout. *J. Fish Biol.* **55**, 675–691.

Valente, L. M. P., Bower, N. I. and Johnston, I. A. (2012). Postprandial expression of growth-related genes in Atlantic salmon (Salmo salar L.) juveniles fasted for 1 week and fed a single meal to satiation. *Br. J. Nutr.* **108**, 2148–2157.

Veggetti, A., Mascarello, F., Scapolo, P. A. and Rowlerson, A. (1990). Hyperplastic and hypertrophic growth of lateral muscle in Dicentrarchus labrax (L.) - An ultrastructural and morphometric study. *Anat. Embryol. (Berl).* **182**, 1–10.

Weatherley, A. H., Gill, H. S. and Lobo, A. F. (1988). Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *J. Fish Biol.* **33**, 851–859.

Wilson, S. J., Ross, J. J. and Harris, A. J. (1988). A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats. *Development* **102**, 815–821.

Won, E. T. and Borski, R. J. (2013). Endocrine regulation of compensatory growth in fis. *Front. Endocrinol. (Lausanne).* **4**, 1–13.

Yakar, S., Werner, H. and Rosen, C. J. (2018). 40 YEARS OF IGF1: Insulin-like growth factors: actions on the skeleton. *J. Mol. Endocrinol.* **61**, T115–T137.

Yang, Y., Zhou, H., Hou, L., Xing, K. and Shu, H. (2019). Transcriptional profiling of skeletal muscle reveals starvation response and compensatory growth in spinibarbus hollandi. *BMC Genomics* **20**, 1–16.

Yates, D. T., Clarke, D. S., Macko, A. R., Anderson, M. J., Shelton, L. A., Nearing, M., Allen, R. E., Rhoads, R. P. and Limesand, S. W. (2014). Myoblasts from intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. *J. Physiol.* **592**, 3113–3125.

Figures and Table

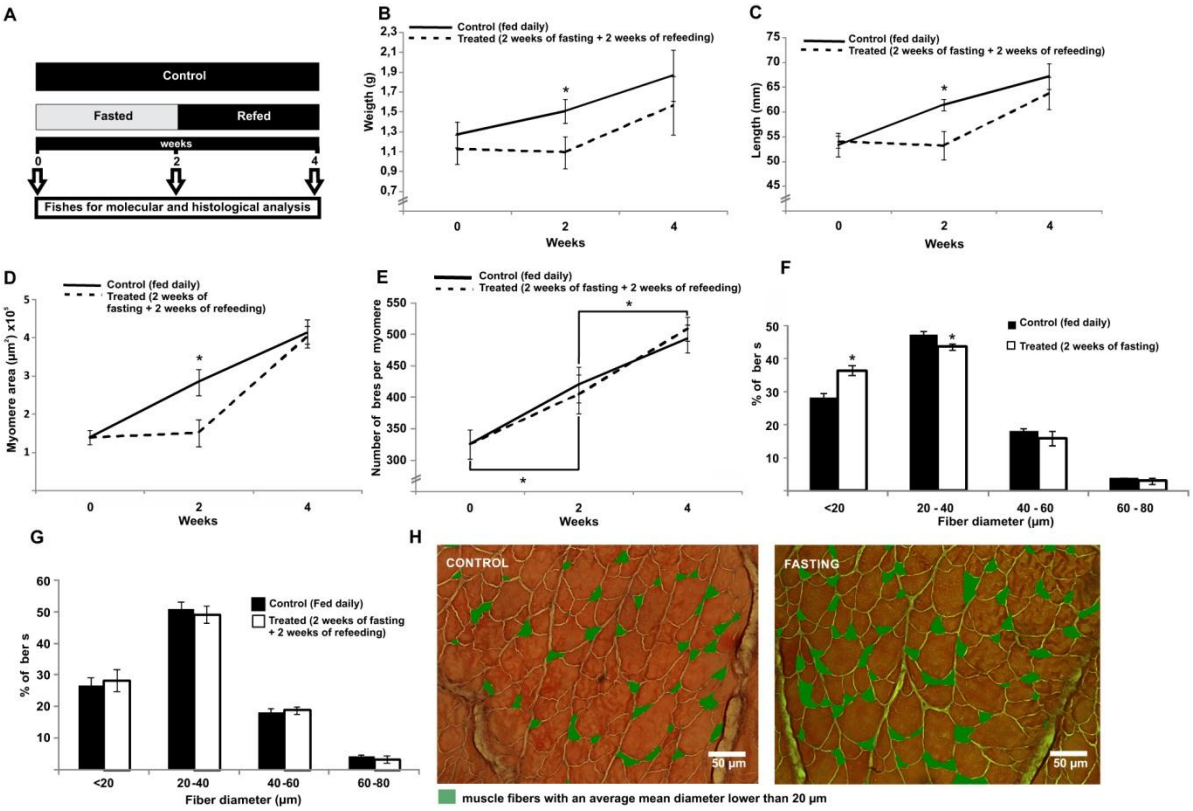

Fig 1. Muscle hypertrophy and hyperplasia are differentially regulated during fasting and refeeding. (A) Schematic experimental protocol. Juvenile pejerrey individuals (53 mm mean body length) in the fast-growing phase were subjected to 2 weeks of fasting, and then to 2 weeks of re-feeding (Fasted-refed group), or were subjected to daily feeding (Control group). (B) Body weight and (C) length curves of pejerrey in fasted-refed and control groups. (D) Myomere area and (E) total number of fibers from a myomere were measure at 0, 2 and 4 weeks in fishes from both groups. (F, G) The diameters of the myofibres were measured and the proportion of fibers of different diameters (<20 µm, 20-40 µm, 40-60 µm and 60-80 µm) per selected myomere were determined after two weeks (F) and four weeks (G) of treatments. (H) Representative image showing the small muscle fibers (<20 µm, in green) in a histological cross section of fishes at two weeks under control or fasting conditions. *Significant difference from controls (Two way nested ANOVA followed by Tukey test for panels B, C, D and E; One way nested ANOVA for panels F and G; $P<0.05$). Data are presented as mean ± s.e.m. and compiled from the analysis of fishes on 3 tanks for treatment, 6 fishes per tank for weight and length measurements, and 3 fishes per tank for histological analysis.

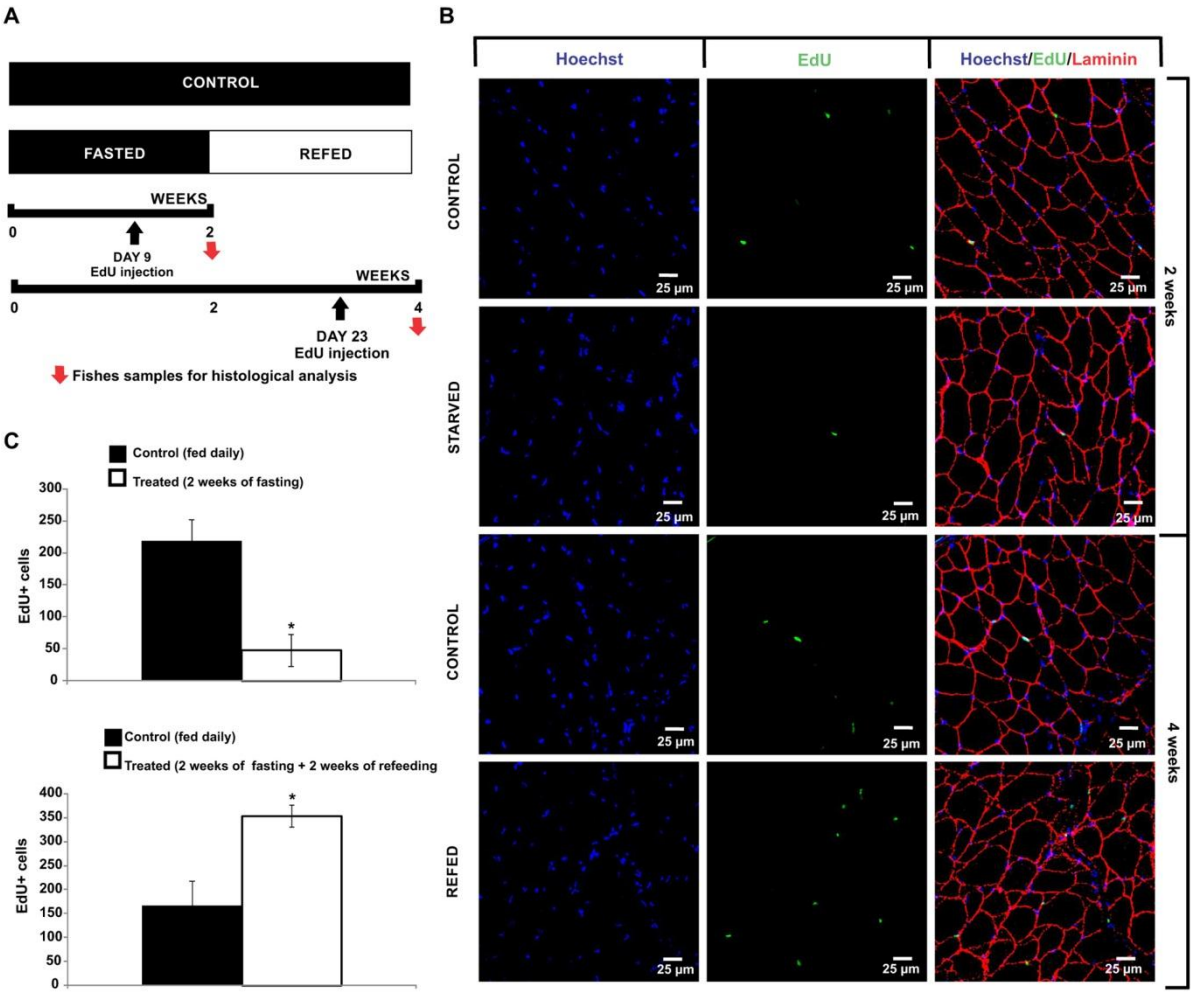

Fig 2. Cell proliferation decreases during fasting and is enhance during refeeding. (A) Schematic experimental protocol. Fishes received an intra peritoneal injection of 5-ethynyl-2'-deoxyuridine (EdU) during fasting and refeeding periods. The same treatment was applied at fishes from control group. (B) Representative images of muscle pejerrey cryo-sections labelled with Hoechst (blue), anti-laminin (red) and EdU (green) after 2 weeks of treatment in control and starved groups, and after 4 weeks of treatment in controls and refed group. (C) Total proliferative EdU positive cells on half epaxial muscle of pejerrey cryo-section of starved and refed fishes and the control groups. * Significant difference from control (One way nested ANOVA, $P<0.05$. Values are expressed as mean ± s.e.m., 3 tanks for treatment, 4 fishes per tank.

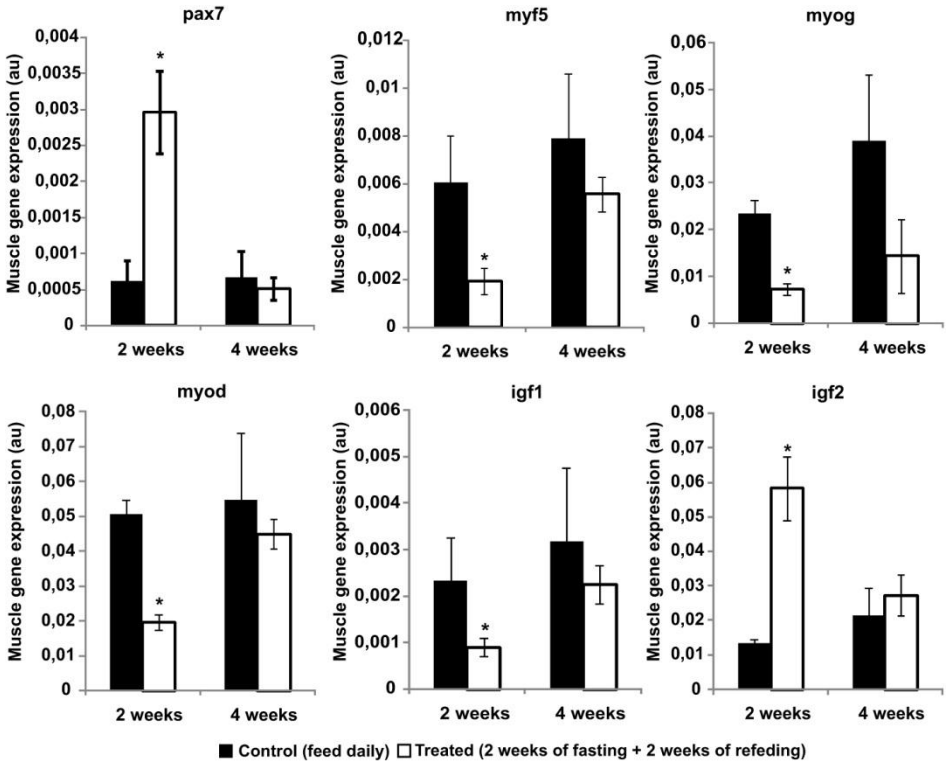

Fig 3. Genes involved in muscle growth are differentially regulated during fasting and refeeding. Relative muscle mRNA expression (arbitrary units) of transcription factors pax7, myf5, myog, myod and growth factors igf1 and igf2 after fasting and refeeding. Standard deviation is depicted with positive and negative error bars. All qPCR graphs show gene expression normalized to ef1a. *Significant difference from control (One way ANOVA, $P<0.05$), n=9 (3 tanks for treatment, 3 fishes per tank).

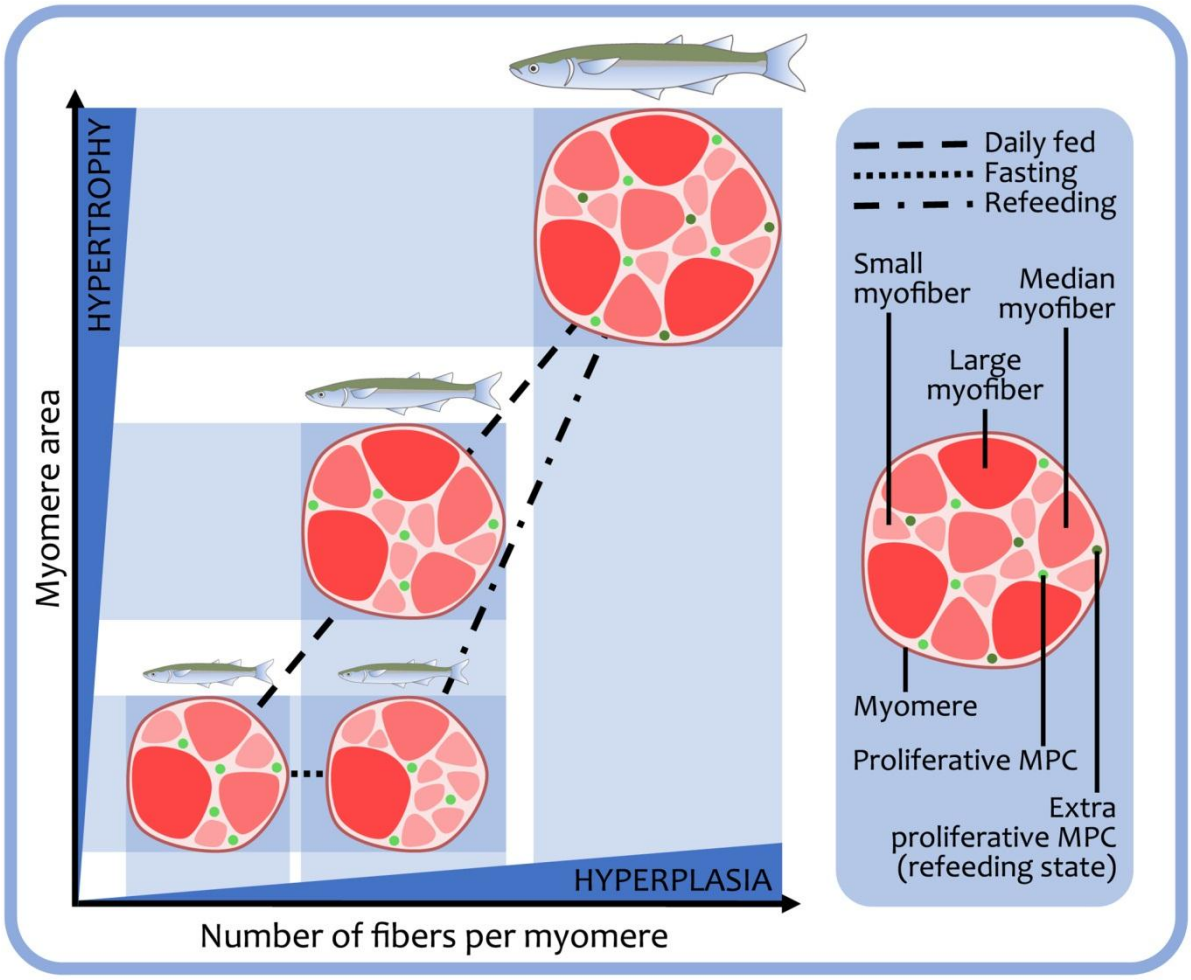

Fig 4. Model depicting the cellular bases of compensatory growth in fish. This model assumes that muscle hypertrophy but not hyperplasia is mainly regulated by nutritional status. A population of muscle progenitor cells proliferate and contribute to new fiber formation during mosaic hyperplasia even under adverse nutritional conditions. Once adverse conditions cease, catch up growth is mainly the result of compensatory hypertrophy of the small muscle fibers formed during fasting, and is partly based on enhancement of MPCs proliferation that contributes to fiber hypertrophy growth.

Table 1. Percentage (mean and standard deviation [sd]) of laminin-attached mononucleated cells isolated from white muscle and revealed for EdU and Myod

Isolated cells markers	Control fish		Fasted fish	
	mean (%)	sd (%)	mean (%)	sd (%)
$Myod^-/EdU^+$	0	0	0	0
$Myod^-/EdU^-$	7.1	1.75	9.96	4.21
$Myod^+/EdU^-$	81.45	4.28	85.96	6.47
$Myod^+/EdU^+$	11.45	1.98	4.08*	2.03

*Significant difference from control (One way ANOVA, $P<0.05$). n=3

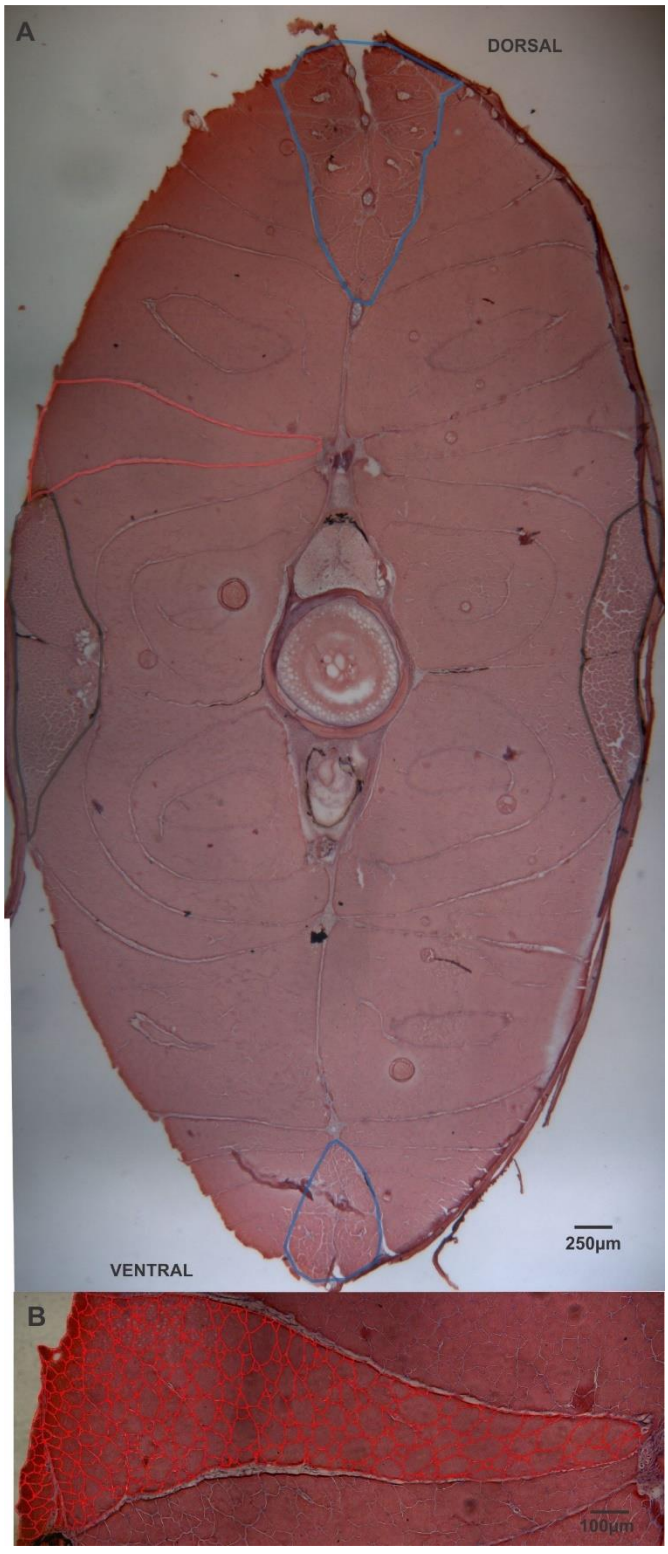

Fig. S1. Hematoxilin-eosin stain of a juvenile pejerrey histological transversal section. Distribution of myofibres and myomeres in a cross-section at position 1/4 of the total length starting from the tail (A). Representative myomere whose muscle fibers mean diameters ranges were found to correlate to the whole section outlined in red. Blue outline shows the red muscle fibers and the muscle involved on fin movement (dorsal and anal fins). (B) Muscle fibers of a myomere were outlined using the Image-Pro Plus 6.0 software.

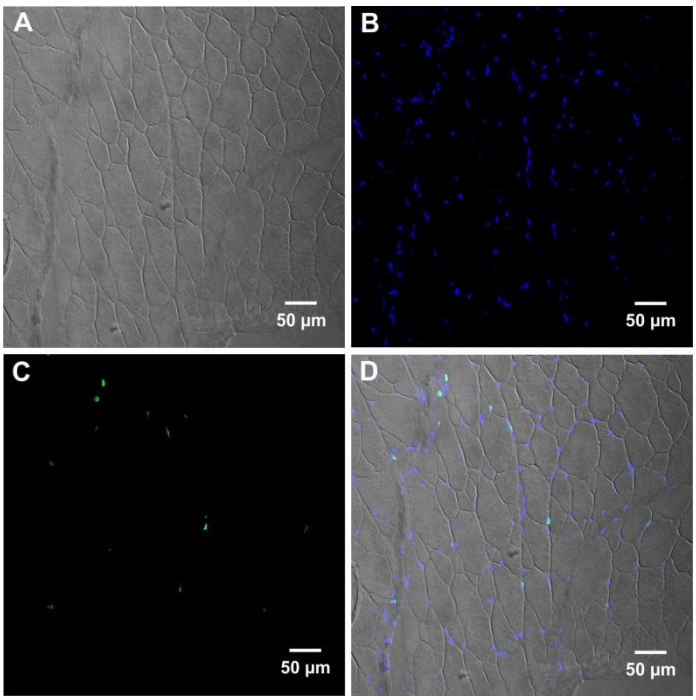

Fig. S2. Validation assay to label pejerrey muscle cell proliferation.
Representative images of muscle pejerrey cryo-sections of white muscle from fishes (50 mm mean body length) 5 days after injections with EdU (75mg/Kg). (A) bright field, (B) labelled with Hoestch, (C) labeled *in vivo* with EdU, and (D) merge images A, B and C.

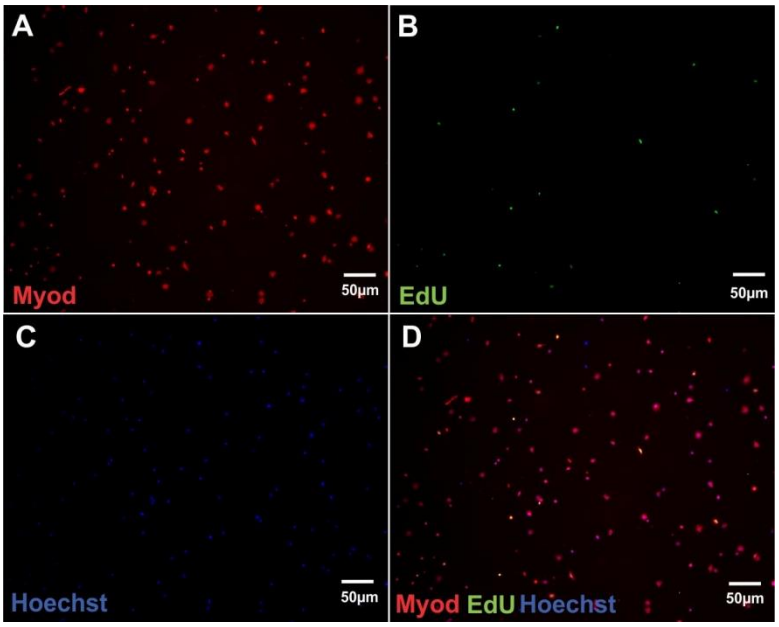

Fig. S3. EdUco-localize with Myod immunodetection in isolated white muscle cells. Representative images of *in vitro*cultured mononucleated cells isolated from white muscle one day after injection with EdU. (A) Myod. (B) EdU. (C) Hoechst. (D) merge images A, B and C.

Table S1. Sequences of the primer pairs forward (F) and reversed (R) used for qPCR assays

Gene	Primer Sequence (5' to 3')
Oligo dT (16)	TTTTTTTTTTTTTTTT
qEF1F	TGGGTGCTGGACAAACTGAAG
qEF1R	CTGTGTCCAGGGGCATCAAT
IGF1-F	AACTGCGGCGCCTGGAAATG
IGF1-R	GTCTTGTCTGGCTGCTGTGCTGTC
qIGF2F	AGAGAACAGCCGAATAAAGGTCA
qIGF2R	TGCTGGTTGGCCTACTGAAAT
qPax7F	CACAAGATAGTGGAGATGGCC
qPax7R	ATCTTAGAGACGCAGCCGT
qMyoDF	CAAAGTGGAGATCCTGCG
qMyoDR	TCCCCGCTGTAGTGTTC
qMyf5F	TGCCATCCAATACATCGAA
qMyf5R	TTGTTTCCAGCCATGCC
qMyoGF	GGTGTTGGTGTTGGAGTG
qMyoGR	CCCTCCTTCCTTTTACACAG