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Miquel Perelló Amorós

REGULATORY MECHANISMS OF MUSCLE GROWTH AND METABOLISM IN FISH: MODULATION BY NUTRITIONAL STATUS, DIET COMPOSITION, EXERCISE AND MUSCULAR REGENERATION

MIQUEL PERELLÓ AMORÓS

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Department of Cell Biology, Physiology and Immunology, University of Barcelona

REGULATORY MECHANISMS OF MUSCLE GROWTH AND METABOLISM IN FISH: MODULATION BY NUTRITIONAL STATUS, DIET COMPOSITION, EXERCISE AND MUSCULAR REGENERATION

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Signed by the PhD Candidate:

Miquel Perelló Amorós

Signed by the Codirectors:

Dr. Joaquim Gutiérrez and Dra. Josefina Blasco

Signed by the Tutor:

Dr. Jaume Fernández Borràs

*"ALS MEUS **PADRINS**, SOQUES ORIGINARIES DE LA MAJORIA DE FAMILIES, VISTES DESDE UN MATEIX. SÓN AQUELLES PERSONES QUÈ, PER ANYS QUE PASSIN, LA CALIDESA QUE ELS CARACTERITZA, ROMAN INTACTE, INSUBSTITUÏBLE I CRUELMENT INDISPENSABLE".*

*"ALS MEUS **PARES**, CULPABLES PRIMERS DE QUE ALGÚ HAGI DE DEDICAR EL SEU TEMPS PERDRE'S EN AQUEST TOM. A VOSALTRES US HO DEC LITERALMENT TOT, PASSAT, PRESENT...I FUTUR".*

*"A NA **MAHBOOB**, SENSE CAP TÍPUS DE DUBTE, EL MILLOR "RESULTAT" IMAGINABLE D'UNA TESI DOCTORAL I EN EL QUE SEGUIRÉ TREBALLANT FINS QUE FACI EL DARRER ALÈ. GRACIES PER SER AL MEU COSTAT, T'ESTIM".*

*"ALS MÉUS **GERMANS**...I **GERMANA**, AMB ELS QUÍ M'HE DE DISCULPAR PER NO SABER SER GERMÀ GRAN, PERÒ ALS QUALS HI TENC UNA SENSIBILITAT DE LA QUE ET FA GELAR EL CLATELL".*

*"A EN **MARTÍ**, NA **BÁRBARA** (SA "MADRINA"), EN **JOSEP** I EN **MARTÍ** ("MARTINET"); ALS QUÈ HE D'AGRAÏR ETERNAMENT QUE M'ACOMPANYASSIN, JUNTAMENT ALS MEUS PARES, AL VIATGE MÉS IMPORTANT QUÈ HE FET MAI, I QUÈ HA MARCAT UN ABANS I UN DESPRÉS EN EL TRANSCURS D'AQUESTA TESI I LA MEVA VIDA".*

*"PER EXTENSIÓ, A LA RESTA DE LA **FAMILIA** QUE M'HA VIST CRÉIXER I AGUANTAT AMB PACIÈNCIA".*

ACKNOWLEDGEMENTS

Què l'únic llegat que quedi d'una tesi doctoral sigui un llibre, engreixat a base de text científic lliure de sentiment, no fa justícia ni és representatiu del què una tesi doctoral representa per a una persona que fa uns anys va decidir intentar afegir l'etiqueta de Dr. o Dra. al costat del seu nom. Si tot va bé, part de la feina realitzada quedarà reflectida en un llistat de publicacions científiques més o menys reconegudes, que deixaran constància del que un hagi pogut assolir durant el trajecte, tot i que malauradament no sempre és així. Del contrari, l'experiència viscuda, roman només a les memòries de les persones que se t'hagin creuat pel camí i "interactuat" amb la teva trajectòria. Com a mallorquí, la meva metàfora de la Tesi Doctoral podria ser ben bé la Ma-10, 112 Km d'asfalt amb un continu serpentejat de corbes de 180º (o més) que discorren entre els barrancs de la vessant litoral de la Serra de Tramuntana de cap a cap, probablement el millor indret per admirar l'illa. Si un es fixa només en la dificultat de la conducció, pot ser tortuós nomes de pensar-ho, però si de tant en tant s'aixeca la vista i un es deixa impressionar per les vistes imponents de Tramuntana, el trajecte haurà valgut més la pena que el destí. Feta la reflexió, no podria continuar sense agrair, encara que sigui en forma de text, a totes les persones i institucions que d'una manera han coincidit amb mi en aquest viatge que continua.

Fent historia, primer de tot haig d'agrair al Dr. Joaquim Gutiérrez per a què em donés l'oportunitat de realitzar el treball de final de grau en el seu grup i posteriorment, juntament amb la Dra. Josefina Blasco i el Dr. Jaume Fernández per deixar-me continuar durant el treball de final de màster, assentant les bases del què ha estat aquesta Tesi. Moltes gràcies per haver dipositat la vostra confiança en mi i espero que el resultat no s'allunyi massa de les expectatives, ja que soc conscient de tots els mals de caps què us hauré ocasionat, han estat 4 anys ben llargs. Agraeixo moltíssim tot el què heu estat a sobre del transcurs dels experiments, publicacions, tesis, etc., i us heu esforçat per ajudar-me quan ho he necessitat. Aquesta qualitat no la puc passar per alt, ja què és el que tot director/a de tesi hauria de tenir. Seguidament, he d'agrair profundament a l'Emilio i a l'Esmail tot el què em van ensenyar durant els meus primers anys al departament. A vosaltres us dec quasi tota la formació bàsica què he rebut, a part de moltes sobretaules, cerveses i rialles (i fava tonka) que des de la vostra partida tant hem enyorat. Culpa vostra han estat molts dels meus mals de caps, vau deixar el llistó molt alt i ara sou dos científics exemplars. No puc deixar d'agrir també a la resta de membres "sènior" del grup, com la Dra. Isabel Navarro, cap del programa de Doctorat què m'ha acollit, i a la omnipresent Encarni, moltíssimes gracies per tot (que no és poc) amb els què m'has ajudat amb les publicacions. Altres, estan lluitant per iniciar la seva carrera com a investigadors/es. Gracies a la Irene, la Sheida, el Borja, l'Ignasi, el Sergio i la Garoa per ajudar-me i ensenyar-me quan les he necessitat. Sense aquestes persones esmentades, ara mateix estaríeu llegint una altra cosa, però ells són només el principi. Seguidament, agrair a la resta de personal investigador, docent i administratiu del la Secció de Fisiologia i Immunologia del Departament que m'han ajudat, d'una manera o altra. Especial menció a la Marga, la Vero i la Cristina. La feina dels tècnics és indispensable en qualsevol laboratori. Moltes gracies també al Dr. Josep Chillarón, per la seva predisposició a ajudar i amabilitat, i al Dani García de la Serrana, per tot el què m'ha ensenyat en qüestió de genòmica. Moltes gracies al personal de l'estabulari humit, el Jordi Guinea, Antonio i companyia, per el suport rebut durant aquests anys. Estic obligat també a agrair a les persones què m'han acollit en les dues estades que he pogut realitzar. Moltes gracies al Dr. Jaume Pérez, Dr. Josep Calduch, i als companys, Paula, Antonio i Erik que van fer molt

agradable (i constructiu) passar un més de febrer a l'IATS, on vaig tenir la oportunitat de veure per primera vegada un model "diferent" de recerca i aprendre una tècnica com el RIA. Some of the most powerful memories that I have of this thesis have the freshness of the weather and the warmness of the people from the French Britain. To Jean-Charles, Cécile and Pierre-Yves to show me the good manners of the INRA Rennes and your massive knowledge, I enjoyed learning in your team, where I studied the two genes with the best-ever name that I know. The full experience was very rewarding. To them and to the other colleagues that I met there (Roland, Guillaume, et al.) I hope to meet you again. Un dels aspectes què han enriquit molt el transcurs de la tesi ha estat el pas desenfrenat d'estudiants i/o col·laboradors/es de diferents indrets del planeta què han passat pel grup en aquests anys. A ells han anat a parar incomptables hores del meu temps a intentar-los ensenyar tot el que sé, de la millor manera que he pogut, però que sense ells, molta de la feina de dintre i fora d'aquest llibre no hagués estat possible. Moltes gracies a TFGs i TFMs com el Gerard, Alba Sauch, Clara, Maria Xandri, Emma, Ruth, Julia, Júlia, Jaume "Dj" Vela, Luís "tiburón", Sebastià, Anna, Mariona, Albert, l'Arnau, la Violeta i l'Aitor, qui m'ha ajudat molt aquest darrer any. Moltes gracies als què venint de fora, m'han internacionalitzat una mica, com l'Antonio, la Yu, la Fatemeh L, la Maryam H, Maryam M i Fatemeh M. Si em deixo algú, que es doni per inclòs/a. Finalment, em toca la part més fàcil i difícil a la vegada; agrair als companys de laboratori i cubicle, què han acabat per convertir-se en la meva família Barcelonina i amb els què he gaudit de incomptables moments dintre i fora del Departament. Tot va començar amb l'Emilio i l'Esmail, però ben d'hora, una colla de persones van anar apareixent per formar aquesta família. Moltes gracies a la Natalia, ordre i eficiència personificades, què em va demostrar el grau de "desordre" què soc capaç de desenvolupar. A la Esti, "fiestis" o "super Esti", com m'agrada més anomenar-la. Flamant nova doctora del departament, amb tots els mèrits possibles i què m'ha deixat un llistó de presentació molt alt. La teva determinació ens serveix a la resta d'exemple. Al Joan, "bro", representant legal d' "el mono", company de "cubi", pis i busseig, tens un "palmarès" a l'altura de pocs en aquest aspecte. Moltes gràcies per les incomptables vegades què m'has donat una mà amb el què faci falta. A la Sara, la nostra "Sari", què amagada sota colors "pastel" i "holis", amenaça amb fer-li ombra al mateix Emilio. Segur que defensaràs una tesi espectacular i tindràs un gran futur científic i personal. Vull ser-hi a prop per veure-ho. Y què he de dir de l'Albert...de les persones més especials què he tingut el plaer de conèixer, un "savi", ment crítica per definició, "estadista", gourmet i amic. M'has ensenyat moltes coses durant aquests anys, dins i fora de la ciència...i les què em queden per aprendre. Aquesta "família" la tanca la Isabel, un relleu necessari i a la què no li puc agrair en paraules l'ajuda què m'ha suposat aquest darrer any de tesi. Tens totes les qualitats per ser una científica excepcional i no dubto què ho seràs. Entre tots els esmentats, heu contribuït a la meva evolució científica i sobretot personal.

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CHAPTER 1: INTRODUCTION

1. INTRODUCTION

The aquaculture in an ancient practice that consists in the culture of aquatic organisms, from aquatic plants and algae to invertebrates and fishes, during the totality or a part of their live and reproductive cycle in confined facilities under controlled rearing conditions. Since its origins situated approximately 5500 years ago to the 19th century, this practice was carried out under the rules of rudimentary knowledge, which started to scientifically evolve since the early 1800s propelled by an arising interest in this practice, which in the mid-1980s slowly became an important economical practice with progressively increasing production rates.

1.1.Actual state of the world and Mediterranean aquaculture

In the last 50 years, an important responsibility has been put on the aquaculture production, as in the early 1990s, the production from extractive capture has reached a plateau, while the demand on these products has exponentially increased as a consequence of a rapidly increasing world population. Soon after this take off of the aquaculture industry, the Food and Agriculture Organization of the United Nations published "The Code of Conduct for Responsible Fisheries", a text released in 1995 which aimed to establish the basic principles and rules to be used as the seed and frame of the subsequent national and international instruments, policies and programs that watch over the fisheries and aquaculture's responsible and sustainable growth and development. Since then, the aquaculture has been gaining weight in the list of the different economical activities that has more potential to contribute to the fight against the big challenges that the humanity has been starting to face in the last decades and that will be magnified in the near future, due to a fast-growing population in an already overexploited world. Thus, one of the most recent milestones that the aquaculture has reached, is to be included since 2015 in "The 2030 Agenda for Sustainable Development", conformed by 17 ambitious Sustainable Development Goals (SDGs) that act as global directives that international community is actually following. In this context, the aquaculture has its own agenda so called "THE 2030 AGENDA AND THE SUSTAINABLE DEVELOPMENT GOALS: THE CHALLENGE FOR AQUACULTURE DEVELOPMENT AND MANAGEMENT and has been recently accepted that this activity has direct implications in all the 17 SDGs (Reviewed by Sampantamit et al. 2020) (Figure 1).

Figure 1: The contributions of capture fisheries and aquaculture production in underpinning the achievement of the Sustainable Development Goals (SDGs). Extracted from Sampantamit et al. (2020).

To this end, the implementation of science-based fisheries and aquaculture management policies, coupled with predictable and transparent regimes for international fish utilization and trade, are widely accepted as minimum substantive criteria for sustainable fisheries and aquaculture. To support evidence-based endeavors, the edition of The State of World Fisheries and Aquaculture presents updated and verified statistics of the sector and analyses current and emerging issues and approaches needed to accelerate international efforts to achieve the goal of sustainable fisheries and aquaculture.

The global fish production is, since the beginning of the recordings, in a constant increase year by year and the last estimations by the FAO situate the 2018 record values at 179 million tons worth USD 401 billion just as a first sale (Figure 2). From this total production, a 46% (82 million tons) worth USD 250 billion, corresponded to aquaculture production. Moreover, from the global production, 87,15% (156 million tons) were directed to human consumption, representing an estimated 20.5 kg per capita annually, while the rest (22 million tons) were destined for non-food uses, mainly to produce fishmeal and fish oil (Figure 2) (FAO 2020).

Aquaculture accounted for 46 percent of the total production and 52 percent of fish for human consumption. Globally, China has remained a major fish producer, accounting for 35 percent of global

fish production in 2018. Excluding China, a significant share of production in 2018 came from Asia (34 percent), followed by the Americas (14 percent), Europe (10 percent), Africa (7 percent) and Oceania (1 percent) (FAO 2020).

The total fish production has increased importantly in all the continents in the last few decades, except in Europe (with a gradual decrease from the late 1980s that is slightly recovering in the last few years) and the Americas (Figure 3).

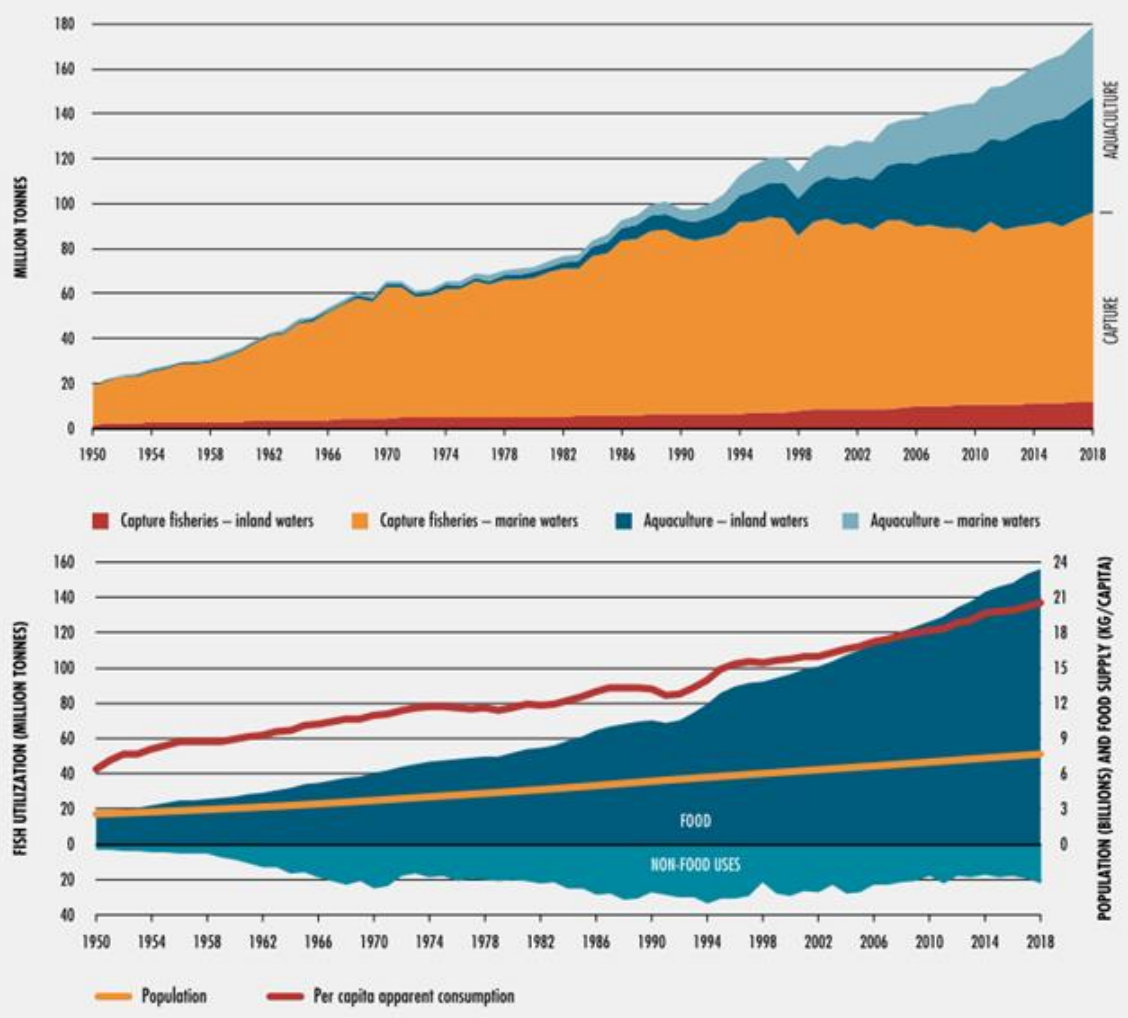


Figure 2: Up, global fish production from the different major sources; down, relationship between global population, fish utilization for food and non-food purposes and overall fish consumption per capita. Extracted from FAO, (2020).

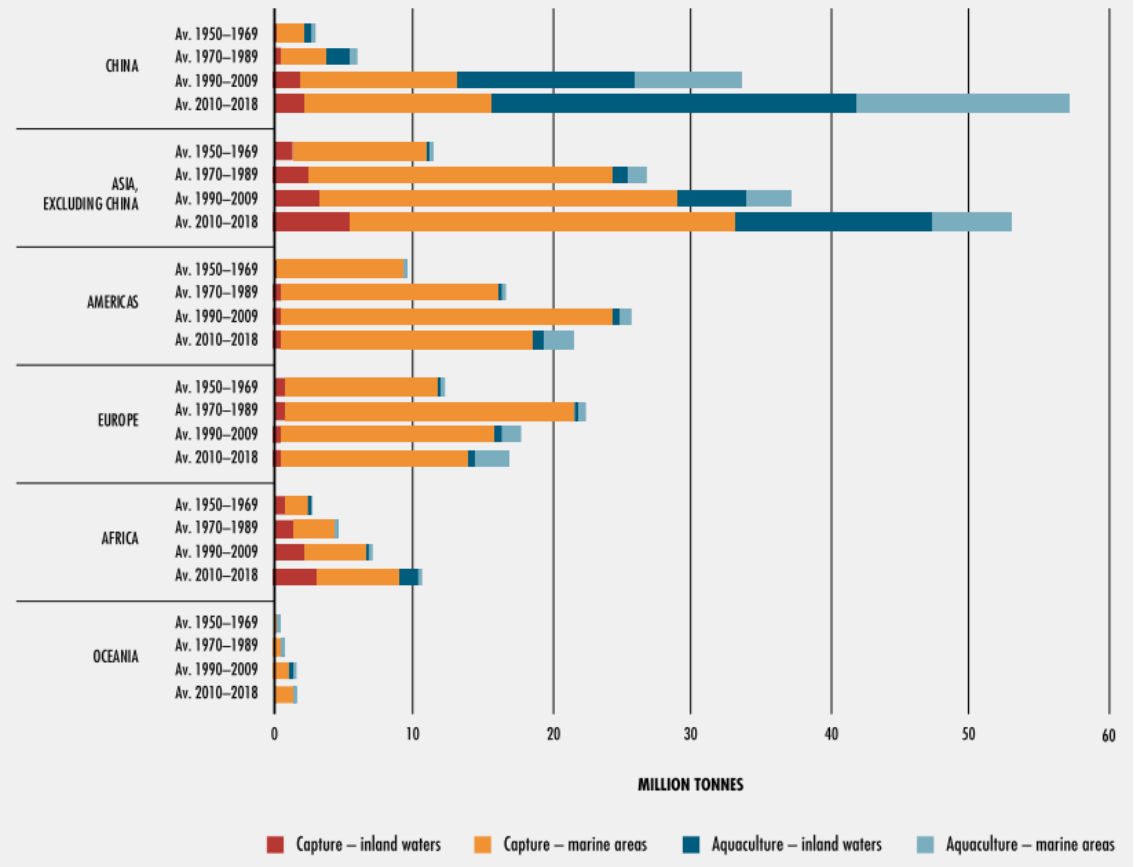


Figure 3: Regional contribution to the world fisheries and aquaculture production. Extracted from FAO, (2020).

In parallel to the production, the global food fish consumption increased annually a 3.1% from 1961 to 2017, doubling the rate of annual world population growth (1.6 %) for the same period, and even being higher than the growth rate of any other animal protein foods (meat, dairy, milk, etc.) which increased by 2.1 percent annually as average (FAO 2020).

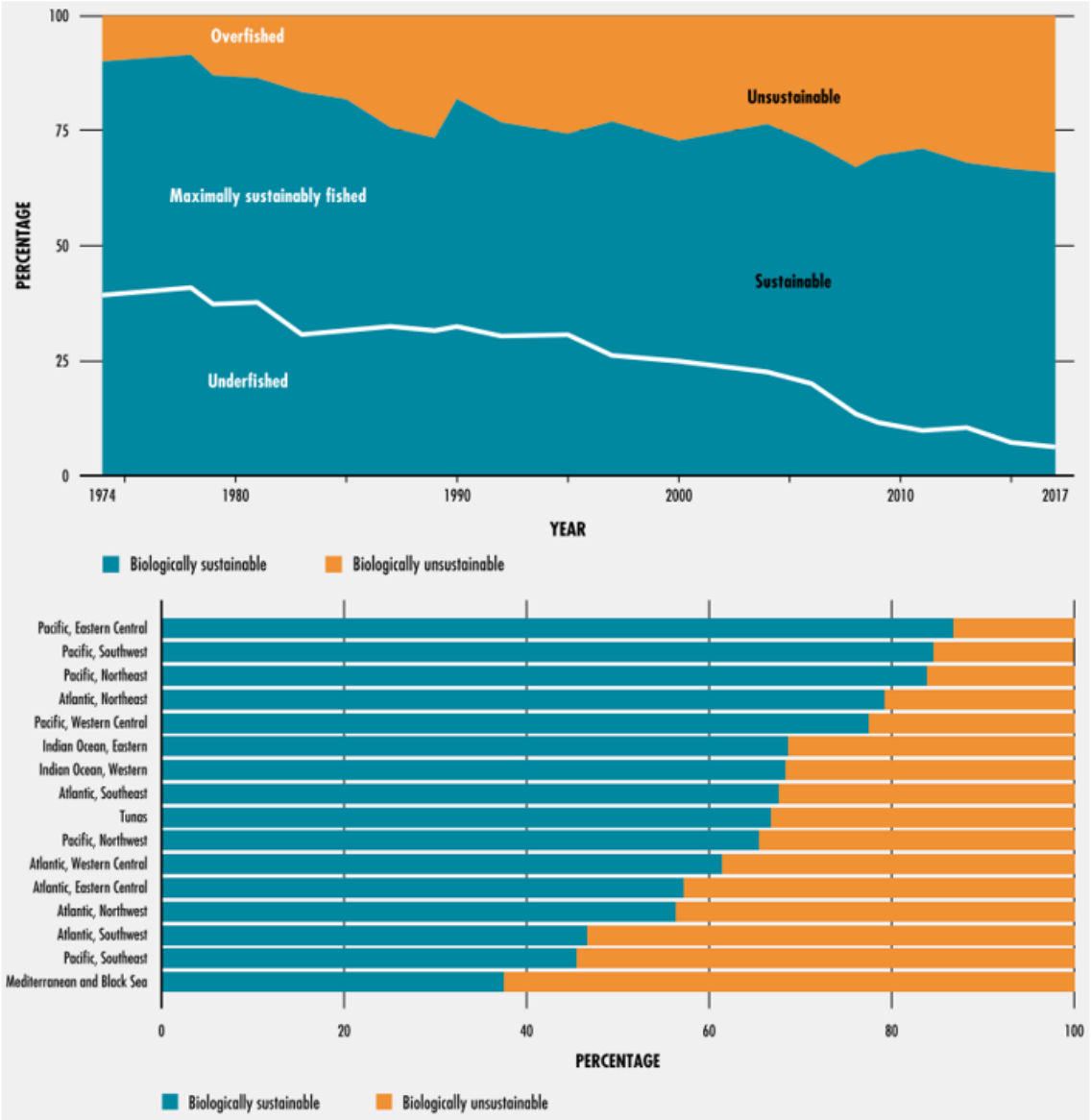


Figure 4: Up, global trends in the state of the world's marine fish stocks; Down, percentages of stocks fished at biologically sustainable and unsustainable levels. Extracted from FAO, (2020).

In 2017, among FAO's Major Fishing Areas, the Mediterranean and Black Sea had the highest percentage (62.5%) of stocks fished at unsustainable levels, followed by the Southeast Pacific (54.5%) and Southwest Atlantic (53.3 %). In contrast, the Eastern Central Pacific, Southwest Pacific, Northeast Pacific and Western Central Pacific had the lowest proportion (13–22 percent) of stocks fished at biologically unsustainable levels. Other areas varied between 21 % and 44% in 2017 (Figure 4) (FAO 2020).

1.2.Gilthead sea bream (*Sparus aurata*) and Rainbow trout (*Oncorhynchys mykiss*)

Gilthead sea bream, *Sparus aurata*, is a euryhaline and eurythermal species found in marine and brackish water and is common in the Mediterranean Sea. This species is a protandrous hermaphrodite which means that during the first or second year of life they are as a male to then turn into a female when they have 33-40 cm body length (Colloca and Cerasi 2011). In late autumn, they migrate to the open sea to spawn (Barbaro et al. 1997), and in early spring, the juveniles migrate to estuaries and protected coastal waters, where there is enough food and milder temperatures. In the larval stage, they feed on phytoplankton and zooplankton, and adults are mainly feed on zoobenthos (Stergiou and Karpouzi 2002), although different dietary patterns can be found within specimens living in the coastal lagoons and at sea (Rosecchi 1985).

Figure 5: morphology of gilthead sea bream and the aquaculture production of this species in 2019 by countries in the Mediterranean concave. Adapted from APROMAR, (2020).

Rainbow trout is one of the most important species of salmonids and is native to the Pacific Ocean in Asia and North America, ranging from Alaska to Mexico. They are found in cold water temperature range of 0 to 25° C, although the best water temperature for spawning and growth are 9-14° C and 16-18° C, respectively. In general, growth and maturity are affected by temperature and food availability, usually reaching maturity at 3-4 years of age (FAO 2009). In the nature, they spawn in

the river and their tributaries from autumn to late spring, although their reproduction in culture systems is based on artificial spawning. Because of its high ability to adapt to wide range of environmental and production conditions, it has been introduced to waters on all the world (FAO 2009). In addition, this species has a simple protocol for reproduction and easily adapting to feeding on artificial food, therefor they are suitable species for aquaculture systems (Vass and Raina 2002) and one of the most cultured and farmed fish species in the world (Stanković, Crivelli, and Snoj 2015).

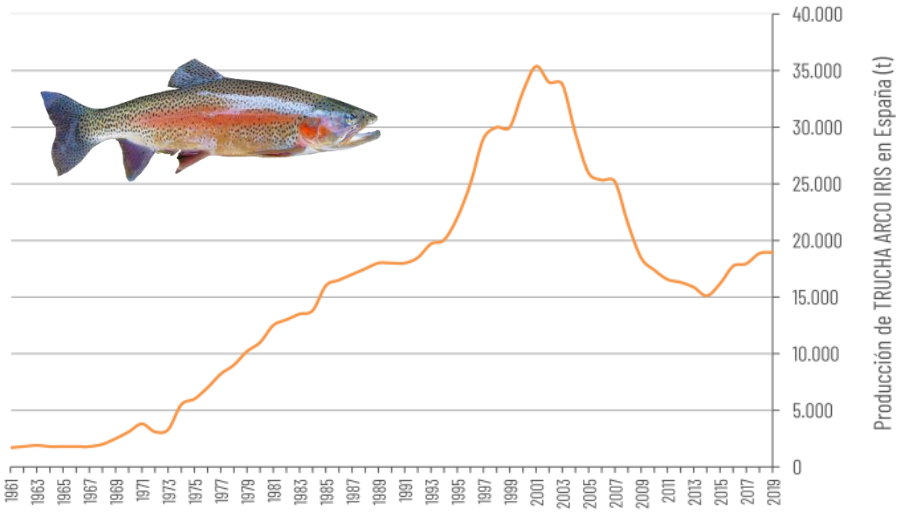


Figure 6: morphology of rainbow trout and the Spanish annual aquaculture production of this species Adapted from (APROMAR 2020).

1.3.Growth: What is it? Determinate vs indeterminate.

Understanding growth may be utterly complicated if all the factors and variables that intervene in its regulation are to be considered, but essentially, at the end growth is an increase in body mass due to consumption of food if the energetic value of the feed intake overcomes the energy expenses (Jobling, 2002). However, for most of the animal species, there is certain limit for somatic growth, which is arrested usually when the animal is ready for reproduction, although differences are species specific. However, a large number of fish species (but not all) are known to be indeterminate growing animals,

which means that in favorable conditions, there is not growth arrest before, during or after reproduction (Biga and Goetz 2021; I. A. Johnston, Bower, and Macqueen 2011) (Figure 7).

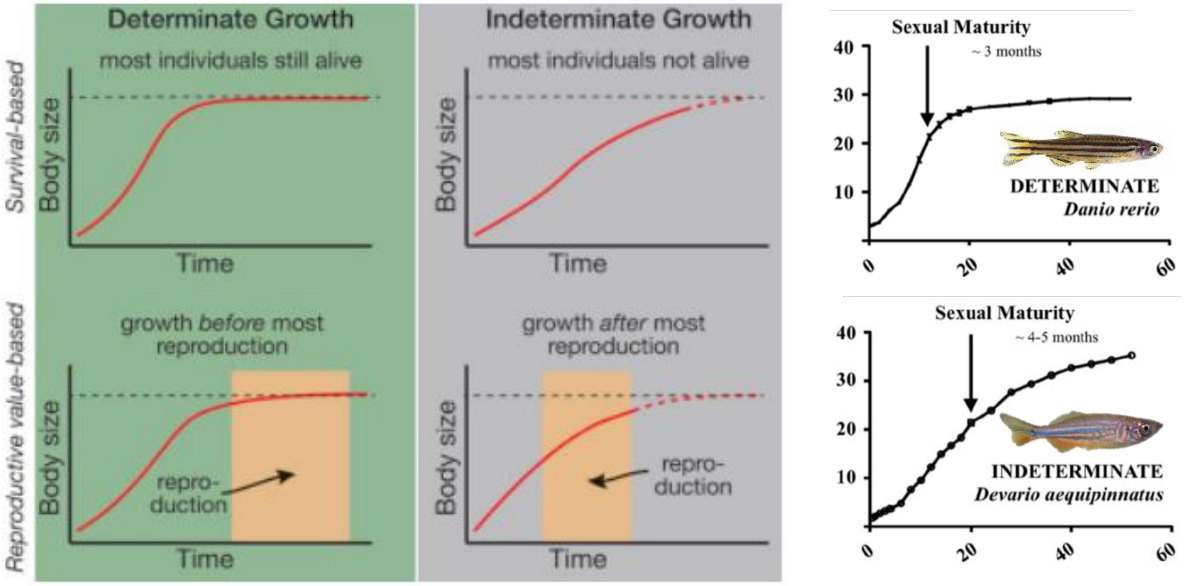


Figure 7: Comparison of survival-based (top) and reproductive value-based (bottom) definitions of growth patterns. Growth curves are in red, zero growth (asymptote) is indicated by black dashed lines, and periods during which reproduction occurs are indicated by orange bars. Determinate growth occurs when most individuals live long enough to reach zero growth (top left, survival-based) or when reduced allocation to growth occurs at the beginning of allocation to reproduction (bottom left, reproductive value-based). Indeterminate growth occurs when most individuals do not survive long enough to reach zero growth (top right, survival-based) or reproduction occurs before zero growth occurs (bottom right, reproductive value-based). Red dashed lines indicate that few or no individuals reach asymptotic size. Adapted from Froehlich *et al.* (2013) and Vitt and Caldwell, (2014).

1.4.Endocrine factors controlling growth

1.4.1. The GH/IGF axis

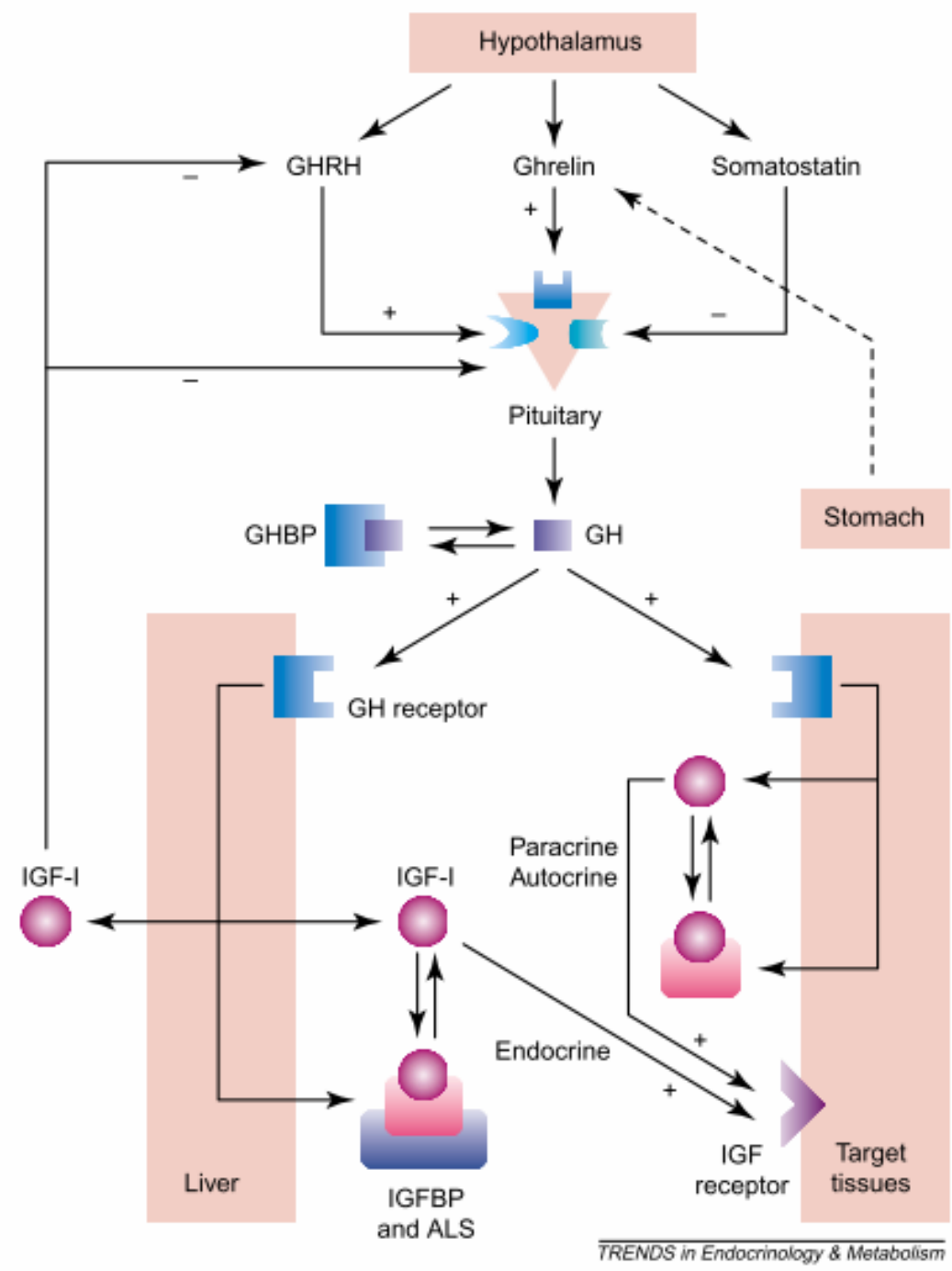


Figure 8: Schematic diagram of the GH–IGF1 system of fishes. As in mammals, the release of growth hormone (GH) from the anterior pituitary is stimulated growth hormone-releasing hormone (GH-RH) and sup- pressed by somatostatin from the hypothalamus. GH released from the anterior pituitary stimulates via its receptor, the GH-R, synthesis of insulin-like growth factor-I (IGF-I) in the liver and its release into the circulation. IGF-I reaches its target cells in numerous organs via the blood stream, where it interacts with the type 1 IGF receptor (IGF-1R) and exerts its effects. Circulating IGF-I is a major negative feedback regulator of pituitary GH secretion because it specifically inhibits GH gene

transcription and secretion. Thus, the physiological role of IGF-I is understood as endocrine. In analogy to mammals, it is classically spoken of as a 'GH–IGF-I axis' in fishes. Furthermore, other organs, such as brain, gills, heart, gastrointestinal tract, pancreas, kidney, spleen, gonads, muscle, cartilage, bone and skin, some of which are shown in the scheme, also produce IGF-I. In these organs, the IGF-I gene occurs in distinct parenchymal cells. GH most probably stimulates the expression and release of IGF-I also in these extrahepatic sites where IGF-I probably acts in an autocrine–paracrine manner. Finally, IGF-I is also expressed in the anterior pituitary and has been suggested also to influence other pituitary endocrine cells via the autocrine–paracrine route. In addition, the environmental factors influencing the GH–IGF-I system at different levels of regulation are included. Extracted from Reinecke, (2010).

Growth hormone (Gh) is a peptide hormone produced by the anterior pituitary gland in response to stimuli from the hypothalamus (Beckman 2011; Manfred Reinecke et al. 2005). Once produced, it is released into the bloodstream, by which will act on diverse organs and tissues that possess Gh receptors (Ghrs) in their cell surfaces. Between the diverse targets of the Gh, the liver is the main one related to the endocrine induction of the Insulin-like growth factors, a second group of peptides produced in response to the Gh. The hepatic Igfs will act endocrinologically over a broad list of tissues, including skeletal muscle and bone where it will trigger growth promoting signaling pathways (Moriyama, Ayson, and Kawauchi 2000; M. Reinecke 2010).

This joint action of Gh and Igf1 for growth hormonal regulation receives the name of Gh/Igf axis, but although these two molecules can be considered the main elements of this hormonal axis, it also requires a series of proteins involved in transport, binding to the target and induction of their effects. Among vertebrates, the Gh/Igf axis is a well-conserved endocrine pathway, though some of its components have diverged greatly and especially in fish, and it must be taken into account that evolutionarily, the teleost fish suffered a complete duplication of the genome (WGD), and even in salmonids, a second round of duplication is reported. Consequently, many of the genes of the Gh/Igf axis among these vertebrates have suffered one or more duplications, becoming paralogues that can end up having different functions. The action of the Gh is due to the binding of this hormone to its receptor, Ghr. As a peculiarity of the Ghr it is necessary to take into account the post transcriptional modifications to which the gene encoding this receptor is subjected. In first, the gene is transcribed in a form called flGhr (Ghr of total length), which is the mRNA that will eventually translate to form functional Ghr (Fuentes et al. 2013). However, this primary transcript may undergo post-transcriptional modifications that result in transcripts of shorter lengths that will result in proteins that induce the expression of certain genes, including those of Igfs (Fuentes et al. 2013; Reindl and Sheridan 2012). Although the main signal transduction pathway that activates Ghrs is mediated by Jak2/Stat5, it has been observed that Ghr2 has a greater tendency to activate the route mediated by

Erk, which participates directly or indirectly in the regulation of the expression of a large number of genes, many of them related to cell survival and metabolism (Jiao et al. 2006; Kittilson, Jones, and Sheridan 2011).

However, this primary transcript may undergo post-transcriptional modifications that result in transcripts of shorter lengths that will result in proteins with different functions. First a tGhr (truncated Ghr) can be generated, which is a plasma membrane anchored protein (like flGhr) that maintains the ability to bind to Gh, but with the deficiency of some of the intracellular domains needed to trigger the signal transduction chain needed to induce the relevant effects. Today, what is known about tGhr suggests that the function of this truncated receptor is to develop a "negative" action in front of a on Gh expression. Thus, in response of high circulating GH levels, there will be a tendency to modify post transcriptionally the flGhr mRNA molecules that will be expressed for this purpose of generating a certain amount of tGhr that will chelate GH without inducing any signal transduction. The other byproduct of the post-transcriptional shortening of flGhr are the so-called GH binding proteins (Ghbps). Ghbps are soluble proteins formed only by the Gh-binding domain of flGhr. The function of these proteins is to bind to circulating Gh in order to increase its average lifespan in blood and regulate their accessibility to GHRs (Fuentes et al. 2013; Reindl and Sheridan 2012).

Evolutionarily, the mammalian orthologue of Ghr suffered a duplication in many fish species, which are described to possess two paralogues of this gene, resulting in the Ghr1 and the Ghr2. Moreover, in salmonids, due to a second complete duplication of the genome, two more analogues of these receptors have been described, resulting in Ghra1 and Ghra2 (equivalent to Ghr1) and GHRb1 and Ghrb2 (equivalent to Ghr2). The exact function of the Ghr paralogues in fish is still nowadays under discussion, as the bibliography is relatively scarce. However, few existing studies indicate that they have complementary functions (Fuentes et al. 2013).

The insulin-like growth factors are proteins with similar structures to that of insulin and in mammals, only two paralogues are described, the Igf1 and Igf2. However, in fish, due to the aforementioned genomic duplications, the paralogues Igf1a, Igf1b, Igf2a and Igf2b has been described, although it seems that not all of them are present in each species (Nornberg, Figueiredo, and Marins 2016). The Igf1 is the most important insulin-like growth factor in the Gh/Igf axis (Moriyama, Ayson, and Kawauchi 2000; Manfred Reinecke et al. 2005). The action of Igf1 on its target cells is through the binding to their specific receptors (Igf1rs), which are very similar also to insulin receptors, allowing

cross-linking between the two molecules, although with an affinity 100 times higher for the Igf1. Binding of Igf1 to its receptors, triggers by its tyrosin-kinase domains, a second chain of phosphorylation's where among the many elements involved are mediated by Pi3k, the serine-threonine Akt and kinase mTor. The response of the activated mTor is to induce, through phosphorylation of 4ebp1 and S6k1, protein synthesis, cell survival, proliferation and differentiation, as well as inhibiting apoptosis among other effects, the sum of the which translates into growth (Figure 9) (Reindl and Sheridan 2012).

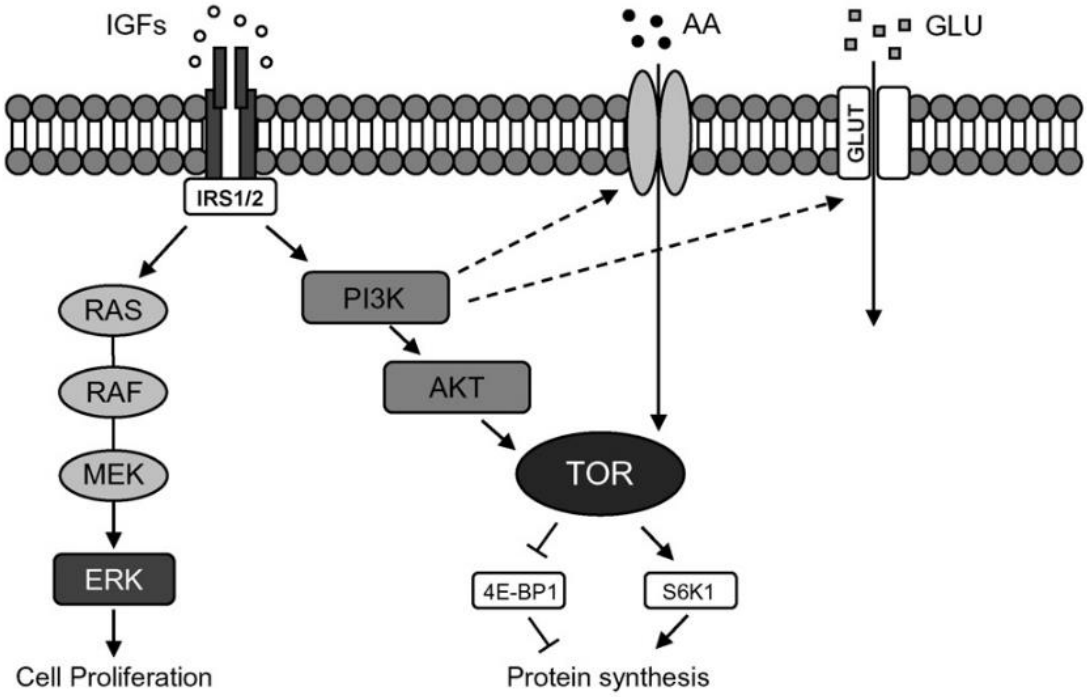


Figure 9: Schematic representation of Tor and Pi3k/Akt signaling pathway Amino acids (AA) uptake stimulate protein synthesis by the activation of Tor phosphorylation and the regulation of its downstream effectors 4ebp1 and S6k1. On the other hand, the binding of insulin or Igfs to their receptors regulates Pi3k phosphorylation to control, among others, substrates uptake (e.g., glucose through GLUT transporters) and the phosphorylation of Akt and Tor. Extracted from (Vélez 2018).

In fish, two paralogues of the Igf1 receptor are described, Igf1ra and Igf1rb in which differential responses of these receptors have been observed in certain situations but with still many clues to uncover (Fuentes et al. 2013; Reindl and Sheridan 2012). As for Igf2, its mechanism of action is very similar to that of Igf1. Its main receptor is the same Igf1r, which ends up triggering the same chain of signal transduction mediated by Akt and mTor. However, Igf2 also can bind to Igf2r, a receptor that, unlike Igf1r, belongs to the mannose receptors. The function of these receptors in mammals is related

to the vesicular transport and the "removal" of Igf2 from the environment, but in fish their function receptors is not yet fully well studied (Méndez et al. 2001; Manfred Reinecke et al. 2005; M. Reinecke 2010). Although Igf1 and Igf2 may appear to have the same function (as they act mainly through the same receptor), they actually have different functions and mechanisms of regulation, although they share many roles. Classically, in mammals it has described in Igf2 as the growth factor that regulates growth during the development, while Igf1 is the major growth hormone regulator post-natal in response to Gh. However, in fish it has been observed that both Igf1 and Igf2 in liver responds similarly to Gh during somatic growth in different conditions. From the different tissues whose growth is regulated by the Gh/Igf axis, the most studied is the muscle tissue, as this tissue represents a higher proportion of body weight in fish. In the growth of muscle tissue, Igf1 has been shown to have a proliferative and differentiating effect and its production in this tissue is directly affected by the nutritional state, especially in relation to fasting and the availability of certain amino acids. On the other hand, Igf2 seems to play a more important role in muscle than Igf1, as there are studies showing that Igf2 has proliferative-enhancing effects than Igf1 (Rius-Francino et al. 2011). As for other regulatory roles of Igfs, as shown in Figure 8, is observed that Igf1 produced by the liver acts as a self-regulator at different levels. First, as a negative feedback regulation, Igf1 acts on the hypothalamus by inhibiting the expression of Ghrh (the neurohormone whose function is to induce Gh expression in the anterior pituitary) and inducing the expression of somatostatin, the neurohormone that inhibits Gh synthesis and induces the expression of other elements with "contrary" effects to the GH-mediated response. Secondly, Igf1 also acts directly on the anterior pituitary gland inhibiting Gh expression. The existence of these feedbacks, together with the regulation of the expression of Igf receptors in the different tissues in response to the physiological state (especially in relation to the nutritional status of the animal), they allow a fine regulation of the growth mediated by the Igfs. This regulation, as will be discussed later, it would be useless in the case of exogenous Gh being administered, already that this system is only valid for Gh actually produced by the individual (Fuentes et al. 2013; M. Reinecke 2010). Finally, the last group of elements on the Gh/Igf axis is a family of proteins called Insulin-like Growth Factor Proteins (Igf Binding) Proteins, Igfbps). Igfbps are a soluble protein that has the function of binding to both Igf1 and in Igf2 with the aim of increasing its half-life in blood and regulating its availability to Igf receptors, thus becoming direct regulators of the growth mediated by Igfs. Its main site of synthesis is the liver, but also they are synthesized in all other tissues where Igfs are produced. Once synthesized and excreted, they bind to Igfs and this Igf-Igfbp complex will be the one to reach the different targets of IGFs where these growth factors will be released by proteolysis of Igfbps by proteases. In mammals, six different

binding proteins are known (Igfbp1-6), but in fish, due to the complete duplication of the genome (two in the case of salmonids) parallels have been observed for some of them. Of the role of Igfbps in fish, in this aspect the literature is not abundant and studies are still needed to finish determining the role that each of them plays in the different fabrics (Garcia de la Serrana and Macqueen 2018; Jaume Pérez-Sánchez et al. 2018). So far, the most relevant are Igfbp1, which in mammals is one classic negative growth regulator and Igfbp3, an Ihgbp (the most abundant) which is usually found bound to a labile acid glycoprotein forming a 150 KDa complex and which positively regulates growth. However, recent studies in fish show that Igfbps 4 and 5 have important functions in regulating tissue growth muscular (Fuentes et al. 2013; Reindl and Sheridan 2012; Manfred Reinecke et al. 2005). In conclusion with respect to Igfbps in fish, due to the scarce literature regarding their function and the added "difficulty" that fish have in evolutionary studies due to genome duplications, cannot be considered yet a well characterized system in these vertebrates.

1.4.2. The ghrelinergic system

Ghrelin is a peptide hormone firstly identified by Kojima, Hosoda and Date, (1999) that is produced mainly by the gastric mucosa in the stomach in vertebrates, but its expression has also been detected in many other tissues (e.g., intestine, heart, pancreas, and especially pituitary and brain).

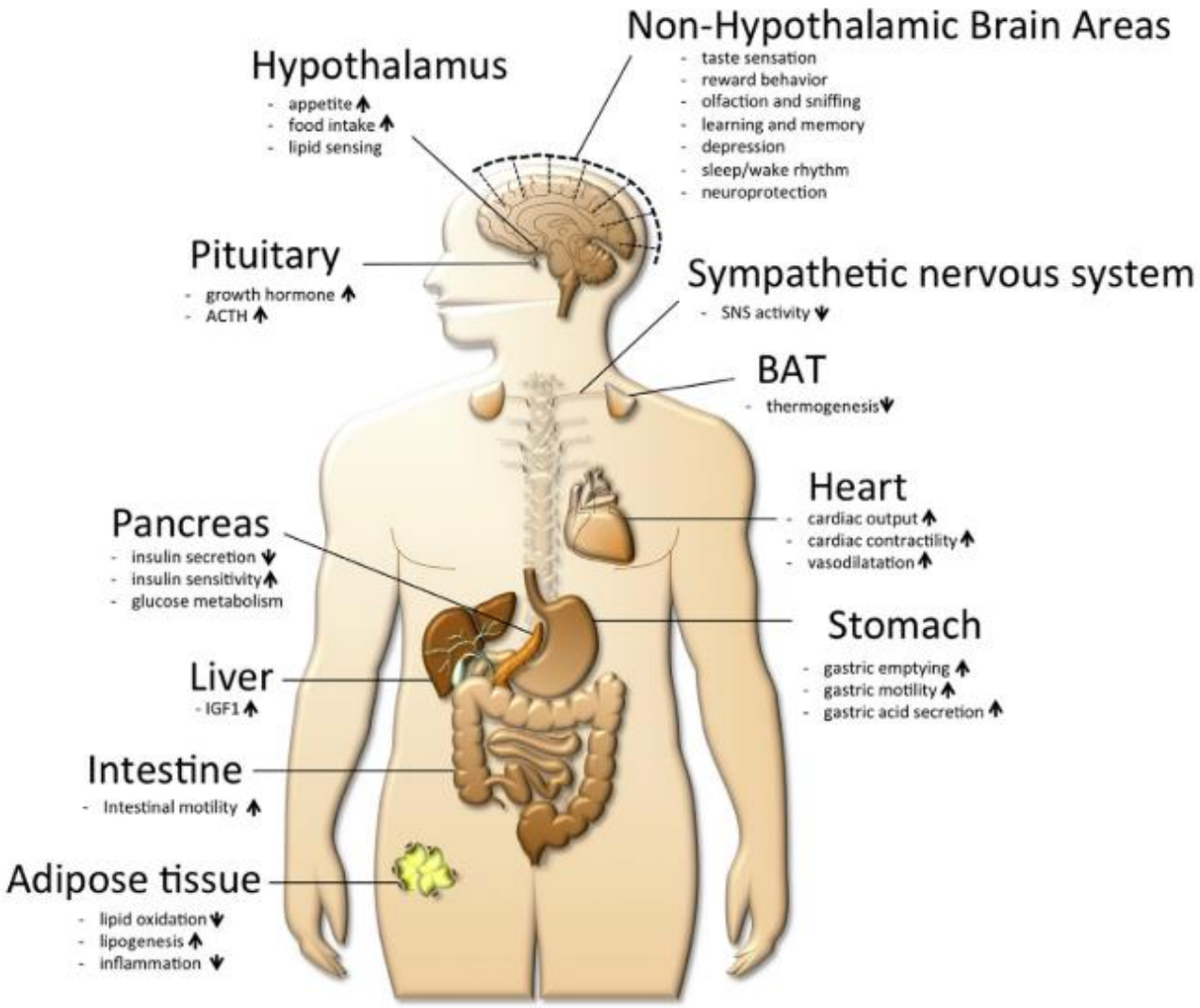


Figure 10: Overview on the main pleiotropic effects of Ghrelin in humans. Extracted from Müller et al. 2015).

This hormone is encoded by the *preproghrelin* gene, which encodes for the proghrelin plus a N-terminal signaling peptide that will be cleaved to the proghrelin peptide which will be further cleaved to a mature peptide of about 12 to 28 amino acids, depending on species, and its antagonic peptide, obestatin. Moreover, for the majority of its functions, the Ghrelin needs a post-traductional acylation

in the Ser3 residue with a C8 to C10 fatty acid. This acylation is carried out by the ghrelin O-acyl-transferase (GOAT), a membrane-bound O-acyltransferase.

The Ghrelin sequence, and specially its more important features, such as the Ser3 residue and the cleaving sites, presents high conservation across vertebrates, including fish (Kojima, Ida, and Sato 2007). Ghrelin was firstly reported to be the natural ligand of the growth hormone secretagogue receptor 1a (Ghsr1a) (Kojima, Hosoda, and Date 1999) and soon after, this hormone received the tag of the "hunger hormone" after being reported to induce adiposity, feed intake and body weight (Tschöp, Smiley, and Heiman 2000). Since then, a vast list of pleiotropic roles has been associated to this hormone as reviewed by Müller *et al.* (2015) and Akalu *et al.* (2020) and displayed in the figure 10. Thus, at least in mammals, Ghrelin is involved the regulation of feed intake, adiposity, growth, energy and glucose metabolism, intestinal motility, digestive enzymes activity, autophagy, oxidative stress response, immunity, bone mass stimulation, sleketal muscle intracellular Ca^{2+} persistent increase, skeletal muscle enhanced mitochondrial oxidation, fatigue resistance, myoblast proliferation, differentiation and regeneration, among others (Akalu et al. 2020).

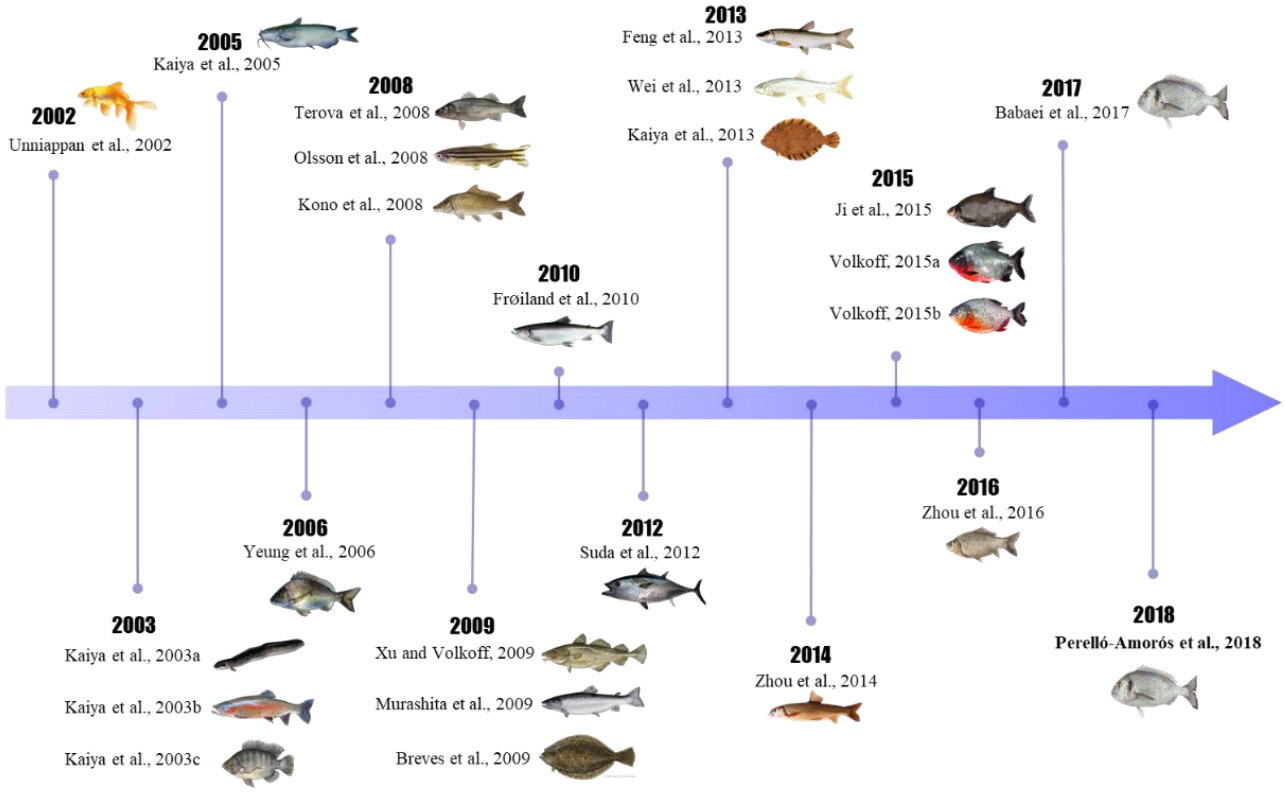


Figure 11: Timeline of the discovery and characterization of the Ghrelin in different fish species.

In fish, Ghrelin was firstly characterized in goldfish (Carassius auratus) (Surajlal Unniappan et al. 2002). Later on, the role of this hormone was reviewed in non-mammalian vertebrates (Kaiya et al. 2008) and recently, different publications have investigated its role in multiple fish species including a first approach to gilthead sea bream (Figure 11) (Kaiya, Kojima, Hosoda, Riley, et al. 2003; Kaiya, Kojima, Hosoda, Moriyama, et al. 2003; Jönsson et al. 2007; Jönsson, Kaiya, and Björnsson 2010; Tinoco et al. 2014; Salmerón, Johansson, et al. 2015; Blanco et al. 2017; Breves, Veillette, and Specker 2009; Babaei et al. 2017).

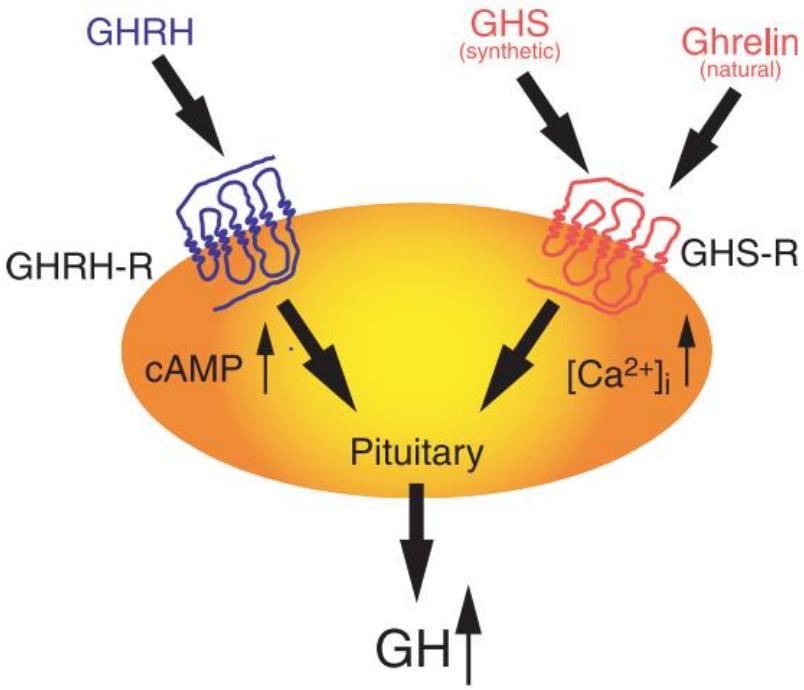


Figure 12: Pathways of growth hormone (GH) release from the pituitary. GH- releasing hormone (GHRH) stimulates GH release through binding to the GHRH receptor (blue) and increasing cAMP levels. By contrast, GH secretagogues (GHSs) stimulate GH release through the GHS receptor (red) to increase intracellular Ca2+ ([Ca2+]i) levels. The endogenous ligand for the GHS receptor was not known until the discovery of ghrelin. Extracted from Kojima *et al.* (2001).

Regarding its receptors, the Ghsrs are G-protein coupled receptors with seven transmembrane domains, firstly discovered in humans and pigs, and two splice variants were described, the Ghsr1a and the Ghsr1b (Howard et al. 1996). The former is the active form the Ghrelin receptor, while the Ghsr1b lacks in its structure two of the transmembrane domains involved in the intracellular Ca^{2+} increase, necessary to initiate intracellular signaling (Figure 12) (Kojima et al. 2001). This Ghsr1b was initially reported to have a simple role as being the negative regulator of the Ghrelin-mediated

Ghsr1a signaling but newer studies suggested that this receptor is involved in a more complex function through its possible heterodimerization with the Ghsr1a and other receptors, such as the orexin receptor 1 (OXI1R), leading to differential signal transduction response (Kaiya, Kangawa, and Miyazato 2013a; 2013b; G. Navarro et al. 2016) but with a still uncomplete information about the exact functions of this receptor.

Since the first reports on the Ghsr1a and Ghsr1b, the Ghsrs family has grown up, with many splice variants and paralogues described in vertebrates (Kaiya, Kangawa, and Miyazato 2013a; 2013b). The Ghsrs are widely expressed, but being the brain, stomach, intestine, and pituitary the tissues with higher expression of these receptors. Among them, the hypothalamus and pituitary gland possesses the higher expression levels of both receptors in most vertebrate's species. In the hypothalamus, Ghrelin stimulates the expression and release of orexigenic neuropeptides like neuropeptide Y and Agouti-related peptide, which are two of the main appetite stimulators in vertebrates, including diverse fish species (Delgado, Cerdá-Reverter, and Soengas 2017). In the pituitary, the high presence of Ghsrs is in agreement with the main role of the Ghrelin as a secretagogue of Gh (Kojima, Hosoda, and Date 1999) which at the end mediated indirectly many other effects atributed to the Ghrelin in vertebrates (Kaiya, Kangawa, and Miyazato 2013a). Although these two main roles of the Ghrelin seem to be the most conserved ones, disparities have been found across vertebrate's groups concerning the Ghrelin role on Gh release and appetite regulation (reviewed by Kaiya, Kangawa and Miyazato, (2013a) and summarized in the Figure 13), leading to a controversy that it is even magnified by the still not clear mechanism on how acylghreliand des-acylghrelin cross the blood brain barrier to exert this role (Edwards and Abizaid 2017), adding another complex level of regulation.

	Mammals	Aves	Amphibian	Fish
				
GH release	Stimulate /No effect	Stimulate	Stimulate	Stimulate /No effect?
Corticosteroids release	Stimulate /No effect	Stimulate	Stimulate	Stimulate
Feeding (ICV)	Stimulate /No effect	Inhibit	-	Stimulate /Inhibit
Feeding (IP, IV)	Stimulate /No effect	Inhibit /No effect	-	Stimulate /No effect
Plasma level during fasting	Increase (Fast response)	Increase (Fast response)	Increase (Slow response)	Increase (Slow response)
GI contraction	Contraction (In vivo)	Contraction (In vitro)	-	No effect (In vitro)
Drinking	Inhibit (IV, ICV)	Inhibit (ICV)	No effect (ICV)	Inhibit (IV, ICV)

Figure 13: Comparisons of ghrelin's effects across vertebrates. Representative physiological effects of ghrelin and the results are summarized. Extracted from Kaiya, Kangawa and Miyazato, (2013a).

1.5.The fasting and refeeding model and the compensatory growth.

The compensatory growth is a special situation of accelerated somatic growth that can occur in many vertebrate species after a growth arrest situation under stress situations (Figure 14). Thus, the physiological regulation and implications of this phenomenon has been extendedly studied in species with interest in animal production, as strategies to stimulate growth rate through enhanced hyperphagia, feed conversion efficiency and specific growth rate. In this regard, fish are probably the group of vertebrates with higher potential of compensatory growth in response to different conditions, alone or in combination with suboptimal temperature, crowding and specially, feed restriction. Most of the fish species are capable to resist long fasting periods which may turn to compensatory growth situations when nutrients are available again (Won and Borski 2013). In this situation, the Gh/Igf1 axis and the ghrelinergic system are supposed to play interconnected roles that participates both in the adaptation to fasting and to promote the compensatory growth upon refeeding where a drastic metabolic transition must be regulated. Then, the ghrelin plays a double role, as a hunger hormone and Gh secretagogue but this interplay between the ghrelinergic system and other physiological mechanisms in fish and specially in gilthead sea bream under fasting and refeeding conditions is still not fully understood (Babaei et al. 2017; Yeung et al. 2006).

During fasting, there is a metabolic switch towards a reduced energy expense coupled to a pro-catabolic status to promote energy obtention from reserves and/or other energy sources, such as proteolysis for amino acid oxidation, which usually leads to decrease in muscle mass, adiposity and body weight with a growth arrest. However, the Ghrelin and Gh increase as an adaptation to fasting leads, upon refeeding, to a hyper-anabolic phase when nutrient availability (especially amino acids) unblocks the liver responsiveness, producing a strong IGF production and Ghrelin and Gh downregulation.

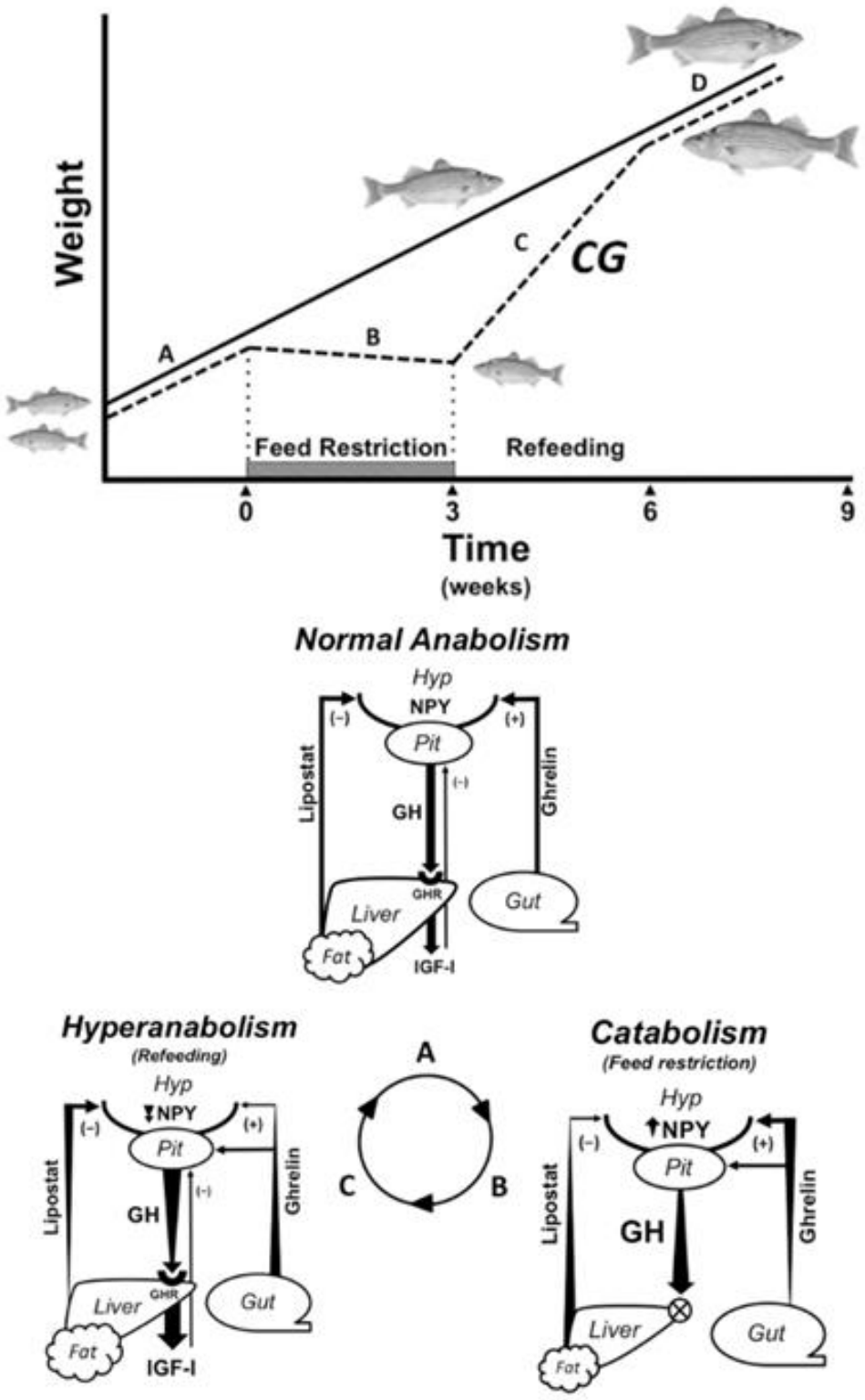


Figure 14: Up, Compensatory growth (CG) paradigm during fasting and refeeding (dashed line) compared to constant growth rate in fed controls (solid line): Normal growth (A) is disrupted by feed restriction (hatched bar), which results in a decline in the growth trajectory (B) and a size disparity compared to control animals fed a constant regimen. When feeding resumes, hyperphagia and enhanced growth axis activity drive a hyperanabolic phase (C) marked by a steeper

growth curve than that of constantly fed animals. The CG response potentially allows stunted animals to fully compensate for lost growth opportunity and re-converge in size with constantly fed controls before the growth rate returns to normal (D); **Down:** Endocrine regulation of growth and appetite during normal anabolism, catabolism, and hyperanabolism (CG) resulting from feeding status. Growth is regulated by the GH/IGF axis; GH secreted into circulation by the pituitary binds its receptor (GHR) to stimulate hepatic IGF-I production, which systemically drives somatic growth and exerts negative feedback on GH secretion. Lipolysis is an alternate function of GH during catabolism. Peripheral signals from a lipostatic mechanism (anorexigenic), possibly leptin, and ghrelin (orexigenic) regulate energy intake by modulating NPY and other neuropeptides in the central feeding center. Ghrelin also functions as a GH secretagogue. Arrows show the direction of regulatory pathways; widening/narrowing of arrows represents a dynamic increase/decrease in a component over the duration of a particular metabolic state. (A) During regular feeding, energy homeostasis is maintained by matching energy intake and expenditure. Peripheral signals counter-regulate appetite centrally. Growth is regulated by nominal levels of circulating GH, which stimulates IGF-I production via hepatic GHRs. (B) Fasting necessitates catabolic processes to provide energy for basal metabolism. Rising ghrelin production stimulates both appetite and circulating GH levels. Elevated lipolytic GH levels exploit stored energy reserves, decreasing lipostatic signaling. Reduced hepatic GHR expression desensitizes the liver to GH-induced IGF-I production. (C) Refeeding signifies the switch from catabolic to anabolic processes. Temporally elevated orexigens carried over from fasting drive hyperphagia. The return to positive energy status is characterized by the resumption of hepatic GH sensitivity and a steep rise in circulating IGF-I levels, which promotes accelerated growth. Eventually, the repletion of energy reserves and negative feedback from IGF-I returns GH and appetite to nominal levels, marking the return to normal growth rates. (PIT, pituitary; HYP, hypothalamus; NPY, neuropeptideY; GH, growth hormone; GHR, growth hormone receptor; IGF-I, insulin-like growth factor I). Extracted from Won and Borski, (2013).

This increased Igf/Gh ratio leads to acceleration of the growth rate (Nebo et al. 2013). Thus, this particular response to fasting and refeeding in fish has been used in applied research towards the optimization of fish production costs and its effects on body growth, metabolism, protein biosynthesis and hormonal responses have been largely studied in fish (Nebo et al. 2013; I. Navarro and Gutiérrez 1995).

As the muscle represents usually more that 40% of the total body weight of fish, it is important to consider it also one of the main energy and protein demanding compartments under anabolic conditions that can revert to an important energy and amino acid source under long-term nutrient deprivation stages. Hence, the adaptations of this large tissue to drastic nutritional changes requires a well-orchestrated and finely regulated metabolic shift and in this regard, as will be reviewed later, this tissue possesses a set of diverse proteolytic molecules that develops the important role of muscular protein breakdown in catabolic situations.

1.6.The model of exercise

1.6.1. Exercise and growth

Most of the fish, as well as many other aquatic animals, spends their time suspended in a water column that moreover usually is not steady, but presenting different degrees of flows. These organisms have the capacity to move through the water column with a precise buoyancy control and especially with the hydrodynamic shape that they have evolutionarily acquired, to reduce the energy expensed of the locomotion through such a dense fluid. This adaptation reached to such a limit to permit to salmonids to live, grow and reproduce in permanently moving waters (William Davison 1997).

This particular adaptation of the fish to tolerate different degrees of natatory activity for extended periods of time with optimized energy expenditure, has put the sustained exercise in the eye of the aquaculture-directed research in the last years. In fact the first researchers found in the sustained exercise a potential approach to optimize growth through reducing stress and optimizing the nutrient usage and welfare in fish. Moreover, a moderate sustained swimming has been reported to reduce the energy expenses that some fish dedicate to social behavior, including drastic direction changes, which in sustained exercise does not occur. This energy saving may be dedicated to growth (Christiansen, Svendsen, and Jobling 1992; Steinhausen, Fleng Steffensen, and Gerner Andersen 2010; P. V. Skov et al. 2011; Jorgensen and Jobling 1993). Such interest reached to the level of deserving an EU COST Action project (FA1304 Fitfish) to study for the first time the natation of fish from a multidisciplinary approach.

The up-to date knowledge about the current state of the research on fish swimming has been reviewed by McKenzie *et al.* (2020). Exercise in fish is usually in the form of intermittent or continuous, long-term swimming at different intensities. The first studies with exercised fish revealed that in some fish species, training may affect muscular hypertrophy and hyperplasia as well as the fueling usage, thus revealing a metabolic shift (B. Y. W. Davison and Goldspink 1977; Rasmussen et al. 2011; Alsop and Wood 1997; Richards et al. 2002a). Since then, the potential of the exercise as a strategy to optimize aquaculture production was clearly stablished and several studies were carried out and reviewed until the last decade (William Davison 1997; Jobling et al. 1993; W Davison and Herbert 2013).

In the actuality, it has been stablished that the capacity of the exercise to improve the growth rate of farmed fish ultimately depend not only in the exercise training program (time and intensity), but also in the nature of each fish species to adapt to the chosen program, based on the nature of their muscle that moreover, depends also in the size of the fish, as larger fish will have a lower performance than smaller fish, under the same exercise regime (Bainbridge, 1958; Palstra *et al.* 2020). In this regard, exercise has been demonstrated to have either positive, null or even negative effects on growth (McKenzie et al. 2020; Palstra, Roque, et al. 2020; Palstra, Kals, et al. 2020).

Taken this in consideration, it has to be noted that at low speeds, in fish usually expressed as body lengths·s^{-1}, the locomotion is mediated mainly by the low-twitch oxidative red muscle, but as soon as the speed increases, the minor red muscle cannot give all the necessary power output, then recruiting progressively the intermediate and finally the fast-twitch anaerobic white muscle fibers. These with fibers areat the end the muscle that is pretended to stimulate in aquaculture-directed research on exercise.

However, as a consequence of increasing the speed, the drag and the cost of swimming enhances exponentially, and since then, different parameters related to the energetics of fish swimming have been established. The total cost of transport T-COT is the sum of the cost of swimming + standard metabolic rate + metabolic cost of digesting and assimilating a meal); the maximum sustainable aerobic speed (U_{ms}); the critical swimming speed (U_{crit}) and the optimal speed (U_{opt}), which is described as the speed where the T-COT is minimum; Hence, different authors have generally postulated that, in small fish, the best speed to promote optimal growth coincides with the U_{opt} for several species including gilthead sea bream (W Davison and Herbert 2013; Palstra, Kals, et al. 2020; Palstra, Roque, et al. 2020).

In gilthead sea bream, beneficial effects of sustained exercise on growth has been already studied in at different experimental conditions. At 1.5 BL·s^{-1}, exercise improved the growth of 90g juveniles approximately 8.9% respect to their controls while having the same feed intake (Antoni Ibarz et al. 2011). In a further experiment in this species using 5g fingerlings swimming at 5 BL·s^{-1} for 5 weeks, a 20% increase in body weight respect to the control fish was observed (Blasco et al. 2015). Recently, (Palstra, Roque, et al. 2020) calculated that the U_{opt} for gilthead sea bream juveniles of 25-45g is around 4.51 BL·s^{-1}. This finding posterior to the experiment carried out by (Blasco et al. 2015) supports the idea that setting the swimming speed close to the U_{opt} will lead to maximal growth

stimulation in fish species that adapt well to the moderate intensity sustained exercise models. However, it is noteworthy that moving big masses of water may represent an important economic impact on the application of the exercise at large scale facilities. Then, suboptimal speeds may be considered, as the beneficial effects of the exercise are not only relying on growth stimulation, but also in nutrient usage and welfare.

Another aspect that is important to consider in the promotion of growth by exercise, is the logical possibility of stimulating the feed intake, as the exercise has been reported to increase the appetite in fish (Reviewed in McKenzie *et al.* 2020). Then there is a necessity to monitor properly the feed intake in both the exercised and the control groups to determine if the exercise induced or not an optimization of the feed conversion ratio (FCR) (reviewed by McKenzie *et al.* (2020)). In this regard, different authors has confirmed the possibility of the exercise to increase the feed conversion rate (Christiansen and Jobling 1990; Jobling et al. 1993; Shrivastava, Blust, and Boeck 2018) and in this adaptation the Gh/Igf1 axis seems to play a main role (reviewed by McKenzie *et al.* 2020), as sustained exercise can alter the Gh and Igf1 in different fish species, including the gilthead sea bream (Barrett and McKeown 1988; Blasco et al. 2015; Shrivastava, Blust, and Boeck 2018).

1.6.2. Exercise, energy, and nutrition

Under exercise, even at optimal speeds, the fish are subjected to a continuous increased energy demand not only from the skeletal muscle that performs the locomotion role, but also from many other tissues and organs as well. Then, if fish have evolved to stand long periods of physical activity, they should have developed the proper metabolic mechanisms to ensure such constant energy and nutrient supply to these organs and hence, a good plasticity is required to select the best energy fuels depending on the diet composition and their availability as the exercise progresses (Reviewed by Magnoni *et al.* 2013).

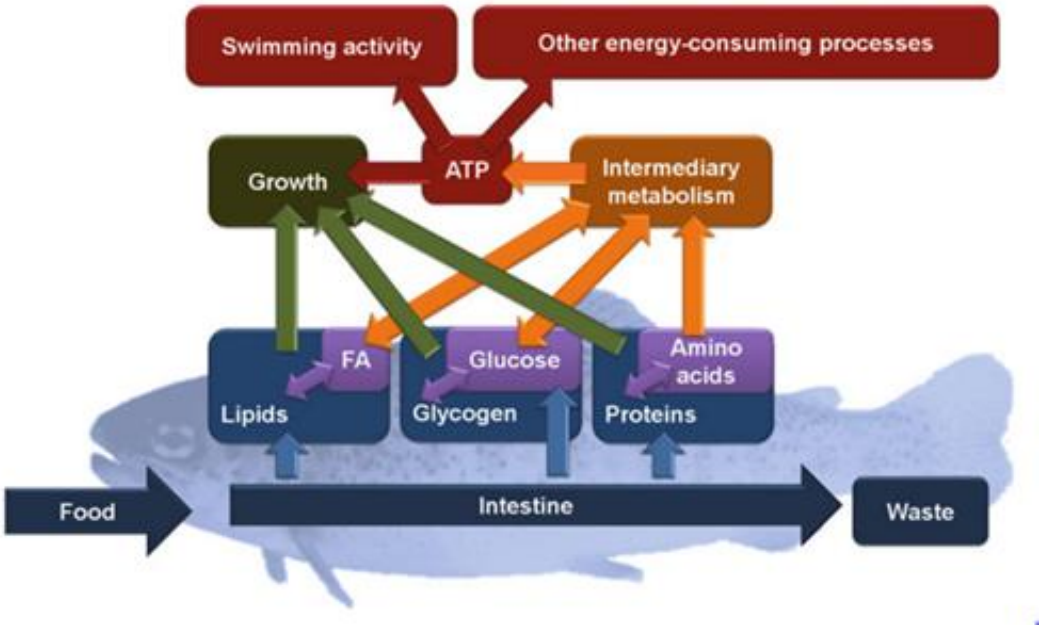


Figure 15: A representation of nutrient use for growth and for energy generation by several metabolic pathways in fish. Lipids, carbohydrates, and proteins provided by the diet can be stored contributing to somatic growth or can be utilized, as they enter in the intermediary metabolism, for several processes requiring ATP, including swimming activity. Extracted from Magnoni *et al.* (2013).

In fish, like in the vast majority of the animals, the ATP is obtained, from the hydrolysis of phosphocreatine, glycolysis, and oxidative phosphorylation and these pathways ultimately rely on the catabolism of lipids, carbohydrates, or proteins that enters to the organism through the diet (Figure 15) and can be accumulated in form of energy reserves when they are in excess (Magnoni, Felip, et al. 2013). In fish, lipids represent the most important energy reserve and are composed mainly of triacylglycerols (TAG) in the skeletal muscle, liver, mesenteries and peri viscerally, being the two later more associated with mechanical and protective functions. Carbohydrates are stored in different

amounts, depending on the capacity of each species to metabolize this kind of nutrients, in the form of muscular and hepatic glycogen. Finally, the last energy fuel are the proteins and free amino acids that are present in the tissues or in circulation. However, the proteins can represent more than 50% of the body weight (BW) of fish and they usually are the most abundant dietary nutrient in omnivorous and carnivorous fish, in a good dietary and metabolic situation they are not contemplated as the preferred energy fuel over lipids and carbohydrates.

As concluded by (McKenzie et al. 2020) in their recent revision, it is necessary to study the interaction between sustained exercise and diet formulation, with a special mention on how the exercise can permit the inclusion of higher % of non-protein and sustainable ingredients to fuel the metabolic costs of swimming, while optimizing the dietary protein retention and growth. This affirmation has been previously claimed by other reviews (Magnoni, Felip, et al. 2013), requesting for research towards the use of exercise to improve the usage of dietary lipids and carbohydrates as energy fuels in fish. Until now, there have been some studies investigating how fish under sustained swimming use fuels (reviewed in McKenzie *et al.* 2020). Alsop and Wood, (1997) initially and later Skov, Lund and Pargana, (2015), observed how rainbow trout under exercise preferred non-protein energy sources as the swimming speed increased. In this regard, it has been observed in gilthead sea bream that moderate exercise can improve growth through making carbohydrates to contribute up to 40% of the metabolic cost of swimming. This fact demonstrates that even if carbohydrates represent usually not more than 10% of the total energy reserves in fish, if supplemented in the diet, these nutrients are preferred as energy fuel, thus provoking a protein-sparing effect for growth (Ibarz *et al.* 2011; Martin-Pérez *et al.* 2012; Leonardo J Magnoni *et al.* 2013), as also reported for rainbow trout (Olga Felip et al. 2012; P. V Skov, Lund, and Pargana 2015).

However, not all the fish species has the same ability to metabolize the carbohydrates, which also depends on the physical state of the animal, the molecular complexity and the quantity of carbohydrates included in the diet, as these properties will determine its digestibility and tolerance (Hemre, Mommsen, and Krogdahl 2002; Stone 2003). Because of these possible intolerance to carbohydrates in some fish species, it is also important the study on the substitution of dietary protein by sustainable lipidic ingredients to be used in fish under sustained exercise.

1.7.Skeletal muscle structure in mammals and fish

The mammalian skeletal muscle is the type of muscle also known as striated muscle due to its particular structure based on long myofibers disposed in parallel orientation to form a muscular unit (Figure 16) (Kraemer, Fleck, and Deschenes 2011). This is the type of muscle involved in the locomotion, both voluntary and involuntary, but also just by the fact of being the largest tissue in almost all the vertebrates species, it is considered a tissue with important systemic metabolic and endocrine and systemic functions (Zanou and Gailly 2013; Sadava et al. 2008). Moreover, there are different types of myofibers, which mainly differ in their mitochondrial content, preferred metabolic pathways and the predominant Myosin ATPases that they express, thus presenting different kinds of muscle contractions (Figure 17C).

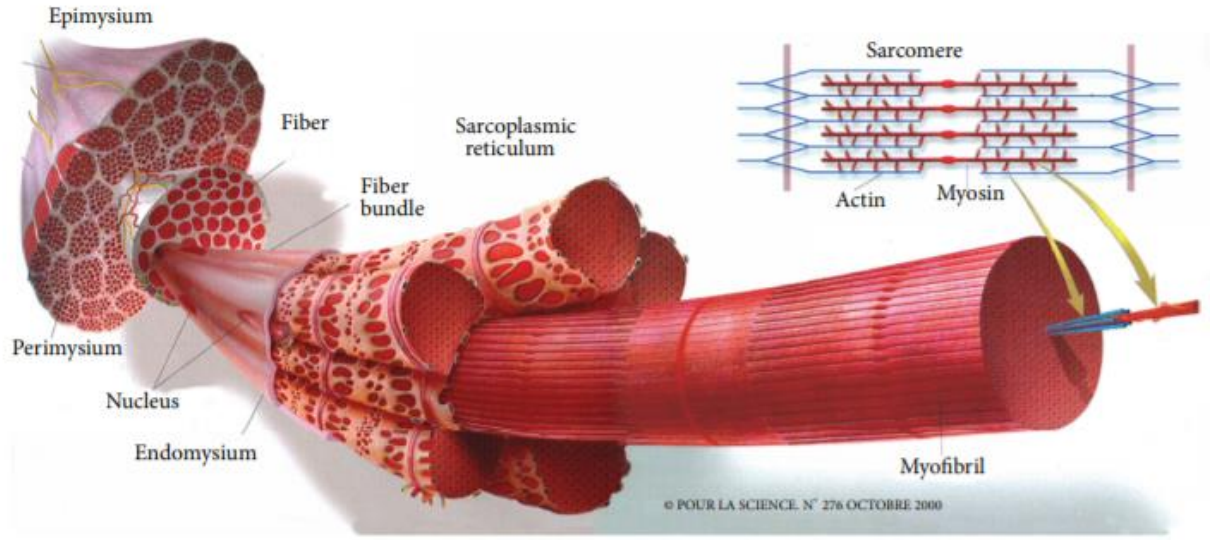
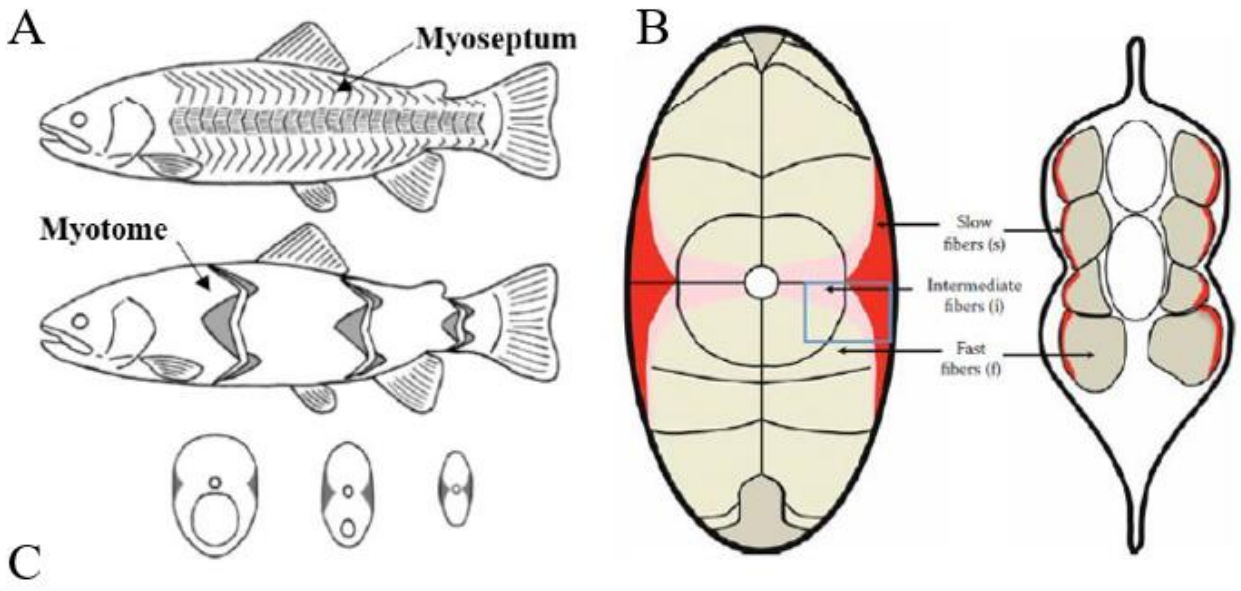


Figure 16: General organization of the muscle. Skeletal muscle predominantly consists of muscle fibers and connective tissue. The latter is distributed on three levels of scale in the muscle: the endomysium, which surrounds each muscle fiber, the perimysium, which compartmentalizes muscle in fiber bundles, and finally the epimysium, which is the external envelope of muscle. Within the fibers, the myofibrils occupy nearly the entire intracellular volume. The contractile unit of the muscle fiber is the sarcomere. Extracted from Listrat *et al.* (2016).



Fiber Type	Dominant MyATPase	Mitochondrial Density	Anaerobic Capacity
Slow	Type I	High	Low
Intermediate	Type IIa	Intermediate	Intermediate
Fast	Type IIb	Low	High

Figure 17: A: Diagram of the fish muscle structure organized in W-shaped myomeres separated by myoseptums. The white muscle is present in the epaxial and hypaxial region with respect to the red muscle, which has a transversally V-shaped distribution along the lateral line. Extracted from Coughlin (2002). B: A third muscle type is formed by pink fibers with an intermediate phenotype between red (slow) and white (fast) fibers. The pink fibers location is also intermediate among that of red and white muscle. C: Summary of the phenotypic differences among fiber types. Adapted from (McClelland and Scott 2014).

In the case of fish, the skeletal muscle possesses a particular axial architecture which is associated to the patterns of force generation necessary for propulsion in an aquatic habitat (Videler 1993). Thus, the fish axial muscle consists of W-shaped muscle blocks known as myomeres or myotomes, which disposed in a parallel form, may represent up to 60% of the total body mass (Figure 17A). Myomeres are composed primarily of multinucleated myofibers. In contrast than in mammal muscle, where the different muscle fibers are distributed in a mosaic-like pattern, the fish axial muscle axial has the

fibers anatomically separated, resulting in two predominant skeletal muscle subtypes: the white muscle (large and powerful fast-twitch glycolytic fibers), which comprises the deep portion of the myomere, representing up to 85% of the muscle mass, and the red muscle (smaller slow-twitch, oxidative fibers), located at the periphery of the myomere. The intermediate fibers are located following the coronal plane of the animal (Figure 17B) (Reviewed by McClelland and Scott, 2014; Rescan, 2019; Dal-pai-silva *et al.* 2020).

Such anatomical separation of the muscle fiber types permitted to understand the implication of each fiber type in different swimming intensities (I. A. Johnston, Bower, and Macqueen 2011), being the slow muscle fibers initially recruited at low speeds, while the intermediate and fast-twitch fibers were recruited with progressive speed increases. Nevertheless, because of the glycolytic nature of the fast muscle, there is a high variability between species depending on the metabolic capacity to metabolize carbohydrates, leading to burst-like natation in some species while allowing sustained swimming at high speeds in others (I. A. Johnston, Bower, and Macqueen 2011; McClelland and Scott 2014). The differential metabolic nature between fast and slow fiber types ensures optimal contraction velocities to provide the correct tension and power output at different swimming speeds (McClelland and Scott 2014). In this regard, white muscle fibers, powered mainly through glycolysis, can provide energy fluxes over 10 times higher than the red muscle ones, but the activity of these fibers is much more unsustainable in time. On the other hand, the red muscle fibers are smaller and perform slower and less powerful contractions, fueled by ATP obtained by oxidative metabolism, a slower energetic pathway for which these fibers are adapted to possess high mitochondrial densities and levels of myoglobin to provide a constant O2 supply.

1.7.1. Skeletal muscle embryonic development, myogenesis and myoblast fusion

The fish muscle ontogeny as well as the mechanisms that control the muscle growth after the embryo is formed, has been largely studied (Reviewed by Rescan, 2019; Dal-pai-silva *et al.* (2020). As the functional unit of the muscle is the myofiber, the muscle development in amniotes as well as in bony fishes comprises the sequential activation, proliferation and differentiation of embryonic cell subpopulations that at the end will end up as the first myofibers and this process is called primary myogenesis.

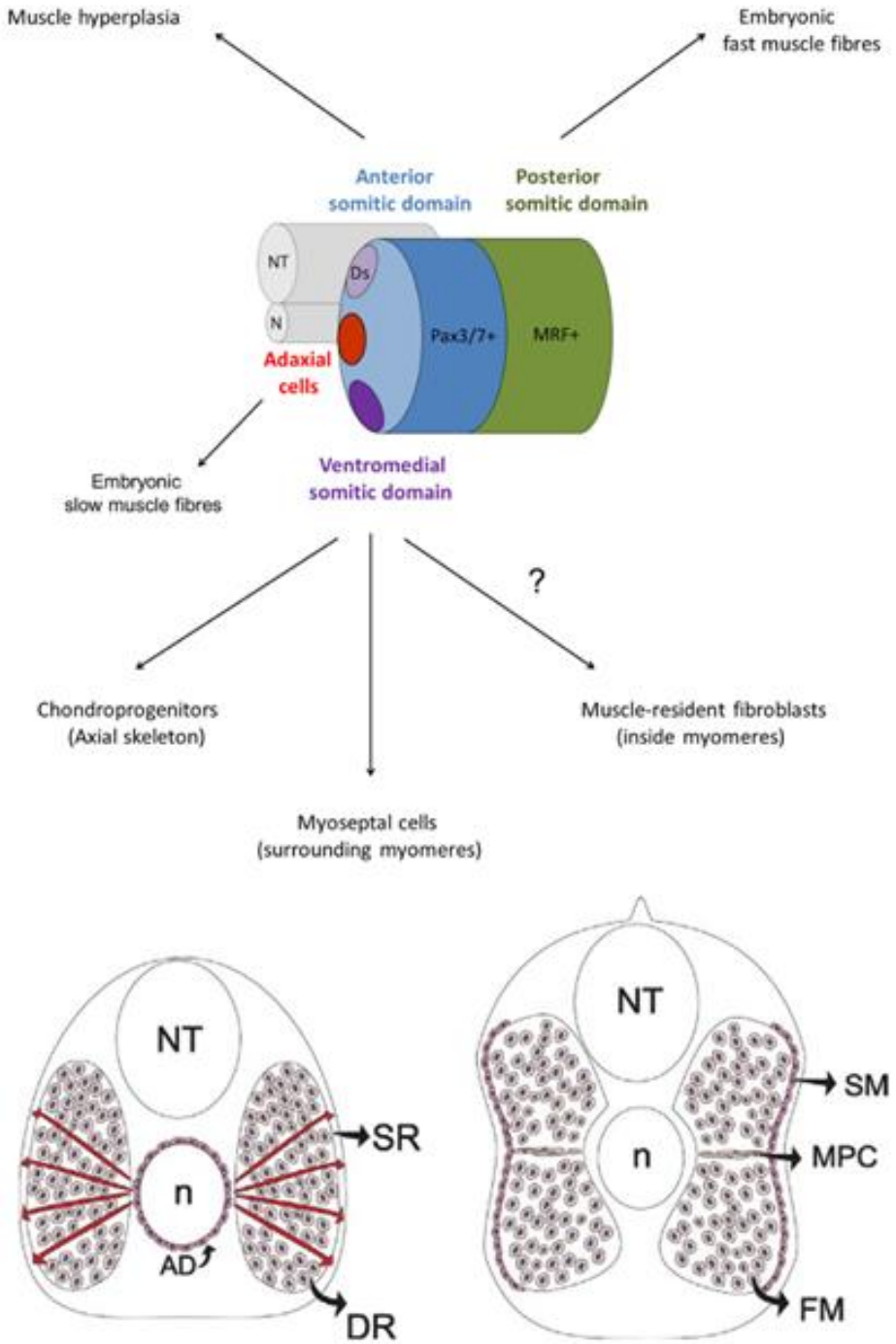


Figure 18: Up, Schematic representation of the compartments of a young zebrafish epithelial somite and their contribution to the formation of the axial musculoskeletal system. Embryonic slow muscle fibers are derived from the adaxial cells (in red) initially adjacent to the axial structures (i.e., the notochord (N) and neural tube (NT)). Posterior cells of the epithelial somite (in green) express myogenic regulatory factors and contribute to the formation of embryonic fast muscle fibers. Anterior cells (in blue) that express pax3/7 form a transient external epithelium that will provide myogenic progenitors necessary for the formation of new muscle fibers immediately following embryonic myogenesis (stratified

hyperplasia) and likely after the post-larval stage (mosaic hyperplasia). The ventral domain of the somite (in purple) gives rise to a ventral sclerotome that produces chondroprogenitors contributing to axial skeleton, myoseptal cells sur- rounding the myotome and possibly connective fibroblasts inside the myotome. A dorsal sclerotome (Ds) that contributes to the formation of myoseptal cells at the dorsal myotendinous junction has been recently identified at the dorsomedial edge of the zebrafish somite; this dorsal sclerotome might also produce muscle resident fibroblasts. Extracted from Rescan, (2019). **Down,** Scheme of the differentiation of slow and fast muscles in fish. Adaxial cells (AD), epithelial-like monolayers flanking the notochord (n), migrate to the superficial region (SR) and differentiate into slow muscle (SM). The cells from the deeper and posterior part of the somites (DR) proliferate and differentiate into fast muscle (FM). A subpopulation of adaxial cells, referred as the slow muscle pioneer cells (MPC), emits cytoplasmic processes in the medial region, possibly to guide the migration of the adaxial cells toward the surface of the myotome. This medial region divides the myotome into the ventral (hypaxial) and dorsal (epaxial) regions. NT, neural tube. Extracted from (Dal-pai-silva *et al.* (2020).

In fish, as well in other vertebrates, myotomes that conform the skeletal muscle are originated during the embryonic development from the segmentation of the somites (Figure 18), bilateral embryonic structures located at both sides of the notochord and the neural tube which condense from the paraxial mesoderm (the mesoderm adjacent to the central body axis) and segment progressively from the rostral to the caudal region of the embryo (Kimmel et al. 1995). Each somite is subdivided generally into two different regions containing specific cell types, the sclerotome, located in the deepest part of the somite, and the dermomyotome, more superficial (P.-Y. Rescan 2019; Dal-pai-silva et al. 2020). The dermomyotome contains cells that are committed to the muscular lineage, known as Muscle Precursor Cells (MPCs), which expresses the paired-box transcription factors Pax3 and Pax7 that characterizes this muscle commitment. These MPCs will be later become primary myoblasts, which will actively proliferate and differentiate in two embryonic subpopulations: the adaxial cells, located in the deep myotome as a monolayer surrounding the notochord, that later will migrate to the surface of the dermomyotome to originate the slow red myofibers; and the posterior domain of the somite, which will concentrate the primary white myocytes. Thus, since the early beginning of the muscle ontogeny, there is a spatial separation of the red and the white muscle in the somite (Dal-pai-silva et al. 2020; P.-Y. Rescan 2019).

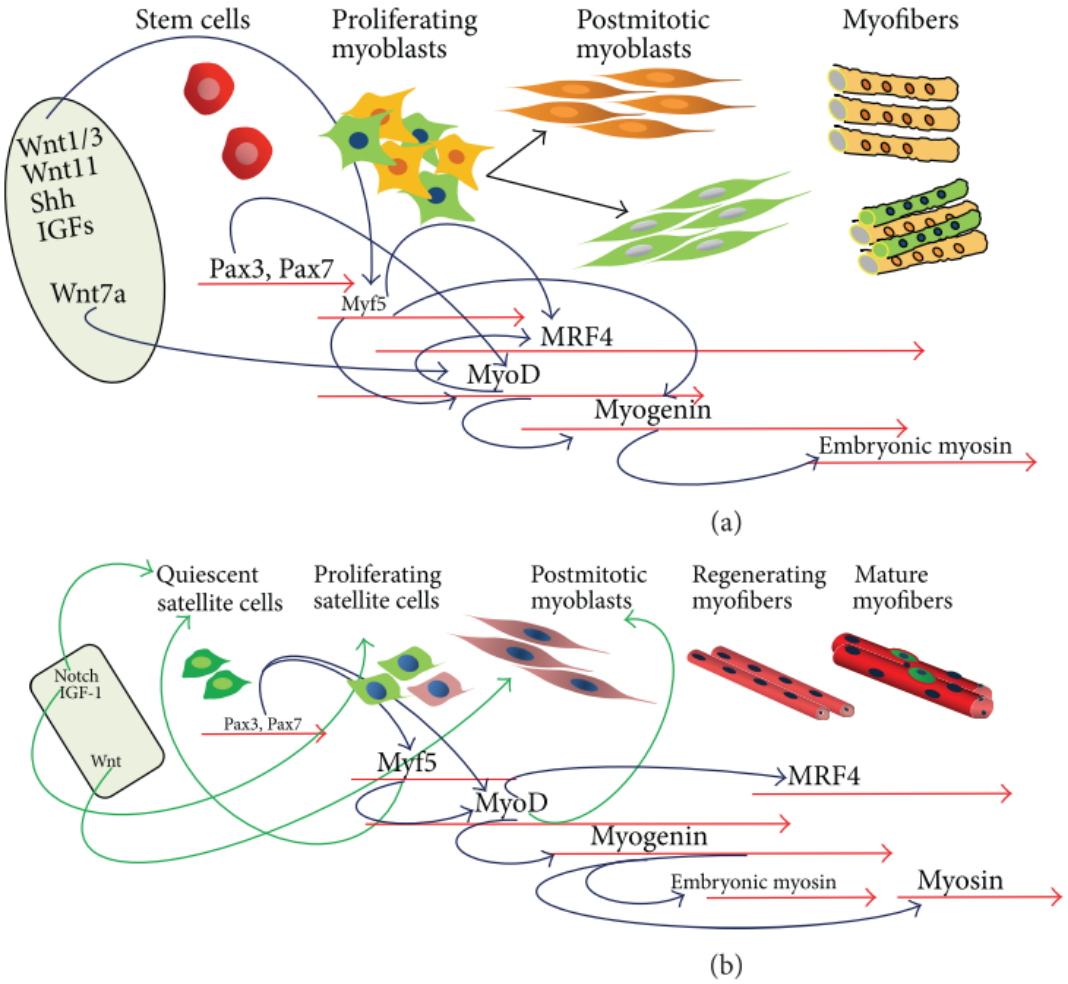


Figure 19: Schematic representation of muscle formation during embryonic development and adult regeneration. (a) Developmental myogenesis occurs in two distinct waves of differentiation that are characterized by a specific and sequential pattern of muscle-related gene expression (red arrows). Skeletal muscles are derived from somites, which receive signals from the neighboring tissues, namely, axial structures (neural tube and notochord), dorsal ectoderm, and lateral mesoderm that in turn induce the activation (blue arrows) of muscle regulatory factors. Shh (from the notochord) andWnt1/3 andWnt11 and IGFs (from dorsal neural tube) signaling have been demonstrated to regulate the expression ofMyf5. Pax3 andMyf5 independently regulate MyoD expression, whereas Myf5 regulates the transient expression of MRF4.Myf5 and MyoD independently activate the expression of Myogenin, which promotes the expression of Myosin. (b) Illustration of the lineage progression of adult myogenesis during muscle regeneration, which recapitulates many of the cellular and molecular aspects of muscle development illustrated in panel (a). Environmental cues (Notch, IGF-1, Wnt, etc.) influence the activity of satellite cells (green arrows). Notch and IGF-1 signaling stimulate the proliferation of satellite cells, whereas Wnt signaling is involved in the transition from proliferation to the differentiation phase of myoblasts. Notch signaling is also necessary for the maintenance of the quiescent state of satellite cells. Depending on MyoD activity, satellite cells can follow one of two fates: they maymaintainMyf5 expression while downregulating MyoD and self-renewing; alternatively, they maintain MyoD expression and differentiate. Quiescent satellite cells express Pax3 and Pax7. Pax7 regulates MyoD and Myf5 expression (blue arrows). Myf5 regulates the expression of MyoD, which in

turn promotes the expression of myogenin and MRF4 (blue arrows). Extracted from Musarò, 2014.Myogenin promotes the expression of Myosin (blue arrows). Extracted from Musarò, (2014).

The myogenic process in fish has been largely studied in the last 4 decades and this crucial regulatory pathway has been recently reviewed to punt in context of other elements that also contribute in the muscle development, growth and regeneration (Figure 19) (Musarò 2014; Dal-pai-silva et al. 2020; Koganti, Yao, and Cleveland 2021). Once the primary myoblasts are activated, the primary round of myogenesis starts. The activation of the myogenic program in embryonic muscle precursors is directed by four basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs), called MyoD, myf5, myogenin, and MRF4 (Buckingham and Rigby 2014; Dal-pai-silva et al. 2020). Moreover, in some fish species, the MyoD has suffered a duplication, thus appearing two paralogues of this gene, the MyoD1 and the MyoD2. In fish somites, the adaxial cells are the first myogenic precursor cells that expresses MRFs in a sequentially manner: MyoD and myf5 in first place, to then express myogenin and mrf4. The expression of MyoD, myogenin and mrf4 rapidly extends to fast myogenic progenitors initially located in the posterior somitic domain (P.-Y. Rescan 2019). The expression of MRFs in the fast myogenic progenitors orchestrates the appearance of a complex molecular machinery that promotes the fusion of the myocytes into multinucleated embryonic fast-twitch muscle fibers. On the other hand, the red slow myogenic progenitors that migrates from the adaxial cells differentiate as single-celled embryonic slow-twitch muscle fibers (Dal-pai-silva et al. 2020). As earlier studied in zebrafish the MRFs may sometimes have redundant roles, while others appears to be specific. This redundancy is more common between the Myods and the Myf5, and to a lesser extent, with Myogenin, as these three MRFs are all paralogues of a common ancestor gene. Thus, the loss of function of MyoD or Myf5 is reflected in a reduced white muscle myogenesis, while the loss of both genes ablates all skeletal muscle (Hinits et al. 2011). In the case of myogenin, its loss of function does not completely prevent white muscle differentiation but reduces drastically the formation of multinucleated myotubes.

This primary myogenesis takes place until the segmentation process finishes, and the larvae starts to form. Is in this moment that the first muscle fibers are formed. Since then, cells Pax7 positive located in the somitic external layer incorporate into the embryonic dermomyotome, where they differentiate and form new myofibers thus increasing the size of the myotome (Dal-pai-silva et al. 2020). This secondary myogenesis is termed stratified hyperplasia (Devoto et al. 1996; Dal-pai-silva et al. 2020; P.-Y. Rescan 2019). Moreover, at the end of the muscle formation, a subpopulation of myogenic precursor cells expressing pax3 and pax7 remain without fusing and stay between the myofibers

(Kassar-Duchossoy et al. 2005). These cells, described both in mammals and fish, receive the name of satellite cells and remain quiescent in the adult skeletal muscle (Hollway et al. 2007; Dal-pai-silva et al. 2020).

The quiescent satellite cells, which can represent from 2 to 10% of the total myonuclei in adult muscle, have a high nucleus to cytoplasm ratio does not express any gene related to muscle differentiation while having a constant expression of Pax7, necessary for the maintenance of this cell population. Apart of Pax-7, other proteins have been described as satellite cell markers. Some of these proteins are specific to the quiescent, activated, or proliferative state of the satellite cell, while others are expressed more widely, such as the cluster of differentiation 34 (Cd34), M-caderin, C-Met receptor tyrosine kinase (c-Met), a7 Integrin, Caveolin-1 and other caveolins, Syndecan-3 and -4, neural cell adhesion molecule (Ncam); 5- Bromo-20-deoxyuridine (BrdU), and proliferating cell nuclear antigen (Pcna) (Dal-pai-silva et al. 2020).

Once the right moment arrives, usually after a growth stimulatory situation or after a muscular injury, satellite cells expressing Pax3 and Pax7 activates through the action of other important proteins, such as the wingless/integrated protein family (WNT) coming from the neural tube and the surface ectoderm, as well as the sonic hedgehog protein (SHH) from the notochord (Hernández-Hernández et al. 2017). Such activation promotes the proliferation and differentiation to myoblasts who will undergo the myogenic process (Bischoff 1986; Dal-pai-silva et al. 2020).

However, this activation of satellite cells to induce myogenesis in adult muscles also happens transiently in the animals that possesses indeterminate growth. This third round of myogenesis is called mosaic hyperplasia, where new small-diameter muscle fibers are generated within the muscle between the pre-existing fibers (I. A. Johnston, Bower, and Macqueen 2011; P.-Y. Rescan 2019). Nevertheless, this mosaic hyperplasia is more active in the juvenile stages for then progressively decrease and eventually cease depending on the age of the animal (P. Y. Rescan et al. 2015; P.-Y. Rescan 2019).

One of the main particularities of the skeletal muscle that happens in all the three possible rounds of myogenesis is the fusion of muscle myoblast into multinucleated myofibers, as extensively reviewed (Krauss 2007; B. Chen, You, and Shan 2019). Briefly, this complex molecular process involves cell-

cell recognition and adhesion, cytoskeleton remodeling for enhancement of cell proximity and membrane rearrangements (Krauss 2007) (Figure 20).

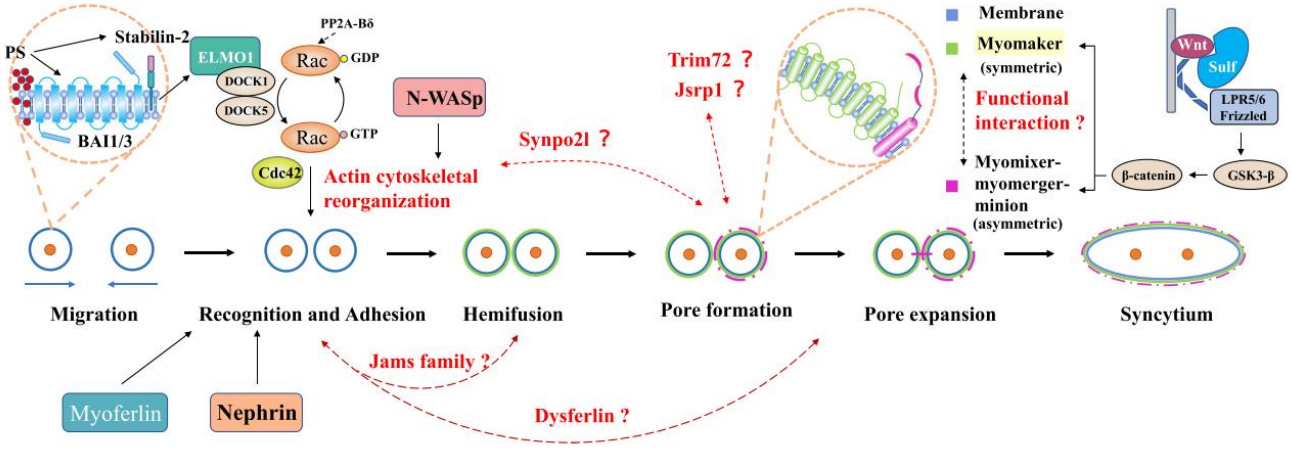


Figure 20: Schematic representation of various steps of myoblast fusion. Myoblast fusion involves migration, recognition, adhesion, remodeling of cytoskeleton, and formation and expansion of the fusion pore. The PS–BAI–ELBO–DOCK–RAC pathway has a certain regulatory effect on cytoskeletal remodeling. Studies have shown that Myomaker is responsible for the establishment of the hemi fusion state, while Myomixer acts in the formation of the fusion pore; both Myomaker and Myomixer are regulated by the Wnt signaling pathway. There may be some interaction between these two proteins, and it is unclear whether such interactions may have an impact on myoblast fusion. Extracted from (B. Chen, You, and Shan 2019).

Even the process of myoblast fusion has been largely studied for decades, still nowadays not all the proteins involved in this process are identified, and from those that are identified, most of them still remain without a clear identified role in order to understand how the muscle development occurs and how it is regulated. Among the most important, are, the immunoglobulin domain containing receptor Kirrel and cytoplasmic proteins Dock1, Dock5 and their adaptor proteins Crk (Srinivas et al. 2007; Moore et al. 2007). Among the most novel molecules that participate in this process, it is necessary to remark the transmembrane protein called Tmem8C/Myomaker and the Myomixer/Myomerger/Minion. The myomaker was discovered in mouse (Millay et al. 2013) and was lately described in zebrafish and in rainbow trout (Landemaine, Rescan, and Gabillard 2014; Landemaine et al. 2019). In all the species, this protein is required for myoblast fusion and muscle regeneration (Landemaine et al. 2019; Landemaine, Rescan, and Gabillard 2014; H. Zhang et al. 2020; Shi et al. 2017). Myomixer is a membrane small peptide that acts as a partner of Myomaker in both mammals and zebrafish (B. Chen, You, and Shan 2019; H. Zhang et al. 2020; Shi et al. 2017; W. Zhang and Roy 2017). However, how these two proteins participate in the myoblast fusion is still poorly known in both mammals and fish.

1.8.Protein degradation

Apart of the endocrine regulation of growth and the molecular program that controls the development of muscle from stem cells to myofibers, there are other molecular mechanisms that play important roles in muscle growth, such as those related with the protein homeostasis and structural remodeling (Nakashima, Ishida, and Katsumata 2011; Bell, Al-Khalaf, and Megeney 2016). These systems coordinate the parallel processes of protein synthesis and degradation on the muscle, based on the availability of amino acids obtained from the diet and the energetic status of the whole-body system. Hence, a positive balance towards protein synthesis is necessary to promote muscular growth, while inadequate balance between feed intake and energy expenditure may trigger the signaling pathways towards protein breakdown to direct amino acids to the catabolic processes thus leading to muscle atrophy and weakness (Bell, Al-Khalaf, and Megeney 2016). Moreover, the proteolytic systems are also involved in the structural remodeling and protein turnover that continually occurs in a growing muscle (myogenesis) and by the natural deterioration that the muscle suffers for being constantly challenged by mechanical, thermal and oxidative stress (Iban Seiliez et al. 2010; I. Seiliez, Dias, and Cleveland 2014; Bell, Al-Khalaf, and Megeney 2016).

The main proteolytic systems that fall into this description can be divided into three main groups: the ubiquitin-proteasome (UbP) system; the calpains, the lysosomal cathepsins (Bell, Al-Khalaf, and Megeney 2016).

1.8.1. The calpains

The calpains are Ca^{2+}-dependent non-lysosomal cysteine proteases that are involved in a large number of cellular processes, including signal transduction, proliferation, cell migration, cell cycle progression, differentiation, apoptosis, membrane fusion and autophagy control, among others (Darrel et al. 2003; Randriamboavonjy, Kyselova, and Fleming 2019; Ono and Sorimachi 2012). The main difference of this proteolytic system with the other two that will be further explained is that Calpain carry out mainly regulatory proteolysis, by specifically cleaving regulatory domains from the functional domains of its target (Figure 21).

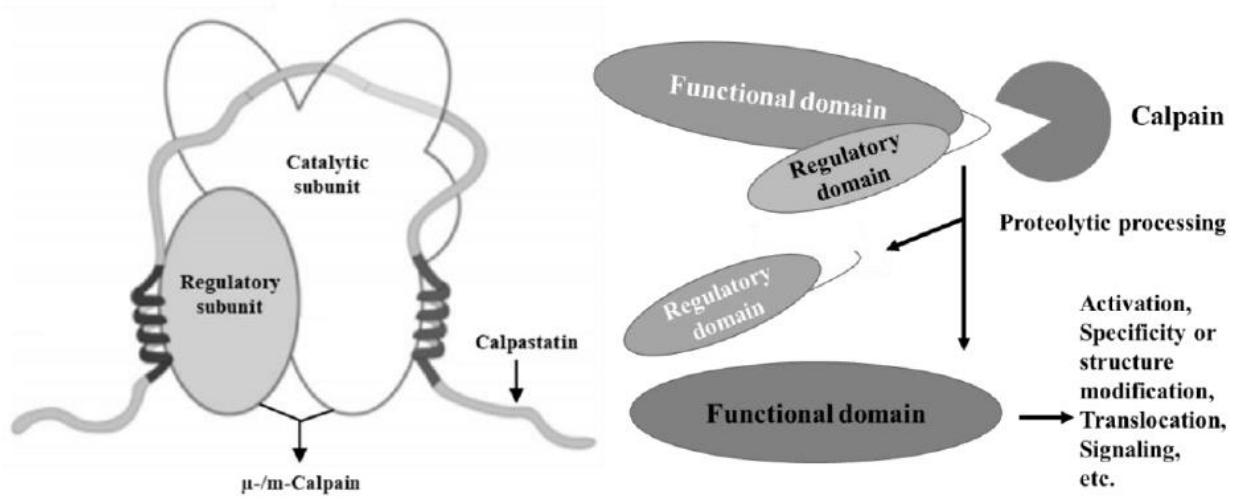


Figure 21: Left, schematic representation of the classical calpain structure and the interaction with the specific inhibitor Calpastatin. Adapted from Kiss et al. (2008). **Right**, example of the proteolytic processing mediated by calpains and different possible effects. Extracted from Vélez, (2018).

In mammals, the calpains are composed of a large catalytic subunit and a small regulatory subunit. Additionally, a third component known as Calpastatin is a specific inhibitor of the calpain activity (Darrel et al. 2003; Randriamboavonjy, Kyselova, and Fleming 2019). The activity of the calpains depends on the formation of heterodimers between the large and the small subunits. However, the catalytic domain required certain concentrations of Ca2+ to be active and in this regard the calpains have been classified depending on the requirements on this bivalent ion. Hence the calpains can be micromolar (μ-) or millimolar (m-) (Darrel et al. 2003). The small subunit of the calpain (also known as calpain 4, Darrel *et al.* 2003) is universal among calpains and has a chaperone-like function for the catalytic subunits, facilitating their correct folding and stability (Ono and Sorimachi 2012).

In mammals there have been identified more than 15 calpain genes (Ono and Sorimachi 2012). Among them, calpains are divided into classical or typical calpains (calpains 1, 2, 3, 8, 9, 11-14), that contain an "EF-hand" type of calcium-binding domain, and non-classical or atypical calpains (calpains 5, 6, 7, 10, 15, and 16), which lacks this domain (Reviewed by Ono and Sorimachi, 2012 and Baudry and Bi, 2016). Regarding the tissue distribution of the calpains, some of them are ubiquitous (calpains 1, 2, 5, 7, 10, 13-16), while others are tissue-specific (calpains 3, 6, 8, 9, 11, and 12). In this regard, the calpain 3 is only expressed in the skeletal muscle (Ono and Sorimachi 2012). In the skeletal muscle development, different calpains have been described to carry out different type of functions (Darrel et al. 2003). Calpain 1 regulates myogenesis by its action on Myogenin, Ezrin,

Vimentin and Caveolin 3 (Moyen et al. 2004); Calpain 2 participates in the fusion of myoblasts to multinucleated myotubes and in cell migration during muscle regeneration (Darrel et al. 2003; Choi, Ferrari, and Tedesco 2020).

In fish, the calpains 1, 2 and 3 have been studied in different species such as gilthead sea bream under different experimental conditions, but because of the still low number of studies featuring this proteolytic genes in conjunction with other physiological systems, their functions on muscle development and regulation remains unclear (Salmerón et al. 2013; Vélez, Azizi, Verheyden, et al. 2017; Vélez, Lutfi, et al. 2017; Vélez, Azizi, Lutfi, et al. 2017).

1.8.2. The Ubiquitin-proteasome system (UbP)

In mammals, the UbP system carries out the degradation of the majority of the proteins (Rock 1994), as its ultimate function is to carry out the degradation of damaged proteins to be further substituted by newly synthetized ones (protein turnover). However, in fish there are some evidences that the role of this system has been relegated to a 17% of the total proteolysis (I. Seiliez, Dias, and Cleveland 2014), thus being necessary to study this important mobilization pathway under different experimental conditions in fish. Briefly and as reviewed by different authors (Bell, Al-Khalaf, and Megeney 2016; Scicchitano, Faraldi, and Musarò 2015), this mechanism consists in the polyubiquitination of the substrate proteins to degrade by the E3/E2 ligases. These tagged proteins will be degraded in the proteasome complex with ATP consumption, leading to protein fragments, amino acids and free ubiquitin as byproducts that can be further reused (Figure 22).

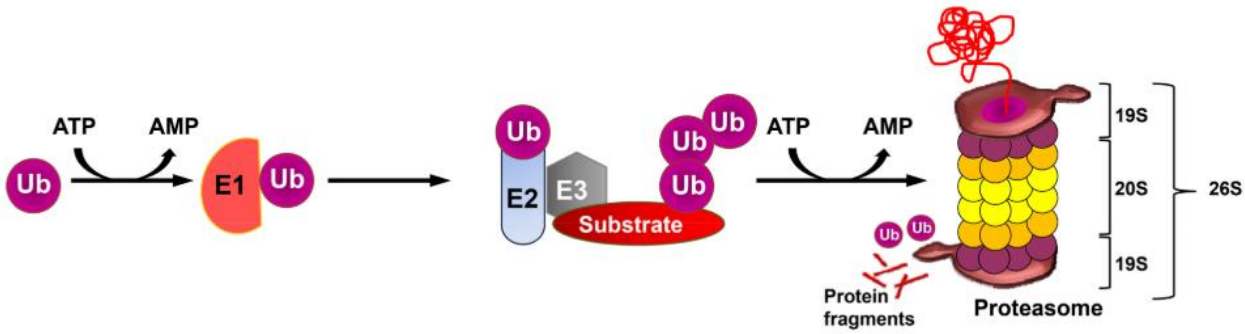


Figure 22: Schematic representation of the ubiquitin-mediated protein degradation. Ubiquitin is activated by the ubiquitin-activating en- zyme-E1 and then transferred to a ubiquitin-conjugating enzyme-E2. The E2 enzyme and the protein substrate both bind to the ubiquitin- protein ligase-E3. The protein substrate becomes polyubiquitinated; the polyubiquitin chain functions as a signal to target the polyubiquitinated protein substrate to the 26S proteasome, which

represents a multi compartmentalized protease formed by two subunits: 20S and 19S, for degradation, generating short peptides and free ubiquitin that can be further reused. Extracted from Scicchitano, Faraldi and Musarò, (2015).

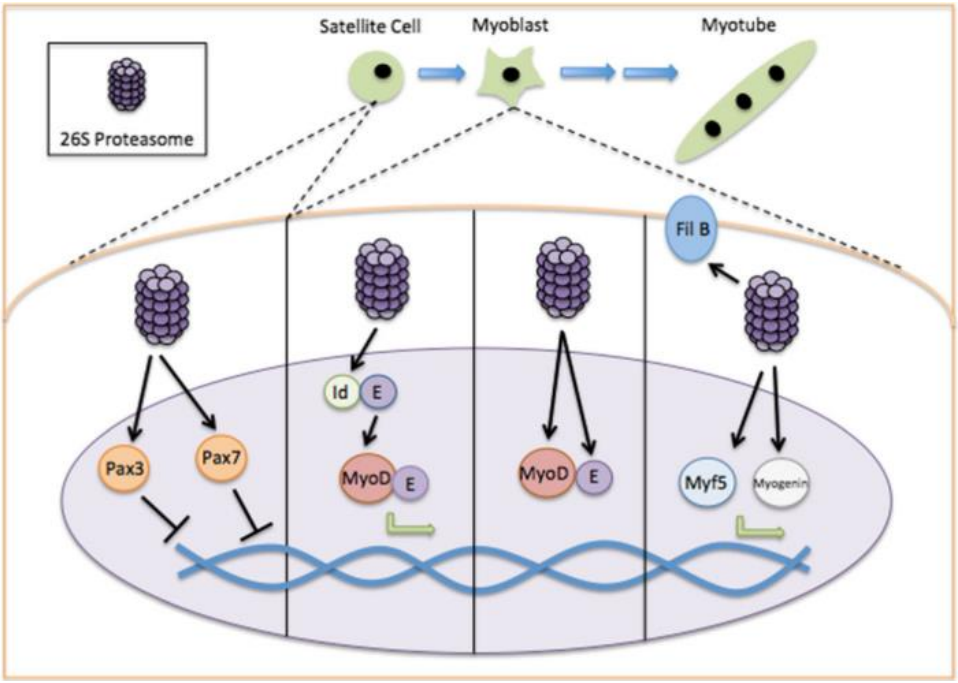


Figure 23: The role of the UPP in skeletal muscle cell differentiation. The fidelity of muscle cell differentiation is dependent upon the spatiotemporal expression of particular myogenic proteins. Indeed, UPP involvement in satellite cell differentiation begins with its role in the removal of Pax3 and Pax7, which maintain satellite cells in their stem cell niche. Further, the 26S proteasome appears critical for the early activation of a key myogenic factor, MyoD, through the removal of an endogenous MyoD inhibitor. The continuation of the myogenic program relies on UPP-dependent degradation of MyoD and its binding partner E2A (E), as well as Myf5, Myogenin, and Filamin B (FilB) during later stages of differentiation. Extracted from Bell, Al-Khalaf and Megeney, (2016).

In muscle, as reviewed by Bell et al. 2016, the UbP system has been described to be implicated in all the main steps of the myogenic process by sequentially degrading the main markers of the different steps, such as the Pax3/7 and the myogenic regulatory factors (Figure 23). The activity of this proteolytic system can be measured either by total ubiquitinated proteins and by measuring the expression of the N3 (Cleveland et al. 2012; Salmerón, Navarro, et al. 2015; I. Seiliez, Dias, and Cleveland 2014). However, in muscle there are other molecules implicated in the UbP system that can also be good markers. Apart of the ubiquitous E3, the muscle possesses two speciffic E3 ligases, the atrogin-1, also known as muscle atrophy F-box protein (MAFbx), and Muscle RING Finger 1 (MuRF1), and both proteins participate in muscle atrophy-derived protein degradation (reviewed by Scicchitano, Faraldi and Musarò, 2015).

These two ligases have been associated with the UbP system implication of muscle response to exercise and fasting-derived muscle atrophy in both mammals and fish (Scicchitano, Faraldi, and Musarò 2015; Bell, Al-Khalaf, and Megeney 2016; Iban Seiliez et al. 2008; Salmerón, Navarro, et al. 2015; Valente, Bower, and Johnston 2012; Garcia de la serrana et al. 2012; Bower, de la serrana, and Johnston 2010; Wang et al. 2011). Both, acute resistance bouts as well as long-term resistance training in mammals, are known to induce both protein synthesis and degradation (Rennie and Tipton 2000; Bell, Al-Khalaf, and Megeney 2016) and part of this protein breakdown is mediated by the UbP system, with upregulation of MAFbx and MuRF1 in both acute and chronic exercise exposures (Bell, Al-Khalaf, and Megeney 2016).

1.8.3. The autophagy and the lysosomal cathepsins

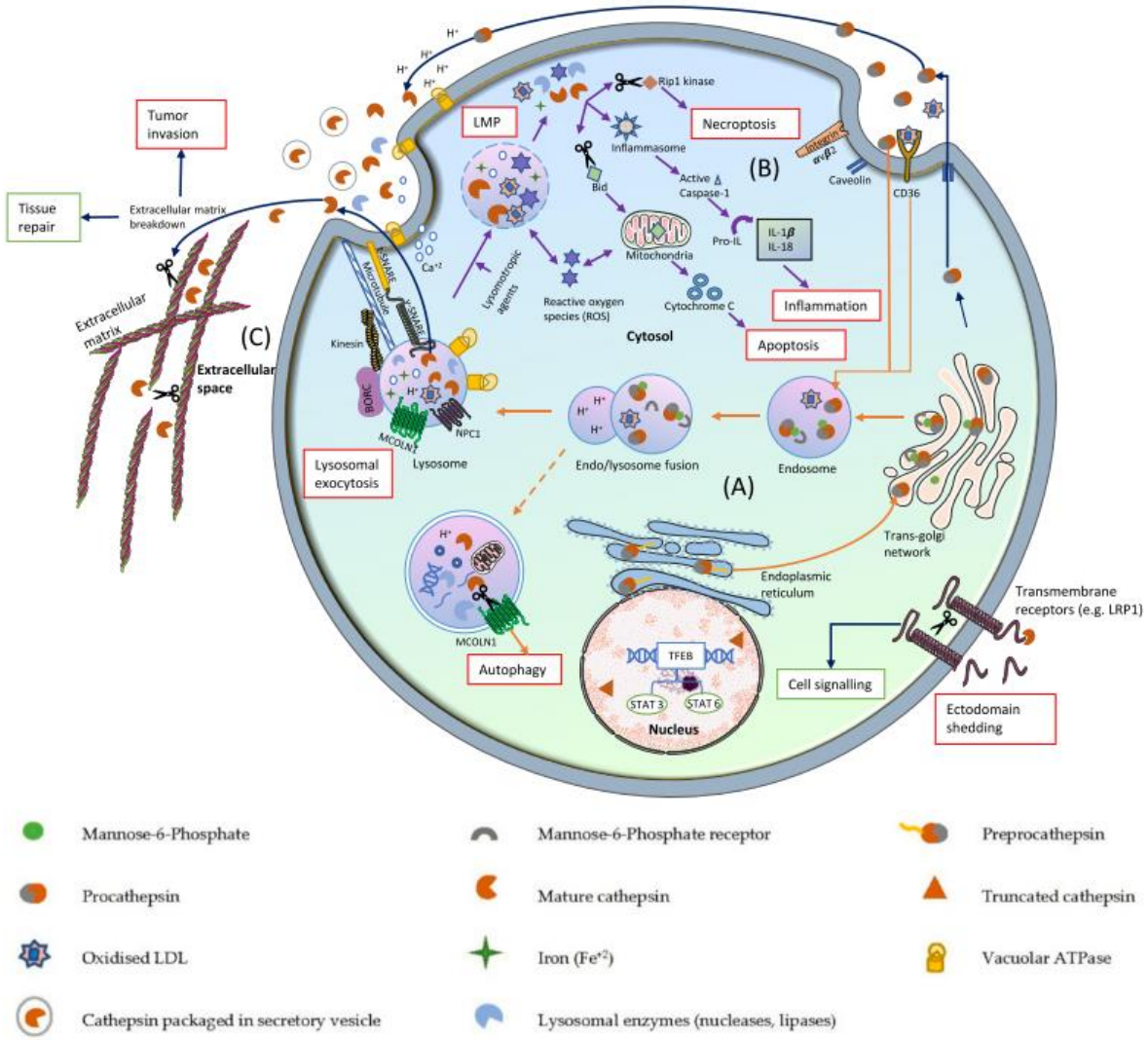


Figure 24: Site-specific functions of cathepsins. (A) cathepsins in the lysosomes (represented by orange arrows): Cathepsins are synthesized as preprocathepsins in the endoplasmic reticulum and transported to endo/lysosomes via Trans-Golgi network where the acidic pH enables their maturation. Cathepsins in the lysosomes are mostly involved in protein degradation besides participating in autophagy. (B) cathepsins in the cytosol (represented by purple arrows): Lysosomotropic agents, ROS or accumulation of modified lipids (oxLDL) leads to lysosomal membrane permeabilization (LMP), releasing cathepsins into the cytosol. Cytosolic cathepsins participate in various activities. For example, cathepsins trigger the inflammasome and promote apoptosis and necroptosis by cleaving various proteins. (C) Cathepsins in the extracellular space (represented by blue arrows): Lysosomal exocytosis involves the secretion of lysosomal contents into the extracellular space with the help of several protein-receptor interactions and Ca+2 ion gradient. Cathepsins are released in the form of procathepsins or enclosed in the secretory vesicles or as active cathepsins. Secreted cathepsins remain attached to the plasma membrane or are released into the extracellular space. Cathepsins on the plasma membrane cleave proteins like integrins. Secreted cathepsins mainly participate in extracellular matrix degradation and thus help in

wound healing. However, excessive ECM cleavage facilitates tumor invasion and promotes cancer. While in the extracellular space cathepsins also shed the ectodomains of transmembrane receptors, leading to either activation or inhibition of cell signaling. ROS: reactive oxygen species; LMP: lysosomal membrane permeabilization; NPC1: Niemann–Pick disease type C1; CD36: cluster of differentiation 36. Extracted from Yadati *et al.* (2020).

Cathepsins are proteolytic molecules that hydrolyze either extracellular proteins or cytoplasmic proteins that has been directed to the auto phagolysosomes, which are cytoplasmic vesicles where low pH (3.8-5) is achieved to permit the action of several hydrolases (Figure 24) (Stoka, Turk, and Turk 2016; Nemova, Lysenko, and Kantserova 2016). However the function of this proteolytic pathway has been largely studied in different pathophysiological scenarios (reviewed by Yadati *et al.* 2020) and most of the studies on the function of this proteins in muscle, focuses on the post-mortem activity of these proteolytic molecules and their contribution to flesh quality (Tesseraud et al. 2020); while the role of cathepsins in muscle growth and regeneration as well its response to nutritional status and physical exercise has been poorly investigated. In mammals there are 11 Cathepsins identified and they have some overlapping functions, while some specific roles have been associated to certain cathepsins (Vidak et al. 2019). In fish, almost all the Cathepsin types have been identified, being the Cathepsin D and the Cathepsin L are among the ones with higher expression and importance in the total proteolysis contribution (Figure 25) (Nemova, Lysenko, and Kantserova 2016).

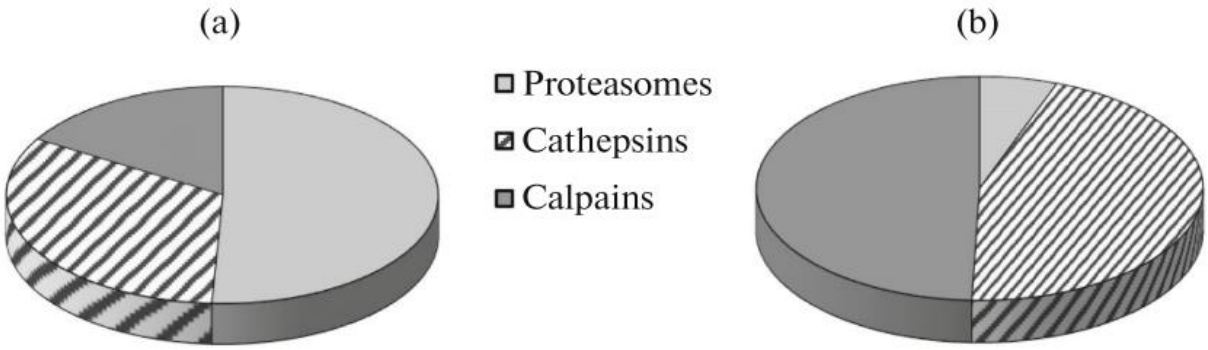


Figure 25: Relative activity of the major proteolytic systems in cultured muscle cells from (a) mammals and (b) fish. Data were obtained using inhibitors of individual proteolytic pathways, therefore, the accuracy of the results is limited by inhibitor cross-reactivity. ATP-independent pathways predominate in muscle protein hydrolysis in fish, in contrast to mammals. Extracted from Nemova, Lysenko and Kantserova, (2016).

The Cathepsin D is an aspartic proteinase that in fish plays the role of the main muscle proteolytic molecule by cleaving native proteins, among them being some of the most important structural and functional proteins of this tissue, such as the myosin heavy chain, actin, and tropomyosin (Nemova, Lysenko, and Kantserova 2016). The Cathepsin L is a lysosomal cystein endoprotease that is involved in the initial phases of the lysis of the phagolysosome content for being later degraded by the

Cathepsin D, which continues with this function (Yadati et al. 2020; Stoka, Turk, and Turk 2016). In mammals there is few information regarding the implications of Ctsl in muscle, however, it has been associated the initial stages in muscle loss under certain pathologic situations such as sepsis or cancer (reviewed by Scicchitano, Faraldi and Musarò, (2015).

The Cathepsins have been studied under certain nutritional experimental approaches in fish. In fasting and refeeding models in gilthead sea bream, rainbow trout and Atlantic salmon, the Cathepsins D and L have been reported to increase their expression during fasting while decreasing after refeeding, along with the tendencies observed in the UbP system (Salmerón, Navarro, et al. 2015; P.-Y. Rescan et al. 2007; Bower, de la serrana, and Johnston 2010). Regarding the regulation of muscular cathepsins by diet in fish, Ctsd decreased with diets with lower digestible protein to digestible energy in rainbow trout and its expression in white muscle increased in rainbow trout fed with diets fully substituted with plant protein (Alami-Durante et al. 2019). Ctsl activity decreased with dietary isoleucin in hybrid bagrid catfish (Jiang et al. 2021) and in grass carp (*Ctenopharyngodon idella*) increased with histidine (Wu et al. 2020).

The Cathepsins in fish muscle have also been studied under exercise conditions, although the information available is still very scarce to fully understand their role in the muscle adaptation to sustained physical activity. In this regard, in gilthead sea bream fingerlings trained for 6 weeks under moderate-sustained swimming conditions, Ctsd gene and protein expression increased in skeletal anterior white muscle, along with several members of the UbP family and Calpains (Vélez, Azizi, Lutfi, et al. 2017). However, moderate exercise has been reported to promote muscle growth with decreased cathepsin activity in Atlantic salmon (Nilsen et al. 2019).

1.9.Bone in fishes

The bone is a connective tissue that in vertebrates was earlier described to have mainly mechanical functions such as providing structural support to other tissues and organs, such as skeletal muscle, with which they perform the locomotory function of the animal. Moreover, it constitutes an important calcium and phosphorus repository (Watkins et al. 2001). The structure of the bone, as it is known for most of vertebrates, including most of the fish species, can be described as a partially mineralized extracellular matrix in which they are included the osteoblasts, osteoclasts (bone resorption cells) and osteocytes (Reviewed in Boglione et al. 2013).

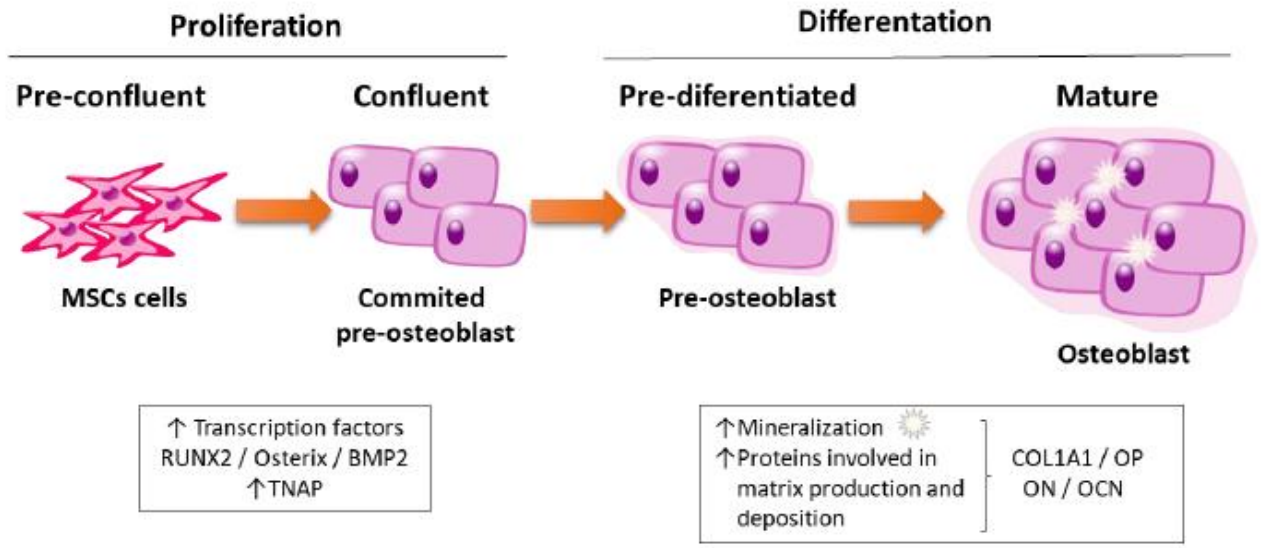


Figure 25: Osteoblast differentiation, maturation and key factors involved. Osteogenesis in vitro of multipotent mesenchymal stem cells (MSCs) that differentiate into mature osteoblasts. During proliferation, cells express transcription factors genes as runt-related transcription factor 2 (Runx2), osterix and bone morphogenetic protein 2 (BMP2) and tissue non-specific alkaline phosphatase (TNAP). In the differentiation phase, cells are expressing proteins involved in matrix production and mineralization such as collagen type 1A (COL1A1), osteopontin (OP), osteonectin (ON) and osteocalcin (OCN). Extracted from Riera Heredia. (2019).

However, some fish species, including the gilthead sea bream, are known to possess a bone in which there are not osteocytes resident in lacunae within the mineralized extracellular matrix (Bonewald 2011; Franz-Odendaal, Hall, and Witten 2006), a phenomenon known as acellular bone (Kolliker 1859). The osteocytes from the cellular bone function as regulators of two major processes in bone: remodeling or bone repair and modelling or bone adaptation (Bonewald 2017; J. Chen 2010; Tatsumi et al. 2007). The osteoblasts, usually in the surface of the bone, proliferate and start to generate extracellular matrix that will be further mineralized. Once entrapped in the matrix, the osteoblasts form a compact cellular network and when the matrix is fully mineralized, they become osteocytes

which remain in continuous communication with their surrounding ones through canaliculi (narrow channels) and cytoplasmatic extensions (Bonewald 2011; Cao et al. 2011; Franz-Odendaal, Hall, and Witten 2006). In the case of the acellular bone, osteocytes are not present (Moss 1961) and the osteoblasts produces and mineralizes extracellular matrix towards the bone surface, thus avoiding being entrapped by the mineralization or dying after being surrounded by mineralized extracellular matrix (Ekanayake and Hall 1987; 1988; Weiss and Watabe 1979; Ofer et al. 2019).

The ontogeny of the bone is known as osteogenesis (Figure 25), and contrarily to the myogenesis that is governed by a series of MRFs, the osteogenesis is transcriptionally controlled by a single transcription factor, the runt related transcription factor 2 (Runx2), which controls osteoblast linage determination and the expression of osteogenic genes (Matsubara et al. 2008). In this process, osteoblasts are originated from mesenchymal stem cells (MSCs) that activates, proliferates and differentiates (Ytteborg et al. 2010) and their ultimate function is, after being recruited for bone formation, to synthesize, secrete, organize, and mineralize the bone matrix, also known as osteoid (Rodan and Noda 1991). The first molecules produced by the commited- and pre-osteoblasts are the tissue non-specific alkaline phosphatase (Tnap) (Reviewed in Infante and Rodríguez, 2018) and the bone morphogenic protein 2 (Bmp2), a TGF-β family member involved in the hedgehog pathway that induces osteoblast differentiation and osteogenesis. Upon osteoblast differentiation, the expression of the proteins involved in the extracellular matrix formation and mineralization occurs (L. Chen et al. 2015; Vieira et al. 2013; Ytteborg et al. 2012). This extracellular matrix has a fibrillar structure and the main organic components of such fiber-like structures are collagen type I (Col1a1) and fibronectin (Fib1a). However, there are also other important structural proteins, such as the Osteopontin (Op), a protein with strong negative charge that quenches Ca salts, thus negatively regulating the mineralization or acting as Ca reservoir; the Osteonectin (On), a glycoprotein with high affinity for Ca ions that binds to the collagen fibers for the initiation of the mineralization of the extracellular matrix; and the Osteocalcin (Ocn), a calcium-binding protein when it is carboxylated, thus promoting the mineralization of the extracellular matrix, but in its non-carboxylated form, acts as an hormone over diverse tissues, including skeletal muscle where induces growth and glucose and lipid uptake (Karsenty and Olson 2016).

In both mammals and fish, there are evidences that this osteogenic process can be impaired by different factors either *in vivo* or *in vitro*, such as the described mutations in the col1a1 gene that causes osteogenesis imperfecta in mammals (Duy et al. 2016). This ontogenic process can be affected

by drugs, which can disrupt COL1A1 and ON in vitro (Humphrey, Morris, and Fuller 2013) and bone loss in patients that used this drug (Nakken and Taubøll 2010). On the other hand, treatment of cultured primary rat calvarial osteoblasts with vitamin A (retinoic acid) induced excessive osteogenesis through decreasing osteoblast proliferation and increasing the activity of TNAP activity and mRNA levels of OP (H. M. Song et al. 2005).

Regarding the nutritional regulation of bone tissue, the effects of long-chain polyunsaturated fatty acids on bone metabolism have been studied in both mammals and fish, including gilthead sea bream *in vivo* and *in vitro* (Poulsen, Moughan, and Kruger 2007; Riera-Heredia et al. 2019; Balbuena-Pecino et al. 2021) and it has been observed that these fatty acids can affect bone either directly or indirectly by affecting tissues closely related to the bone, such as the skeletal white muscle (Wauquier et al. 2015). The effects of fasting and refeeding have been studied in cellular-boned fish, such as rainbow trout (Takagi et al. 1992; Persson et al. 1997), but not inacellular-boned fish species same as the gilthead sea bream. Nevertheless, the bone growth is affected directly by the Gh/Igf axis in mammals (Schoenle et al. 1982; He et al. 2006; Beattie et al. 2018; Ohlsson, Bengtsson, Bengt-Ake; Isaksson, and Andreassen, Troels T.; Slootweg 1998) and low Igf concentrations have been associated with bone loss in fasting or undernutrition situations (Merimee, Zapf, and Froesch 1982; Crane and Cao 2014). In fish, Igf stimulates bone growth in *Anguila japonica* and Atlantic salmon (Wargelius et al. 2005) and in cultured gilthead sea bream osteoblasts, insulin and Igfs stimulates cell proliferation (Capilla et al. 2011), thus, known the implication of the Gh/Igf axis in the fasting and refeeding regulation, further studies need to be performed in fish under these conditions and in conjunction with other related tissues.

If the osteogenesis is the process by which bone is created, the bone is a dynamic tissue that nay undergo periods of remodeling and resorption, a process carried out by a specialized bone cells known as osteoclasts. The osteoclast is a tissue-specific macrophage polykaryon originated from the differentiation of monocyte/macrophage precursors cells residing in or near of the bone surface.

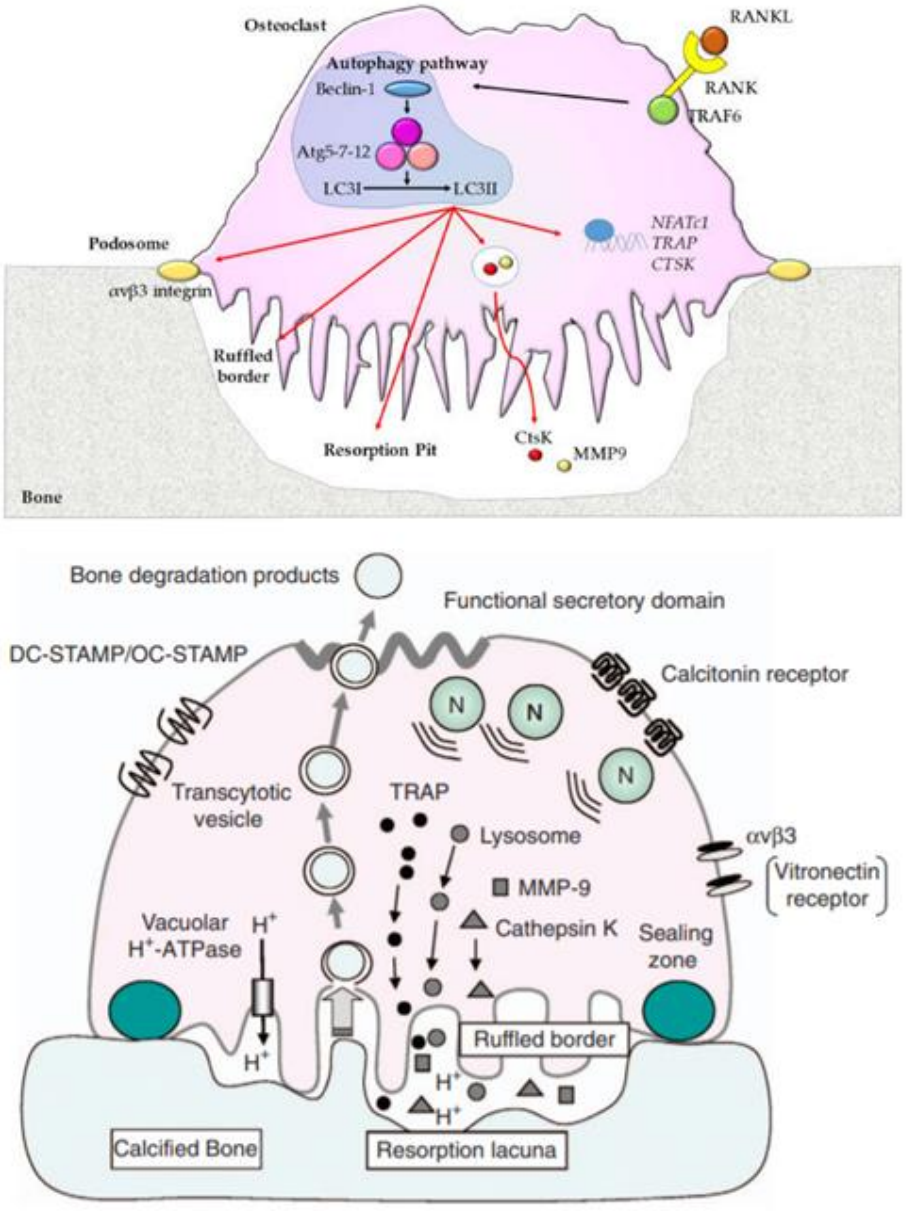


Figure 26: Up, The role of autophagy in osteoclasts. Osteoblast-secreted and osteocyte-secreted RANKL that binds to its receptor RANK on osteoclasts leads to the recruitment of TRAF6 and an increase of Beclin-1 and Atg5/7/12 with enhanced conversion from LC3I to LC3II. This autophagic pathway regulates the expression of osteoclast genes [NFATc1, TRAP, and CTSK (Cathepsin K)], podosome and ruffled border formation, and bone resorption activity stimulating the secretion of CtsK and MMP9. Extracted from Montaseri *et al.* (2020). **Down,** Ultrastructure and function of osteoclasts. Osteoclasts have several characteristics, such as multiple nuclei, abundant mitochondria and a large number of vacuoles and lysosomes. Bone-resorbing osteoclasts form ruffled borders and sealing zones. The resorbing area under the ruffled border is acidic. Vacuolar H+-ATPase localized in the ruffled border is involved in the transport of protons into the resorption lacunae. Enzymes such as cathepsin K, MMP9 and TRAP are secreted into the resorption lacuna to degrade bone matrix proteins. Matrix degradation products are endocytosed from the central portion of the ruffled border, packaged into transcytotic vesicles and secreted from the functional secretory domain. Osteoclasts express large numbers

of calcitonin receptors and αvβ3 vitronectin receptors. Osteoclasts also express DC-STAMP and OC-STAMP, which are involved in the cell–cell fusion of osteoclasts. Extracted from Takahashi, Udagawa and Suda, (2014).

The CSF-1 and RANKL are the receptors required to induce the expression of genes that typify the osteoclast lineage, among which the lytic proteins tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), and MMP9 are secreted to the external vacuole (also known as Howchip's lacunae), that will be further acidified and promote the degradation of the adjacent mineralized bone. The degradation byproducts, such as collagen fragments and solubilized calcium and phosphate, will be processed within the osteoclast and released into the circulation (Figure 26).

However, the information regarding the nutritional regulation of the osteoclastogenesis is very scarce, (Van Der Velde et al. 2012) reported in mammals that under fasting, Ghrelin may induce the osteoclastogenic process, thus making of high-interest the co-study of the bone resorption systems in parallel with the osteogenic process, the Gh/Igf axis and specially, the ghrelinergic system.

1.10. Mitochondria.

Mitochondria are cellular organelles that are implicated in uncountable physiological process in eukaryotic cellular physiology, such as ATP production, redox homeostasis, biosynthesis of macromolecules, intracellular calcium regulation and apoptosis, among others. Probably among them, the main physiological implication of these organelles in health and disease is to function as redox and stress sensors of the cell, as because the oxidative nature of this cellular compartment, is also the source of most of the ROS species when the cell undergoes metabolic impairments or toxic insults. In this scenario, mitochondria under constant stress as these organelles are the first affected by any of those impairments and they have to adapt in order to reestablish the energetic homeostasis of the cell. Such constant stress and adaptations shorten the shelf life of the elements (double membrane and proteins) that conforms these organelles, thus being necessary a high turnover rate that increases with the energetic demand of the cell under oxidative conditions. This turnover is subdivided in two processes, the mitochondrial biogenesis and the mitochondrial dynamics.

1.10.1. Mitochondrial biogenesis and dynamics

The mitochondrial biogenesis can be defined as the *de novo* generation of new mitochondria, either for increasing the mitochondrial mass of a tissue in response to an increased energy demand, or to promote mitochondrial turnover in substitution of old damaged mitochondria that will be degraded in the mitophagy process. In mammals, the mitochondrial biogenesis has been largely studied and reviewed under different physiological scenarios, among them is noteworthy to highlight the physical aerobic/endurance exercise, a situation where the energy demand increases massively, as the skeletal muscle represents around 40% of body mass in humans and up to 60% in fish (Scarpulla 2011; Popov 2020; Memme et al. 2021). However, the biogenesis is only one side of the protein turnover, as it goes hand by hand with the mitochondrial dynamics, which briefly is the combination of the fusion and fission processes that mitochondria can undergo. Mitochondrial fusion occurs to build larger and more efficient mitochondria, which is beneficial in high energy demanding conditions, while fission processes are required to exscind overused and stressed mitochondria to direct the most damaged fractions to the mitophagy degradation while keeping the healthier portions of the mitochondria (Memme et al. 2021). Thus, in a healthy mitochondrial network under high energy demand, both fusion and fission processes may be parallelly activated with the mitochondrial biogenesis in order to guarantee maximal energy production and ROS dissipation with increase mitochondrial turnover.

The process of mitochondrial biogenesis and dynamics induced by exercise in mammals is summarized in the figure 27. One of the particularities of the mitochondrial biogenesis is that, because of the endosymbiotic origin of the mitochondria, the genes that codifies for the nearly 1200 proteins (in mammals) are divided between the nuclear (nDNA) and mitochondrial chromosomes (mtDNA) (Calvo, Clauser, and Mootha 2016). However, the tiny (16.5kb) mitochondrial chromosome, encodes only for 13 proteins belonging to the electron transport chain (ETC), along with 2 rRNAs and 22 tRNAs (Memme et al. 2021). Thus, over 99% proteins required to build up new mitochondria are nuclear encoded, which means that after their translation, they will need to be imported via mitochondrial chaperones and protein import channels. Hence, the mitochondrial biogenesis requires a finely tuned coordination of both mtDNA and nDNA transcription which is achieved with a series of highly regulated transcription factors. Among the proteins that regulates the expression of the nuclear-encoded genes, in most of the vertebrates there is one factor that has been well stablished by the literature as the master regulator of the mitochondrial biogenesis, which is the transcriptional coactivator peroxisome proliferator activated receptor γ coactivator 1α (Pgc1α) (Scarpulla 2011; Scarpulla, Vega, and Kelly 2012; Memme et al. 2021). The Pgc1α and its family members, Pgc1β

and Pgc-related co-activator (Prc), upregulate the gene transcription by docking with other generic transcription factors (TFs) and additional proteins on DNA promoters to regulate the nuclear genes encoding for mitochondrial proteins (NuGEMPs) (Puigserver et al. 1999; Scarpulla 2011; Scarpulla, Vega, and Kelly 2012; Memme et al. 2021). Among these nuclear encoded genes, two of the most widely studied are the Citrate Synthase, which is one of the most representative enzymes of the Kreebs' Cycle, and the Cytochrome c oxidase subunit IV, which is the only subunit of the electron transport chain encoded by the nuclear genome.

The consideration of the Pgc1α as the master regulator of this complex process is because of the extreme level, almost unprecedented, of regulation that this transcription coactivator is subjected (Figure 28), which combines, a part of the transcriptional and translational regulation, a double activation process that combines phosphorylation and deacetylation that permits the entrance of this coactivator to the nucleus and the docking with the other necessary transcription factors (Popov, 2020). Acute exercise was one of the firstly demonstrated inductors of the Pgc1α activation process, which can happen in response to different extrinsic signals, such as: (1) activation of calcium/calmodulin-dependent protein kinase (CaMK) in response to increased intracellular Ca2+ concentration; (2) activation of p38 mitogen-activated protein kinase (p38 MAPK), which is activated in response to multiple stressors, such as reactive oxygen species (ROS); and (3) AMP-activated protein kinase (AMPK) phosphorylation, activated by high AMP:ATP ratio and by myosin ATPases (Memme et al. 2021). Finally, the expression of Pgc1a can be mediated by cAMP through adrenergic signaling (Handschin et al. 2003). Once activated, Pgc1α interact with a variety of TFs, being the most importants the nuclear respiratory factors 1 and 2 (NRF)-1/2, which induce the expression of the mitochondrial transcription factor A (Tfam), which is the most important transcription factor of the mtDNA-derived genes as well as promoter of the mtDNA duplication (Scarpulla 2011; Memme et al. 2021). Thus, this Pgc1α-Nrf-Tfam axis permits the coordination of the nuclear and mitochondrial gene expression required for the mitochondrial biogenesis. Any imbalance in this sequential activation of transcription between both genomes can lead to the disruption of the proteostasis in the mitochondria which can trigger the mitochondrial unfolded protein response (UPRmt).

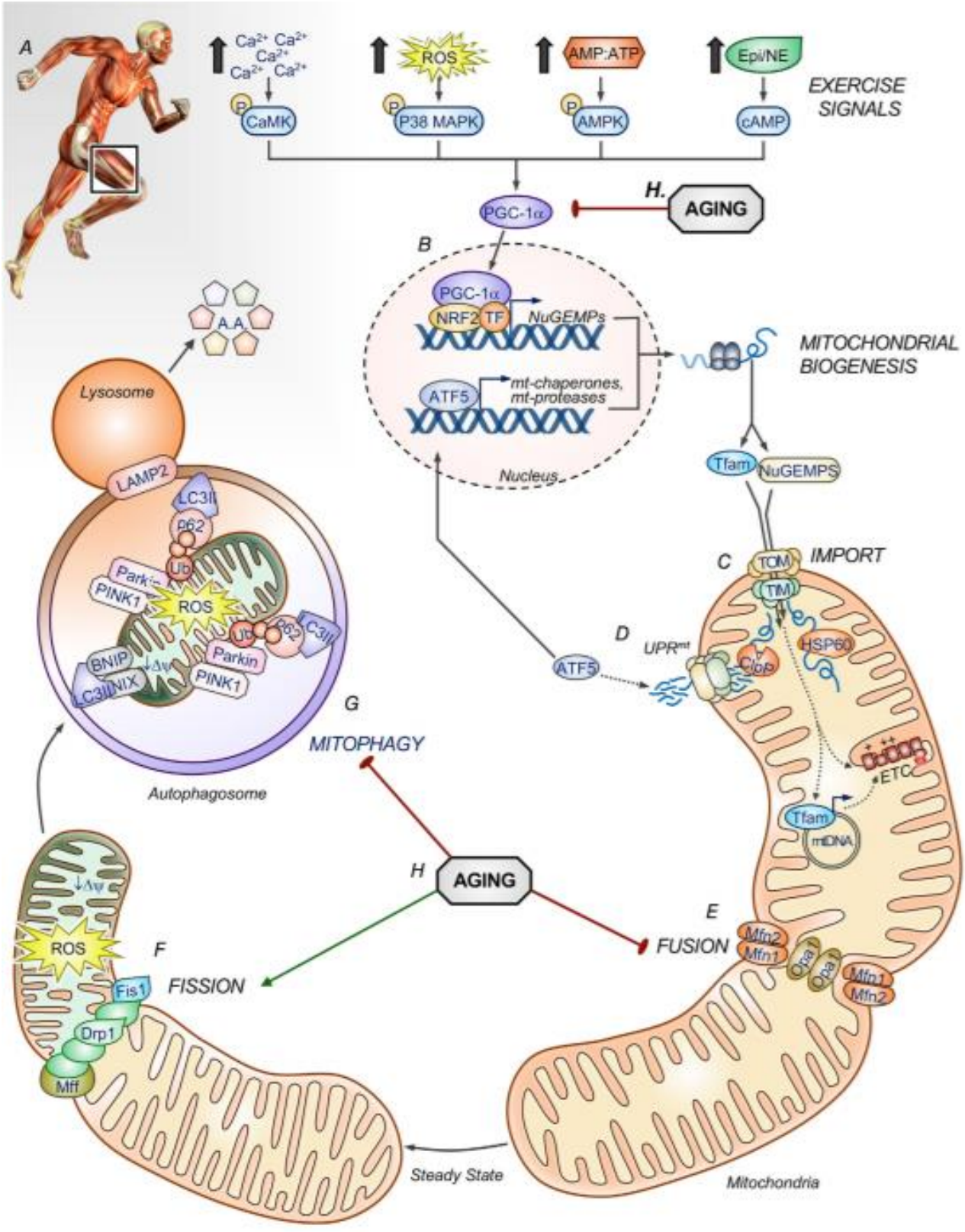


Figure 27: Regulation of mitochondrial health with exercise and the influence of age Exercise induces robust changes in mitochondrial content and quality that are beneficial for metabolic health. **A,** motor unit recruitment of myofibers produces action potentials that induce Ca2+ release from the SR. Increased cytosolic Ca2+ prompts myosin-actin interactions, crossbridge cycling, and the hydrolysis of ATP to produce muscle force, as well as the generation of ATP

and ROS from respiring mitochondria. **B,** consequently, these events activate the signalling kinases that converge on Pgc1α, allowing its translocation to the nucleus where it coactivates TFs to increase the expression of NuGEMPS, such as the mtDNA transcription factor, Tfam. **C,** newly expressed nuclear-derived proteins are then imported into mitochondria through translocases of the outer, and then inner membranes (TOM and TIM, respectively). D, the exercise stimulus increases protein levels within mitochondria, which promotes the activation of the UPRmt. The cleavage of terminally misfolded protein aggregates in the matrix and the release of their peptide fragments, block ATF5 mitochondrial entry, thus redirecting it to the nucleus to upregulate transcription of mitochondrial chaperones and proteases and equip the organelle with an augmented capacity for protein folding. E, mitochondrial fusion proteins Mfn1/2 along with Opa1 facilitate the fusion of the outer and inner membranes, respectively, allowing for improved sharing of metabolites amongst neighboring organelles. F, regions of the mitochondrial network may become dysfunctional as they are incapable of matching the metabolic needs of the tissue and require segregation from the reticulum in order to spare their adjacent organelles from further impairment. These damaged organelles undergo fission, mediated by the interaction of proteins Fis1 and Mff with Drp1 to constrict and remove the organelles, allowing for their clearance via mitophagy. G, once separated, dysfunctional mitochondria with a low accumulate PINK1 on their outer membrane. PINK1 recruits the E3-ligase Parkin, which subsequently ubiquitinates outer membrane proteins to flag the organelle for removal via mitophagy. The adapter protein p62 binds to the ubiquitin on the tagged cargo, as well as to LC3-II embedded in the phagophore membrane, and promotes the formation of the autophagosome, which fuses with the lysosome to degrade the mitochondria and release the constituent amino acids for cellular recycling. H, aged muscle displays attenuated Pgc1α signalling for mitochondrial biogenesis (red line), as well as increased fission:fusion protein ratio (green line), thus promoting a fragmented network of organelles, as well as suppressed mitophagy flux. Extracted from Memme, (2021)

As mentioned above, the mitochondrial biogenesis needs to be coupled with mechanisms that guarantees the overall health of the mitochondrial population of the cell and tissue by promoting the fusion of mitochondria and the excision of overused and damaged mitochondria via mitophagy (Memme et al. 2021). Classically, the mechanism by which mitochondrial fusion occurs involves the activity of the proteins mitofusin-1/2 (Mfn1/2) and the optical atrophy protein 1/2 (Opa1/2), which facilitate the fusion of the adjacent outer and inner membranes, respectively (Mishra and Chan 2016). Similarly, mitochondrial fission requires the joint action dynamin related protein 1 (Drp1), mitochondrial fission factor (Mff or Miffb) and fission protein 1 (Fis1), which in combination will promote the constriction and cleavage of mitochondrial fragments from the network to direct the exscinded fragments to mitophagy (Toyama et al. 2016; Memme et al. 2021).

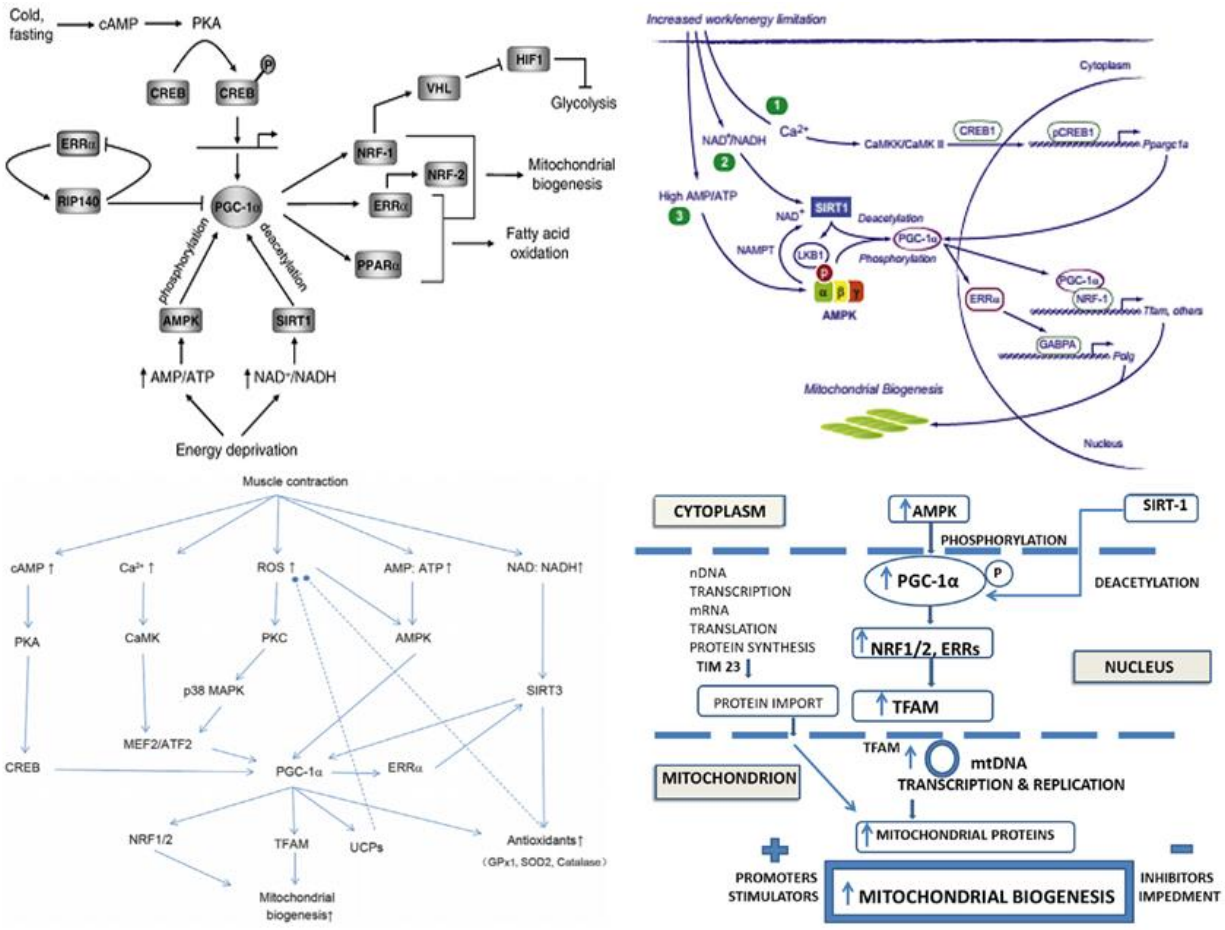


Figure 28: The central role of Pgc1α activation in the mitochondrial biogenesis. AMP:ATP = adenosine monophosphate/adenosine triphosphate ratio; AMPK = AMP-activated protein kinase; ATF = activating transcription factor; CaMK = Ca2+/Calmodulin-activated protein kinases; cAMP = cyclic adenosine monophosphate; CREB = cAMP response element-binding protein; ERRa = estrogen-related receptor a; GPx1 = glutathione peroxidase 1; MAPK = mitogen-activated protein kinases; MEF2 = myocyte enhancer factor-2; NAD:NADH = nicotinamide adenine dinucleotide / nicotinamide adenine dinucleotide hydrogen ratio; NRF = nuclear respiratory factor; PGC-1α = peroxisome proliferator-activated receptor g coactivator 1-a; PKA = protein kinase A; PKC = protein kinase C; ROS = reactive oxygen species; ;SIRT1 = sirtuin 1; SIRT3 = sirtuin 3; SOD = superoxide dismutase; TFAM = mitochondrial transcription factor A; UCPs = uncoupling proteins. Adapted from (Scarpulla 2011; Piantadosi and Suliman 2012; Popov 2020; L. Li et al. 2020).

Briefly, mitophagy, a kind of autophagy, occurs when the double-membraned vesicles known as autophagosomes engulf damaged organelles that are tagged for degradation by specialized protein. Such tagging occurs when these organelles exhibit a decreased membrane potential and/or excessive increases in ROS production (Wei, Liu, and Chen 2015; Memme et al. 2021). The main mitophagy pathway, at least under exercise conditions, involves the joint action of the proteins Pink1 and Parkin (Geisler et al. 2010; Memme et al. 2021). In healthy mitochondria, Pink1 is imported into

mitochondria and degraded by resident proteases, but when there is a disruption of the mitochondrial membrane potential and consequently, of the protein import systems, Pink1 accumulates on the outer membrane (Geisler et al. 2010; Memme et al. 2021). This accumulation of Pink1 facilitates the recruitment of the E3-ubiquitin ligase Parkin to mitochondria, which will carry out the ubiquitination different outer membrane proteins, such as Mfn2, tagging the organelle for degradation (Geisler et al. 2010; Memme et al. 2021).

Mitophagy is induced by numerous cellular stresses, including the energetic imbalance that occurs under acute exercise (Vainshtein & Hood, 2016; Erlich et al. 2018; Chen et al. 2018b; Hood et al. 2019). During exercise, the AMP/ATP ratio increases, causing the aforementioned activation of AMPK that in this case, will activate the first proteins involved in the mitophagy independently of the Pgc1α. Parallely, mTORC1, is a known suppressor of the process that will be inhibited in pro-mitophagy conditions (J. Kim et al. 2011; Memme et al. 2021).

In fishes, literature about mitochondrial biogenesis regulation is very scarce and recent and is necessary to assume that this process has certain degree of conservation among vertebrates despite the metabolic differences that exist between these two vertebrates' group. As well as in mammals, the Pgc1α has been one of the earliest mitochondrial biogenesis-related proteins studied in fish but in this case, the role of this master regulator is under current discussion in fish as initially, discrepant responses in comparison to mammals were observed in fish under exercise or cold exposure (McClelland et al. 2006; LeMoine, Genge, and Moyes 2008; Magnoni et al. 2012; Magnoni, Palstra, and Planas 2014; McClelland and Scott 2014). Moreover, later, Bremer et al. 2016 postulated that the important Ampk-Pgc1α-Nrf1 axis that governs the mitochondrial biogenesis in mammals may be disrupted by insertions in the Pgc1α sequence region involved in the docking with Nrf1. Nevertheless, recent studies in rainbow trout demonstrated again that the Pgc1α does respond to swimming, but the response of this axis depend on the exercise duration and intensities, being the higher intensity programs those who induce more mitochondrial biogenesis in white muscle as a metabolic adaptation from anaerobic to aerobic metabolism (Pengam et al. 2020a; 2020b).

The main studies on overall mitochondrial function in fish have focused mainly on the effects of nutritional (Enyu and Shu-Chien 2011), osmotic (Tse, Chow, and Wong 2012), thermal (Beck and Adam Fuller 2012; Mueller et al. 2011; O'Brien 2011) and chemical stress (Peter et al. 2013). Mitochondrial function has been reported to be involved in growth regulation by diet composition in

both channel catfish (Eya et al. 2012) and in rainbow trout (Eya et al. 2017). Among the mitochondrial proteins assessed in nutrition-related studies, the UCPs received a high degree of interest and the nutrient levels on the gene expression of respiratory chain uncoupling proteins, UCP2 and UCP3, has been studied in rainbow trout (Coulibaly et al. 2006) and gilthead seabream (Bermejo-Nogales et al. 2011; Bermejo-Nogales, Calduch-Giner, and Pérez-Sánchez 2014). Moreover, the role of these UCPs under environmental stressors has been also studied in gilthead sea bream (Bermejo-Nogales et al. 2014). Regarding the mitochondrial dynamics in fish, the available information is very scarce, but it has been reported, in yellow catfish (Y. Song et al. 2020) and snout bream (X. Li et al. 2019), that diet composition can modulate fusion and fission, as well as biogenesis and oxidation processes.

Finally, the Cox, Cs and their ratio at gene expression and enzyme activity level has been studied in white muscle of exercised gilthead seabream fed with different diets, richer in protein (Blasco et al. 2015) or carbohydrates (Martin-Perez et al. 2012), but because of the complex regulation of these two enzymes in the whole context of the metabolism of fish, further studies are required.

CHAPTER 2: OBJECTIVES

With the most absolute purpose of understanding what the "simple" concept of "growth regulation" implies, the present thesis is a compilation of original works that aims to deepen in how the growth and metabolism of fish, and most concretely, its muscle, behaves in different metabolically challenging conditions. Hopefully, the knowledge generated in these works and integrated in this multi-perspective thesis, can contribute to somehow help to improve the Aquaculture sector in the Mediterranean Sea and abroad. This ambitious goal is structured in different objective:

1) To establish the regulation of the ghrelinergic system in bream through the fasting and feedback model:

a) Characterization of the sequences of Preproghrelin, Ghsr1a and Ghsr1b in gilthead sea bream (Article 1).

b) Characterizing Preproghrelin and its two receptors in acute and long-term feeding responses and its interaction with the Gh/Igf1 axis (Article 1).

2) To deepen in the beneficial effects of exercise on growth and its modulation through changes in the dietary protein/lipid ratio:

a) Determining how this dietary modification alters different growth parameters, muscular proximal composition and reserves turnover (Article 3).

b) Determining how the mitochondrial proteins in red and white muscle and in the liver respond to this dietary modification in order to infer changes in the nutritional efficiency and growth improvement (Article 3).

3) To make a comparative study about the response of the main musculoskeletal growth regulation systems under two different challenging experimental models, the fasting and refeeding and the sustained exercise under two different diets.

a) Studying the gene expression of specific proteins of the ontogenetic and growth pathways (myogenesis and osteogenesis), as well as the degradation pathways (proteolysis and osteoclastogenesis) in white muscle and bone (Articles 2 and 4).

b) Determining its effects on white muscle growth (myogenesis/proteolysis balance) and meat quality (Article 4).

4) To complete the characterization of the new Myomaker and Myomixer fusogens in rainbow trout and gilthead sea bream and its involvement in the myogenic process *in vivo* and *in vitro* and in the in vivo muscle regeneration.

a) Characterization of the sequence of Myomixer in rainbow trout (Article 5) and Muomaker and Myomixer in gilthead sea bream (Article 6).

b) To study the involvement of Myomaker (in gilthead sea bream) and Myomixer (in rainbow trout and gilthead sea bream) in the myogenic process *in vivo* and *in vitro* as well as during the muscle regeneration *in vivo* (Article 5 and 6).

CHAPTER 3: DIRECTORS' REPORT

UNIVERSITAT DE BARCELONA

Departament de Biologia Cel·lular, Fisiologia i Immunologia
Facultat de Biologia

Edifici Ramon Margalef (plantas 3-A & 4-A)
Av. Diagonal, 643
E-08071 Barcelona
Tel: +34 934021531 Fax: +34 934110358
e-mail: dept-fisiologia@ub.edu

B:KC Barcelona Knowledge Campus

El Dr. Joaquin Gutiérrez Fruitós y la Dra. Josefina Blasco Mínguez, como director y co-directora de la tesis presentada por Miquel Perelló Amorós titulada "Regulatory mechanisms of muscle growth and metabolism in fish: modulation by nutritional status, diet composition, exercise and muscular regeneration" manifiestan la veracidad del factor de impacto y la implicación del doctorando en los artículos científicos publicados o pendientes de publicación presentados en esta tesis.

Los directores también declaran que la participación de Miquel Perelló Amorós en la elaboración de los artículos ha sido muy activa, tal y como queda reflejado en todos ellos en la relación de autores, ya que consta como primer autor en la mayoría de ellos (art. 1, 3, 4, 5 y 6). Comparte coautoria con implicación equivalente a la primera autora, Fátima Lavajoo (art. 2) o a la segunda autora, Isabel García (art. 4). Los datos de estos dos artículos no han sido presentados en ninguna otra tesis doctoral. Miquel ha contribuido de manera principal en el planteamiento y realización de los experimentos, obtención de datos, análisis de los resultados, así como en la redacción y elaboración de dichos artículos. Por otro lado, el experimento realizado en el artículo 5 deriva de una estancia de 3 meses realizada en el Laboratoire de Physiologie et Genomique des Poissons con el grupo del Dr. Jean-Charles Gabillard, Instituto Nacional para la Investigación Agronómica (INRA), Campus Beaulieu en Rennes (Francia).

Article 1 (Chapter I): "Characterization and nutritional regulation of ghrelin and its receptors in gilthead sea bream."
Authors: Perelló-Amorós, M., Vélez, E.J., Vela-Albesa, J., Sánchez-Moya, A., Riera-Heredia, N., Heden, I., Fernández-Borràs, J., Blasco, J., Calduch-Giner, J.A., Navarro, I., Capilla, E., Jönsson, E., Pérez-Sánchez, J. and Gutiérrez, J.
Journal: Frontiers in Endocrinology 9:399.
DOI: 10.3389/fendo.2018.00399
Impact Factor: 3.634 JCR (2018) Q2

Article 2 (Chapter II): "Regulatory mechanisms involved in muscle and bone remodeling during refeeding in gilthead sea bream."
Authors: 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. and Gutiérrez, J.
Journal: Scientific Reports (2020) 10:184.
DOI: 10.1038/s41598-019-57013-6
Impact Factor: 4,379 JCR (2020) Q1

Article 3 (Chapter III): "Mitochondrial Adaptation to Diet and Swimming Activity in Gilthead Seabream: Improved Nutritional Efficiency."
Authors: Perelló-Amorós, M., Fernández-Borràs, J., Sánchez-Moya, A., Vélez, E. J., García-Pérez, I., Gutiérrez J. and Blasco J.
Journal: Frontiers in Physiology. 12:678985.
DOI: 10.3389/fphys.2021.678985.
Impact Factor: 4,566 JCR (2020) Q1

Article 4 (Chapter IV): "Diet and exercise modulate Gh-Igfs axis, proteolytic markers and myogenic regulatory factors in juveniles of gilthead sea bream (Sparus aurata)."
Authors: Perelló-Amorós, M., García-Pérez, I., Sánchez-Moya, A., Innamorati, A., Vélez, E.J., Achaerandio, I., Pujolà, M., Calduch-Giner, J., Pérez-Sánchez, J., Fernández-Borràs, J., Blasco, J. and Gutiérrez, J.
Journal: Animals. Accepted (21 july 2021).
Impact Factor: 2,752 JCR (2020) Q1

Article 5 (Chapter V): "Myomixer is expressed during embryonic and post-larval hyperplasia, muscle regeneration and differentiation of myoblats in rainbow trout (Oncorhynchus mykiss)."
Authors: Perelló-Amorós, M., Rallière, C., Gutiérrez, J. and Gabillard, J. C.
Journal: Gene (202) 790:145688.
DOI: 10.1016/j.gene.2021.145688.
Impact Factor: 3,688 JCR (2020) Q1

Article 6 (Chapter VI): "Myomaker and Myomixer characterization in Gilthead Sea bream under different myogenesis conditions."
Authors: Perelló Amorós, M., Tarrazón, A., Jorge, V., Garcia-Pérez, I., Sanchez-Moya, A., Gabillard, J.C., Moshayedi, F., Navarro I., Capilla, E., Fernandez-Borràs, J., Blasco, J., Chillaron, J., Garcia de la serrana, D., Gutiérrez, J.
Journal: En preparación para Scientific Reports.

Barcelona, Julio 2021

Dr. Joaquin Gutiérrez

Dra. Josefina Blasco

CHAPTER 4: ARTICLES

ARTICLE 1

Ghrelin and Its Receptors in Gilthead Sea Bream: Nutritional Regulation

***FRONTIERS IN ENDOCRINOLOGY* (2018): 9, 1–14**

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Ghrelin and Its Receptors in Gilthead Sea Bream: Nutritional Regulation

*Miquel Perelló-Amorós[1], Emilio J. Vélez[1], Jaume Vela-Albesa[1], Albert Sánchez-Moya[1], Natàlia Riera-Heredia[1], Ida Hedén[2], Jaume Fernández-Borràs[1], Josefina Blasco[1], Josep A. Calduch-Giner[3], Isabel Navarro[1], Encarnación Capilla[1], Elisabeth Jönsson[2], Jaume Pérez-Sánchez[3] and Joaquim Gutiérrez[1]**

[1] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain, [2] Fish Endocrinology Laboratory, Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, [3] Nutrition and Fish Growth Endocrinology, Institute of Aquaculture Torre de la Sal (CSIC), Castellón, Spain

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National Cerebral and Cardiovascular Center, Japan

***Correspondence:**
Joaquim Gutiérrez
jgutierrez@ub.edu

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Ghrelin is involved in the regulation of growth in vertebrates through controlling different functions, such as feed intake, metabolism, intestinal activity or growth hormone (Gh) secretion. The aim of this work was to identify the sequences of *preproghrelin* and Ghrelin receptors (*ghsrs*), and to study their responses to different nutritional conditions in gilthead sea bream (*Sparus aurata*) juveniles. The structure and phylogeny of *S. aurata preproghrelin* was analyzed, and a tissue screening was performed. The effects of 21 days of fasting and 2, 5, 24 h, and 7 days of refeeding on plasma levels of Ghrelin, Gh and Igf-1, and the gene expression of *preproghrelin*, *ghsrs* and members of the Gh/Igf-1 system were determined in key tissues. *preproghrelin* and the receptors are well conserved, being expressed mainly in stomach, and in the pituitary and brain, respectively. Twenty-one days of fasting resulted in a decrease in growth while Ghrelin plasma levels were elevated to decrease at 5 h post-prandial when pituitary *ghsrs* expression was minimum. Gh in plasma increased during fasting and slowly felt upon refeeding, while plasma Igf-1 showed an inverse profile. Pituitary *gh* expression augmented during fasting reaching maximum levels at 1 day post-feeding while liver *igf-1* expression and that of its splice variants decreased to lowest levels. Liver Gh receptors expression was down-regulated during fasting and recovered after refeeding. This study demonstrates the important role of Ghrelin during fasting, its acute down-regulation in the post-prandial stage and its interaction with pituitary Ghsrs and Gh/Igf-1 axis.

Keywords: ghrelin, GHSR1a, growth hormone, IGF-1, fasting and refeeding

INTRODUCTION

Ghrelin is a peptide hormone secreted mainly by the stomach in vertebrates, but also detected in many other tissues (e.g., intestine, heart, pancreas, and especially pituitary and brain). Ghrelin is synthesized as Preproghrelin, and the mature peptide varies between 12 and 28 amino acids, depending on species and form of Ghrelin, but it shows high sequence homology across vertebrates, including fish (1). Since its discovery, Ghrelin has been involved in many physiological processes like the regulation of feed intake, adiposity, growth, energy and glucose metabolism, intestinal

motility and digestive enzymes activity, among others (2). The first characterization of Ghrelin in a fish species, the goldfish (*Carassius auratus*), was done by Unniappan et al. (3). Later, Kaiya et al. (4) reviewed its function in non-mammalian vertebrates and recently, different publications have investigated its role in other fish species (5–12), but very little is known about this hormone in gilthead sea bream (*Sparus aurata*) (13).

Ghrelin functions through binding to its receptors, which are also known as the growth hormone secretagogue receptors (Ghsrs). The Ghsrs are a family of transmembrane G-protein coupled receptors, and the Ghsr1a isoform, discovered a few years before Ghrelin (14), is known as the active form. An alternative splice variant named Ghsr1b, was also described by the same authors, but its structure lacks two transmembrane domains leading to the impossibility of this isoform to initiate intracellular signaling. Since the discovery of these two receptors, the Ghsrs family has been widely studied and other numerous isoforms (splice variants and paralogues) have been described in vertebrates (15, 16).

ghsrs mRNA is found in many tissues, including brain, stomach, intestine, and especially pituitary gland. The high expression levels detected in the pituitary in vertebrates confirms the role of Ghsrs in the regulation of growth hormone (Gh) production (17). Gh is one of the key elements of the Gh/insulin-like growth factor-1 (Igf-1) axis, which is the main regulator of growth in vertebrates. Depending on factors such as nutritional state, Gh can directly stimulate anabolic or catabolic processes by binding to the Gh receptors (Ghrs). Moreover, systemic Gh mainly acts in the liver, where it stimulates the production of Igf-1. This growth factor in turn acts in many peripheral tissues stimulating growth-related processes (18). Thus, most of the physiological peripheral roles of Ghrelin appear to be mediated indirectly by the modulation of Gh release (19). In addition, Ghrelin has been described to have an important role in the hypothalamus in mammals, where it acts on different *ghsrs*-expressing cell populations, leading to enhanced expression and release of orexigenic neuropeptides like neuropeptide Y and Agouti-related peptide, hence stimulating appetite in most vertebrates, including diverse fish species (20). Moreover, it has been recently reported, at least in mammals, that Ghrelin acts over the hypothalamic Gh releasing hormone neurons (21). Although there is a controversy on how the different forms of Ghrelin (acylated and unacylated) cross the blood brain barrier to exert this role (22), adding another complex level of regulation.

Fish are capable to resist long fasting periods (23) and the Gh/Igf-1 system, displays interesting changes to adjust metabolism and growth to nutrient supply. Ghrelin in its double role as a hunger hormone and Gh secretion regulator should play an important role in fasting and refeeding physiology, although these aspects are poorly known in fish, especially in gilthead sea bream (13, 24).

The objective of the present work was to identify and characterize Ghrelin and its receptors by analyzing sequences, phylogeny and gene expression through a tissue screening, and to study their responsiveness upon fasting and refeeding in relation with the Gh/Igf-1 axis in gilthead sea bream juveniles.

MATERIALS AND METHODS

Fish Maintenance and Distribution

Gilthead sea bream juveniles (initial body weight 50 ± 3 g; length 15.3 ± 0.68 cm) were obtained from a commercial hatchery (Piscimar, Borriana, Spain) and reared in the facilities of the Faculty of Biology. Forty-two fish were randomly distributed in six 200 L seawater tanks (7 fish/tank) and some extra fish for tissue screening were kept in another 200 L tank. Fish were kept in a seawater recirculation system at a constant temperature of 23 ± 1°C and at 12 h light/12 h dark photoperiod through the whole experiment. During the acclimation period (2 weeks), fish were fed to apparent satiety twice a day with a commercial diet (Optibream, Skretting, Burgos, Spain). This study was carried out in accordance with the recommendations of the EU, Spanish and Catalan Government-established norms and procedures. The protocol was approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 110/17 and DAAM 9488).

Experimental Design

After acclimation, a period of 21 days of fasting and 7 days of refeeding was designed, according to previous experience (25). During the refeeding period, fish were fed once a day to apparent satiety. Samplings were made at the beginning and end of the fasting period (−21 and 0 days, respectively), and at 2, 5, 24 h and 7 days upon refeeding. The −21 days, 24 h and 7 days samplings were made just before feeding, representing one day fasting. The day 0 sampling was done at the same time of the day, and fish were fed right after to start the refeeding. In each sampling, 6 fish were first anesthetized with MS-222 (0.08 g/L), and once blood was extracted, were sacrificed by an overdose of MS-222 (>0.1 g/L). Then, brain, pituitary, liver and stomach were dissected and stored in liquid nitrogen. Before sacrifice, body weight, body length (standard), and liver and viscera weight were measured to calculate distinct biometric indexes: condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI).

Additionally, 3 fish were sacrificed and sampled for 17 distinct tissues and/or organs. RNA was obtained from tissue samples (30–100 mg) or from the whole pituitary gland and brain with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) and reverse transcribed following the procedures previously described (26). Briefly, 1 µg of RNA was treated with DNase I (Life Technologies, Alcobendas, Spain) following the manufacturer's instructions to remove genomic DNA. After DNase treatment, retrotranscription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain) for 10 min at 25°C, 60 min at 50°C and 5 min at 85°C. Samples were immediately stored at −20°C for further analysis.

Preproghrelin and *ghsrs* Characterization

Primers for the amplification of the complete codifying sequences of *preproghrelin*, *ghsr1a* and *ghsr1b* were designed using Primer3Plus software (27) with the nucleotide sequences obtained from the Nutrigroup-IATS nucleotide database

of gilthead sea bream at http://www.nutrigroup-iats.org/seabreamdb (28, 29)]. The three sequences are deposited in GenBank (NCBI) under accession numbers: MG570187 for *preproghrelin*; MG570188 for *ghsr1a*, and MG570189 for *ghsr1b*. Sequences specificity was confirmed by PCR amplification of transcribed RNA samples from the tissue screening that were run on an agarose gel for size verification.

A multiple Preproghrelin sequence alignment was performed using the default settings of the MAFFT tool online (server) version (http://mafft.cbrc.jp/alignment/server/). The phylogeny was inferred using the JTT + G + I model substitution method and an unrooted tree was constructed using the MEGA X software with a bootstrapping value of 1,000. Previously, using the same software, a test was performed to determine which substitution model was the best for our data (data not shown). Unequivocal identity of *ghsr1a* and *ghsr1b* was verified by Blast and BlastX searches, as well as by transmembrane domain analysis by means of TMHMM transmembrane helixes prediction program (http://www.cbs.dtu.dk/services/TMHMM-2.0).

Ghrelin, Gh and Igf-1 Plasma Levels

Plasma levels of acylated Ghrelin were measured using the Ghrelin N- radioimmunoassay (RIA) protocol originally described by Hosoda et al. (30) and modified by Jönsson et al. (7) with the exception that plasma was not extracted, just quickly centrifuged (1,000 rpm, 1 min) before pipetting to the RIA tubes, and iodinated human Ghrelin (NEX388010UC, PerkinElmer, USA) was applied as tracer. Anti-rat Ghrelin [1-11] antisera, which specifically recognizes the conserved n-octanoylated Ser3 epitope on Ghrelin, was used at a final dilution of 1:500000 (gift from Dr. Hiroshi Hosoda, Japan). Standard was made using synthetic rainbow trout acylated Ghrelin (Peptide institute, Japan).

All samples were assayed in duplicate and included in one assay. The Ghrelin RIA was validated for gilthead sea bream, and the slopes of the standard curve and of a serial dilution of plasma samples were parallel (Supplementary Figure 1). Plasma levels of Gh and Igf-1 were measured by corresponding RIAs, as previously described (31, 32).

Gene Expression

The mRNA transcript levels were examined by quantitative real-time PCR (qPCR) according to the requirements of *MIQUE guidelines* (33) in a CFX384^{TM} Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). All reactions were performed in the conditions previously described (26). The primers used are listed in **Table 1**. To amplify the two *ghsrs* the forward primer was designed in a common region, and the reverse primer for *ghsr1a* in a region overlapping exon 1 and 2, and for *ghsrb1* in a region including the differential nucleotides at the end of translation and the 3′-UTR. In addition, elongation factor 1 alpha (*ef1a*), ribosomal protein S18 (*rps18*) and *b-actin* (only in brain) were analyzed and served as reference genes in order to calculate the relative expression of the target genes (34). Both, reference genes stability and relative expression calculation were determined with the Bio-Rad CFX Manager Software (v2.1).

Statistical Analyses

Data was analyzed using IBM SPSS Statistics 22 and are showed as mean ± standard error of the mean (SEM). Normality and homogeneity of variances were tested with Shapiro-Wilk Test and Levene's, respectively. When data did not follow a normal distribution or did not have homoscedasticity, it was converted by logarithm transformation. Differences among groups were tested by one-way analysis of variance (ANOVA) followed by Tukey HSD or LSD, as *post-hoc* tests. In case of no homoscedasticity, the non-parametric Kruskal-Wallis test was used with the Dunnett's T3 as *post-hoc*. The confidence interval for all analyses was set at 5%.

RESULTS

Preproghrelin and Ghsrs Characterization

Translation of *preproghrelin* nucleotide sequence (907 nucleotide in length) resulted in a 107 amino acid sequence that presented 97% identity with that of another sparid, the blackhead sea bream (*Acanthopagrus schlegelii*), as the most significant result in a BlastX search. The predicted sequence of gilthead sea bream Preproghrelin contained the conserved N-terminal signal peptide (26 amino acids), that yields Proghrelin after cleavage. In the Proghrelin region, the sequence contained the characteristic Ser3 residue, which is the octanonylation target, as well as the GlyArg amidation and cleavage site to obtain the N-terminal mature Ghrelin (20 amino acids) and the C-terminal Proghrelin peptide (**Figure 1A**).

The nucleotide sequences of *ghsr1a* and *ghsr1b* (1708 and 1793 nucleotide in length, respectively) encoded for 384 (Ghsr1a) and 292 (Ghsr1b) amino acids sequences that shared a 98% of identity with their respective orthologs in the blackhead sea bream (35). In the same way, the TMHMM transmembrane helixes program predicted the presence of the characteristic seven transmembrane domains in Ghsr1a, whereas Ghsr1b did not retain the last two due to alternative gene splicing (**Figure 1B**).

The phylogenetic analysis of the Preproghrelin amino acid sequence is shown in **Figure 2**. The unrooted tree highlights the conservation of this protein in vertebrates, although it presents clusters that separate the different vertebrate classes and fish orders. Results of the *preproghrelin* and *ghsrs* gene expression screening are shown in **Figures 3A,B**, respectively. *preproghrelin* was mainly expressed in stomach, but weak expression was also detected in many other tissues (i.e., spleen and head kidney). Regarding the receptors, brain, pituitary and liver were the tissues with highest expression of both, *ghsr1a* and *ghsr1b*, although low levels of expression were also found in many other. In pituitary and brain, the expression levels of *ghsr1a* were very similar, but the expression of *ghsr1b* was higher in liver. Thus, in pituitary and brain the most abundant isoform was *ghsr1a* while in liver was *ghsr1b*.

Fasting and Refeeding Experiment

Growth and Morphometric Parameters

Morphometric parameters results are shown in **Figures 4A–D**. Mean body weight (which was not significantly affected) and CF presented a similar pattern along the fasting/refeeding

TABLE 1 | Sequences, melting temperatures (Tm) and GenBank accession numbers of the primers used for qPCR.

Primer list (*Sparus aurata*)			
Gene	**Sequence (5′-3′)**	**Tm (°C)**	**Accession Number**
ef1a	**F:** CTTCAACGCTCAGGTCATCAT	60	AF184170
	R: GCACAGCGAAACGACCAAGGGGA		
rps18	**F:** GGGTGTTGGCAGACGTTAC	60	AM490061.1
	R: CTTCTGCCTGTTGAGGAACCA		
b-actin	**F:** TCCTGCGGAATCCATGAGA	60	X89920
	R: GACGTCGCACTTCATGATGCT		
preproghrelin	**F:** CCCGTCACAAAAACCTCAGAAC	60	MG570187
	R: TTCAAAGGGGGCGCTTATTG		
ghsr1a	**F:** GTCGGCGGCTGTGGCAAAGA	60	MG570188
	R: GGCCAACACCACCACCACCAAC		
ghsr1b	**F:** CGCACACGCATAACTTTGTC	60	MG570189
	R: GAGGAGGATGAGCAGGTGAA		
gh	**F:** GCCCCATCGACAAGCACG	60	FJ827496
	R: GAGTCTACATTTTGCCACCGTCAG		
ghr-1	**F:** ACCTGTCAGCCACCACATGA	60	AF438176
	R: TCGTGCAGATCTGGGTCGTA		
ghr-2	**F:** GAGTGAACCCGGCCTGACAG	60	AY573601
	R: GCGGTGGTATCTGATTCATGGT		
igf-1a	**F:** AGGACAGCACAGCAGCCAGACAAGAC	60	AY996779
	R: TTCGGACCATTGTTAGCCTCCTCTCTG		
igf-1ab	**F:** AGTCATTCATCCTTCAAGGAAGTGCATCC	60	EF688015
	R: TTCGGACCATTGTTAGCCTCCTCTCTG		
igf-1abc	**F:** ACAGAATGTAGGGACGGAGCGAATGGAC	60	EF688016
	R: TTCGGACCATTGTTAGCCTCCTCTCTG		
igfbp1a	**F:** AGTGCGAGTCCTCTCTGGAT	60	KM522771
	R: TCTCTTTAAGGGCACTCGGC		
igfbp2a	**F:** CGGGCTGCTGCTGACATACG	60	AF377998
	R: GTCCCGTCGCACCTCATTTG		
igfbp4	**F:** TCCACAAACCAGAGAAGCAA	60	F5T95CD
	R: GGGTATGGGGATTGTGAAGA		02JMZ9K
igfbp5b	**F:** TTTCTCTCTCGGTGTGC	60	AM963285
	R: TCAAGTATCGGCTCCAG		
igf-rb	**F:** GCTAATGCGAATGTGTTGG	55	KT156847
	R: CGTCCTTTATGCTGCTGATG		

experiment, decreasing after fasting and slightly increasing afterwards, partially recovering at day 7. Regarding HSI, a significant decrease was observed after fasting, but was significantly increased at day 7 post-refeeding. At 2 h post-prandial the stomach was clearly full, but no food was found in the intestine, whereas at 5 h the stomach had emptied almost all its food content. Thus, VSI was significantly lower after the fasting period. With refeeding, it increased at 2 and 5 h, but at 1 and 7 days the VSI values returned to baseline levels.

Plasma Ghrelin, Gh and Igf-1

Ghrelin, Gh, and Igf-1 plasma concentrations are presented in **Figures 5A–C**. Plasma Ghrelin showed maximum levels after fasting and at 2 h post-prandial, and a significant dip at 5 h, but then returned to high levels after 1 and 7 days. However, it should be taken into account that those samples, as well as the one before the whole fasting period, were taken after a 24 h fast, which appears to be a potential stimulus for Ghrelin secretion. Circulating Gh increased significantly with fasting. Then, there was no acute post-prandial change but a gradual decrease upon refeeding returning to basal after 7 days. Plasma Igf-1 levels had an inverse pattern to that of Gh; showing significantly lower values after the 21 days fasting period compared to day 0 and then returning to basal levels at 7 days post-refeeding.

Gene Expression

Preproghrelin and ghsrs

Stomach *preproghrelin* gene expression (**Figure 6A**) did not show any change after fasting, but a significant difference was observed after 1 day of refeeding. In the brain, *preproghrelin* gene expression was much lower than in the stomach (**Figure 3A**);

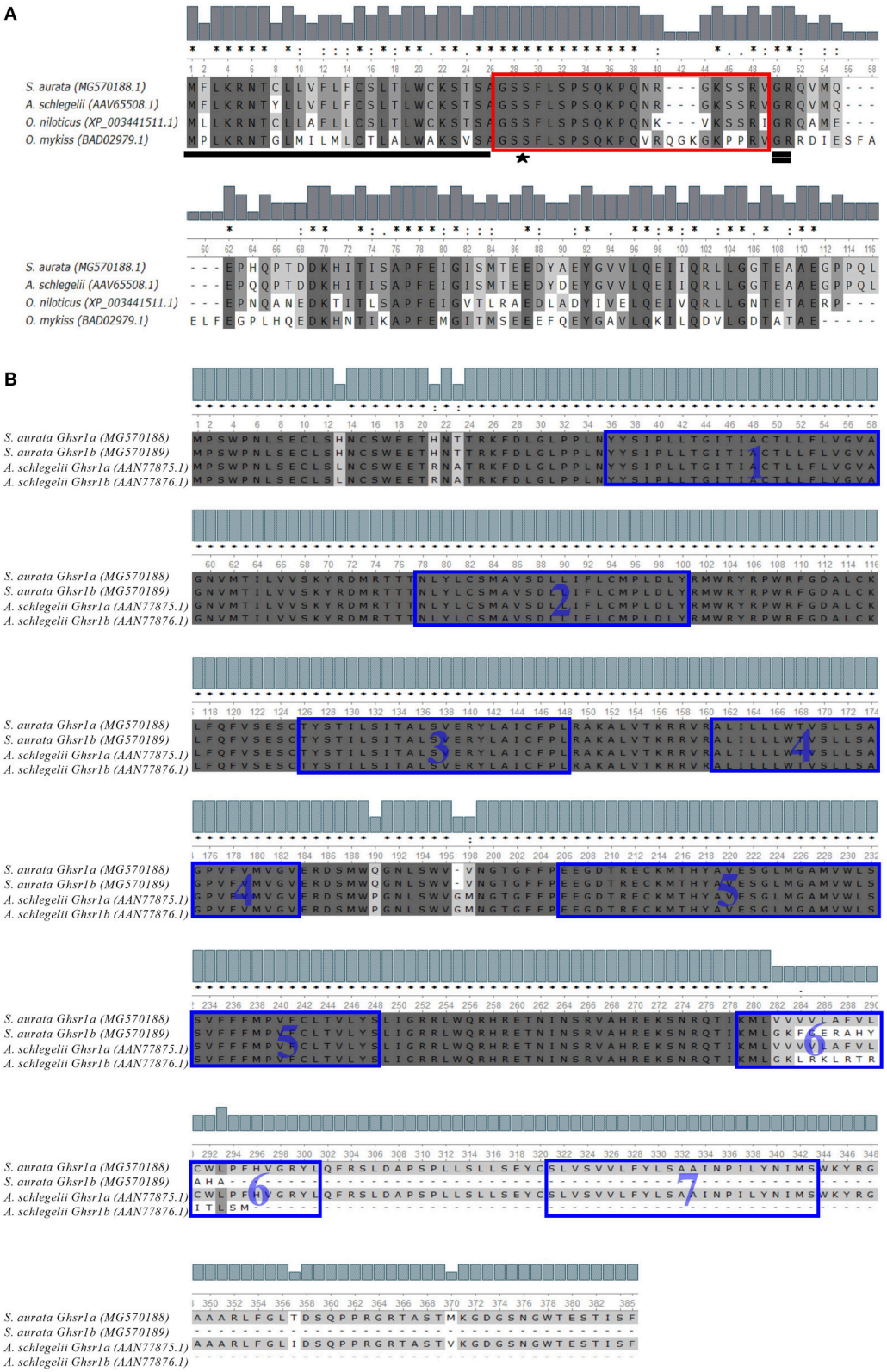

FIGURE 1 | (A) Multiple alignment of the Preproghrelin amino acid sequences corresponding to members of sparidae perciformes (*S. aurata* and *A. schlegelii*), cichlidae perciforme (*O. niloticus*) and salmoniformes (*O. mykiss*). From N- to C-terminal, the signal peptide (underlined), the mature Ghrelin (red boxed) and C-terminal Proghrelin peptide (rest of the sequence) are highlighted. Moreover, the acylation target Ser3 residue (starred) and the GlyArg amidation and cleavage signal

(Continued)

FIGURE 1 | (double underlined) are identified and conserved. **(B)** Amino acid alignment of the translated sequences of *S. aurata* Ghsr1a and Ghsr1b with their respective orthologs of the sparidae perciforme (*A. schlegelii*). Predicted transmembrane domains are blue boxed. Percentage of identity is indicated in grey scale. "*" indicates positions which have a single, fully conserved residue; ":" indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix and "." indicates conservation between groups of weakly similar properties - scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

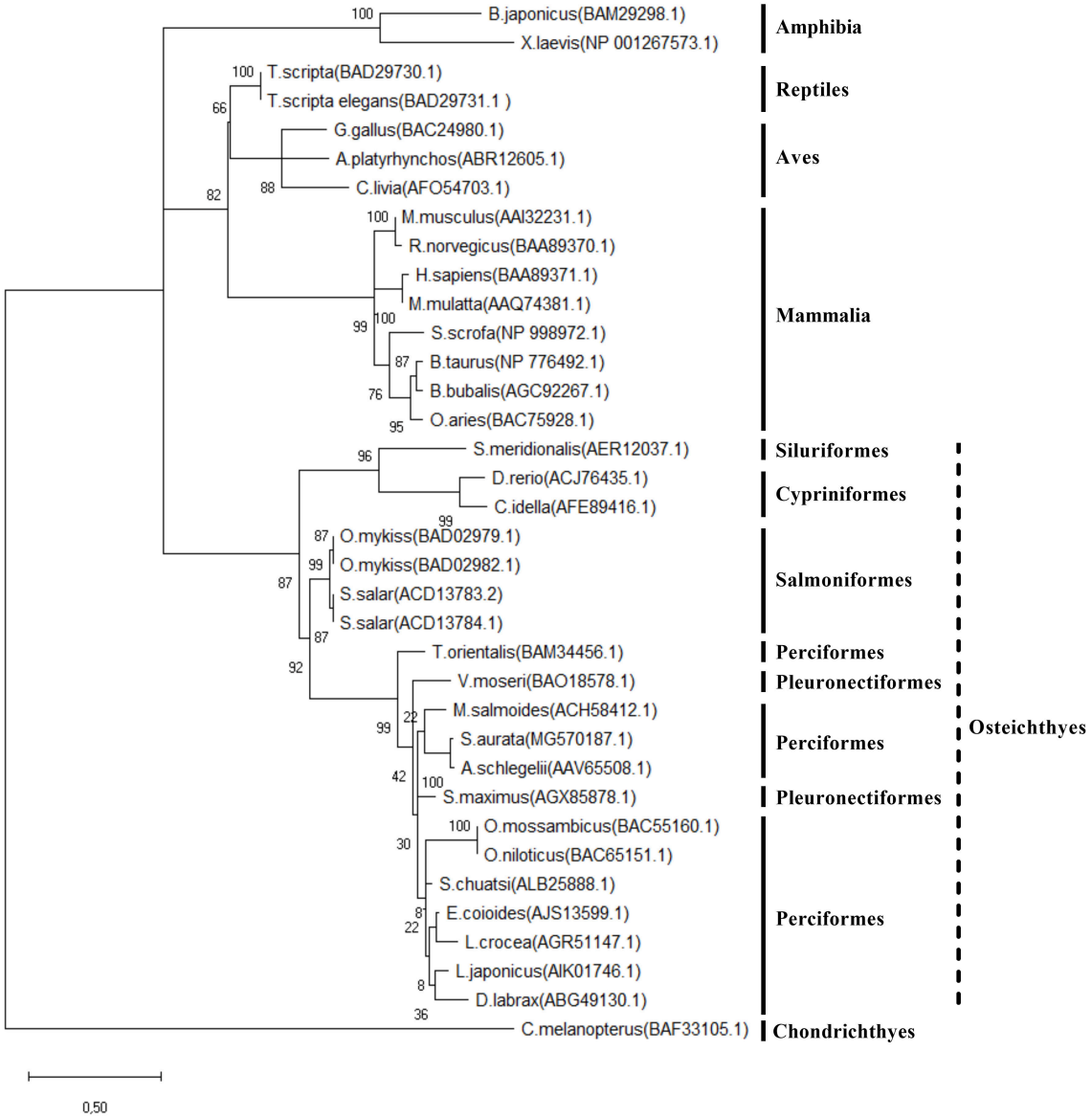

FIGURE 2 | Phylogenetic analysis (unrooted tree) of Preproghrelin among different vertebrates. Multiple alignment was performed using the default settings of the MAFFT tool online (server) version (http://mafft.cbrc.jp/alignment/server/) and a phylogenetic tree by Maximum Likelihood phylogeny was built with the MEGA X tool using the JTT + G + I substitution model. *B. japonicus* (*Bufo japonicus*), *X. laevis* (*Xenopus laevis*), *G. Gallus* (*Gallus gallus*), *A. platyrhynchos* (*Anas platyrhynchos*), *C. livia* (*Columba livia*) *T. scripta* (*Trachemys scripta*), *M. musculus* (*Mus musculus*), *R. norvegicus* (*Rattus norvegicus*), *H. sapiens* (*Homo sapiens*), *M. mulatta* (*Macaca mulatta*), *S. scrofa* (*Sus scrofa*), *O. aries* (*Ovis aries*), *B. Taurus* (*Bos Taurus*), *B. bubalis* (*Bubalus bubalis*), *S. meridionalis* (*Silurus meridionalis*), *D. rerio* (*Danio rerio*), *C. idella* (*Ctenopharyngodon idella*), *O. mykiss* (*Oncorhynchus mykiss*, *S. salar* (*Salmo salar*), T. orientalis (*Thunnus orientalis*), *V. moseri* (*Verasper moseri*), *M. salmoides* (*Micropterus salmoides*), *S. maximus* (*Scophthalmus maximus*), *S. aurata* (*Sparus aurata*), *A. schlegelii* (*Acanthopagrus schlegelii*), *L. japonicus* (*Lateolabrax japonicus*), *D. labrax* (*Dicenthrachus labrax*), *E. coioides* (*Epinephelus coioides*), *L. crocea* (*Larimichthys crocea*), *S. chuatsi* (*Siniperca chuatsi*), *O. mossambicus* (*Oreochromis mossambicus*), *O. niloticus* (*Oreochromis niloticus*), *C. melanopterus* (*Carcharhinus melanopterus*). Length of the branches corresponds to number of substitutions per site and confidence values (based on a bootstrap number of 1,000) are shown above and below the lines, respectively.

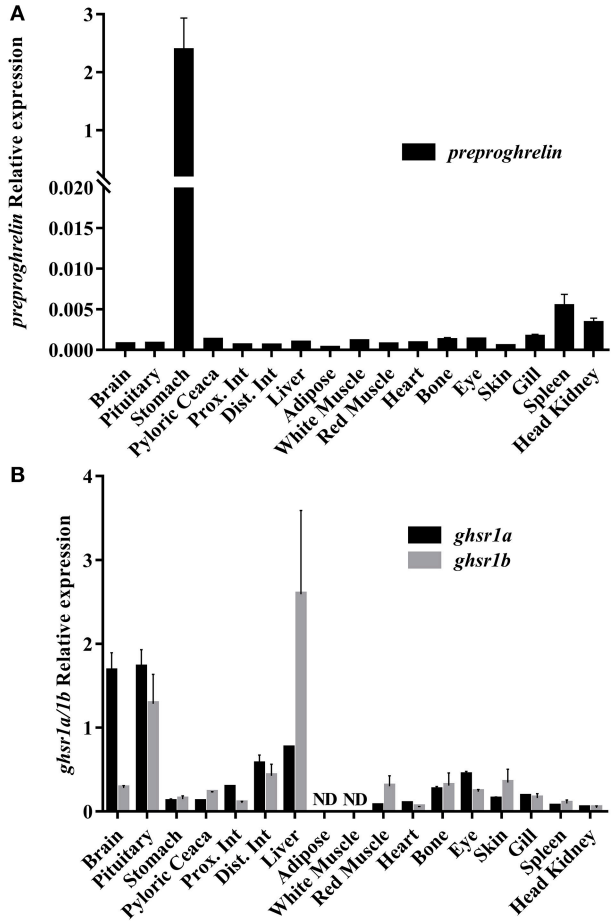

FIGURE 3 | Relative gene expression across a tissue screening of *preproghrelin* **(A)** and Ghrelin receptors *ghsr1a* and *ghsr1b* **(B)**. Data are shown as means ± SEM from three individual fish ($n = 3$). Prox. Int, proximal intestine; Dist. Int, distal intestine.

fasting effects were not found either but at 5 h post-prandial the expression levels in the brain were significantly down-regulated (**Figure 6D**) compared to the initial sampling (−21 days), and similar low expression values were maintained at 1 and 7 days post-feeding.

The mRNA expression profile of both pituitary *ghsrs* isoforms (**Figures 6B,C**) was similar along the experiment, stable during fasting and down-regulated significantly at 5 h refeeding. After 1 and 7 days, the expression of *ghsr1a* increased significantly reaching the levels as before fasting, while the *ghsr1b* expression remained low until the end. Moreover, the gene expression patterns of both *ghsrs* in the brain (**Figures 6E,F**) were almost identical, being practically irresponsive to either 21 days of fasting or the onset of feeding.

Gh/Igf-1 axis members

Pituitary *gh* gene expression (**Figure 7A**), similarly to plasma Gh, progressively increased to reach maximum levels at 1 day post-refeeding, decreasing back to basal levels at day 7.

The liver gene expression of total *igf-1* (**Figure 7D**) remained stable after fasting and in the early post-prandial period, but after 1 day, the lowest levels were observed, and at day 7 returned to baseline. The *igf-1* splice variants (**Figure 7E**) showed a similar gene expression profile than that of total *igf-1*, especially *igf-1a* with little effects of fasting and lowest expression levels at 1 day post-refeeding, recovering basal values after 7 days. Moreover, *igf-1b* and *igf-1c* showed a significant post-prandial dip at 2 h, maintaining still lower values at day 1, to return to basal levels at day 7.

Concerning liver Gh receptors, both were significantly down-regulated due to fasting (**Figures 7B,C**). However, different post-prandial responses were observed: *ghr-1* stopped decreasing at 2 and 5 h, while *ghr-2* expression continued to decline until 2 h, remaining low up to 1 day post-refeeding. The expression of both receptors was then up-regulated at day 7 in comparison to early post-prandial measurements. In the case of Igf-1 receptors, the only isoform detected in liver was *igf-1rb* (**Figure 7F**). Its expression was not affected by fasting but was significantly down-regulated at 2 h of refeeding, to then recover at 7 days initial expression levels.

The gene expression of four *igfbps* is shown in **Figures 7G,H**. *igfbp1a* and *igfbp2a* expression had similar stable patterns, except that *igfbp1a* showed a significant abrupt peak in expression 2 h post-refeeding, returning to basal levels at 5 h. The expression of *igfbp4* and *igfbp5b* was detected for the first time in gilthead sea bream liver. Both presented a similar profile, but *igfbp5b* did not show significant changes while the response for *igfbp-4* was more pronounced, with a significant decrease at 5 h and 24 h post-prandial compared to the onset of refeeding. Then, such low expression level was maintained after 1 day post-refeeding and basal levels were recovered after 7 days.

DISCUSSION

Preproghrelin and Ghsrs Characterization

Since its discovery, the *preproghrelin* nucleotide and amino acid sequences have been described in many vertebrate species (36). In the present study, phylogenetic analysis of the gilthead sea bream translated sequence highlighted the conservation of the most characteristic features. In fact, Preproghrelin is considered a well-conserved protein, but with a perceptible evolution among classes and orders. The gilthead sea bream Preproghrelin resulted more closely related to other Sparidae species, flatfishes and European sea bass (*Dicentrachus labrax*), but more distant to salmonids, cypriniformes, siluriformes and chondrictyes.

The expression of *preproghrelin* was detected mainly in stomach and pyloric caeca, which agrees with previous studies in mammals and other fish species, establishing that the main source of Ghrelin is the stomach (3, 4, 6, 12, 13). Moreover, weak *preproghrelin* expression was detected in other tissues and organs as in different fish species (5, 6). One of the main targets of Ghrelin is the brain, where it is reported to act in appetite-regulating areas to induce (or decrease in some species) feed

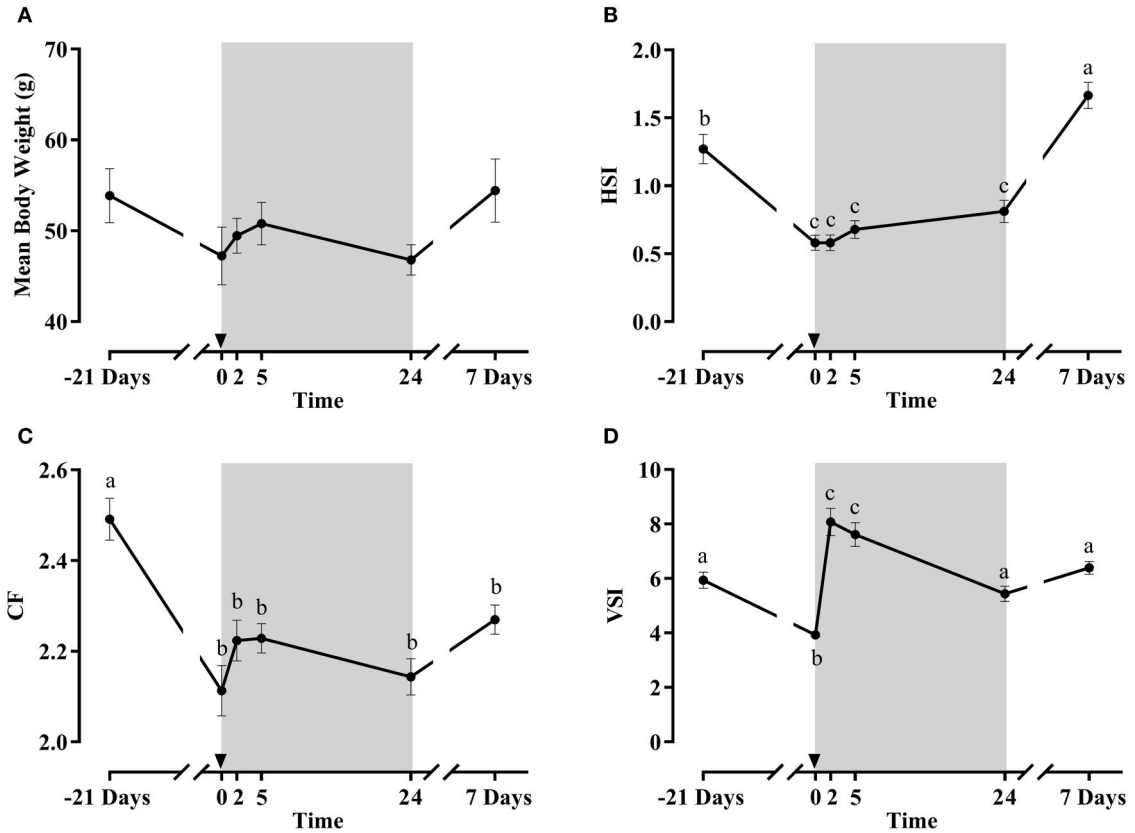

FIGURE 4 | Mean body weight **(A),** condition factor (CF) **(B)**, hepatosomatic index (HSI) **(C)** and viscerosomatic index (VSI) **(D)** of fish during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means ± SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

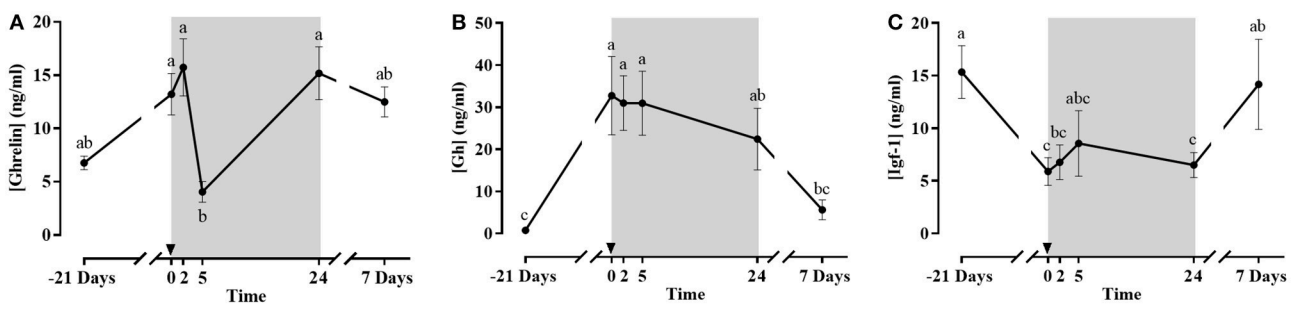

FIGURE 5 | Plasma concentration of Ghrelin **(A)**, Gh **(B)** and Igf-1 **(C)** during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means ± SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

intake (19, 22). Thus, the detection of *preproghrelin* mRNA expression locally in the brain may also contribute to confirm the existing hypothesis that Ghrelin is synthetized both peripherally and centrally (22). In our screening, the low *preproghrelin* mRNA levels detected in the brain may be due to the fact that the whole brain was taken, instead of only the hypothalamus, which is supposed to be the main production site and target in the brain.

The gene expression screening of the two *ghsrs* showed that both are widely distributed among multiple tissues and organs, in line with previous research (16). The tissues with higher expression were pituitary, brain and liver, which support that these are the main targets of Ghrelin action in gilthead sea bream, as in many other vertebrate species (15, 37). Furthermore, as far as we know, this is the first time that it is observed that isoform *a* is more abundant in brain and pituitary, while isoform

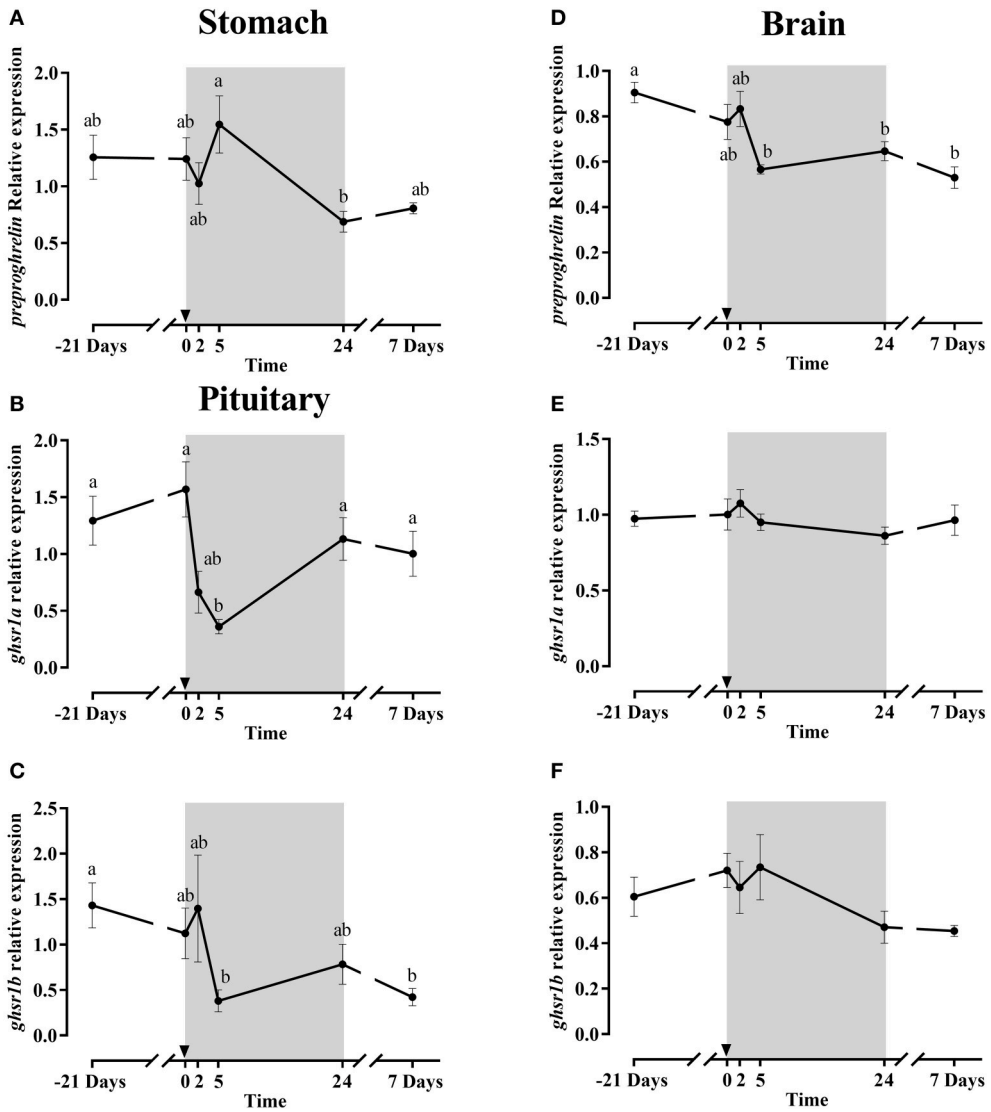

FIGURE 6 | Relative gene expression of stomach *preproghrelin* **(A)**, pituitary *ghsr1a* **(B)** and *ghsr1b* **(C)** and brain *preproghrelin* **(D)**, *ghsr1a* **(E)** and *ghsr1b* **(F)** during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means ± SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

b is more abundant in liver. Such differential expression in these tissues could suggest that Gh secretion requires the presence of the truncated isoform to achieve better regulation, as suggested (15, 16).

Fasting and Refeeding Effects on Growth Performance and Ghrelin

Although gilthead sea bream tolerates long periods of food deprivation well (25, 38–40), the morphometric parameters reduction after 21 days of fasting confirmed that the fish had entered in a catabolic state, which was progressively reverted upon refeeding, as demonstrated by the recovery of the body indexes at the end of the experiment.

The existing literature reveals that the response of Ghrelin to fasting may be, especially in fish, species-specific. Thus, fasting has been reported to up-regulate, down-regulate or unchange the gastrointestinal tract and brain *ghrelin* mRNA levels in diverse fish species (7, 41–45). Such a variety of responses could indicate that other factors, such as sex and age of individuals (44), temperature (46), fasting duration (42) or diet (13) may also affect Ghrelin production. Interestingly, during the development of the present work, Babaei et al. (13) also reported a tissue-specific *preproghrelin* expression response to fasting in gilthead sea bream.

The different response observed to 21 days of fasting with Ghrelin plasma levels and *preproghrelin* mRNA levels in stomach, is consistent with previous fish studies (45, 47) and suggests

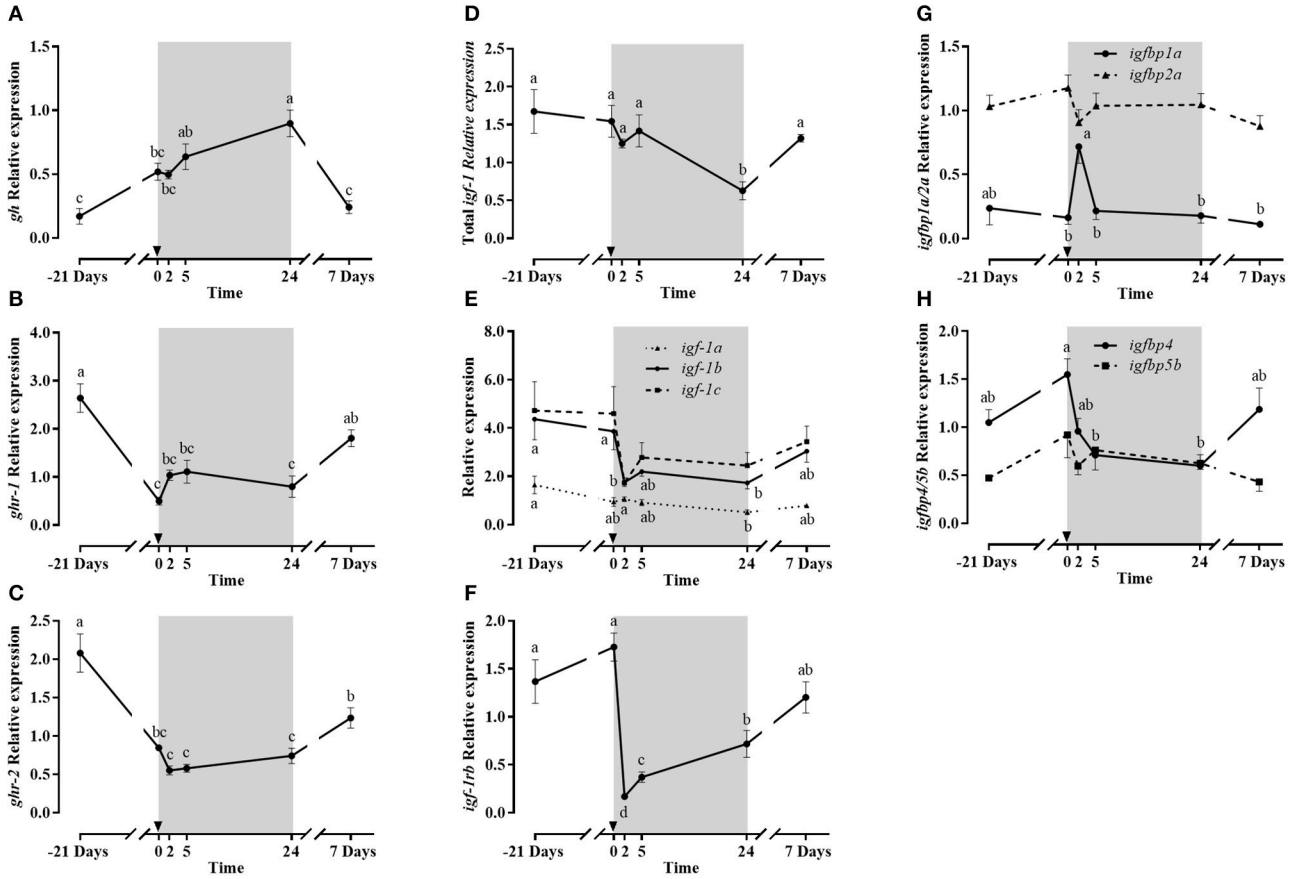

FIGURE 7 | Relative gene expression of pituitary *gh* **(A)**, liver *ghr-1* **(B)** *ghr-2* **(C)** total *igf-1* **(D)**, *igf-1* splice variants **(E)** *igf-1rb* **(F)**, *igfbp1a* and *igbp2a* **(G)** and *igfbp4* and *igfbp5b* **(H)** during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means ± SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

that post-transcriptional mechanisms are in place. However, Ghrelin plasma levels were also high at 1 and 7 days post-refeeding probably due to the 24 h fast. In sea bass, a rise in *preproghrelin* expression was observed during the first days of fasting, to then decrease progressively to fed control values after 21 days of fasting (48). In grass carp, a peak of intestinal *ghrelin* expression was described after 7 days of fasting (49). In goldfish, Unniappan et al. (42) found that fasting for 3 and 5 days significantly increased Ghrelin plasma levels, while in gut or hypothalamus *preproghrelin* expression did not increase until after 7 days of fasting. Moreover, in Atlantic salmon, Ghrelin levels were significantly increased after 2, but not 14 days of fasting (50). Together, these observations support the idea that in diverse fish species, the response increasing Ghrelin plasma levels occurs mainly during the early stage of fasting and is not always related to changes in gut gene expression.

Besides, with refeeding Ghrelin plasma levels that were still high at 2 h, were followed by a significant decrease at 5 h, suggesting an inhibitory effect on Ghrelin secretion as food enters the stomach. These decrease in Ghrelin coincided with the beginning of circulating Gh decline, suggesting the relationship between these two hormones. A similar decrease was also observed at 1 h post-prandial in tilapia (51), and in refed striped bass (52). Moreover, such reduced plasma levels coincided with the peak in stomach *preproghrelin* mRNA levels, whereas the minimum expression 1 day after refeeding corresponded with the recovery of Ghrelin plasma levels, indicating an inverse relationship between the regulation of the gene expression and the circulating hormone. Thus, it appears that during this specific postprandial stage (2, 5, and 24 h) *preproghrelin* gene expression could be regulated by Ghrelin plasma levels.

Unniappan et al. (42) also observed that in goldfish, *preproghrelin* mRNA levels (in gut and hypothalamus) and Ghrelin plasma levels were sensitive to feeding when analyzed periprandially. At 3 h pre-meal, Ghrelin plasma and mRNA levels were high, and 1 and 3 h after feed intake were down-regulated in both tissues. Similar results were observed by Hatef et al. (53) in zebrafish, in which *preproghrelin* mRNA levels in brain and gut were down-regulated 3 h post-meal and increased in fasted fish. These studies are in accordance with the observed decrease in plasma Ghrelin and brain *preproghrelin* mRNA at 5 h

post-feeding in the present experiment, indicating that Ghrelin may be mainly regulated by feed intake also in gilthead sea bream.

Fasting and Refeeding Effects on Ghsrs

The *ghsrs* responded differentially to refeeding in brain and pituitary. The expression in brain remained constant, while in the pituitary decreased progressively up to 5 h to recover at 1 or 7 days of refeeding the expression of *ghsr1a*, and to a lesser extent of *ghsr1b*. In rats, brain and pituitary *ghsrs* were up-regulated in fasting and decreased after refeeding (15, 54, 55). However, the function of Ghsrs in fish and other non-mammalian vertebrates is still not fully understood. Thus, although Ghsrs have crucial roles in the ghrelinergic system and their expression is finely regulated by nutritional condition, hormonal status and environmental factors, their response is highly variable depending on the species especially in fish, in which a higher number of Ghsrs isoforms has been described (19).

Peddu et al. (51) did not find in Mozambique tilapia brain a clear response to fasting in *ghsrs* expression, but at feeding time (just before food administration) both receptors were up-regulated to decrease at 1 and 3 h post-feeding. In the same species, a significant change was not observed in brain *ghsr1a* expression between 1 and 7 days of fasting, while *ghsr1b* increased after 3 but not 5 fasting days (56). In Atlantic salmon, a fasting period of 2 or 14 days did not change *ghsr1a* brain expression (50), neither it did 15 days of fasting in zebrafish *ghsrs* (57). Contrarily, Kaiya et al. (58) found that 7 days of fasting induced a decrease in the expression of *ghsr1a* in the vagal lobe of goldfish. Thus, although species differences exist it seems that there is regulation of *ghsrs* depending on the alimentary condition.

Ghrelin receptors in fish pituitary have been poorly investigated, but low basal expression levels have been found in tilapia (56), goldfish (59, 60) or yellow catfish (61). In the case of grass carp, 14, 21, and 28 days of fasting resulted in increased pituitary gene expression of *ghsr1a* that correlated with increased plasma Gh and *preproghrelin* pituitary gene expression (62). Moreover, these authors found that Ghrelin administration provoked an increase in pituitary *ghsr1a* expression. In the present study, the decrease in *ghsrs* expression during the post-prandial stage was noticeable and related with circulating Ghrelin, pointing to a slowdown of the system during food intake. To summarize, Ghrelin receptors expression in the brain do not show a uniform regulation among fish species and seem to be less influenced by the nutritional condition in comparison to mammals. Furthermore, less is known about pituitary Ghsrs dynamics during fasting in fish, but in gilthead sea bream, both isoforms present a similar response that parallels Ghrelin plasma levels.

Fasting and Refeeding Effects on the Gh/Igf Axis

The rise of circulating Gh during fasting was parallel to *gh* mRNA levels in the pituitary, being significantly high at 5 h post-feeding. The expression of *gh* remained high until 1 day of refeeding, and similarly to plasma Gh, returned to basal values after 7 days, thus indicating the important and extended effect of fasting in this hormone. This response of Gh to fasting and refeeding has been observed in previous studies in various fish species, such as Chinese perch (*Siniperca chuatsi*), tilapia and black sea bream (*Spondyliosoma cantharus*) (63–65). Plasma Igf-1 also responded to nutritional state, presenting an inverse pattern to that of Gh, decreasing with fasting and slowly increasing with refeeding. Liver total *igf-1* gene expression as well as its splice variants partially recovered after 7 days of refeeding. These results are in line with previous works (63, 66). The inverse correlation between Gh and Igf-1 plasma levels during fasting was pointed out in gilthead sea bream previously (38, 67, 68), and has been described in several other fish species (e.g., coho salmon, chinook salmon, channel catfish, Nile tilapia or gilthead sea bream) in diverse conditions (26, 47, 66, 69–71). Moreover, the results support that the circulating Gh/Igf-1 ratio is a good indicator of metabolic state in gilthead sea bream and that it is clearly affected by feeding condition (67, 72). Picha et al. (52) suggested that during fasting in striped bass, high Ghrelin levels contribute to counteract the negative feedback normally exerted by Igf-1 on Gh release, in order to maintain Gh secretion.

The gene expression of *ghrs* in the liver also reflected the nutritional status. The dramatic down-regulation of both *ghr-1* and *ghr-2* expression, along with increased Gh plasma levels, suggests a Gh liver desensitization during the fasting period (23). After refeeding, a rapid increase in the mRNA levels of *ghr-1*, the isoform mostly related with anabolic processes in this species was observed, and later in the expression of *ghr-2*, indicating that ingested nutrients may have initiated growth promotion (23). Furthermore, liver *igf-1rb* showed a similar tendency to that of *ghrs* after refeeding and its abrupt post-prandial expression drop at 2 h was not recovered until the end of the trial. It is interesting that this response is parallel to *igf-1b* and *c* hepatic gene expression. Down-regulation of liver *igf-1rb* expression was also observed in gilthead sea bream during exercise (26), but as far as we know, this is the first time that this effect is found in refed fish.

The expression of *igfbps* was stable during fasting while 7 days of refeeding recovered their basal values. Nevertheless, *igfbp-4* presented the highest expression after 21 fasting days in agreement with its Igf-1 conservative function, while the increase of *igfbp-1a* at 2 h post-feeding fitted well with its recognized role in mobilization conditions in this species. Similarly, in a fasting and refeeding experiment in rainbow trout, Gabillard et al. (73) observed different responses for *igfbps*. Hevrøy et al. (50) described the effects of fasting on Ghrelin and Gh/Igf-1 system in Atlantic salmon, in which Igfbp-1 seemed to be a marker of catabolic state. Breves et al. (63) demonstrated different roles of *Igfbps* during fasting, and indicated that Igfbp-1b may operate to reduce Igf-1 signaling during fasting in tilapia. The functional relationship between Gh, Igf-1 and Ghrelin during fasting in fish needs to be further investigated.

To summarize, the full *preproghrelin, ghsr1a and ghsr1b* nucleotide sequences and their response during fasting/refeeding have been described for the first time in gilthead sea bream.

Both, long term (21 days) and short term (24 h) fasting increased circulating Ghrelin, which showed the lowest values few hours post-prandial. The plasma Ghrelin dip was also reflected by pituitary *ghsrs,* suggesting that Ghrelin's stimulatory action on Gh secretion is modulated by feeding. Plasma Gh levels were elevated in parallel with its pituitary gene expression returning to basal levels after 7 days of refeeding, although at this time circulating Ghrelin was again increased. Taken together, the data suggest that Ghrelin can be a regulator of Gh secretion in gilthead sea bream, but the metabolic state itself and other regulatory molecules may exert important effects. Finally, this study indicates that in gilthead sea bream, Ghrelin secretion is mainly related to the progress of the digestive process, showing a down-regulation in the post-prandial period to rise again just before feeding.

AUTHOR CONTRIBUTIONS

MP-A, EV, and JG conceived and designed the experiments; MP-A, EV, JV-A, AS-M, NR-H, JF-B, JB, IN, EC, and JG performed the experiments; MP-A, EV, JV-A, AS-M, NR-H, IH, JF-B, JB, JC-G, IN, EC, EJ, JP-S, and JG analyzed the data and interpreted the results; JF-B, JB, IN, EC, EJ, JP-S, and JG contributed reagents, materials, analysis tools; MP-A, EV, JV-A, AS-M, NR-H, IH, JF-B, JB, JC-G, IN, EC, EJ, JP-S, and JG wrote and revised the paper.

DATA AVAILABILITY STATEMENT

The three sequences obtained in the present study are deposited in GenBank (NCBI) under accession numbers: MG570187 for *preproghrelin*; MG570188 for *ghsr1a*, and MG570189 for *ghsr1b.* All relevant data is contained within the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00399/full#supplementary-material

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ARTICLE 2

Regulatory mechanisms involved in muscle and bone remodeling during refeeding in gilthead sea bream

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Regulatory mechanisms involved in muscle and bone remodeling during refeeding in gilthead sea bream

F. Lavajoo[1,2,4], M. Perelló-Amorós[1,4], E. J. Vélez[1,3], A. Sánchez-Moya[1], S. Balbuena-Pecino[1], N. Riera-Heredia[1], J. Fernández-Borràs[1], J. Blasco[1], I. Navarro[1], E. Capilla[1,4] & J. Gutiérrez[1,4*]

The tolerance of fish to fasting offers a model to study the regulatory mechanisms and changes produced when feeding is restored. Gilthead sea bream juveniles were exposed to a 21-days fasting period followed by 2 h to 7-days refeeding. Fasting provoked a decrease in body weight, somatic indexes, and muscle gene expression of members of the Gh/Igf system, signaling molecules (*akt*, *tor* and downstream effectors), proliferation marker *pcna*, myogenic regulatory factors, myostatin, and proteolytic molecules such as cathepsins or calpains, while most ubiquitin-proteasome system members increased or remained stable. In bone, downregulated expression of Gh/Igf members and osteogenic factors was observed, whereas expression of the osteoclastic marker *ctsk* was increased. Refeeding recovered the expression of Gh/Igf system, myogenic and osteogenic factors in a sequence similar to that of development. Akt and Tor phosphorylation raised at 2 and 5 h post-refeeding, much faster than its gene expression increased, which occurred at day 7. The expression in bone and muscle of the inhibitor myostatin (*mstn2*) showed an inverse profile suggesting an inter-organ coordination that needs to be further explored in fish. Overall, this study provides new information on the molecules involved in the musculoskeletal system remodeling during the early stages of refeeding in fish.

Gilthead sea bream (*Sparus aurata* Linnaeus 1758) is one of the most important marine fish species in Mediterranean aquaculture, which has expanded over the past two decades[1] in parallel with the scientific research and the knowledge of its physiology. Unlike mammals, fish are able to adapt to relatively long periods of starvation and it is possible to use fasting as a practice to improve product quality by reducing body lipid content, and refeeding as a way to induce compensatory growth[2–4]. Under normal feeding conditions, fish grow and store energy reserves, while in fasting body stores are mobilized to maintain life processes[5]. During fasting, the metabolism switches to a catabolic status, resulting in low growth rate, and the following refeeding reverts the situation towards a hyper-anabolic phase when organisms attempt to accelerate the growth rate[6]. Both approaches, fasting and refeeding, can be very informative in fish basic and applied research.

The effects of fasting and refeeding on body growth, metabolism, protein biosynthesis and hormonal responses have been largely studied in fish[6,7]. The muscle mass of fish species is an important tissue that considerably depends during fasting and refeeding on protein degradation and synthesis. During these stages of nutritional changes, metabolism and growth are adapted to resist the restrictions and rapidly adjust to the arrival of new nutrients. All these determine changes on the endocrine status and in the regulation of substrates mobilization by muscle and bone[6,8]. In compensatory growth studies, it has been described that refeeding stimulates proliferation of fish myogenic cells[9,10].

The growth hormone and insulin-like growth factors (Gh/Igfs) are both, in vertebrates including fish, key factors regulating growth. Muscle and bone are widely regulated by this system and the presence of Gh and Igf1 receptors (Ghrs and Igf1Rs) and Igf isoforms as well as binding proteins (Igfbps) are well described in fish, especially in these tissues[11,12]. Moreover, in gilthead sea bream, the function of Gh/Igfs and its response to diverse

[1]Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain. [2]Present address: Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, I.R., Iran. [3]Present address: Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B4, Canada. [4]These authors contributed equally: F. Lavajoo, M. Perelló-Amorós, E. Capilla and J. Gutiérrez. *email: jgutierrez@ub.edu

conditions has been characterized and the ratio of the circulating levels of Gh and Igf1 is considered a good marker of growth quality in terms of its relation with body indexes or muscle fibers composition[12,13].

Muscle growth is also controlled by myogenic regulatory factors (MRFs) (MyoD, Myf5, Myog and Mrf4), and the negative endogenous regulator myostatin, as well as other factors that control sequentially the process of development and growth[6,14,15]. The effects of fasting and refeeding on myogenesis have been studied in diverse fish species[6,10,16] demonstrating the function of the different MRFs during the metabolic arrest caused with food limitation and the subsequent activation when feeding is restored. Furthermore, during fasting and refeeding, proteolytic molecules play a remarkable role to adapt to the changes in amino acids supply. This is more important in fish that have a specific and enhanced protein metabolism. The main endogenous proteolytic systems, each one performing specific degradative or regulatory functions according to the nutritional conditions are, calpains, cathepsins and ubiquitin-proteasome, all of which are well-known in fish, especially salmonids[17–19], but also in gilthead sea bream[20–22].

In addition to muscle, bone is also an important tissue for skeletal and locomotion functions, but also, as a reservoir of minerals that is clearly affected when nutrition is restricted. Essential during development, synchronicity between bone and muscle is required for proper musculoskeletal growth[23,24]. Besides to being induced by the Gh/Igf system, the process of osteogenesis is also regulated by skeleton-derived factors that control specific stages of osteoblasts development and bone building. Although less known in fish than in mammals, most of these molecules have been identified in gilthead sea bream[25,26]. Thus, while the Runt-related transcription factor 2 (*runx2*) and the structural molecule fibronectin 1a (*fib1a*) have a key role in the early development of the tissue, the non-collagenic molecules such as osteopontin (*op*) and osteocalcin (*ocn*), play a main role in bone maturation and matrix mineralization. However, little information exists concerning the effects of fasting and refeeding in the regulation of these molecular factors and bone development in fish species.

The objective of this study is to analyze the effects that fasting and refeeding provoke in muscle and bone in *S. aurata*, characterizing the expression pattern of various genes of interest of the musculoskeletal system to improve our knowledge of the regulatory mechanisms involved in these processes, and to explore their contribution to vertebrate compensatory growth. Specifically, we were interested in the early refeeding (within 24 h) when the mechanisms of tissue remodeling are triggered by the arrival of the first nutrients after 21 days of fasting, which offers an interesting scenario of regulatory molecules that restart their activity gradually after a period of latency.

Materials and Methods

Animals, experimental design and ethics statement. Gilthead sea bream (*Sparus aurata* L.) juveniles (initial body weight 50 ± 3 g; standard length 15.3 ± 0.68 cm; sexually immature) were obtained from a commercial hatchery (Piscimar, Burriana, Spain) and kept in the facilities of the Faculty of Biology (Universitat de Barcelona, Spain) during four weeks before acclimation period. Forty-two fish were randomly distributed into six two-hundred-liters tanks (7 fish per tank) with a recirculation system. Fish were kept at 23 ± 1 °C and a photoperiod of 12 h of light and 12 h of dark during the whole experiment. During the acclimation period (2 weeks), the fish were fed *ad libitum* twice a day with a commercial diet (Optibream, Skretting, Burgos, Spain).

After the acclimation period, a 28-day experiment was designed as previously described[27]. Briefly, it consisted in two parts: a 21-days fasting period and a 7-days refeeding period. Samples were taken at the beginning of fasting (-21 days), and at the end of it at 0, 2, 5, and 24 h and 7 days after refeeding. During the refeeding period, the fish were given the same commercial diet (Optibream, Skretting) once a day until apparent satiety. The -21 days, 24 h and 7 days samplings were performed 24 h after the last feeding. During the experiment, all the environmental conditions (temperature, photoperiod, salinity: 21 °C; 12 L: 12D; 38‰, respectively) were maintained stable.

In each sampling, six individuals (one per tank) were anesthetized and sacrificed per condition. The fish were anesthetized with MS-222 (0.08 g/L) (Sigma-Aldrich, Tres Cantos, Spain), body length (standard) and body weight were measured and blood was drawn from the caudal vein. The plasma levels of Gh and Igf1 were measured by corresponding radioimmunoassays as previously described[27]. Then, the fish were sacrificed by an overdose of the same anesthesia (0.3 g/L) and, bone from the vertebral column and skeletal white muscle from the dorsal area were dissected and snap frozen in liquid nitrogen immediately. All samples were stored at −80 °C until further analysis. This study was carried out in accordance with the recommendations of the EU, Spanish and Catalan Government-established norms and procedures. The specific protocol was approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 110/17 and DAAM 9488).

Gene expression analyses. As previously described[27], total RNA was extracted from 100 mg of skeletal white muscle and vertebral column samples using TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) and a Precellys® Evolution and Cryolys system (Bertin Technologies, Montigny-le-Bretonneux, France) for tissue homogenization. Quantity, quality and integrity were determined with a NanoDrop2000 (Thermo Scientific, Alcobendas, Spain), and a 1% agarose gel (w/v), respectively. Then, one µg RNA was treated with DNase I (Life Technologies, Alcobendas, Spain) and retrotranscribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain).

Next, the mRNA transcript levels were examined by quantitative real time PCR (qPCR) according to the requirements of the MIQUE guidelines[28] in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). All reactions were performed in the conditions previously described[27]. The primers used for each tissue are listed in Supplementary Table 1. Moreover, in both tissues the reference genes elongation factor 1 alpha (*ef1α*), ribosomal protein S18 (*rps18*), beta actin (*β-actin*) and ribosomal protein L27 (*rpl27*) were analyzed and the combination of the two most stable (*ef1α* and *rps18*) was used to calculate the relative expression of the genes of interest following the Pfaffl method[29]. Both, reference genes stability and relative expression calculation were determined with the implemented Bio-Rad CFX Manager Software (v2.1).

Protein expression analyses. Protein was extracted from 100 mg of either muscle or bone samples. Tissue homogenates were made in 1 mL of RIPA buffer supplemented with phosphatase (PMSF and NA_3VO_4) and protease inhibitors (P8340, Santa Cruz) using the Precellys® Evolution coupled to a Cryolys cooling system (Bertin Technologies, Montigny-le-Bretonneux, France).

Protein quantification was performed following the Bradford's method using BSA (Sigma Aldrich, Tres Cantos, Spain) for the standard curve. Next, 15 µg of the soluble protein fraction were prepared in a loading buffer (containing SDS and β-mercaptoethanol), heated at 95 °C for 5 min and run in a 12% polyacrylamide gel. Following, the proteins were transferred overnight to Immobilon® PVDF-FL 0.2 µm Transfer Membranes (Merck Millipore Ltd., Tullagreen, Cork, Ireland), previously activated in methanol. Total transferred protein was determined by 5 min incubation with REVERT™ Total Protein Stain (LI-COR, Lincoln, Nebraska, USA) and reading at 700 nm using the Odyssey Fc Imaging System (LI-COR). Membranes were blocked in Odissey Blocking Buffer (diluted 1:1 in TBS) (LI-COR) for 1 h at room temperature, and then overnight at 4 °C and in agitation in the corresponding diluted primary antibody. The primary antibodies used were as follows: Goat polyclonal anti cathepsin L (anti-Ctsl D-20 antibody; catalog no. sc-6501), goat polyclonal anti-cathepsin D (anti-Ctsd; catalog no. sc-6486) all from Santa Cruz Biotechnolgy (Santa Cruz, California, USA), rabbit polyclonal anti-phospho Akt (cat- no. 9271), anti-total Akt (cat. no. 9272) and anti-phospho Tor (cat. no. 2971) from Cell Signaling Technology (Beverly, MA, USA) and anti-total Tor (cat. no. T2949) from Sigma-Aldrich (Tres Cantos, Spain). All these antibodies have been previously demonstrated to cross-react successfully with the proteins of interest in gilthead sea bream[22,23,30]. Subsequently after washing with TBS-T, the membranes were incubated with the corresponding secondary antibodies: goat anti-rabbit (Cat. No. 925-32211, Servicios Hospitalarios) or donkey anti-goat (Cat. No. 925-32214, Servicios Hospitalarios) diluted at 1:10000 in the same blocking buffer. After incubation, membranes were washed with TBS-T and fluorescence signal was measured at 800 nm using the Odyssey Fc Imaging System (LI-COR). Stripping was performed using a commercial stripping buffer (NewBlot PVDF 5X Stripping Buffer (LI-COR). Detailed information about Western blot membranes distribution can be found in Supplementary Information 3.

Statistical analyses. The data obtained was analyzed using IBM SPSS Statistics vs. 22 and is presented as a mean ± standard error of the mean (SEM). Normality and homogeneity of the variances were tested with the Shaphiro-Wilk's test and the Levene's test respectively. When the data did not show a normal distribution or homoscedasticity, were transformed by logarithm. Differences among groups were tested by one-way analysis of variance (ANOVA) followed by Tukey HSD or LSD, as *post-hoc* tests. In the case that normality and/or homoscedasticity were not found even after logarithmic data transformation, the non-parametric Kruskal-Wallis test or the Dunnett's T3 as *post-hoc*, were used. The confidence interval for all analyses was set at 5%.

Results

Somatic parameters and GH/IGF-I ratio. As shown in Supplementary Table 2, fasting for 21 days provoked an arrest of growth (i.e. body weight and condition factor, CF) that tended to recover after 7 days refeeding. Similarly, hepatosomatic index (HSI) felt down significantly during fasting to reach normal values after 7 days refeeding. Moreover, the viscerosomatic index (VSI) was also significantly reduced by fasting, and refeeding induced an acute increase to recover the basal levels after 1 day. Fasting resulted also in a high Gh/Igf1 ratio that only recovered basal values after 7 days refeeding. In fact, as we previously reported[27], plasma Gh levels raised from 0,77 ± 0,03 to 32,77 ± 0,79 ng/ml in response to 21 days of fasting with a progressive decrease to basal levels with refeeding; while circulating Igf1 levels showed an inverse pattern, decreasing from 15,35 ± 2,51 to 5,9 ± 1,32 ng/ml after fasting and recovering only after a long-term refeeding.

Muscle responses to fasting and refeeding. *GH and IGF family.* Total *igf1* gene expression showed a significant decrease after 21 days of fasting recovering basal levels by day 7 of refeeding (Fig. 1A); the same effect was observed for the *igf1* splice variants *igf1a* and *igf1b*, but not for *igf1c* (Fig. 1B–D). The *igf2* (Fig. 1E) profile was similar although a significant decrease was observed from 2 h refeeding and basal levels recovered at 7 days refeeding. Between the *igf1rs*, *igf1ra* was not affected by fasting but decreased significantly at 24 h refeeding recovering later (Fig. 1F), while *igf1rb* decreased during fasting, and continued dropping at 2 and 5 h refeeding reaching then significantly the lowest levels that remained low after 7 days refeeding (Fig. 1G). The expression of the *igfbps*, *igfbp1* and *igfbp4* was unaltered (data not shown), but *igfbp5b* decreased significantly at 2 h refeeding, and recovered basal levels 7 days after refeeding (Fig. 1H). Concerning *ghrs*, they showed an inverse profile, with *ghr1* decreasing significantly during fasting and at 2 and 5 h refeeding, to increase at 1 and 7 days refeeding; while *ghr2*, although not significantly, progressively increased through the experiment up to 24 h of refeeding, decreasing afterwards at day 7 (Fig. 1I).

Signaling molecules. The mRNA levels of *akt*, *tor*, and the downstream molecules *70s6k* and *4ebp1* (Fig. 2A–D) showed a similar gene expression profile. With the exception of *akt*, all molecules exhibited a significant decrease with fasting and a progressive increase during the refeeding period, significant at day 7 for *akt*, *tor* and *4ebp1*. Interestingly the postprandial response for Akt and Tor was much faster when their phosphorylation was studied (Fig. 2E,F), being significantly increased at 2 and 5 h refeeding compared to time zero and decreasing later at 1 and 7 days. *foxo3* did not show any significant difference along the experiment (data not shown).

Muscle growth-related factors. The profiles of *pax7* and *pcna* expression were similar and correlated very well with those of *myf5* and *myod1*, decreasing significantly during fasting and partially recovering after 7 days refeeding (Fig. 3A–D). The expression of other myogenic genes also decreased significantly during fasting or early refeeding (*myog*, *mrf4*, *mstn2*) (Fig. 3E–G), or showed similar tendencies (*myod2*, *mstn1*, data not shown). At

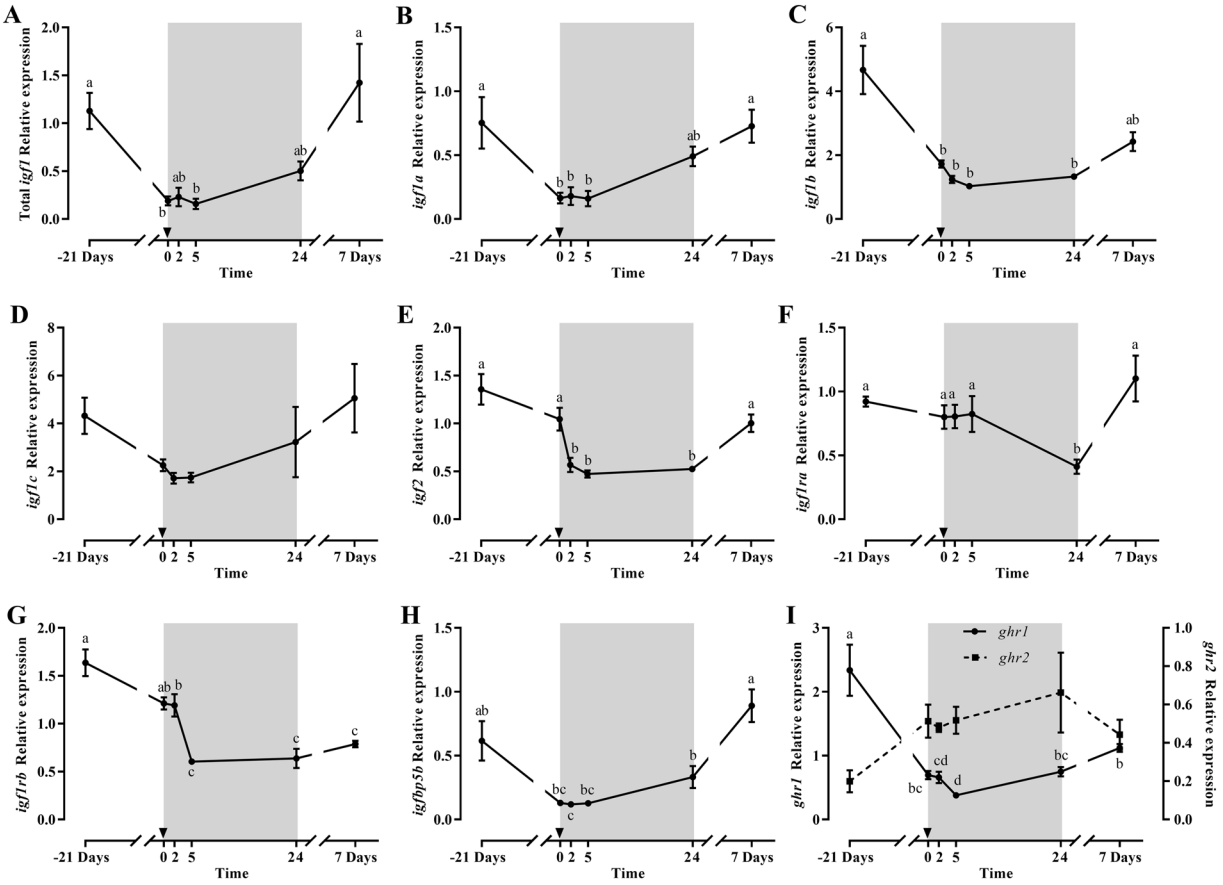

Figure 1. Relative gene expression of skeletal white muscle total *igf1* (**A**), *igf1a* (**B**), *igf1b* (**C**), *igf1c* (**D**), *igf2* (**E**), *igf1ra* (**F**), *igf1rb* (**G**), *igfbp5* (**H**) and *ghr1* and *ghr2* (**I**) in gilthead sea bream during the fasting and refeeding experiment. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

7 days of refeeding, *mrf4* started to increase again while *myog* and *mstn2* remained at low levels. Moreover, the structural myosin light chains, *mlc2a* and *mlc2b* showed an inverse profile with a significant increase for *mlc2a* at 24 h refeeding, returning to basal levels at day 7, and maintained expression for *mlc2b* (Fig. 3H–I).

Proteolytic systems' genes. Most of the proteolytic genes expression was significantly downregulated during fasting or early refeeding, recovering basal levels after 7 days refeeding (Fig. 4). This was the case for *capn1*, *capns1b*, *capn3*, *ctsda* and *ub*, and a similar tendency was observed for *capns1a*. Moreover, *capn2* and *ctsl* expression was also significantly downregulated with fasting but after 7-days refeeding were not able to recover basal values. It is remarkable how *mafbx* and *murf1* increased or were maintained after 21 days of fasting to significantly decrease after 5 or 24 h refeeding, reaching basal levels at the end of the experiment, while *n3* showed only minor changes on expression along the whole experiment (Fig. 4G–I). Regarding protein expression, only Ctsd decreased significantly with fasting, but both Ctsd and Ctsl presented similar patterns postprandially with a single peak at 2 h (Fig. 5).

Bone responses to fasting and refeeding. *GH and IGF family.* Bone total *igf1* and *igf1a* mRNA levels showed significant lower values after the 21 days of fasting, returning to basal levels at 24 h and 7 days refeeding (Fig. 6A,B). Differently, *igf1b* and *igf1c* expression was not significantly affected by fasting or refeeding (Fig. 6C,D); and *igf1ra* expression was maintained at fasting but significantly decreased at 1 and 7 days refeeding (Fig. 6E). Neither i*gf1rb* nor *igfbp*s were affected by fasting or refeeding (data not shown). *ghrs* expression presented, as observed in muscle, a reverse profile. Thus, while *ghr1* expression decreased significantly during fasting and early refeeding, *ghr2* expression increased significantly during the same period. Then, both *ghrs* returned to initial levels at 24 h or 7 days refeeding (Fig. 6F).

Bone-related genes. The expression of the osteogenic factors *runx2*, *fib1a*, *col1a1*, *ocn* and *on* showed a significant diminution during fasting or early refeeding (Fig. 7A–E). Then, *runx2*, *fib1a* and *col1a1* recovered the basal mRNA levels after 1 or 7 days of refeeding, while *ocn* and *on* presented a similar tendency but without reaching basal levels and still remaining low at day 7.

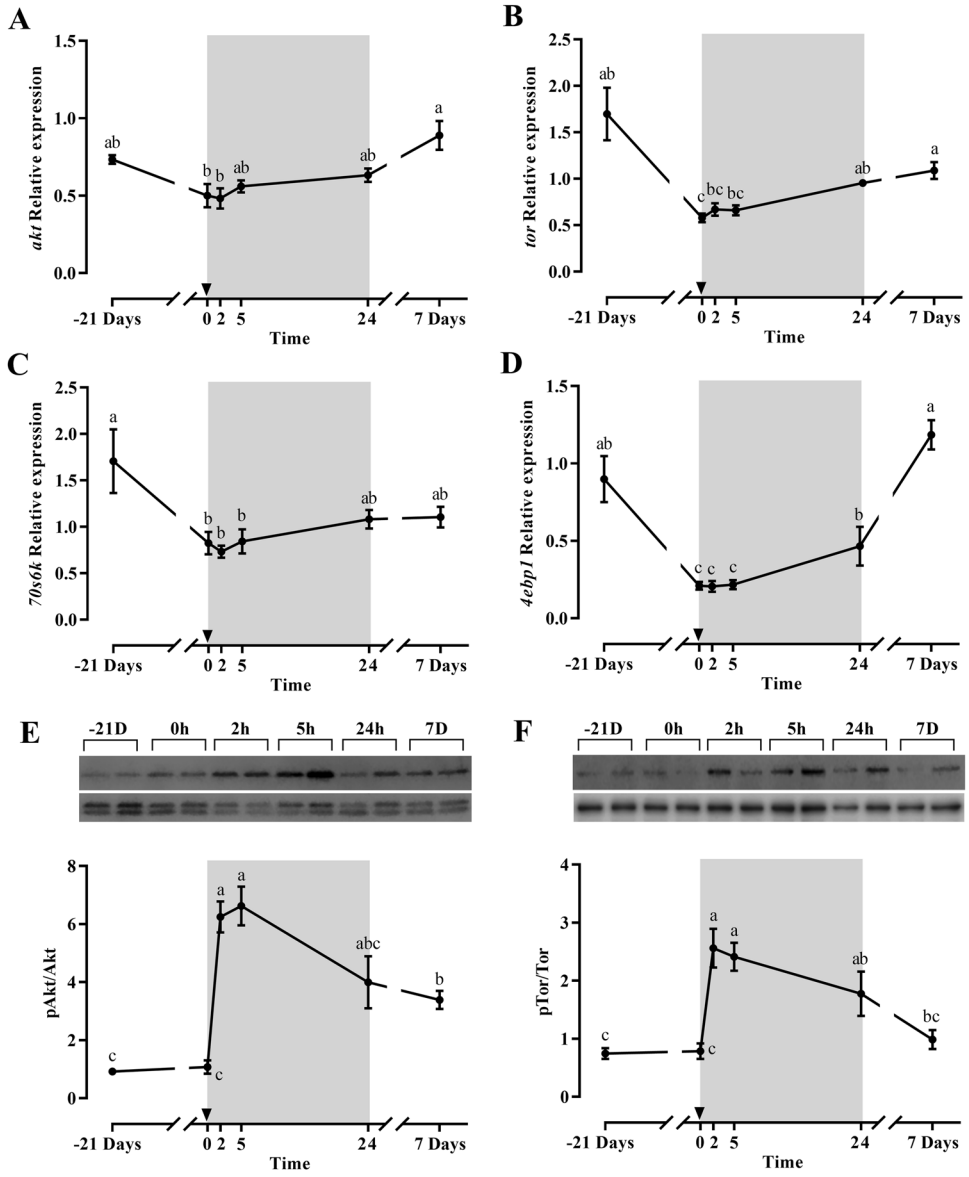

Figure 2. Relative gene expression of skeletal white muscle *akt* (**A**), *tor* (**B**), *70s6k* (**C**) and *4ebp1* (**D**) and representative blot and densiometric analysis of the phosphorylation ratios of Akt (**E**) and Tor (**F**) in gilthead sea bream during the fasting and refeeding experiment. For the Western blots, the same membranes cropped in two were used to analyze Tor (top part) and Akt (bottom part). The phosphorylated forms were analyzed first and after stripping, the corresponding total forms were determined in the same membranes. The intensity of the phosphorylated form was normalized by its total form, and the intensity of each specific band was normalized by the total transferred protein for the corresponding well. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

Concerning the osteoclastogenic genes, the expression level of *ctsk* increased after 21 days of fasting, to recover the basal levels at 2 h refeeding, while *mmp9* showed a tendency to decrease at 21 days fasting, peak at 2 h refeeding to then significantly decrease at 7 days refeeding (Fig. 7F,G). *trap* showed a very similar profile to *mmp9* but without significant changes throughout the experiment (data not shown).

Muscle growth-related factors. First, *pax7* gene expression was studied in bone samples to confirm purity of the tissue and the results obtained showed very low mRNA levels, close to undetectable showing a basal line without changes throughout the experiment (data not shown). Then, the other myogenic genes studied in bone (i.e. *myod1*, *myod2*, *mrf4*, *mstn1* and *mstn2*) did not show significant responses to either fasting or refeeding (data not shown) with the exception of *myod2* and *mstn2* (Fig. 7H,I). *myod2* expression decreased during fasting showing significant low levels at early refeeding (2 and 5 h), and recovered basal values at the end of the experiment; *mstn2*

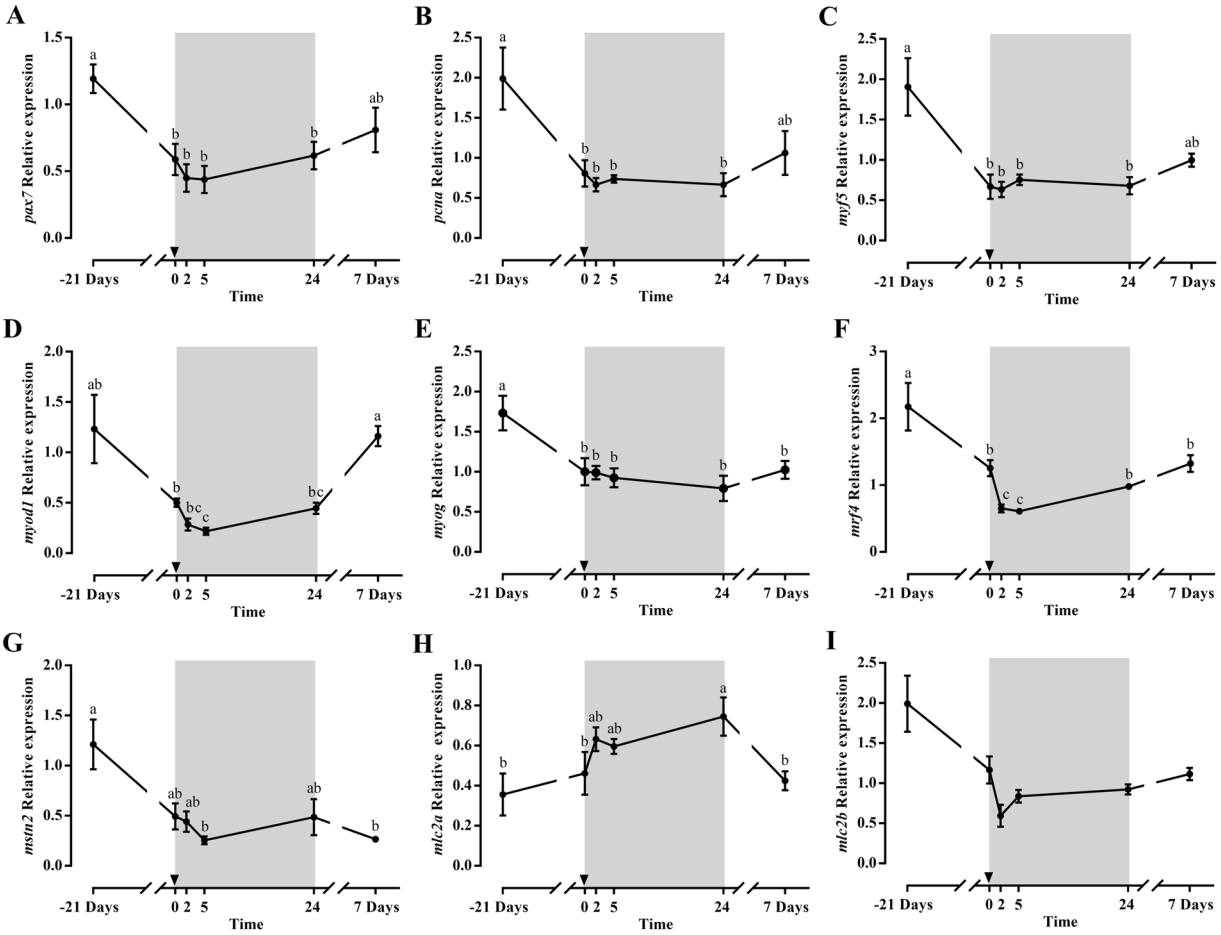

Figure 3. Relative gene expression of skeletal white muscle *pax7* (**A**), *pcna* (**B**), *myf5* (**C**), *myod1* (**D**), *myog* (**E**), *mrf4* (**F**), *mstn2* (**G**), *mlc2a* (**H**) and *mlc2b* (**I**) in gilthead sea bream during the fasting and refeeding experiment. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

showed an almost contrary profile, since it was stable during fasting but peaked at 5 h postprandial to progressively return to basal values at 24 h and 7 days refeeding.

Discussion

Fish are well adapted to resist fasting periods and this characteristic has been used to study the mechanisms that regulate the balance between anabolism and catabolism in these vertebrates[7]. The present study continues the previous one already published by Perelló-Amorós and coworkers[27] in which the function of ghrelin during fasting and refeeding was characterized in gilthead sea bream. That paper reported also the effects on *gh* and *igf1* pituitary and liver expression, hormone plasma levels and the fish biometric indexes. Briefly, fasting provoked an increase in ghrelin plasma levels and a decrease on liver *igf1* expression, while refeeding reversed progressively the condition. In the present study, we focused on the Gh/Igf axis and other regulatory systems in muscle and bone, providing valuable information on the regulation of the musculoskeletal system, especially in bone, a tissue still poorly explored in fish. Refeeding reversed the fasting condition and the sequential sampling permitted to analyze how regulatory mechanisms returned gradually to the growth condition. Although the absence of a pair-fed control group does not allow differentiating among refeeding and postprandial effects, the present data suggest that early refeeding after 21-days fasting amplifies the responses likely occurring postprandially at an attenuated magnitude. Thus, the experimental model would emphasize the main steps of skeletal muscle remodeling that take place in gilthead sea bream when food is restored.

Igfs play an important role on growth regulation in vertebrates[12,31–33] and during adverse conditions are downregulated to preserve the survival of the animal over the body growth. Gh secretion is dependent of nutrients and both, Gh and Igf1 regulate metabolism in fish[7,34]. In gilthead sea bream, 21 days of fasting resulted in a general decrease in the muscular gene expression of total *igf1* and its splice variants. This is in agreement with the significant decrease of liver total *igf1* expression previously described in the same animals[27], indicating altogether that both, systemic and local Igf1 production, might contribute to arrest muscle growth under these catabolic conditions. Similarly, Peterson and coworkers[33] in channel catfish (*Ictalurus punctatus*) reported that fasting for 30 days reduced fish weight by approximately 60% and decreased *igf1* mRNA in muscle, and this has been also seen in other fish species[11,35]. This decrease in Igf1 during a nutrient-starvation period can stop muscle proliferation

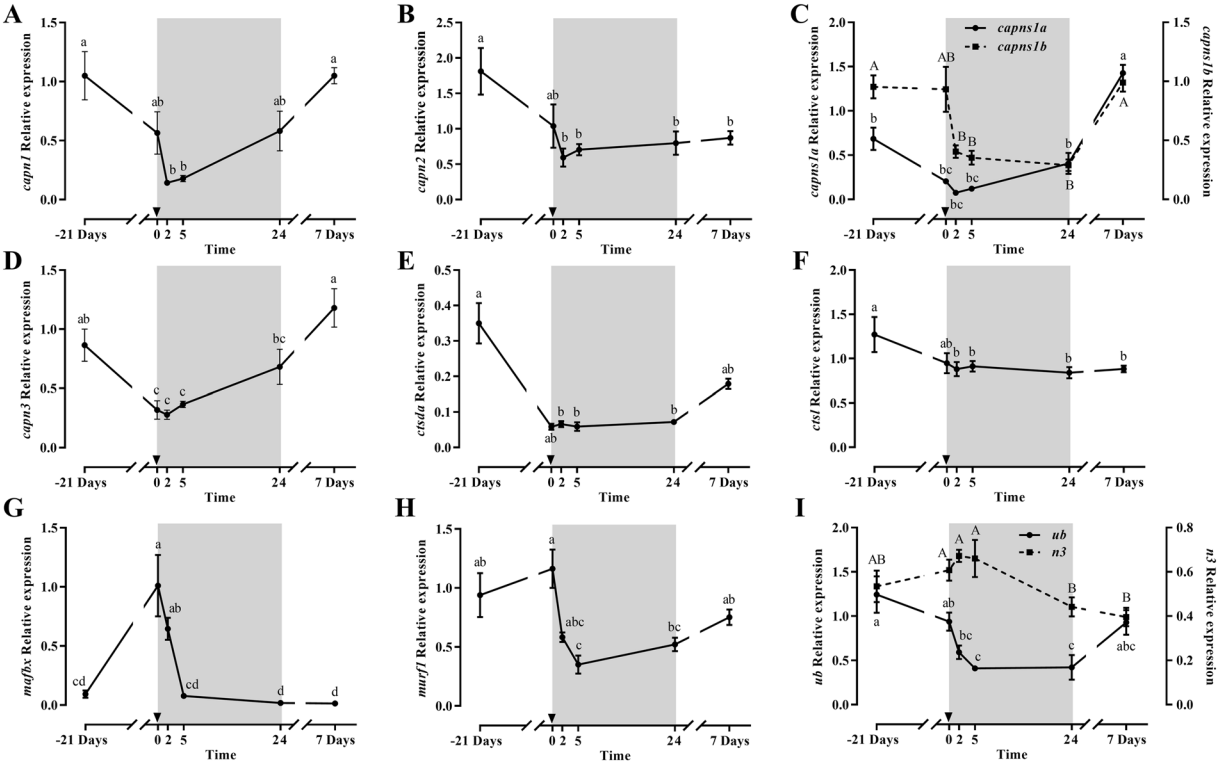

Figure 4. Relative gene expression of skeletal white muscle *capn1* (**A**), *capn2* (**B**), *capns1a* and *capns1b* (**C**), *capn3* (**D**), *ctsda* (**E**), *ctsl* (**F**), *mafbx* (**G**), *murf1* (**H**) and *ub* and *n3* (**I**) in gilthead sea bream during the fasting and refeeding experiment. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

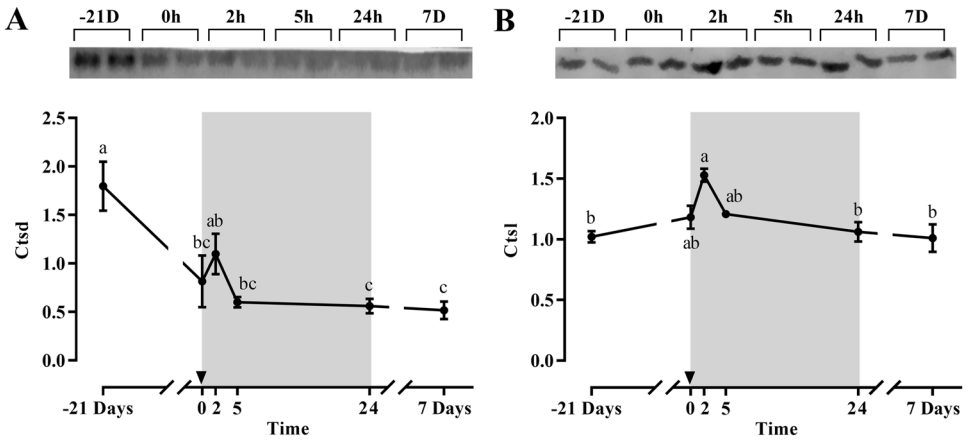

Figure 5. Representative blot and densiometric protein levels of skeletal white muscle Ctsd (**A**) and Ctsl (**B**) in gilthead sea bream during the fasting and refeeding experiment. Each protein was analyzed in cropped membranes of different Western blots along with other proteins (data not shown). The intensity of each specific band was normalized by the total transferred protein. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

and development as observed in an *in vitro* muscular model of gilthead sea bream under specific amino acids limitation (i.e. lysine)[36]. In the current study, both *igfrs* were affected differently; thus, *igf1ra* did not respond to fasting but to refeeding, recovering the expression levels at day 7, while the decreased expression levels of *igf1rb* in fasting and early refeeding were maintained until the end of the experiment. Such a differential response has been reported in several species using *in vivo* or *in vitro* models[8,10,32,36,37] and suggests a functional split between

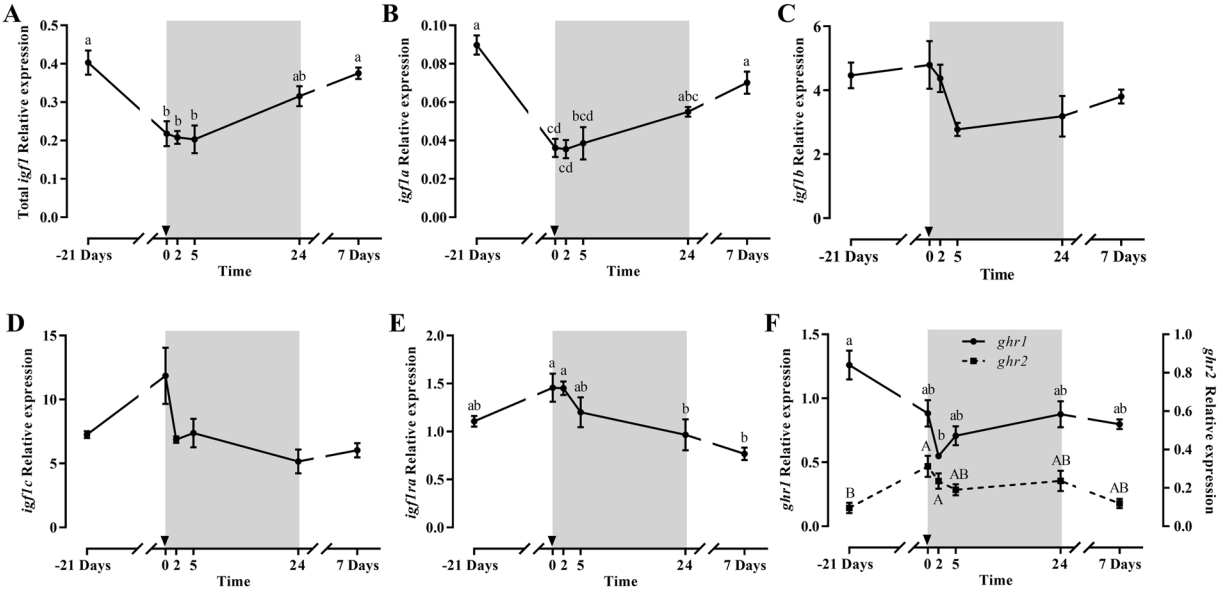

Figure 6. Relative gene expression of bone total *igf1* (**A**), *igf1a* (**B**), *igf1b* (**C**), *igf1c* (**D**), *igf1ra* (**E**) and *ghr1* and *ghr2* (**F**) in gilthead sea bream during the fasting and refeeding experiment. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

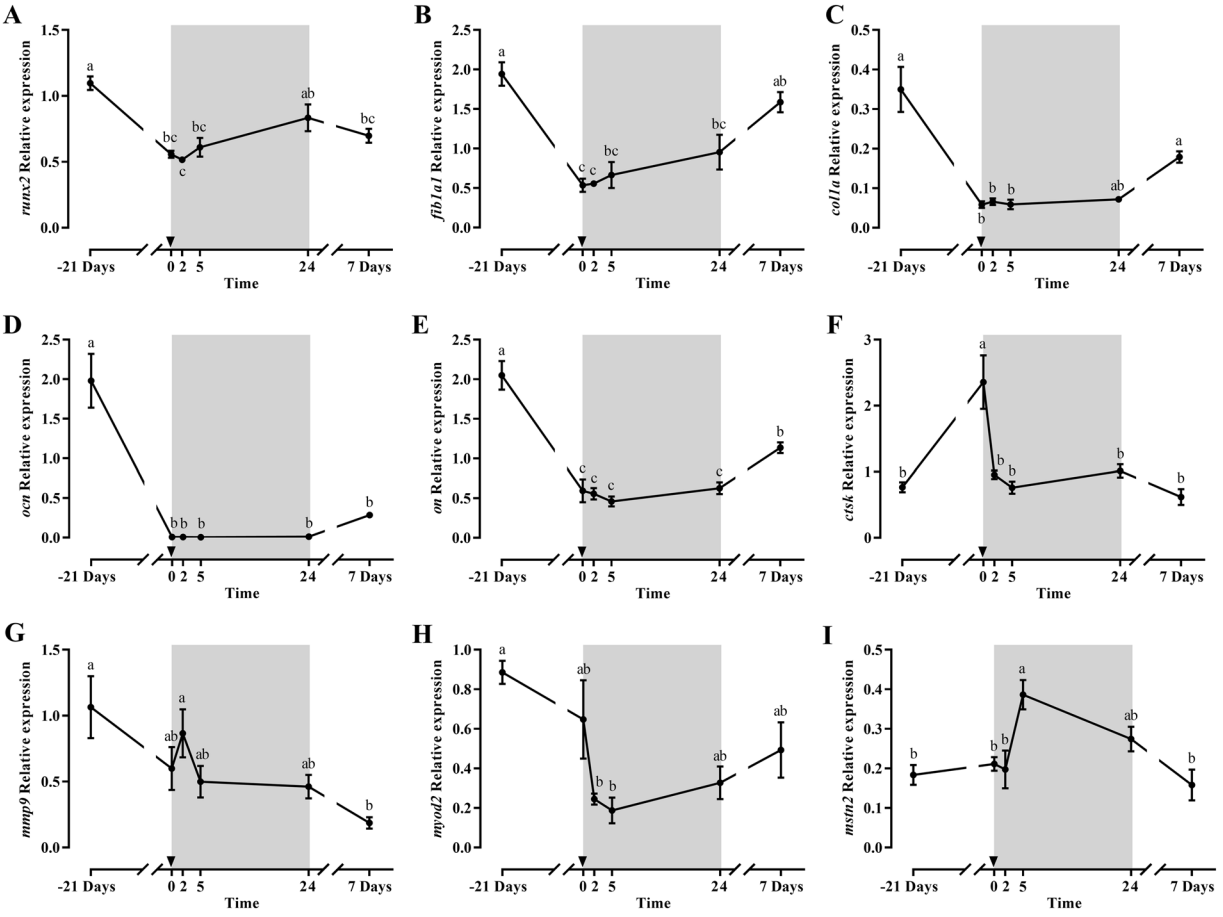

Figure 7. Relative gene expression of bone *runx2* (**A**), *fib1a* (**B**), *col1a1* (**C**), *ocn* (**D**), *on* (**E**), *ctsk* (**F**), *mmp9* (**G**), *myod2* (**H**) and *mstn2* (**I**) in gilthead sea bream during the fasting and refeeding experiment. The postprandial period is shown in grey and the time in hours. Data are shown as means ± SEM (n = 6). Letters indicates significant differences ($p < 0.05$) by one-way ANOVA, LSD and Tukey HSD test.

both *igfrs* isoforms. Furthermore, these findings point out that during a fasting or a low food intake period, Igf1 production decreases, but contrarily, some of the *igfrs* isoforms can be maintained, suggesting a rise in Igf sensitivity[8,32,38,39].

Muscle *igfbps* also responded differently to the treatment and while expression of *igfbp1* or *igfbp4* was unchanged, *igfbp5* significantly decreased in fasting and recovered progressively after 7 days of refeeding. This is in agreement with the anabolic function attributed to *igfbp5*, which has been identified as a good growth marker in gilthead sea bream[40]. Similar responses were detected in some species[41,42]; but not in others[35,43,44], although such a decrease is analogous to the Igfbp3 plasma levels determined in fasted coho and chinook salmon[45–47], suggesting a similar response of these two *igfbps* to long term food deprivation in different fish species. Thus, Igfbps in teleost are differently affected by the various metabolic conditions[42] and in the case of gilthead sea bream muscle, *igfbp5b* seems to be the most sensitive form to food availability. Moreover, *ghrs* expression showed in this study a dual role, with the decrease of *ghr1* until 5 h refeeding and a partial increase at 24 h and 7 days refeeding and the increase of *ghr2* expression up to 24 h refeeding. These patterns coincide with the differential function attributed to each receptor in gilthead sea bream and other fish species, being Ghr1 anabolic and Ghr2 catabolic[23,48]. Thus, in gilthead sea bream the growth enhancement caused by sustained exercise was followed by an increase in *ghr1* expression and a decrease in *ghr2* expression in muscle[40]. Similarly in this species, Gh treatment induced in muscle an increase of *ghr1* expression but not *ghr2*[23]. In summary, fasting depresses the components involved in growth promotion (*ghr1*, *igf1*, *igf1rb*, *igfbp5*), while refeeding reverts progressively the situation to activate muscle recovery.

Next, the expression of key elements of the main signaling pathways regulating protein synthesis and muscle growth (*akt* and *tor*) was significantly diminished during fasting. Such a scenario has been also observed in different fish species exposed to food deprivation[11,22]. The present study also demonstrates the sequential activation of those pathway components with the recovery of nutrition; however, it is very interesting to compare such a progressive response in gene expression with the quick phosphorylation of Akt and Tor within 2 h post-feeding. Similar responses were previously observed in fine flounder (*Paralichthys adspersus*)[11], rainbow trout[19,49] and even in gilthead sea bream both *in vivo*[50], as well as *in vitro*[30]. Thus, the comparison of *akt* and *tor* mRNA levels with their corresponding protein phosphorylation status during fasting and refeeding in the present study corroborates the different timely regulation of these pathways at protein and gene levels. Nevertheless, according to our results, it has to be taken into account that while refeeding is likely a major factor in the gene/protein expression and/or phosphorylation responses, they may also occur to some degree (especially those related with protein activation), during the introduction of nutrients in a common postprandial period. Therefore, future studies should consider these overlapping responses to specifically identify the dynamic nature of how nutrients regulate these processes in fish.

The information on MRFs under nutritional restriction in gilthead sea bream is scarce and current results could be useful to understand muscle growth regulation in such catabolic situations. Similarly to that observed in cultured muscle cells with lysine deficiency[36], in this study the expression of *myod1*, *myf5*, *myog*, *mrf4* and *mstn2* decreased during fasting or early refeeding, while late refeeding recovered basal expression of *myods* and *myf5*. Thus, making noticeable that the sequence of MRFs up-regulation during refeeding follows the same characteristic order as during myogenesis activation[15]. The parallelism between the profile of *myf5*, *myod1* and *myod2* with *pcna* or *pax7* agrees with the involvement of these MRFs in the first stage of myogenesis. Interestingly, similar patterns of recovery after starvation have been reported for several MRFs, mostly *myod* and *myog*, in salmonids[16,32,51,52] and in juvenile Nile tilapia (*Oreochromis niloticus*)[6]; overall, confirming the beginning of muscle remodeling at that early stage of refeeding. On the other hand, *mstn2* expression decreased with fasting and maintained significant low levels after 7 days refeeding, which is in agreement with previous results found in rainbow trout[52] and sea bass (*Dicentrarchus labrax*)[53]. Thus refeeding provoked a clear down-regulation of *mstn* gene expression to favor muscle recovery, in parallel to the increase observed of *pax7*, *pcna*, and MRFs expression.

Concerning muscle structural components, *mlc2a* and *mlc2b* showed an inverse profile, with *mlc2a* expression not changing with fasting but showing a significant increase at 24 h refeeding to return to basal levels at day 7. Bower and Johnston[37] also found in Atlantic salmon an increase of *mlc2* after 14 days of refeeding, and previous studies in gilthead sea bream[22,23,54] showed that an increase in specifically, *mlc2a* expression, indicates a condition that favors muscle proliferation. In this sense, in the present study the *mlc2a* peak at 24 h refeeding paralleled the tendency to enhance the expression of *pax7*, *pcna*, *tor* and the MRFs involved in early myogenesis (*myod2* and *myf5*), facilitating the muscle to grow during this period of compensatory growth.

Several members of the main proteolytic systems have been characterized and their responses upon different experimental challenges investigated in different fish species[18,55–60], including gilthead sea bream[20–22]; however, due to the different experimental conditions (i.e. duration of periods, fish age, etc.) among studies, it is difficult to reach clear explanation of their respective roles. In the present study, two different expression patterns were observed concerning proteolytic genes; those that were downregulated during fasting and recovered with refeeding, like calpains and some cathepsins, in a similar trend to that observed for *igf1* or MRFs; and those belonging to the ubiquitin-proteasome system, like *murf1* or *mafbx*, which increased in response to fasting and decreased with refeeding.

Regarding the calpains, in agreement with our findings, channel catfish fasted for 35 days presented a strong down-regulation of *capn1* and *capn3* genes, which recovered basal expression after a refeeding period[58]. Contrarily, Salmerón and coworkers[20] did not find significant effects on calpains expression in gilthead sea bream and suggested that these molecules could have a secondary role in the adaptation to food deprivation; and in fact, observed later that *ctsd* and *ctsl* increased significantly under the same conditions[21], similarly as observed in halibut for *ctsb* and *ctsd*[8]. Although we did not observe an increase in the gene expression of *ctsda* or *ctsl* in this study, amino acids limitation in *in vitro* myocytes upregulated both cathepsins expression[61]. Moreover, in the present study, Ctsl protein expression increased in fasting or early refeeding while Ctsd decreased, suggesting

this different response certain distribution of their regulatory functions. Concerning the ubiquitin-proteasome system, in a previous experiment in gilthead sea bream, 30 days of fasting upregulated the expression of the same genes that were increased in the present study[21]. Different authors have found similar responses of these genes with fasting[19,44,50,60,62] and, specific amino acids limitation increased also the expression of *mafbx* and *murf1*[61]. Interestingly, results were also supported at a protein level *in vivo*, since fasted rainbow trout muscle presented an increase in the total amount of polyubiquitinated proteins[19].

As an overview, in the present fasting and refeeding model, it seems that there is a clear difference between the expression of genes of the three major proteolytic pathways, being the ubiquitin-proteasome system the one that may have a stronger role in response to food restriction. Refeeding reverts the condition with calpains recovering high basal levels, while cathepsins present a variable role depending on the molecule considered. All this suggests a coordinated distribution of the proteolytic functions during the mobilization of reserves (fasting) and the remodeling associated with the compensatory muscle-growth induced by refeeding.

The skeletal system has multiple physiological functions in vertebrates[63], and the high incidence of skeletal deformities is still an important bottleneck for the sustainability of aquaculture[64]. Information about the effects of fasting and refeeding on bone has been mainly studied in cellular-boned fish, like the rainbow trout[65,66], while in fish species with acellular bone such as gilthead sea bream knowledge is limited. In mammals, it is well known that Igfs are important mediators of bone growth[67,68], being present at high concentrations in the bone matrix and, any deficiency on the Igf genes affecting skeletal growth[69]. Igfs stimulate differentiation of osteoblasts regulating the balance between bone accretion and resorption, which occurs throughout life[70]. So, in a fasting condition, or under severe chronic undernutrition, Igf1 concentrations decrease in association with low bone turnover and significant bone loss[71,72]. In fish, early studies also demonstrated the growth stimulatory effect of Igf1 in branchial arches of Japanese eel (*Anguilla japonica*)[69] and more recently, in an *in vitro* model of bone-derived cells from vertebra of gilthead sea bream, Igf1 as well as insulin, were demonstrated to stimulate cell proliferation[70].

In the current study, the effects of fasting and refeeding in bone were, in terms of the Gh/Igf system, similar to those found in muscle. Thus, the expression of *ghr1* and *ghr2* was inverse at fasting and refeeding, but the profiles were similar between the two tissues, supporting the conservation of Ghrs' role as well as their coordination in muscle and bone to adapt to the changes in alimentary conditions. About peptides, fasting resulted in a decrease of total *igf1* and *igf1a* expression, supporting reduced bone growth during catabolic conditions, while the other splice variants or the *igfbps* did not respond to the treatments. In agreement to these data, several authors previously demonstrated the proliferative effects of Igf1 *in vitro* in gilthead sea bream osteoblasts, and in embryonic zebrafish ZF-4 cells[73–75]. Thus, the bone total *igf1* downregulated expression observed in fasting is parallel to the decrease in *igf1* and *pcna* expression in muscle and, the general arrest of growth. On the other hand, during refeeding the upregulated expression of total *igf1* and *ghr1* in bone as in muscle, pointed out also to the importance of the coordination between the two tissues in this period, for the harmonic growth of the muscle-skeletal system for the fish to properly increase its size.

Several genes regulate the osteogenic process, and *runx2* is the key transcription factor determining bone lineage and inducing the expression of genes more involved in matrix production and mineralization (i.e. *fib1a*, *col1a1*, *ocn* and *on*)[26,76]. The results of the current study showed that during the fasting period, all osteogenic genes decreased in agreement with results on biometric indexes, MRFs in muscle and, the Gh/Igf system. During starvation, active osteoblasts were not observed in rainbow trout pharyngeal bone, and in Nile tilapia, an imbalance between bone formation and resorption (i.e. osteoblasts *versus* osteoclasts activity) was reported, resulting in reduced bone mass[65,77]. Moreover, Van der Velde and coworkers[78] suggested also that during fasting, the orexigenic hormone ghrelin increases osteoclastogenesis in mice. In the animals of the present study, ghrelin levels increased during fasting to diminish in an acute way at 5 h post-feeding[27]. Thus, during the fasting period, the levels of circulating ghrelin can contribute to arrest the osteogenic process increasing bone degradation. The decrease in osteoclastic activity fits well with the significant enhancement of *ctsk* expression observed after 21 days of fasting and its down-regulation in refeeding, since Ctsk is secreted by osteoclasts and is an important factor of bone resorption[79]. In this sense, the present study demonstrates such an equal role in gilthead sea bream.

In osteoblast-like cell lines of this species, fish serum is more suitable than bovine serum to induce bone formation causing changes in *col1a*, *ocn* and *on* gene expression[80]. In the present study, the expression of osteogenic genes was restored with refeeding in a sequential order, being *runx2* the first gene upregulated while the other genes followed progressively (*fib1a*, *col1a1*, *on* and *ocn*) in a sense that resembled the osteogenic process[26], indicating that bone growth and matrix mineralization were being reinstated. In agreement, in goldfish (*Carassius auratus*), it was found that while *col1a* continually increased after day 7 of scale regeneration, *ocn* increased only after day 14[81]. Based on our results, 7 days of refeeding were not enough to recover the latest genes *on* and *ocn*, like in muscle for *myog* or *mrf4*, suggesting that both tissues recovered in parallel to guarantee an harmonic growth; besides indicating that more than 7 days of refeeding are necessary for complete restoration of the musculoskeletal system anabolism.

The expression of different myogenic genes in mammalian bone is known and their role in coordinating both skeletal tissues has been described[82,83] but these studies in fish are scarce. In the present study, *pax7*, a marker of muscle satellite cells[15,84], was evaluated in order to verify the purity of the tissue, which was corroborated since *pax7* expression was undetectable in the bone samples. Then, the gene expression of other MRFs was determined. García de la serrana and coworkers[85] in a transcriptional study in gilthead sea bream, found for the majority of genes analyzed a faster and more pronounced response to leucine injection in skeletal muscle than in bone. A similar tendency was found in the present study when comparing both tissues, since *myod2* expression showed in bone still low levels at 5 and 24 h or 7 days refeeding, while in muscle the levels were already recovered by the end of the period studied. On the other hand, bone *mstn2* expression, showed an opposite response compared to muscle with a significant increase at 5 h post-feeding, which could be inhibiting bone growth at this specific stage. In humans, the role of myostatin in bone resorption and rheumatoid arthritis pathology was recently

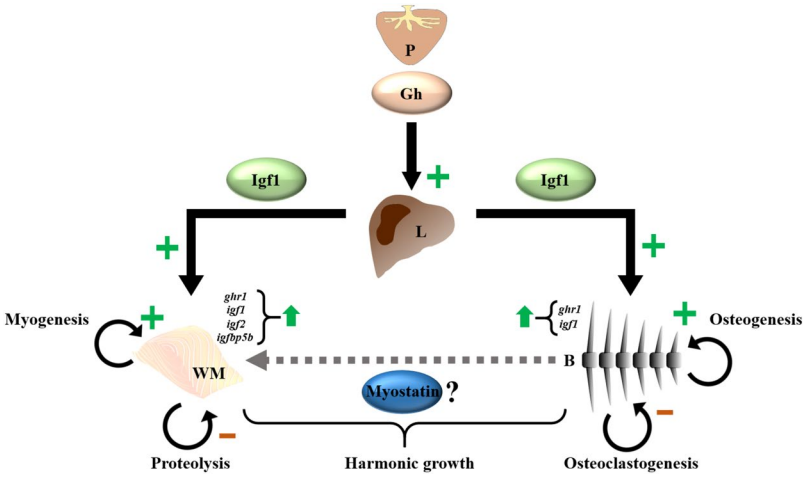

Figure 8. Schematic representation of the proposed changes occurring during early refeeding in gilthead sea bream. The Gh/Igf system recovers the synthetizing role with the Gh plasma levels still elevated activating now the hepatic expression/secretion of *igf1*, in parallel with the progressive up-regulation of the anabolic system components (*ghr1*, *igf1*, *igf2* and *igfbp5b*). This condition contributes to the activation of the myogenic (*pax7*, *myf5*, *myod1* and *mrf4*) and osteogenic (*runx2*, *fib1a*, *col1a1* and *on*) genes, while downregulates the proteolytic (*mafbx* and *murf1*) and osteoclastogenic (*ctsk*) genes in muscle and bone, respectively. This early stage of refeeding may require a fine regulation of the different molecules involved, being myostatin a good candidate for bone and muscle crosstalk to assure harmonic musculoskeletal growth. P: pituitary; L: liver; WM: white muscle; B: bone.

demonstrated[82]; thus, the inhibitory function of Mstn2 in fish, could serve also in this early refeeding period as a regulator to an overly rapid recovery of bone growth. In fact, the inter-tissue inhibitory role of myostatin has been already described in mammals[83,86] serving as a coordinator among skeletal tissues. This function still needs to be demonstrated in fish, but myostatin could contribute not only to control muscle mass, but also to coordinate the growth of both tissues avoiding miss-matches and potentially reducing the appearance of skeletal deformities.

In summary, 21 days of fasting in gilthead sea bream depressed Igf1 as well as most of the synthesis, myogenic and osteogenic mechanisms at the same time that activated several proteolytic molecules from the ubiquitin-proteasome system to mobilize muscle resources. Refeeding triggered rapidly compensatory mechanisms starting with the Igf1 system, and progressively activated the regulatory factors of myogenesis and osteogenesis in a sequence that repeats the processes of development and growth in either tissue, overall restoring proper musculoskeletal growth (Fig. 8). Furthermore, the expression in bone of myostatin suggests an interesting tissue coordinative function that deserves future investigation in this and other fish species. All this can help to better understand the role of these regulatory factors and their crosstalk in the early phase of somatic growth recovery after a period of food limitation.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

J.G. conceptualized the study; F.L., M.P.-A., E.J.V., A.S.-M., S.B.-P., N.R.-H., J.F.-B. and J.B. performed the sampling; F.L. and M.P.-A. performed the laboratory analyses; F.L., M.P.-A., E.J.V., E.C. and J.G. analyzed and interpreted the data; J.B., I.N., E.C. and J.G. acquired funding; F.L., M.P.-A., E.J.V., E.C. and J.G. drafted and critically reviewed the manuscript. All authors read and approved the final paper.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to J.G.

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ARTICLE 3

Mitochondrial adaptation to diet and swimming activity in gilthead seabream: Improved nutritional efficiency

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Mitochondrial Adaptation to Diet and Swimming Activity in Gilthead Seabream: Improved Nutritional Efficiency

*Miquel Perelló-Amorós[1], Jaume Fernández-Borràs[1], Albert Sánchez-Moya[1], Emilio J. Vélez[2], Isabel García-Pérez[1], Joaquin Gutiérrez[1] and Josefina Blasco[1]**

[1] Department of Cell Biology, Physiology, and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain, [2] Université de Pau et des Pays de l'Adour, E2S UPPA, INRAE, UMR 1419 Nutrition Métabolisme et Aquaculture, Saint-Pée-sur-Nivelle, France

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***Correspondence:**
Josefina Blasco
jblasco@ub.edu

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Sustained exercise promotes growth in different fish species, and in gilthead seabream we have demonstrated that it improves nutrient use efficiency. This study assesses for differences in growth rate, tissue composition and energy metabolism in gilthead seabream juveniles fed two diets: high-protein (HP; 54% protein, 15% lipid) or high energy (HE; 50% protein, 20% lipid), under voluntary swimming (VS) or moderate-to-low-intensity sustained swimming (SS) for 6 weeks. HE fed fish under VS conditions showed lower body weight and higher muscle lipid content than HP fed fish, but no differences between the two groups were observed under SS conditions. Irrespective of the swimming regime, the white muscle stable isotopes profile of the HE group revealed increased nitrogen and carbon turnovers. Nitrogen fractionation increased in the HP fed fish under SS, indicating enhanced dietary protein oxidation. Hepatic gene expression markers of energy metabolism and mitochondrial biogenesis showed clear differences between the two diets under VS: a significant shift in the COX/CS ratio, modifications in UCPs, and downregulation of PGC1a in the HE-fed fish. Swimming induced mitochondrial remodeling through upregulation of fusion and fission markers, and removing almost all the differences observed under VS. In the HE-fed fish, white skeletal muscle benefited from the increased energy demand, amending the oxidative uncoupling produced under the VS condition by an excess of lipids and the pro-fission state observed in mitochondria. Contrarily, red muscle revealed more tolerant to the energy content of the HE diet, even under VS conditions, with higher expression of oxidative enzymes (COX and CS) without any sign of mitochondrial stress or mitochondrial biogenesis induction. Furthermore, this tissue had enough plasticity to shift its metabolism under higher energy demand (SS), again equalizing the differences observed between diets under VS condition. Globally, the balance between dietary nutrients affects mitochondrial regulation due to their use as energy fuels, but exercise corrects imbalances allowing practical diets with lower protein and higher lipid content without detrimental effects.

Keywords: high-fat, high-protein, exercise, mitochondrial dynamics, turnover, stable isotopes, *Sparus aurata*

INTRODUCTION

The growth capacity of animals is determined in a multifactorial way, involving characteristics inherent to the specific population of the reared animals as well as the physicochemical properties of the environment and the culture practices and regimes. In aquaculture, water flow is an important factor that can influence the daily energy expenditure associated with the swimming activity of fish. Thus, the study of how metabolic fuels are used to satisfy the energy demands for activity is important for the aquaculture sector to optimize feeds for optimal growth (Magnoni et al., 2013a). Exercise can stimulate growth and food conversion in various fish species (Palstra and Planas, 2013; McKenzie et al., 2020). In general, fish swimming below their maximum aerobic capacity is qualitatively similar to mammals performing aerobic exercise, showing a trend toward a more aerobic phenotype (Johnston and Moon, 1980; McClelland et al., 2006; LeMoine et al., 2010; McClelland and Scott, 2014). This positive effect of sustained moderate exercise in promoting growth also occurs in rainbow trout (Felip et al., 2012) and gilthead seabream fingerlings and juveniles (Ibarz et al., 2011; Blasco et al., 2015). This effect is regulated by the growth hormone/insulin-like growth factor (GH/IGF-I) axis (Blasco et al., 2015; Vélez et al., 2016), since there is an adjustment in the use of nutrients as the aerobic capacity of white muscle is increased and the mesenteric fat deposits are reduced. We previously demonstrated that sustained moderate swimming stimulated the use of carbohydrates, optimizing protein retention, and growth in both rainbow trout (Felip et al., 2012) and gilthead seabream (Ibarz et al., 2011; Felip et al., 2012; Martin-Perez et al., 2012). For these reasons, the exercise induced through sustained moderate swimming can be useful when formulating protein-adjusted diets, due to the more efficient use of non-protein energy. Indeed, the need to consider the formulation of fish diets in the effects of exercise has recently been pointed out (McKenzie et al., 2020), but there are not studies about it.

If growth improvement after exercise occurs through improved metabolic efficiency, this should be reflected in mitochondrial adaptation associated with the physical status of the host. The metabolism's ability to adapt efficiently by substrate sensing, trafficking, storage, and utilization, dependent on availability and requirement, is known as metabolic flexibility (Smith et al., 2018). Mitochondria undergo constant adaptive changes in response to fluctuations in energy demand and supply, both in their absolute numbers (biogenesis) and their morphology (through fusion/fission processes), to accommodate demand for ATP (oxidative metabolism) (Liesa and Shirihai, 2013). It is known as metabolic flexibility (Smith et al., 2018). Different mitochondrial proteins participate in this process.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1a, is a transcriptional factor that enhances mitochondrial biogenesis and oxidative function (Puigserver et al., 1998; Wu et al., 1999), being the main regulator of the cytochrome c oxidase (subunit IV of OXPHOS complex) (Scarpulla, 2011). Single bouts of exercise lead to transient increases in PGC-1a levels (Baar et al., 2002) and a high-fat diet in humans leads to decreased gene expression (Sparks et al., 2005). Uncoupling proteins (UCPs) reduce electrochemical gradients responsible for ATP generation, uncoupling oxidative phosphorylation (Divakaruni and Brand, 2011), and participate in tissue nutrient sensing and regulation of nutrient metabolism (Diano and Horvath, 2012). In response to a high-lipid diet in rats, *ucp2* upregulation and *pgc1a* downregulation have been observed (Rius-Pérez et al., 2020). Mitochondria also undergo constant fusion and fission dynamics, through changes in mitofusin (*mit1*, *mit2*) and fission (*fis1*) proteins expression. In general, fusion is stimulated where there is energy balance (demand = supply), while fission of the mitochondrial network into individual units is necessary to eliminate damaged mitochondria when cellular stress is present (Smith et al., 2018). In rats, a high fat diet induces fatty deposition in the liver and impaired mitochondrial function (Gonçalves et al., 2014, 2015), but endurance training effectively prevents these pathological processes.

Although the cellular and physiological implications of mitochondrial dynamics have been studied extensively in mammals (Westermann, 2010, 2012; Ni et al., 2015; Meyer et al., 2017; Chandhok et al., 2018), there is a scarcity of such data in fish. Mitochondrial homeostasis ensures that metabolism and physiological function persists through a global balance of mitochondrial processes (Milder and Petel, 2012). Despite extensive knowledge about this metabolic flexibility in mammals (Chen et al., 2018; Smith et al., 2018), this is not the case for fish, even though mitochondrial protein function is highly conserved. A relationship between growth, diet composition, and mitochondrial function has been observed both in channel catfish (Eya et al., 2012) and in rainbow trout (Eya et al., 2017). The influence of nutrient levels on the gene expression of respiratory chain uncoupling proteins, UCP2 and UCP3, is well documented in rainbow trout (Coulibaly et al., 2006) and gilthead seabream (Bermejo-Nogales et al., 2011, 2014), with these being the first to be modified in response to both environmental and nutritional (by caloric restriction) stressors (Bermejo-Nogales et al., 2014). Diet can modulate fusion, fission, biogenesis, and oxidation processes in yellow catfish (Song et al., 2020) and snout bream (Li et al., 2019). The ratio of cytochrome c oxidase (COX) to citrate synthase (CS) mitochondrial enzyme activity in the white muscle of exercised gilthead seabream is modulated differently depending on diet and life stage, with more protein (Blasco et al., 2015) and more carbohydrates (Martin-Perez et al., 2012) in fingerlings and juveniles, respectively (Martin-Perez et al., 2012). However, the possible regulatory role of PGC1a on oxidative metabolism is less clear than in mammals (McClelland and Scott, 2014).

Partial replacement of dietary protein with non-protein energy sources (lipids and carbohydrates) is a common practice in aquaculture, saving money on expensive ingredients and increasing nitrogen retention, and then reducing the environmental impact (by lowering nitrogen discharges). However, the efficiency of the substitution depends on

endogenous factors of the fish (e.g., nutrient requirements of the species, life stage or the reproductive phase). Moreover, high-energy diets for aquaculture may reduce the period until the market size is reached for some species, and decrease feed costs by reducing the amount of money spent on proteins (Leaver et al., 2008). In fish, however, undue increases in lipid content have been associated with high fat deposition (Caballero et al., 1999; Company et al., 1999) and increased oxidative stress (Sánchez-Nuño et al., 2018). An optimal diet should provide the necessary nutrients in appropriate proportions for maintenance, tissue repair and growth, without excessive deposits of fuel reserves. Stable isotope analysis of fish nutrition has been revealed to be useful in the evaluation of reserve turnover (Martínez Del Rio et al., 2009) because enzymes involved in catabolic processes, such as decarboxylation and deamination show a preference for light isotopes (Gannes and Marti, 1998). This causes tissues to become enriched with heavier isotopes (e.g., ^{13}C and ^{15}N). This factor of discrimination is known as fractionation. We have evaluated the use of stable isotopes as an indicator of feeding balance when assessing the optimal nutritional conditions for growing fish (Beltrán et al., 2009; Martín-Pérez et al., 2011; Martin-Perez et al., 2012; Martin-Pérez et al., 2013). Our studies have demonstrated that nitrogen dietary fractionation ($\Delta\delta^{15}N$) is a good marker of protein balance because it reflects protein turnover and retention efficiency (Martínez Del Rio et al., 2009; Martín-Pérez et al., 2011). In gilthead seabream, we observed an inverse relationship between dietary protein content and muscle nitrogen fractionation, and that this fractionation was also inversely related to the specific growth rate (SGR) (Martin-Pérez et al., 2013). This reflects a direct relationship between diet protein content and SGR.

The practice of using lipid supplementation to decrease dietary protein levels in order to reduce production costs is not common during the early life-stages because it may cause imbalances that negatively affect these periods of rapid growth and compromise the efficiency of the culture and the quality of the product. Understanding how changes in diet composition related to swimming activity affect mitochondria can help to clarify the molecular mechanisms that underpin growth performance and body composition. Therefore, the aim of the present study was to analyze the beneficial effects of sustained swimming in gilthead seabream fingerlings that were fed commercial diets with different proportions of macronutrients (substitution of proteins with lipids), especially their effects on the mitochondrial homeostasis of the main tissues. We measured growth parameters, body indices and the principal components of the main tissues (liver, and white and red muscle). Since the balance and availability of nutrients may affect cell metabolism, we analyzed the gene and protein expression of mitochondrial proteins and the ^{15}N and ^{13}C isotope relationships in the liver and both types of skeletal muscle. Two groups of fish maintained under similar conditions that performed voluntary swimming were also analyzed for the same variables, acting as the reference values for the diets.

TABLE 1 | Chemical composition of the diets.

	HP DIET	HE DIET
Digestible energy (MJ/kg)	18	19.9
Protein (% DM)	54	50
Lipids (% DM)	15	20
DHA (% DM)	15	20
EPA (% DM)	1	1.4
ARA (% DM)	2.5	3
DHA/EPA/ARA	5/12.5/1	3.5/7.5/1
Cellulose (% DM)	1.6	1.7
Ash (% DM)	10.5	6.4
Total P (% DM)	1.4	1.2
Estimated nitrogen-free extract	20.5	23.5

DM, dry matter; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; P, elemental phosphorus.

MATERIALS AND METHODS

Experimental Design

Nine hundred eighty gilthead seabream fingerlings (mean body weight, 4.1 ± 0.1 g) were obtained from a commercial hatchery (Piscimar, Burriana, Spain) and reared in the facilities of the Faculty of Biology (University of Barcelona) at 23 ± 1°C under a 15L:9D photoperiod. After 1 week of acclimation, the fish were anesthetized with MS-222 and their individual weight and length measured. The fish were distributed between eight 200-L (n = 80 for each tank) and two 400-L (n = 160 for each tank) tanks at the same biomass density (1.5 kg/m^3). A representative number of fish (40 undertaking voluntary swimming (VS) and 120 performing sustained swimming (SS) for each diet group) were fitted with a passive integrated transponder (PIT, size:1.25 mm × 7 mm) tag (Trovan Electronic Identification Systems, Madrid, Spain) near the dorsal fin to enable their identification and individual monitoring of the SGR. The 400-L tanks were kept in rearing conditions, where the fish swam spontaneously (voluntary swimming group, VS). In the 200-L tanks, a circular laminar flow was created, placing a plastic column at the center of each tank and using a plastic flute to direct the water exit in a tangential direction toward the wall of the tank. The flow of each tank was regulated to achieve an initial speed of 2.5 body lengths (BL)/second to force the fish to perform sustained moderate swimming (sustained swimming group, SS). The flow speed of these tanks was adjusted during the experiment to maintain the speed as the fish body length increased. The fish food was obtained from Skretting España S.A. (Burgos, Spain), and two different diets were chosen based on their composition (**Table 1**). One was the high protein diet (HP diet, 54P/15L), while the other was the high energy diet (HE diet, 50P/20L). Each diet was used to feed half (one 400-L and four 200-L) of the tanks. The daily ration was set as 5% of the total biomass for each tank divided into three meals. The pellet size was increased 3 weeks after the beginning of the trial.

The biometric parameters (weight and standard length) of all the fish were measured at the end of the experiment (6 weeks) and used to calculate the Condition Factor (CF) [CF = W/L^3], where

W is the body weight in grams and L is the body length in cm. At this point and after 12 h of fasting, the PIT-tagged fish from each tank were sacrificed by sectioning the spinal cord and eviscerated. The weights of the mesenteric fat and liver were obtained, and the mesenteric fat index (MFI) and hepatosomatic index (HSI) were calculated. Specific growth rate was calculated for each PIT-tagged fish [SGR = 100 × ((ln (Wf) - ln (Wi))/T)], where Wf and Wi are the final and initial body weight of the fish in grams, respectively, and T is the time of experiment in days. Samples of liver and red and white skeletal muscle were taken and stored in liquid nitrogen until further analysis.

All animal handling procedures were conducted following the norms and procedures established by the European Union Council (86/609/EU) and the Spanish and Catalan Governments, with approval obtained from the Ethics and Animal Care Committee of the University of Barcelona (permit number DAAM 7644).

Muscle Proximate Composition and Isotopic Composition Analysis ($\delta^{15}N$ and $\delta^{13}C$)

Samples of white muscle were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Aliquots of each sample were taken for use in isotopic analyses and to assess the lipid, protein, glycogen, and water contents. Water content was determined gravimetrically after drying the samples at 95°C for 24 h. Lipids were extracted as described by Bligh and Dyer (1959). The lipid extracts were dried under an N_2 atmosphere and total lipids determined gravimetrically. Proteins were purified from defatted tissue samples via precipitation with 10% (v/v) trifluoroacetic acid. The extracts were dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, United States) and the protein content was calculated from the total N content obtained by elemental analysis (Elemental Analyzer Flash 1112, Thermo Finnigan, Bremen, Germany), assuming 1 g of N for every 6.25 g of protein. Glycogen was extracted and purified from the tissues following alkaline hydrolysis by boiling with 30% KOH and an alcoholic precipitation, as described by Good and coworkers (Good et al., 1933). The glycogen content was then assessed using the anthrone-based colorimetric method described by Fraga (1956).

Samples of diet and white muscle were lyophilized and ground into a homogenous powder for isotopic analysis. Aliquots of the diet and their purified fractions (lipid and protein) and of white muscle and their purified tissue fractions (glycogen, lipid, and protein), which ranged from 0.3 to 0.6 mg, were weighed in small tin capsules. Samples were analyzed to determine the carbon and nitrogen isotope composition using a Mat Delta C isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyzer. Isotope ratios (15N/14N, 13C/12C) determined by isotope ratio mass spectrometry are expressed in delta (δ) units (parts per thousand, ‰), as follows:

$$\delta = [(Rsa/Rst) - 1] \times 1000$$

where, Rsa is the 15N/14N or 13C/12C ratio of the samples and Rst is the 15N/14N or 13C/12C ratio of the international standards (Vienna Pee Dee Belemnite for C and air for N). The same reference material analyzed during the experimental period was measured with ± 0.2‰ precision. Nitrogen isotopic fractionation values ($\Delta\delta^{15}N$) were calculated as the difference between the δ value of the tissue and the corresponding δ value of the diet.

RNA Extraction, cDNA Synthesis, and q-PCR

To perform the gene expression analysis, tissue homogenization was performed using 100 mg of white muscle. Tissue samples were homogenized in 1 ml of TRI Reagent® (Applied Biosystems, Alcobendas, Spain), using the Precellys® Evolution Homogenizer cooled with Cryolys® (Bertin-Corp, Montigny-le-Bretonneux, France) at 4–8°C. After homogenization, RNA extraction was performed following the protocol of the TRI Reagent® manufacturer. The RNA concentration of each sample was measured using Nanodrop2200 (Thermo Fisher Scientific, Alcobendas, Spain). The RNA integrity of each sample was checked using 1% agarose gel electrophoresis with 3% SYBR® Safe DNA Gel Stainer (Bio-Rad, El Prat de Llobregat, Spain). Then, one μg of RNA was treated with DNase I (Life Technologies, Alcobendas, Spain) and retrotranscribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain).

Gene expression analysis was performed by quantitative polymerase chain reaction (q-PCR) with the cDNA samples according to the requirements of the MIQE guidelines (Bustin et al., 2009), using the iTAQ Universal SYBR® Green Supermix (Bio-Rad, El Prat de Llobregat, Spain) in Hard-Shell® 384-well PCR plates (Bio-Rad, El Prat de Llobregat, Spain) and the CFX384TM Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The q-PCR program was: 3 min at 95°C, 39 × (30 s at 95°C, 30 s at primer melting temperature and fluorescence detection), 5 s at 55°C followed by a melting curve ranging from 55 to 95°C with an increase of 0.5°C every 30 s.

The primer sequences and GenBank accession numbers are shown in the **Table 2**. The reference genes elongation factor 1 alpha (*ef1α*), ribosomal protein S18 (*rps18*), ribosomal protein L27 (*rpl27*), and mitochondrial import receptor subunit TOM20 (*tom20*) were analyzed and the combination of the most stable ones was used to calculate the relative expression of the genes of interest following the Pfaffl method (Pfaffl, 2001). The stability of the reference genes (assessed with the geNorm algorithm) and the relative expression to the geometric mean of the reference genes were calculated with the Bio-Rad CFX Manager™ 3.1 software.

Protein Extraction and Western Blot Analysis

Protein was extracted from 100 mg of white skeletal muscle in 1 mL of RIPA buffer supplemented with phosphatase (PMSF and NA_3VO_4) and protease inhibitors (P8340, Santa Cruz) using the Precellys® Evolution homogenizer coupled to a Cryolys cooling system (Bertin Technologies, Montigny-le-Bretonneux, France).

Soluble protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) (Sigma Aldrich,

TABLE 2 | Primers used for real-time quantitative PCR.

Gene	Sequences 5′-3′	Ta (°C)	Accession number
cox4a	F: ACC CTG AGT CCA GAG CAG AAG TCC R: AGC CAG TGA AGC CGA TGA GAA AGA AC	60	JQ308835
cs	F: TCC AGG AGG TGA CGA GCC R: GTG ACC AGC AGC CAG AAG AG	60	JX975229
pgc1a	F: CGT GGG ACA GGT GTA ACC AGG ACT C R: ACC AAC CAA GGC AGC ACA CTC TAA TTC T	60	JX975264
ucp1	F: GCA CAC TAC CCA ACA TCA CAA G R: CGC CGA ACG CAG AAA CAA AG	60	FJ710211
ucp2	F: CGG CGG CGT CCT CAG TTG R: AAG CAA GTG GTC CCT CTT TGG TCA T	60	JQ859959
ucp3	F: AGG TGC GAC TGG CTG ACG R: TTC GGC ATA CAA CCT CTC CAA AG	60	EU555336
mit1	F: CAT CGT TGG AGG AGT GGT GTA R: CCG TAC AGT GAG GCT GAG AG	60	JX975250
mit2	F: GGG ATG CCT CAG CCT CAG AAC CT R: CTG CCT GCG GAC CTC TTC CAT GTA TT	60	JX975251
fis1	F: TCT CAG GAA CGA GCC AGG GAA CA R: CCT TGT CGA TGA GTT TCT CCA GGT CCA G	60	JX975249
miffb	F: CGC AGC AGC ATT CCC TTC R: CTC GTA CTG GAT TCG GTT CAT CT	60	JX975252
ef1a	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	60	AF184170
rps18	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	60	AM490061.1
tom20	F: TGT TCA TCG GGT ACT GCA TC R: TTC TGC TTT CTC CTC CGT TC	60	FM146454.1
rpl27	F: AAGAGGAACACAACTCACTGCCCCAC R: CTTCTGCCTGTTGAGGAACCA	68	AY188520

Ta, annealing temperature in the qPCR.

Tres Cantos, Spain) for the standard curve. 20 μg of the soluble protein fraction were added to a loading buffer (containing SDS and β-mercaptoethanol), heated at 95°C for 5 min and run in a 12% polyacrylamide gel. The proteins were then transferred overnight to Immobilon®-FL PVDF 0.2-μm transfer membranes (Merck Millipore Ltd., Tullagreen, Cork, Ireland) that had been previously activated in methanol. Total transferred protein was determined by a 5-min incubation with REVERT™ Total Protein Stain (LI-COR, Lincoln, Nebraska, United States). The signal was read at 700 nm using the Odyssey Fc Imaging System (LI-COR). After total protein quantification, the membranes were blocked with Odyssey Blocking Buffer (diluted 1:1 in TBS) (LI-COR) for 1 h at room temperature before being incubated overnight at 4°C and in agitation with the corresponding primary antibody diluted in blocking buffer + 0.05% Tween20. The primary antibodies used were purchased from ABCAM (Cambridge, United Kingdom), as follows: rabbit polyclonal anti-COX IV antibody (ab16056; 1:1000), rabbit polyclonal anti-CS antibody (ab96600; 1:2000), mouse monoclonal anti-mitofusin 1 + mitofusin 2 antibody [3C9] (ab57602; 1/1000) and rabbit polyclonal anti-UCP3 antibody (ab180643; 1/500 for WM and 1/1000 for RM). The cross-reactivity of these antibodies with gilthead seabream was confirmed by the molecular weight of the bands. In the case of COX4a and CS, the blots were performed from the same membranes which were split prior to the primary antibody incubation. After washing with TBS-T, the membranes were incubated with the corresponding secondary antibodies diluted in blocking buffer + 0.05% Tween20 at 1:10000 dilution: IRDye® 800CW Goat anti-Rabbit (925-32211), Li-Cor, Lincoln, Nebraska, United States) and IRDye® 800CW Goat anti-Mouse (925-32210, Li-Cor, Lincoln, Nebraska, United States). After incubation, the membranes were washed with TBS-T and the fluorescence of the immunoreactive bands was measured at 800 nm using the Odyssey Fc Imaging System (LI-COR). Stripping was performed using a commercial stripping buffer (NewBlot PVDF 5X Stripping Buffer) (LI-COR).

Statistics

Data for all parameters are presented as means ± standard error of the mean (SEM). Data normality and homoscedasticity through groups were checked with the Shapiro–Wilk test followed by Levene's test. Data were analyzed by a two-way analysis of variance (ANOVA) with diet (HP, HE) and swimming activity (VS, SS) set as independent factors, and their interaction. Pairwise comparisons were analyzed by unpaired student *t*-tests. Differences among groups were considered

TABLE 3 | Somatic growth parameters of gilthead seabream fed the HP or HE diet under conditions of voluntary swimming (VS) or sustained swimming (SS).

	VS		SS		
	HP DIET	HE DIET	HP DIET	HE DIET	
BW	16.42 ± 0.25	15.01 ± 0.21***	16.12 ± 0.20	15.99 ± 0.17†††	D: < 0.001 A: NS D × A: 0.003
CF	1.46 ± 0.01	1.54 ± 0.01***	1.54 ± 0.01†††	1.52 ± 0.01**†	D: < 0.001 A: < 0.001 D × A: < 0.001
HSI	1.42 ± 0.04	1.93 ± 0.05***	1.55 ± 0.03†††	1.89 ± 0.06***	D: < 0.001 A: NS D × A: NS
MFI	1.20 ± 0.10	1.07 ± 0.08	1.01 ± 0.09	1.29 ± 0.10*	D: NS A: NS D × A: 0.024
SGR	3.24 ± 0.06	3.19 ± 0.04	3.14 ± 0.03	3.05 ± 0.03*†	D: 0.010 A: NS D × A: NS

*Data are presented as the mean ± SEM. Body weight (BW) and Condition factor: n = 154 for VS and n = 315 for SS conditions for each diet group (all fish). Hepatosomatic index (HSI), mesenteric fat index (MFI), and specific growth rate (SGR): n = 40 for VS and n = 120 for SS conditions for each diet group (PIT-tagged fish). The p-values of the two-way ANOVA analysis are displayed in the right column. D, diet; A, Activity; D × A, Interaction; n.s., p-value > 0.05. Pairwise comparisons were assessed by unpaired Student's t-test. *p < 0.05, **p < 0.01, and ***p < 0.001 between diet groups (HP and HE). †p < 0.05 and †††p < 0.001 between swimming activity groups (VS and SS).*

TABLE 4 | Proximal composition and stable isotopes analysis in skeletal white muscle.

	VS		SS		
	HP DIET	**HE DIET**	**HP DIET**	**HE DIET**	
% moisture	77.41 ± 0.13	77.25 ± 0.20	77.31 ± 0.22	77.17 ± 0.16	D: NS A: NS D × A: NS
% glycogen	0.37 ± 0.02	0.35 ± 0.03	0.27 ± 0.02†	0.28 ± 0.02†	D: NS A: < 0.001 D × A: NS
% lipid	1.45 ± 0.02	1.55 ± 0.02***	1.47 ± 0.03	1.54 ± 0.01	D: < 0.001 A: NS D × A: NS
% protein	21.22 ± 0.24	20.95 ± 0.24	21.05 ± 0.025	21.02 ± 0.37	D: NS A: NS D × A: NS
δ^{15}N DM[1]	11.52 ± 0.07	10.85 ± 0.08***	11.62 ± 0.05	10.84 ± 0.07***	D: < 0.001 A: NS D × A: NS
δ^{13}C DM[1]	−19.33 ± 0.03	−20.49 ± 0.05***	−19.43 ± 0.04	−20.49 ± 0.03***	D: < 0.001 A: NS D × A: NS
δ^{13}C glycogen	−20.74 ± 0.12	−21.89 ± 0.12***	−20.92 ± 0.12	−21.73 ± 0.13***	D: < 0.001 A: NS D × A: NS
δ^{13}C lipid	−24.45 ± 0.18	−25.08 ± 0.08**	−24.20 ± 0.06	−25.09 ± 0.05***	D: < 0.001 A: NS D × A: NS
δ^{15}N protein	12.56 ± 0.06	11.81 ± 0.10**	12.74 ± 0.02†	11.92 ± 0.07***	D: < 0.001 A: NS D × A: NS
$\Delta\delta^{15}$N DM[1]	3.42 ± 0.07	3.88 ± 0.08***	3.52 ± 0.06	3.86 ± 0.07***	D: < 0.001 A: NS D × A: NS

*Data are presented as the mean ± SEM (n = 12). The p-values of the two-way ANOVA analysis are displayed in the right column. D, diet; A, activity; D×A, interaction; n.s., p-value > 0.05. Pairwise comparisons were assessed by unpaired Student's t-test. **p < 0.01 and ***p < 0.001 between diet groups; †p < 0.05 between physical activity groups. [1]DM, dry matter. $\Delta\delta^{15}$N, nitrogen fractionation, was calculated based on the δ^{15}N DM measured for both diets, which were 8.07 ± 0.6 and 6.97 ± 0.09 for the HP and HE diets, respectively.*

significant at $p < 0.05$. Data were analyzed using IBM SPSS version 25 (IBM Corp., Armonk, NY, United States).

RESULTS

Somatic Parameters and Muscle Composition

Table 3 shows the results for somatic parameters. Diet type affected final body weight, CF, and SGR, with an interaction being present for the two first parameters. The HP diet group presented a significantly higher body weight and lower CF than the HE diet group. Swimming activity significantly increased body weight in the HE diet group, with observed differences between the two groups disappearing. The CF also decreased with the HE diet, but differences were maintained. The final body weight of the PIT-tagged fish was comparable to the body weight based on the total biomass of each group (VS: HP = 17.90 ± 0.31, HE = 15.95 ± 0.38, $p < 0.001$; SS: HP = 16.12 ± 0.48, HE = 15.94 ± 0.48, ns). Diet affected the HSI, with this being significantly higher in HE group. The MFI was also significantly higher in the HE group under the SS condition, because significant interaction was observed.

Regarding muscle composition (**Table 4**), diet affected the lipid content of white muscle (significantly higher in the HE diet group) and swimming activity significantly decreased muscle glycogen content, irrespective of diet. Protein content was not affected by any factor. The isotopic composition (δ^{13}C and δ^{15}N) of muscle and its reserves was significantly different between the HP and HE diet groups, regardless of the swimming condition (**Table 4**). The δ^{15}N and δ^{13}C values of bulk muscle, and its components (δ^{15}N-protein, δ^{13}C-glycogen, and δ^{13}C-lipid), were significantly lower in the HE diet group compared with the HP diet group (δ^{15}N: HP diet, 8.07 ± 0.06; HE diet, 6.97 ± 0.09). Nitrogen fractionation ($\Delta\delta^{15}$N) was significantly higher in the HE diet group, indicating a higher protein turnover in fish fed the HE diet, but the difference between the two diet groups decreased under SS conditions ($\Delta\delta^{15}$N HP – Δ^{15}N HE: 3.42–3.88 = −0.46 in VS; 3.52–3.86 = −0.34 in SS) because of greater fractionation in the HP diet group.

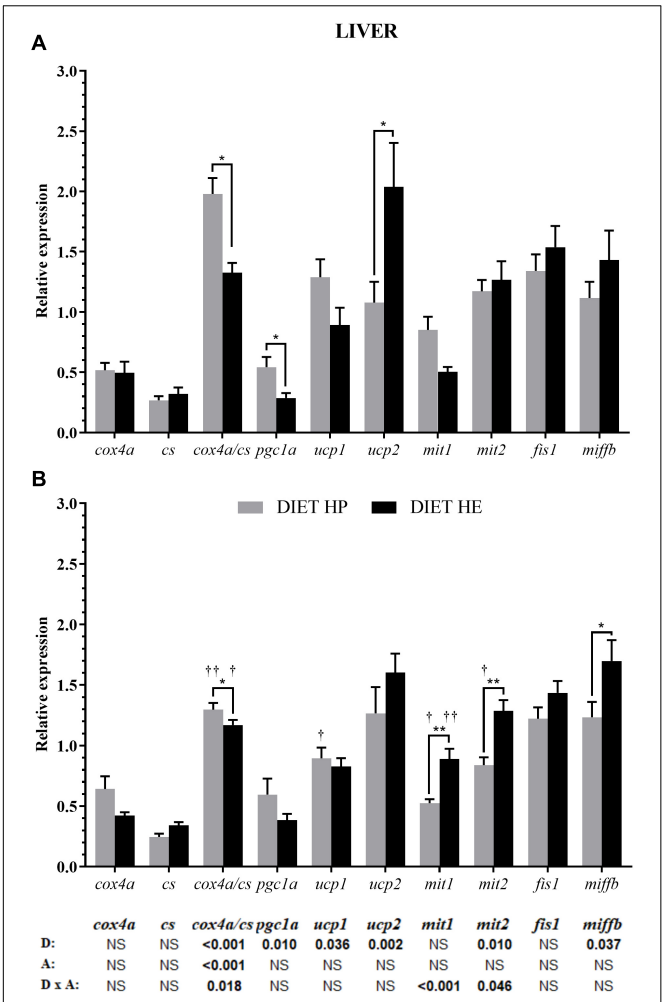
Gene Expression

Liver

Diet modified the expression of genes for mitochondrial proteins related to energy metabolism (*ucps*, *pgc1a, and cox4a/cs* ratio) and mitochondrial fusion/fission (*mit2* and *miffb*) (bottom of **Figure 1**). Despite the lack of significant differences in *cox* and *cs* gene expression by diet or activity, minor changes in these genes caused significant effects over their ratio (*cox4a/cs*) by both factors as well as their interaction. Interestingly, the uncoupling proteins *ucp1* and *ucp2* displayed inverse expression patterns. The gene expressions of *pgc1a* and *ucp1* were higher in fish fed the HP diet, while *ucp2* expression was higher in fish fed the HE diet (**Figure 1A**). Lower *mit2* and *mffb* gene expressions were found in the HP diet group, but under SS conditions, a significant interaction was found for *mit1* and *mit2* expressions, resulting in higher expression in the HE diet group (**Figure 1B**).

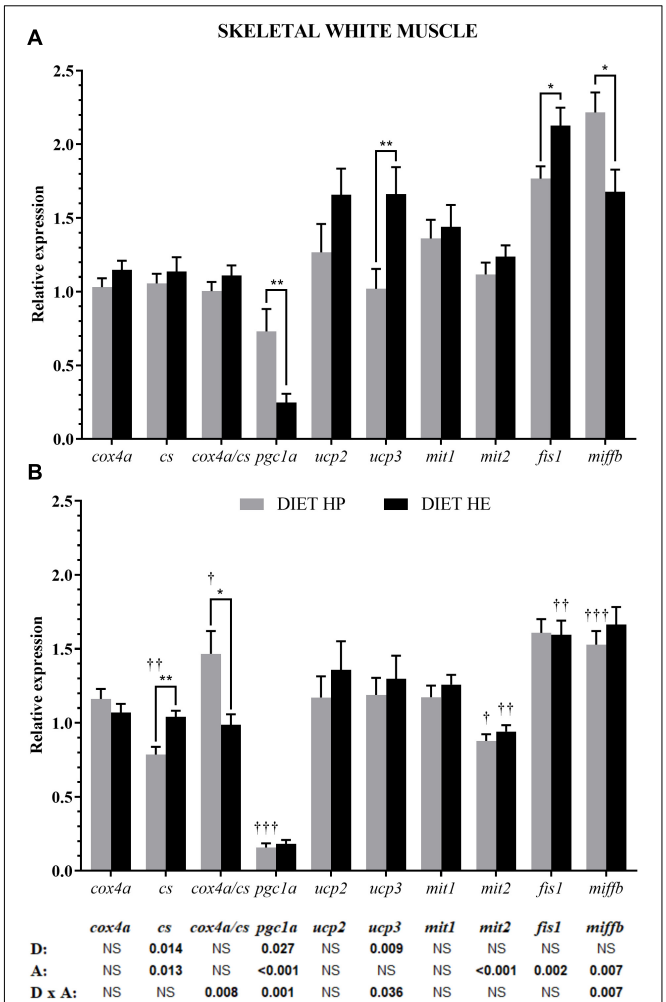
White and Red Skeletal Muscle

In white muscle, both diet and swimming activity induced changes in the expression of genes related to energy metabolism, but only activity modified the fusion/fission proteins (bottom of **Figure 2**). Diet modified the expressions of *cs, pgc1a,* and *ucp3*. Under VS conditions, *pgc1a* expression in fish fed the HP diet was significantly higher than in those fed the HE diet, whereas the reverse pattern was true of *ucp3* expression (**Figure 2A**). The expressions of *cs* and *pgc1a*, as well as three proteins related to fusion (*mit1*) and fission (*fis1* and *miffb*), were also modified by activity (**Figure 2B**). Interaction was observed for *cox/cs* ratio, *pgc1a*, *ucp3*, and *miffb*. Activity significantly decreased the expressions of *cs* and *pgc1a* in the



	cox4a	cs	cox4a/cs	pgc1a	ucp1	ucp2	mit1	mit2	fis1	miffb
D:	NS	NS	**<0.001**	**0.010**	**0.036**	**0.002**	NS	**0.010**	NS	**0.037**
A:	NS	NS	**<0.001**	NS	NS	NS	NS	NS	NS	NS
D x A:	NS	NS	**0.018**	NS	NS	NS	**<0.001**	**0.046**	NS	NS

FIGURE 1 | Relative gene expression of the enzymes in energy metabolism, markers of mitochondrial biogenesis and markers of mitochondrial dynamics in the liver of gilthead seabream fed the HP or HE diet under conditions of voluntary swimming **(A)** or sustained swimming **(B)**. Data are shown as the mean ± SEM (n = 12 for VS and n = 16 for SS conditions). The factorial statistical analysis was assessed by two-way ANOVA and the p-values of the diet (D), swimming activity (A) and the interaction (D × A) are displayed at the bottom of the figure. Pairwise comparisons were assessed by unpaired Student's t-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ between diet groups (HP AND HE); $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ between swimming activity groups (VS and SS).



	cox4a	cs	cox4a/cs	pgc1a	ucp2	ucp3	mit1	mit2	fis1	miffb
D:	NS	**0.014**	NS	**0.027**	NS	**0.009**	NS	NS	NS	NS
A:	NS	**0.013**	NS	**<0.001**	NS	NS	NS	**<0.001**	**0.002**	**0.007**
D x A:	NS	NS	**0.008**	**0.001**	NS	**0.036**	NS	NS	NS	**0.007**

FIGURE 2 | Relative gene expression of the enzymes in energy metabolism, markers of mitochondrial biogenesis, and markers of mitochondrial dynamics in the white skeletal muscle of gilthead seabream fed the HP or HE diet under conditions of voluntary swimming **(A)** or sustained swimming **(B)**. Data are shown as the mean ± SEM (n = 12 for VS and n = 16 for SS conditions). The factorial statistical analysis was assessed by two-way ANOVA and the p-values of the diet (D), swimming activity (A) and the interaction (D × A) are displayed at the bottom of the figure. Pairwise comparisons were assessed by unpaired Student's t-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ between diet groups (HP and HE); $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ between swimming activity groups (VS and SS).

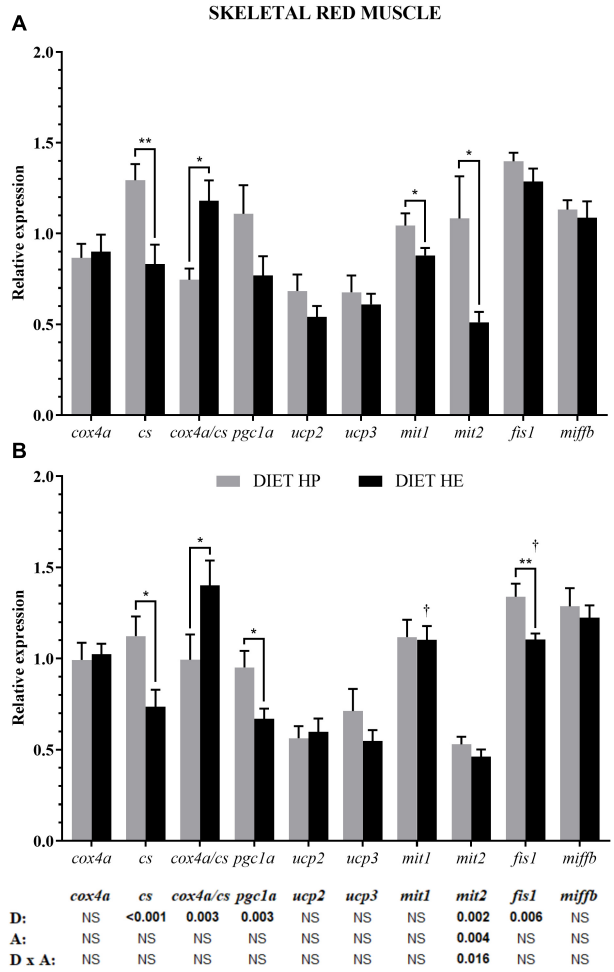
HP group, explaining the observed interaction for these and *ucp3* in the HE group. Activity significantly decreased *mit2* expression in both diet groups, *fis1* expression in the HE group, and *miffb* expression in the HP group; this latter gene showed interaction.

In red muscle, diet modified *cs* and *pgc1a* expression related to energy metabolism, *mit2* expression related to fusion, and *fis1* expression related to fission. Activity significantly modified the expressions of *mit2* (bottom of **Figure 3**) and *fis1* ($p < 0.052$). The HP group had significantly higher *pgc1a* and *cs* expressions, and a lower *cox4a/cs* ratio, compared with the HE group (**Figures 3A,B**). Notably, *mit2* expression was significantly higher in the HP group, but the difference was lost with activity (**Figure 3B**) due to interaction. Because of the swimming activity, a significant reduction in *fis1* expression was found in the HE group under the VS condition, being significantly lower than in the HP group (**Figure 3B**).

Protein Expression

White Skeletal Muscle

Diet only modified the protein expression of PGC1a, and there was an interaction with activity (bottom of the **Figure 4**). Under the VS condition, the HE group presented significantly higher levels of PGC1a, whereas under the SS condition, PGC1a levels were significantly decreased (i.e., comparable between the



	cox4a	cs	cox4a/cs	pgc1a	ucp2	ucp3	mit1	mit2	fis1	miffb
D:	NS	<0.001	0.003	0.003	NS	NS	NS	0.002	0.006	NS
A:	NS	NS	NS	NS	NS	NS	NS	0.004	NS	NS
D x A:	NS	NS	NS	NS	NS	NS	NS	0.016	NS	NS

FIGURE 3 | Relative gene expression of the enzymes in energy metabolism, markers of mitochondrial biogenesis and markers of mitochondrial dynamics in the red skeletal muscle of gilthead seabream fed the HP or HE diet under conditions of voluntary swimming **(A)** or sustained swimming **(B)**. Data are shown as the mean ± SEM (n = 12 for VS and n = 16 for SS conditions). The factorial statistical analysis was assessed by two-way ANOVA and the p-values of the diet (D), swimming activity (A) and the interaction (D × A) are displayed at the bottom of the figure. Pairwise comparisons were assessed by unpaired Student's t-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ between diet groups (HP and HE); $^{\dagger}p < 0.05$ between swimming activity groups (VS and SS).

two diet groups) (**Figure 4D**). Activity affected COX levels, and consequently, the COX/CS ratio changed significantly (**Figures 4A–C**). Both COX and CS levels, and their ratio, showed interactions (bottom of **Figure 4**). Under the VS condition, COX4 protein levels were significantly higher in the HE group. However, COX and CS levels decreased significantly in the HE group under SS conditions compared with VS conditions. Interaction was observed in UCP3 protein levels (**Figure 4F**).

Red Skeletal Muscle

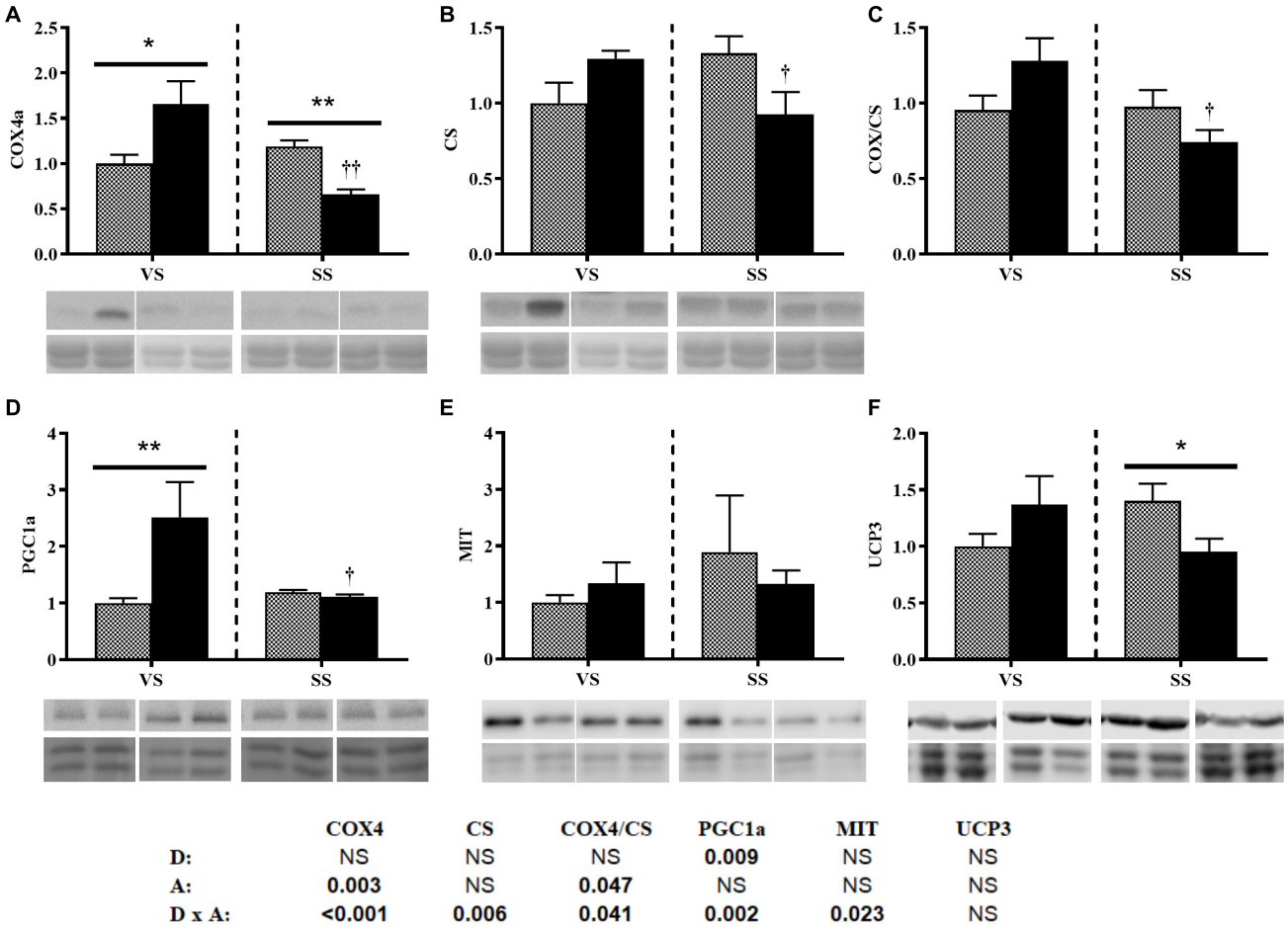
In red muscle, diet only affected COX4 levels, and this resulted in changes to the COX/CS ratio (bottom of **Figure 5**). Activity altered the levels of COX, CS (**Figures 5A–C**), and UCP3 (**Figure 5F**), related to energy metabolism. Diet and activity interacted for the three proteins (bottom of **Figure 5**). Under VS conditions, COX4 and CS levels, as well as the COX/CS ratio, were significantly higher and UCP3 levels were significantly lower in the HE group; by contrast, SS conditions significantly decreased these enzyme levels and increased UCP3 levels in this group. Activity led to increased MIT levels in the HP group, with these being significantly higher than in the HE group; an interaction was observed between the two factors (**Figure 5E**). PGC1a synthesis was not affected by diet or activity (**Figure 5D**).

DISCUSSION

Somatic Parameters and Muscle Composition

It has been shown for some fish species that sustained moderate exercise promotes growth and improves food conversion rates in several teleost fish species (Davison, 1997; Palstra and Planas, 2013; Blasco et al., 2015). However, the potential growth enhancement induced by this type of exercise is strictly related to the natural physical activity pattern of each species, showing effects that are more marked in pelagic fish species (Palstra and Planas, 2013). Recently, it has been postulated that the beneficial effects of exercise on growth are achieved when the swimming speed is close to the optimal swimming speed of each species (U*opt*) (McKenzie et al., 2020), with the feed intake covering all the energy costs of swimming. It has been established that the U*opt* of gilthead seabream juveniles weighing 25–45 g is 4.5 $BL{\cdot}s^{-1}$ (Palstra et al., 2020). As the fish in the present study were 2–4 g, their U*opt* should be around 5 $BL{\cdot}s^{-1}$ (0.35–0.4 $m{\cdot}s^{-1}$). In the present study, sustained moderate-to-low-intensity exercise at a speed near 50% of the U*opt* was used (as this speed is considered more practical for fish farming) to determine if the different proportions of protein and lipid in commercial diets met the increased metabolic demands of swimming activity without compromising growth.

The different composition of the diets used (HP and HE diets) was reflected in the growth and body composition of the fish fed each diet. Thus, the lower protein and higher lipid content of the HE diet group induced lower growth, increased liver size and elicited a higher percentage of muscle lipid when compared to the HP diet group. Long-term high dietary fat intake provokes a gradual deposition of lipids in several organs of this species, including the liver (Company et al., 1999). Moreover, the negative effect of high lipid diets on growth has been reported previously in gilthead seabream (Company et al., 1999), as well as in other species like dentex (Espinós et al., 2003) and sea bass (Peres and Oliva-Teles, 1999). However, improved growth occurred with the HE diet, meaning that no differences in growth were observed between the two diet groups when fish were subjected to sustained swimming in the present study. The HE group did maintain a significantly higher HSI, but this was probably due to increased fat deposition in this organ. Our group previously observed improved growth in seabream fingerlings fed a diet with a similar composition to that of the HP diet, but swimming at 5 BL/s^{-1} (Blasco et al., 2015). Furthermore,

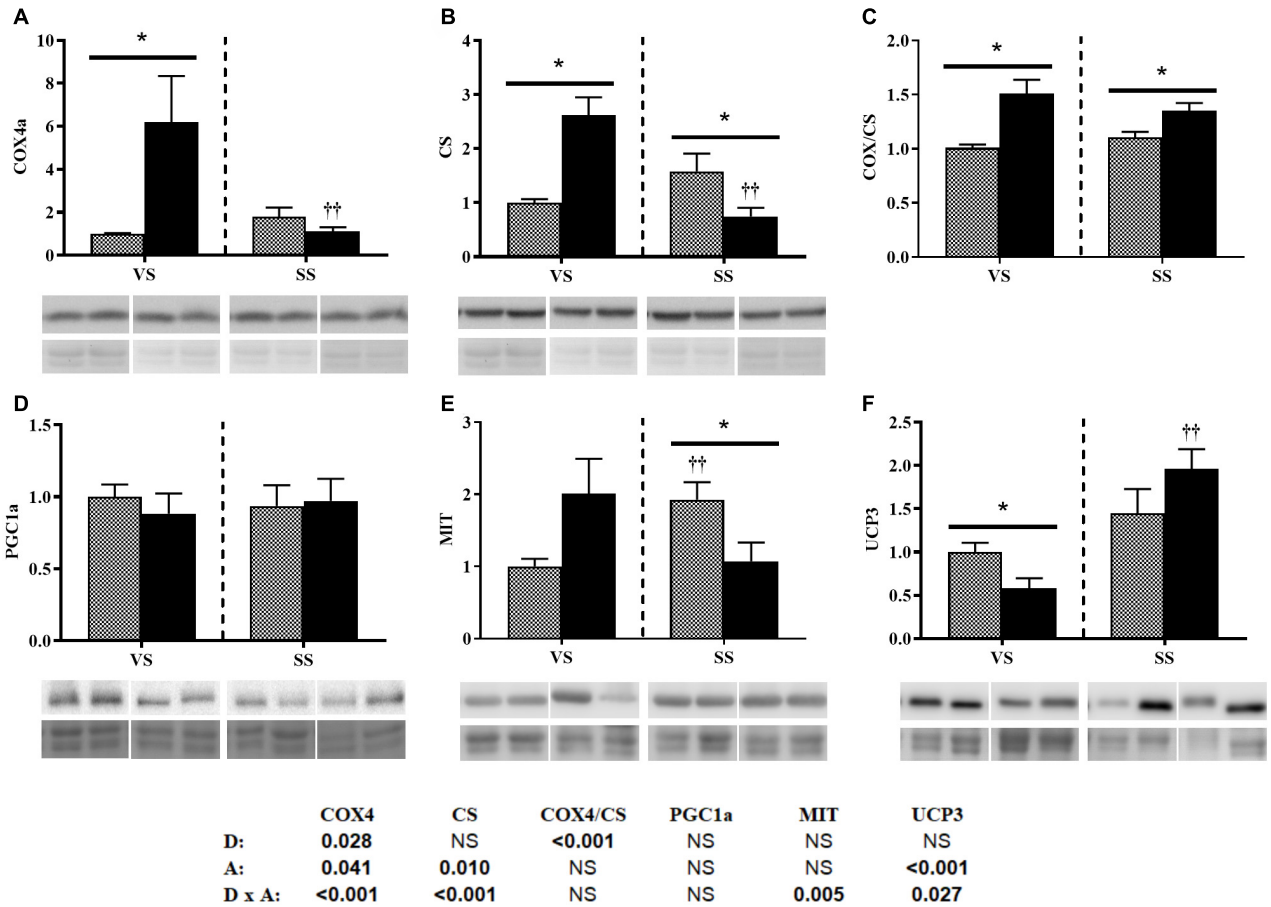


	COX4	CS	COX4/CS	PGC1a	MIT	UCP3
D:	NS	NS	NS	**0.009**	NS	NS
A:	**0.003**	NS	**0.047**	NS	NS	NS
D x A:	**<0.001**	**0.006**	**0.041**	**0.002**	**0.023**	NS

FIGURE 4 | Representative western blot and densitometric analysis of the protein levels of COX4 **(A)**, CS **(B)**, the COX/CS ratio **(C)**, PGC1a **(D)**, MIT1/2 **(E)**, and UCP3 **(F)** in the white skeletal muscle of gilthead seabream fed the HP or HE diet under conditions of **(A)** voluntary swimming (VS) or **(B)** sustained swimming (SS). Bands were normalized to their Revert^{TM} total protein staining (the corresponding well is shown). To eliminate intermembrane variability, the relative intensity of each specific band was normalized by the geometric mean of the intensity of that of the VS HP diet group of the corresponding membrane. For COX4a and CS, the blots were performed from the same membranes which were split before the primary antibody incubation. Data are shown as the mean ± SEM ($n = 6$). The factorial statistical analysis was assessed by two-way ANOVA and the p-values of the diet (D), swimming activity (A) and the interaction (D × A) are displayed at the bottom of the figure. Pairwise comparisons were assessed by unpaired Student's t-test. *$p < 0.05$ and **$p < 0.01$ between diet groups (HP and HE); †$p < 0.05$ and ††$p < 0.01$ between swimming activity groups (VS and SS).

we previously reported a reduction in the lipid content of white muscle in exercised juveniles fed a high carbohydrate/low protein diet swimming at 1.5 BL/s^{-1} (Martin-Perez et al., 2012). These results indicated that gilthead seabream adapts their metabolism to the type of activity they are performing and their nutritional regime to prioritize the best fuel, as reviewed by Magnoni and coworkers (Magnoni et al., 2013b). The fuel selection depends on the type of exercise, with lipids the preferred fuel at low or medium velocities (i.e., aerobic training) and carbohydrates the preferred fuel as the velocity (and the energy demand) increases (Richards et al., 2002). The glycogen content of white muscle was reduced by the SS condition, regardless of the diet. These results corroborate the findings observed in the exercised juveniles of this species (Sánchez-Gurmaches et al., 2013), confirming that gilthead seabream subjected to exercise increases glucose oxidation to meet the high energy demands (Felip et al., 2013).

The isotopic composition of white muscle reveals clear differences in the effect of diet by reflecting the different proportions of vegetable-type components from a given feed (Beltrán et al., 2009; Martín-Pérez et al., 2011). Then, the $\delta^{15}N$ values were lower in the HE diet group than in the HP diet group, both in the total muscle and in its protein fraction, reflecting the lower $\delta^{15}N$ values of the HE diet probably due to the higher proportion of vegetable protein. The reduced ^{13}C values in the lipid fraction of the HE diet group were due to the high lipid content in this tissue, which is in agreement with the findings of previous studies (Martín-Pérez et al., 2011; Martin-Pérez et al., 2013). Nitrogen dietary fractionation ($\Delta\delta^{15}N$) is a good marker of protein balance because it reflects the protein



	COX4	CS	COX4/CS	PGC1a	MIT	UCP3
D:	0.028	NS	<0.001	NS	NS	NS
A:	0.041	0.010	NS	NS	NS	<0.001
D x A:	<0.001	<0.001	NS	NS	0.005	0.027

FIGURE 5 | Representative western blot and densitometric analysis of the protein levels of COX4 **(A)**, CS **(B)**, the COX/CS ratio **(C)**, PGC1a **(D)**, MIT1/2 **(E)**, and UCP3 **(F)** in the red skeletal muscle of gilthead seabream fed the HP or HE diet under conditions of **(A)** voluntary swimming (VS) or **(B)** sustained swimming (SS). Bands were normalized to their Revert^{TM} total protein staining (the corresponding well is shown). To eliminate intermembrane variability, the relative intensity of each specific band was normalized by the geometric mean of the intensity of that of the VS HP diet group of the corresponding membrane. For COX4a and CS, the blots were performed from the same membranes which were split before the primary antibody incubation. Data are shown as the mean ± SEM (n = 6). The factorial statistical analysis was assessed by two-way ANOVA and the p-values of the diet (D), swimming activity (A) and the interaction (D × A) are displayed at the bottom of the figure. Pairwise comparisons were assessed by unpaired Student's t-test. $^{*}p < 0.05$ between diet groups (HP and HE); $^{\dagger\dagger}p < 0.01$ between swimming activity groups (VS and SS).

turnover and the protein retention efficiency (Martínez Del Rio et al., 2009; Martín-Pérez et al., 2011). Interestingly, fish fed the HP diet exhibited a lower fractionation than those fed the HE diet, indicating that less transformations of the protein from the diet (less protein turnover) were needed before being deposited into the muscle proteins (Gaye-Siessegger et al., 2003, 2007). The lower protein content of the HE diet with respect to the HP diet could contribute to this higher fractionation. In fact, a negative correlation between the protein percentage of the diet and nitrogen fractionation has been established for this species (Martin-Pérez et al., 2013). The lower growth of the HE diet group under VS conditions could reflect this higher fractionation, which is in agreement with that observed in gilthead seabream fed different proportions of plant proteins (Beltrán et al., 2009).

The SS condition did not modify the differences in isotopic composition of the diet or nitrogen fractionation of white muscle. Although the fractionation was different for each diet group under SS conditions, the final growth did not differ between the two groups and interaction was observed. This suggests different scenarios for each group. In the HE group, the greater use of dietary lipids for energy purposes might have induced a protein-sparing effect. In the HP diet group, the greater use of dietary protein for energy purposes might have resulted in less growth. This higher amino acid oxidation from the diet in the HP diet group under SS conditions is reflected in the higher nitrogen fractionation when compared to that under VS conditions, reducing the differences in this parameter between the two diet groups (VS: −0.46; SS: −0.34).

Mitochondrial Adaptation in the Liver

As a key tissue in regulating metabolism, the liver presented changes in the gene expression patterns of mitochondrial proteins related to energy metabolism and fusion/fission balance in response to diet and exercise. In the present study, the significant

decrease in the cox4/cs ratio in the HE group under VS conditions suggests that lipid oxidation was greater in this group. In agreement with this, LeMoine and coworkers (LeMoine et al., 2008) reported that the liver of goldfish fed a high-fat diet showed an increase in *cs* mRNA levels, but not in *cox* gene expression. Furthermore, Sánchez-Nuño and collegues (Sánchez-Nuño et al., 2018) demonstrated that gilthead seabream fed a high-fat diet showed an increase in the activity of CS, but not of COX. The COX/CS activity ratio indicates modifications of the mitochondrial surface/volume ratio (Ibarz et al., 2010; Martin-Perez et al., 2012), which reflects shifts in the use of nutrients. Thus, the significant decrease in the COX/CS ratio in the livers of fish fed a HE diet showed a clear metabolism shifting toward increased lipid oxidation in this group, which reflects that this tissue possesses a good plasticity for modifying the nutrient utilization depending on the diet composition. As a consequence of this shift, we observed increased reactive oxygen species (ROS) levels (data not shown, Sánchez-Moya et al., 2017), which could explain the increase in *ucp2* expression to uncouple oxidative phosphorylation. UCPs have a role as redox sensors that can serve to attenuate the effects of the ROS (Rial and Zardoya, 2009). The expression of *ucp1* increases when lipid flux to the liver increases in carp (Jastroch et al., 2005) and in gilthead seabream (Bermejo-Nogales et al., 2010). In our study, *ucp1* and *ucp2* showed different expression profiles, suggesting different roles of these proteins in achieving a balance between uncoupling activity and oxidative capacity. Although the role and tissue specificity of UCPs in gilthead seabream have been investigated, some overlap and redundancy might occur among UCPs, especially in relation to UCP2 (Bermejo-Nogales et al., 2010, 2014). High mRNA levels of *ucp2* and lower mRNA levels of *pgc1a* in the liver of the HE diet group under VS conditions are consistent with that observed in mammals presenting metabolic syndrome with increased lipid accumulation and ROS production (Rius-Pérez et al., 2020). By contrast, the higher mRNA levels of *pgc1a* in the HP diet group indicated a higher oxidation rate of dietary protein, yielding a lower energy output and explaining the lower expression of *ucp2.*

Under SS conditions, energy demands increased and the cox/cs ratio for mitochondrial energy proteins was affected with an interaction between diet and activity. Compared with VS conditions, a significant decrease in the cox/cs ratio, notably in the HP group, was associated with a reduced cox/cs ratio between the two diet groups. In other energy demanding conditions such as hypothermal stress, the COX/CS ratio has been reported to decrease, usually due to the increased expression and/or activity of CS (Lucassen et al., 2003; Eckerle et al., 2008; Ibarz et al., 2010; Orczewska et al., 2010; Sánchez-Nuño et al., 2018). Although swimming activity did not modify *ucp2* gene expression, no differences in *ucp2* expression were observed between the two diet groups, indicating a better balance between the TCA cycle and oxidative phosphorylation in the HE group under SS compared with VS conditions.

Diet induced changes in the expression of mitofusin, *mit 2*, and fission protein, *miffb*). No effect of swimming activity were observed in the expression of markers of mitochondrial dynamics. However, the significant interaction observed in the two mitofusins, *mit1* and *mit2* would indicate that the fusion processes are modulated by swimming activity depending of diet. In the present study, the simultaneous increase in the mRNA levels of *mit1*, *mit2* and *miffb* in the HE diet group under SS conditions indicated an increased mitochondrial turnover requiring both fusion and fission processes. This new balance represents an adaptation of the mitochondrial network in HE group toward a much higher oxidative capacity of the liver. In general, simultaneous mitochondrial fusion and fission processes indicate an improvement in the health status of a mitochondrial population (Scorrano, 2013), but the specific effects of exercise involve high expression of *mit1* and *mit2*, promoting the formation of a stronger network of healthy mitochondria (Liesa and Shirihai, 2013; Scorrano, 2013; Gonçalves et al., 2014, 2015). Interestingly, rats fed a high fat diet developed impaired mitochondrial function related to fatty deposition in liver, but endurance training was noted to prevent this pathological process (Gonçalves et al., 2014, 2015).

In mammals, it is postulated that mitofusins are directly regulated by *pgc1a* (Chandhok et al., 2018). We found that *pgc1a* expression was modulated by diet, being higher in the HP group and lower in the livers of fish in the HE group. The lower levels in the HE group may have indicated that this transcription factor was previously activated to promote *mit1*, *mit2*, and *miffb* upregulation before undergoing negative feedback. However, the close relationship between *pgc1a* and the downstream genes was not observed in the livers of fish under VS conditions, suggesting that *pgc1a* may not be the only regulator of these molecules in fish. The role of *pgc1a* in fish is currently under discussion [reviewed by Bremer and coworkers (Bremer et al., 2016)].

Thus, diet composition modified the liver oxidative capacity, because a higher content of lipids in the diet induced the uncoupling of the respiratory chain and decreased oxidation. Under SS conditions, uncoupling disappeared, lipid oxidation increased, and the mitochondria fusion process was promoted.

Mitochondrial Adaptation in White and Red Skeletal Muscle

White muscle mitochondrial proteins related to energy metabolism (i.e., CS, UCP3, and PGC1a) were affected by diet. The higher lipid concentration in the muscle of fish fed a HE diet, together with the "oxidative overload," modified the mitochondrial protein profile in comparison to the HP group, showing higher *cs, ucp3*, and lower *pgc1a* expressions. The higher oxidative state of white muscle would explain the increase in *ucp3* mRNA levels and its higher protein expression in this tissue, preventing the overproduction of ROS (Bermejo-Nogales et al., 2014). The differences between the gene and protein expression of PGC1a suggested that changes were already induced by the diet in the first weeks of feeding in the HE diet group. Thus, in the earlier weeks of the experiment, the high energy level of the HE diet might have triggered AMPK activation, which would then induce *pgc1a* expression to increase the oxidative capacity of the white muscle. The subsequent increase in the protein expression of PGC1a observed in the muscle would induce a strong negative feedback regulation on its gene expression, which is consistent with that observed in goldfish fed a high-fat

diet (LeMoine et al., 2008). This regulation of *pgc1a* has been observed in mammals fed high-fat diets [in mice as reported by Bonnard and coworkers (Bonnard et al., 2008) and in humans as reported by Sparks and coworkers (Sparks et al., 2005)]. The lack of correlation between *cs* gene and CS protein expression illustrated the importance of post-transcriptional processes regulating this protein (Bustin et al., 2009).

Sustained moderate exercise induces a more aerobic phenotype in the white muscle of gilthead seabream (Martin-Perez et al., 2012; Blasco et al., 2015) and other species (Johnston and Moon, 1980; McClelland et al., 2006; Anttila et al., 2010), and this phenotype should be reflected by changes in the gene and protein expression of COX and CS enzymes. Swimming activity modified the expression of these metabolic proteins and related proteins (e.g., UCPs and PGC1a.) In this scenario of greater energy demand, the gene expression and protein expression of mitochondrial proteins associated with energy metabolism changed differently in white muscle depending on the diet (most showed interaction). The downregulation of cs expression in the HP group under SS conditions increased the cox/cs ratio when fed a HE diet; by contrast, reduced COX4 protein levels in the HE group decreased the COX/CS ratio. The significant decrease in *pgc1a* expression in HP group under the SS condition could be involved in *cs* downregulation. These results indicate that there is increased protein oxidation in HP group and increased lipid oxidation in the HE group. Interestingly, the increased *ucp3* expression found in the white muscle of fish fed a HE diet disappeared when shifting from VS to SS conditions. Activity appears to decrease the oxidative uncoupling associated with the HE diet and increases aerobic ATP production, consistent with observation in endurance trained humans (Russell et al., 2003; Schrauwen-Hinderling et al., 2003). There are no data about *ucp3* regulation in fish subjected to exercise, but in mammals, an inverse relationship has been observed between UCP3 and PGC1a, the latter being this a transcription factor stimulated by exercise in mammals but having an unclear role in fish (McClelland, 2012; Bremer et al., 2016). In zebrafish, a transient increase in PGC1a expression has been observed to occur at the beginning of exercise (LeMoine et al., 2010). It needs to be demonstrated whether such a response occurred in the first days of exercise in gilthead seabream. Diet did not modify the gene expression of fusion/fission proteins, but activity decreased the expression of *mit2* in both diet groups and of the *fis1* and *miffb* (related the fission processes), changing the balance between the fusion and fission processes in the VS condition. A significant reduction in *fis1* gene expression due to activity in HE group could indicate that the mitochondrial machinery works more efficiently with a higher lipid content during exercise. In mammals, lipid accumulation disrupts mitochondrial homeostasis (Lowell and Shulman, 2005), and is accompanied by a greater increase in fission compared to fusion processes in skeletal muscle (Holmström et al., 2012; Huertas et al., 2019). This increases mitochondrial degradation by mitophagy, promoting mitochondrial turnover (Chandhok et al., 2018).

It is interesting to note how the expression profile of genes and mitochondrial proteins in red muscle differed from that of white muscle between the two diet groups. These differences would be due to the nutrients that the muscles prefer to use to obtain energy, with red muscle preferring lipids and white muscle preferring carbohydrates (Magnoni et al., 2013b). Moreover, red muscle is an oxidative tissue with a high proportion of mitochondria. COX4 and CS activities are 3 and 7 times higher, respectively, in red muscle than in white muscle in gilthead seabream (Martin-Perez et al., 2012). Diet modified *cs* expression, and consequently the *cox/cs* ratio, as well as *pgc1a* expression, with *cs* and *pgc*1a expression being lower in HE group. The higher protein expression of CS and COX in this group would indicate that the high lipid content of HE diet did not exceed the oxidative capacity of this tissue. The downregulation of the *cs* gene expression might have occurred as a response to the high increase of its protein expression. Moreover, the significantly higher COX/CS ratio, both at the gene and protein level in HE diet group, indicated increased lipid oxidation in this tissue. In contrast to white muscle, red muscle has a lipid level that is 10 times higher in this species (Martin-Perez et al., 2012). In red muscle, which is adapted to lipid oxidation, no changes in the expression of *ucp3* gene or its protein induced by diet were observed. This indicated that the high lipid content of HE diet not exceed the oxidative capacity of this tissue.

The activity did not provoke changes in the expression of these genes, *cs* and *cox*. But the increase in UCP3 levels in the red muscle of the HE group under SS conditions suggests that these enzymes are regulated to adapt to a high lipid overload, such as that observed in the skeletal and cardiac muscle of mammals (Nabben et al., 2014). The impact of swimming on mitochondrial proteins related to energy metabolism was greater in the white muscle compared with the red muscle of gilthead seabream. It agrees with a previous study in our group showing that the red muscle workload under sustained exercise is reduced, but that increased white muscle activity compensates for this (Martin-Perez et al., 2012). It is interesting to know that the composition of the diet modifies more than the activity, the balance between the processes of fusion and fission, but more research is needed to understand the meaning of the changes.

The composition of the diet affected the oxidative capacities of both white and red muscles, increasing their oxidative rates when fish were fed a higher lipid content, especially in the red muscle. Increased lipid oxidation eventually caused uncoupling in white muscle, but not in red muscle. The SS condition was able to reverse these changes, promoting the uncoupling in red muscle and eliminating it in white muscle. In this sense, the decrease in the *fis1* gene expression in white muscle would indicate the improvement of mitochondrial health by moderate, sustained exercise.

Conclusion

In this study, we show how changes in diet composition (lower protein/lipid ratio) and average physical activity (voluntary swimming versus sustained exercise) affected the body growth of juvenile sea bream differently. Lower protein/lipid ratio affected growth through unbalanced availability and use of nutrients, especially indicated by changes in key mitochondrial proteins

related to energy metabolism, mitochondrial turnover, and biogenesis in the liver and white and red and white muscles. The beneficial effects of sustained swimming reversed the altered mitochondrial functions by increasing the use of lipids, along with a protein sparing effect of enhanced growth. From an applied point of view, a cheaper (reducing the protein/lipid ratio) and sustainable (reducing nitrogenous waste) diet can be used in combination with moderate swimming to preserve fish growth, even in the period of fast growth of juvenile sea bream, and to improve the welfare of fish.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Ético de Experimentación Animal de la Universidad de Barcelona (CEEA 663/13 and DAAM 7644).

AUTHOR CONTRIBUTIONS

JB, JF-B, and JG conceived and designed the study. MP-A, EV, AS-M, and IG-P performed the experiment and laboratory analysis. MP-A and JB analyzed the data and drafted the manuscript. All authors contributed to the writing and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.678985/full#supplementary-material

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ARTICLE 4

Diet and Exercise Modulate GH-IGFs Axis, Proteolytic Markers and Myogenic Regulatory Factors in Juveniles of Gilthead Sea Bream (*Sparus aurata*)

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Article

Diet and Exercise Modulate GH-IGFs Axis, Proteolytic Markers and Myogenic Regulatory Factors in Juveniles of Gilthead Sea Bream (*Sparus aurata*)

Miquel Perelló-Amorós [1,†], Isabel García-Pérez [1,†], Albert Sánchez-Moya [1], Arnau Innamorati [1], Emilio J. Vélez [2], Isabel Achaerandio [3], Montserrat Pujolà [3], Josep Calduch-Giner [4], Jaume Pérez-Sánchez [4], Jaume Fernández-Borràs [1], Josefina Blasco [1] and Joaquim Gutiérrez [1,*]

1 Departament de Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain; miquelperelloamoros@gmail.com (M.P.-A.); isabelgarcia@ub.edu (I.G.-P.); alsanchezmo@ub.edu (A.S.-M.); arnauic98@gmail.com (A.I.); jaume.fernandez@ub.edu (J.F.-B.); jblasco@ub.edu (J.B.)

2 Université de Pau et des Pays de l'Adour, E2S UPPA, INRAE, UMR1419 Nutrition Métabolisme et Aquaculture, F-64310 Saint-Pée-sur-Nivelle, France; emilio-jose.velez-velazquez@inrae.fr

3 Department d'Enginyeria Agroalimentària i Biotecnologia, Escola Superior d'Agricultura de Barcelona, Universitat Politècnica de Catalunya BarcelonaTech, 08860 Barcelona, Spain; maria.isabel.achaerandio@upc.edu (I.A.); montserrat.pujola@upc.edu (M.P.)

4 Nutrigenomics and Fish Growth Endocrinology, Institute of Aquaculture Torre de la Sal (CSIC), Ribera de Cabanes, 12595 Castellón, Spain; j.calduch@csic.es (J.C.-G.); jaime.perez.sanchez@csic.es (J.P.-S.)

* Correspondence: jgutierrez@ub.edu; Tel.: +34-934-021-532

† These authors contributed equally to this work.

Simple Summary: The effects of exercise and diet on growth markers were analyzed in gilthead sea bream juveniles. Under voluntary swimming, fish fed with a high-lipid diet showed lower growth, growth hormone (GH) plasma levels, flesh texture, and higher expression of main muscle proteolytic markers than those fed with a high-protein diet. However, under sustained exercise, most of the differences disappeared and fish growth was similar regardless of the diet, suggesting that exercise improves nutrients use allowing a reduction of the dietary protein, which results in an enhanced aquaculture production.

Abstract: The physiological and endocrine benefits of sustained exercise in fish were largely demonstrated, and this work examines how the swimming activity can modify the effects of two diets (high-protein, HP: 54% proteins, 15% lipids; high-energy, HE: 50% proteins, 20% lipids) on different growth performance markers in gilthead sea bream juveniles. After 6 weeks of experimentation, fish under voluntary swimming and fed with HP showed significantly higher circulating growth hormone (GH) levels and plasma GH/insulin-like growth-1 (IGF-1) ratio than fish fed with HE, but under exercise, differences disappeared. The transcriptional profile of the GH-IGFs axis molecules and myogenic regulatory factors in liver and muscle was barely affected by diet and swimming conditions. Under voluntary swimming, fish fed with HE showed significantly increased mRNA levels of *capn1*, *capn2*, *capn3*, *capns1a*, *n3*, and *ub*, decreased gene and protein expression of Ctsl and Mafbx and lower muscle texture than fish fed with HP. When fish were exposed to sustained exercise, diet-induced differences in proteases' expression and muscle texture almost disappeared. Overall, these results suggest that exercise might be a useful tool to minimize nutrient imbalances and that proteolytic genes could be good markers of the culture conditions and dietary treatments in fish.

Keywords: aerobic training; muscle remodeling; endocrine regulation; hypercaloric-diets; fillet quality

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1. Introduction

One of humanity's greatest challenges is feeding a constantly growing population, in a situation in which the availability of natural resources is limited and respect for ecosystems

must be a priority. In this context, the aquaculture sector can help face this demand by providing aquatic products like fish, one of the healthiest sources of high-quality protein, fat, vitamins, and oligo elements [1]. However, there is still a need to develop the sector towards a better sustainability combined with the improvement of fish growth and product quality. In this sense, the reduction of dietary protein was a priority in many nutritional studies since it is the most expensive component in aquafeeds, its catabolism is the main source of water nitrogen loading [2–5], and the ecological impact (overfishing and habitats destruction) that the use of fish components accounts. The maintenance and handling of fish during rearing is another area in which improvements can be made, and in this regard, the effects of exercise as a tool to increase growth rate and flesh quality were widely studied in different species [4,6–16]. Moderate and sustained swimming results in clear benefits in total fish growth, muscle structure, and both metabolic and endocrine status [4,6,9–14]. Interestingly, exercise changes nutrient requirements and utilization, and this activity may increase the inclusion of more sustainable nutrients in the diet, such as lipids or carbohydrates, which allow the reduction of nitrogen discharges and feeding costs. In fact, this study is an extension of a previous work which studied how swimming activity modulate the effects of different dietary protein/lipid ratios on growth rate, tissue composition, and energy metabolism in the same experimental fish [5]. In that study, it was reported that the lower protein/lipid ratio affected growth through unbalanced availability and use of nutrients, especially indicated by changes in key mitochondrial proteins related to energy metabolism; however, the sustained exercise counteracted most of these alterations.

Somatic growth in vertebrates is mainly regulated by the hypothalamic-pituitary axis through the growth hormone (GH) and the insulin-like growth factors (IGFs) system [17], and many authors reviewed its role in fish species [18–21]. In gilthead sea bream, changes in the GH-IGFs axis were studied through the seasonal cycle [22], life stages [23], nutritional status [24,25], as a response to environmental factors [26,27], or even after several weeks of sustained exercise [14,28]. All these works highlighted the important regulatory functions of those endocrine factors in this species. Once GH is secreted by the anterior pituitary gland, it is transported via the blood, reaching the target tissues in which it interacts with the corresponding membrane receptors (GHR-1 and GHR-2) to trigger its effects. Differential regulation of the GH-IGFs axis components by nutrients and environmental factors was reported at a transcriptional level in fish liver and muscle (reviewed by Pérez–Sánchez et al. [29]). In liver, GH stimulates the production and release of IGFs, which are subsequently sequestered by the IGF-binding proteins (IGFBPs) to modulate their distribution and bioavailability [20]. In gilthead sea bream, Tiago et al. [30] identified three splice variants of *igf-1* (*igf-1a*, *igf-1b*, and *igf-1c*) that were differentially expressed in tissues. They observed that *igf-1c* was the most expressed isoform in the hepatic tissue, suggesting an important systemic role for this splice variant. IGFs exert their effects through the IGF-1 receptor(IGF-1R) and the IFG-2 receptor (IGF-2R), which should be also considered to better understand how all these factors are implicated in growth control.

Besides the GH-IGFs axis, other important molecules are involved in fish muscle development, such as the proteolytic systems and the myogenic regulatory factors (MRFs) that contribute sequentially directing muscle fibers recycling and formation [31,32]. Proteolytic systems participate in protein degradation and amino acids recycling or catabolism [33,34], and are considered key regulatory factors controlling growth potential with a greater importance during periods of intensive growth (i.e., fingerlings vs. juveniles or adult fish) [13,35]. The main proteolytic systems include the calpains, the lysosomal cathepsins, and the ubiquitin-proteasome (UbP) system [32]. These proteolytic markers were characterized in gilthead sea bream and pointed out as useful molecules to detect muscle remodeling episodes that can affect muscle structure, tenderness, and flesh quality [13,35–37]. Moreover, they are essential, especially during exercise, since physical activity seems to affect protein turnover in skeletal muscle to restructure the myofilaments and prevent exercise-induced muscle damage, thus facilitating somatic growth [13]. Furthermore, the

exercise was shown to have different effects on the anterior and caudal muscle regions, reflecting the progress of remodeling through the muscle trunk [6,13].

Moreover, the MRFs are involved in muscle hyperplasia (i.e., the recruitment of new fibers) and hypertrophy (i.e., the increase in fiber size), which are processes that occur continuously during the whole fish life, as many species have indeterminate growth [38,39]. Some of these MRFs are crucial to control cell proliferation and muscle lineage determination [myogenic factor 5 (Myf5) and myogenic determination factor (Myod)]; lead to myoblasts fusion and differentiation (myogenin); or are responsible for myotubes maturation [myogenic regulatory factor 4 (Mrf4)] [36,39]. Besides MRFs, myostatin (Mstn) is also important in this myogenic developmental process since it negatively regulates myocytes proliferation and differentiation [40,41].

In this framework, the main objective of this work was to study whether sustained exercise could compensate a low dietary amount of protein balanced with a high lipid composition in juveniles of gilthead sea bream. To this end, changes in the GH-IGFs axis, proteolytic systems and MRFs will be evaluated at transcriptional and protein levels.

2. Materials and Methods

2.1. Experimental Design

Nine hundred and eighty gilthead sea breams (4.1 $\pm$ 0.1 g body weight) were obtained from a commercial hatchery (Piscimar SL, Burriana, Spain) and reared in the facilities of the Faculty of Biology (University of Barcelona) in a semiclosed recirculation system with a weekly renewal of 20–30%, at 23 $\pm$ 1 °C, a salinity of 35–37‰ and a photoperiod of 15 h light/9 h dark. The fish were randomly distributed between two 400 L and eight 200 L tanks at the same biomass density (1.5 kg $\times$ m^3). In the 400 L tanks, fish were kept in voluntary swimming conditions, while in the 200 L tanks, fish were forced to a sustained swimming. To achieve this sustained activity, a tangential laminar flow was created by placing a plastic column in the center of each tank and connecting the water inlet to a vertical tube with lateral holes. The initial flow speed was set at 2.5 body lengths (BL) $\times$ s^{-1}. Fish were fed with two different commercial diets provided by Skretting Spain SA (Burgos, Spain). Both diets differed mainly in protein and fat content (as illustrated in Table 1) and were named as high-protein (HP: 54% protein/15% lipid) or high-energy diet (HE: 50% protein/20% lipid). Each diet was used to feed half of the tanks, one of 400 L and four of 200 L, thus establishing four experimental conditions. Fish were fed with a ration of 5% of the total biomass of each tank divided in 3 meals per day. The pellet size was increased from 1.5 mm to 1.9 mm after three weeks of experiment, according to the larger size of the fish. Biometric parameters (weight and length) were determined at the beginning of the experiment, after three weeks, and at the end of the trial (6 weeks) for monitoring fish growth. Fish were fasted for 12 h before any manipulation and sampling. As previously reported [5], for the gene and protein expression analysis in the final sampling at week 6, 12 fish from each voluntary swimming tank and 4 fish from each exercise tank (16 fish for each diet) were properly anaesthetized (MS-222, Sigma-Aldrich, Tres-Cantos, Spain), measured, weighed and sacrificed by severing the spinal cord and then eviscerated. Blood from these fish and the fish used for proximal composition [5] was taken from caudal vessels using EDTA-Li (Sigma-Aldrich, Tres-Cantos, Spain) as an anticoagulant and centrifuged (13,000$\times$ *g*, 10 min, 4 °C) to separate the plasma, which was stored at −80 °C until further analysis. Samples of liver and both anterior and caudal regions of white muscle were collected and immediately frozen in liquid nitrogen and stored at −80 °C until being processed for the analysis of either gene or protein expression. Moreover, additional 10 fish from the voluntary swimming tanks and 4 fish from the sustained swimming tanks (16 for each diet) were equally sacrificed to obtain a piece of 1.5 $\times$ 1.5 cm of white muscle that was extracted from the anterior-dorsal region, bagged, and kept on ice for the evaluation of texture, as explained below.

Table 1. Composition of diets used in experimental trial.

Item	HP Diet	HE Diet
Digestible energy (MJ/kg)	18	19.9
Protein (% dry mass)	54	50
Lipids (% dry mass)	15	20
DHA (% dry mass)	15	20
EPA (% dry mass)	1	1.4
ARA (% dry mass)	2.5	3
DHA/EPA/ARA	5/12.5/1	3.5/7.5/1
Cellulose (% dry mass)	1.6	1.7
Ashes (% dry mass)	10.5	6.4
Total P (% dry mass)	1.4	1.2
Estimated nitrogen-free extract	20.5	23.5

MJ: megajoules; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; P: elemental phosphorus.

All animal-handling procedures were conducted following the guidelines of the Council of the European Union (EU 2010/63), the Spanish and Catalan governments and with the approval of the Ethics and Animal Care Committee of the University of Barcelona (CEEA 663/13 and permit number DAAM 7644).

2.2. GH and IGF-1 Plasma Levels

Plasma GH was determined by a homologous gilthead sea bream radioimmunoassay (RIA) as previously described [42]. The sensitivity and midrange (ED50) of the assay were 0.15 and 1.8 ng/mL, respectively. Plasma insulin-like growth factors (IGFs) were extracted by acid–ethanol cryoprecipitation [43], and the concentration of IGF-1 was measured by means of a generic fish IGF-1 RIA validated for Mediterranean perciform fish [44]. The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

2.3. Gene Expression

2.3.1. RNA Extraction and cDNA Synthesis

To perform the gene expression analysis, tissue homogenization was carried out from 30 or 100 mg of liver or white muscle, respectively. The samples were homogenized in 1 mL of TRI Reagent® Solution (Applied Biosystems, Alcobendas, Spain) using Precellys® Evolution Homogenizer cooled at 4–8 °C with Cryolys® (Bertin Technologies, Montigny-le–Bretonneux, France). After homogenization, RNA extraction was performed following the manufacturer's TRI Reagent® protocol. The RNA concentration and purity of the samples were determined using the Nanodrop 2200™ (ThermoScientific, Alcobendas, Spain). The RNA integrity was checked in a 1% (*w*/*v*) agarose gel stained with SYBR-Safe® DNA Gel Stain (Life Technologies, Alcobendas, Spain). The RNA samples were stored at −80 °C.

For cDNA synthesis, 1.1 µg of total RNA was treated with DNase I Amplification Grade (Life Technologies, Alcobendas, Spain) and retrotranscribed with the Transcriptor First Strand cDNA Synthesis Kit® (Roche, Sant Cugat del Vallès, Spain). The cDNA obtained was stored at −20 °C until further analysis.

2.3.2. Quantitative Real-Time PCR (qPCR)

The qPCR was carried out following the MIQE guidelines [45] in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain) using iTAQ Universal SYBR® Green Supermix (Bio Rad, El Prat de Llobregat, Spain) and Hard-Shell® 384-well PCR plates (Bio-Rad, El Prat de Llobregat, Spain). The analyses were carried out in triplicate, using for each well: 2.5 µL of iTAQ Universal SYBR® Green Supermix (Bio–Rad, El Prat de Llobregat, Spain), 1 µL of cDNA, 250 nM (final concentration) of forward and reverse primers and 1.25 µL of DEPC water. The qPCR program consisted of 3 min at 95 °C, 39 × (10 s at 95 °C, 30 s at the melting temperature of the primers and fluorescence detection), followed by an amplicon dissociation analysis from 55 to 95 °C with an increase of 0.5 °C each 30 s.

In the liver, the mRNA transcript levels of total *igf-1*, its three splice variants (*igf-1a*, *igf-1b*, and *igf-1c*), *igf-2*, *igfbp-1a*, *igf-1rb*, *ghr-2*, plus four reference genes (*ef1a*, *rps18*, *rpl27a,* and *tom20*) were analyzed. In the anterior and caudal white skeletal muscle, the gene expression of the same GH-IGFs axis components examined in liver was analyzed, plus *igf-1ra* and *ghr-1*; as well as the expression of proteolytic markers (*ctsl*, *ctsda*, *capn1*, *capn2*, *capn3*, *capns1a*, *capns1b*, *mafbx*, *murf1*, *n3,* and *ub*), MRFs (*myod1*, *myod2*, *mrf4,* and *myogenin*), and growth inhibitors (*mstn1* and *mstn2*), plus four reference genes (*ef1a*, *rps18*, *rpl27a,* and *tom20*). All the primers used in the qPCRs are shown in Table S1.

Transcript abundance of each studied gene was calculated with the Bio–Rad CFX Manager™ 3.1 software (Hercules, CA, USA) relative to the geometric mean of the combination of the two most stable reference genes (confirmed by the geNorm algorithm), using the method described by Pfaffl [46].

2.4. Western Blot Analysis

Protein was extracted from 100 mg of anterior white skeletal muscle in 1 mL of radioimmunoprecipitation assay buffer (RIPA) supplemented with both phosphatases and proteases inhibitors (i.e., PMSF, NA_3VO_4 and the cocktail P8340, Santa Cruz, CA, USA) using the Precellys® Evolution Homogenizer cooled with Cryolys®. Concentration of soluble protein was determined by the Bradford's method using bovine serum albumin (BSA, Sigma-Aldrich, Tres Cantos, Spain) for the standard curve. Twenty to forty µg of the soluble protein fraction were prepared in a loading buffer (containing SDS and β-mercaptoethanol), heated at 95 °C for 5 min, and run in a 12% polyacrylamide gel. Following, the proteins were transferred overnight a 4 °C to Immobilon® PVDF-FL 0.2 µm Transfer Membranes (Merck Millipore Ltd., Cork, Ireland), previously activated in methanol. Total transferred protein was determined by 5 min incubation with Revert™ Total Protein Stain (LI-COR, Lincoln, NE, USA) and the signal was read at 700 nm using the Odyssey Fc Imaging System (LI-COR, Lincoln, NE, USA). Membranes were blocked in Odyssey Blocking Buffer (diluted 1:1 in TBS) (LI-COR) for 1 h at room temperature, and then overnight at 4 °C and in agitation in the corresponding diluted primary antibody. The primary polyclonal antibodies used and corresponding dilutions were: Capn1 (sc-7530; 1/100), Ctsda (sc-6486; 1/200), Ctsl (sc-6501; 1/200), and Mafbx (sc-33782; 1/400) (Santa Cruz, CA, USA). These primary antibodies were previously validated for gilthead sea bream [13,37,47]. The antibody species were Rabbit for Mafbx and goat for the other proteins. After washing with TBS-T, the membranes were incubated with the goat antirabbit fluorescence secondary antibody for Mafbx and donkey antigoat for the other proteins (Cat. No. 925-32211 and Cat. No. 925-32214, respectively, Servicios Hospitalarios) for 1 h at room temperature at a dilution of 1/10,000 for Mafbx and 1/5000 for the other proteins. After incubation, the membranes were imaged at 700 nm (Mafbx) or 800 nm (the other membranes). To reuse the membranes, they were immersed in a commercial stripping buffer (NewBlot PVDF 5X Stripping Buffer, LI-COR, Lincoln, NE, USA) for 20 min at room temperature. From the images obtained, the quantification of the proteins was performed using the Odyssey Software Image Studio v. 5.2.5. (LI-COR, Lincoln, NE, USA). The raw images captured for the Western Blot analysis are compiled in the supplementary Figure S1.

2.5. Muscle Texture Measurement

Texture analyzes were performed at the Departament d'Enginyeria Agroalimentària i Biotecnologia of the Universitat Politècnica de Catalunya (ESAB, Castelldefels, Spain) using a TA.XT2i Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK) coupled to a Mini Kramer HDP/MKO5 (Stable Micro Systems Ltd., Godalming, UK). As texture analysis, maximal strength and elasticity were measured using the muscle pieces (1.5 × 1.5 cm) extracted from the anterior-dorsal region. Maximal strength is defined as the maximal force applied to cut the sample completely. Elasticity is the capacity of the muscle to recover its initial aspect following the application of force, and it coincides with the linear portion

of the texture curve before break point. Both parameters were measured as previously described [36,48].

2.6. Statistical Analysis

Data were analyzed using IBM SPSS Statistics v. 25 (IBM Corp., Armonk, NY, USA) and presented as means + standard error of the mean (SEM). A Shapiro–Wilk test was performed to analyze the normality of the data, and homogeneity of the variances was tested with a Levene's test. Data were analyzed by a two-way analysis of variance (ANOVA) with diet (HP, HE) and swimming activity (VS: voluntary swimming; EX: exercise) set as independent factors, and it was followed by a Tukey's post-hoc test. Differences among groups were considered significant at $p < 0.05$.

3. Results

3.1. GH and IGF-1 Plasma Levels

The GH plasma levels as well as the GH/IGF-1 ratio were affected by the diet composition, the swimming activity, and most importantly, by the interaction between both factors. Hence, in fish under voluntary swimming, the circulating GH plasma levels and the GH/IGF-1 ratio were significantly higher in fish fed with HP in comparison to those fed with HE. However, exercise significantly decreased both parameters in HP-fed fish, thus disappearing the differences among both dietary groups. The IGF-1 levels were not affected by any factor (as illustrated in Figure 1).

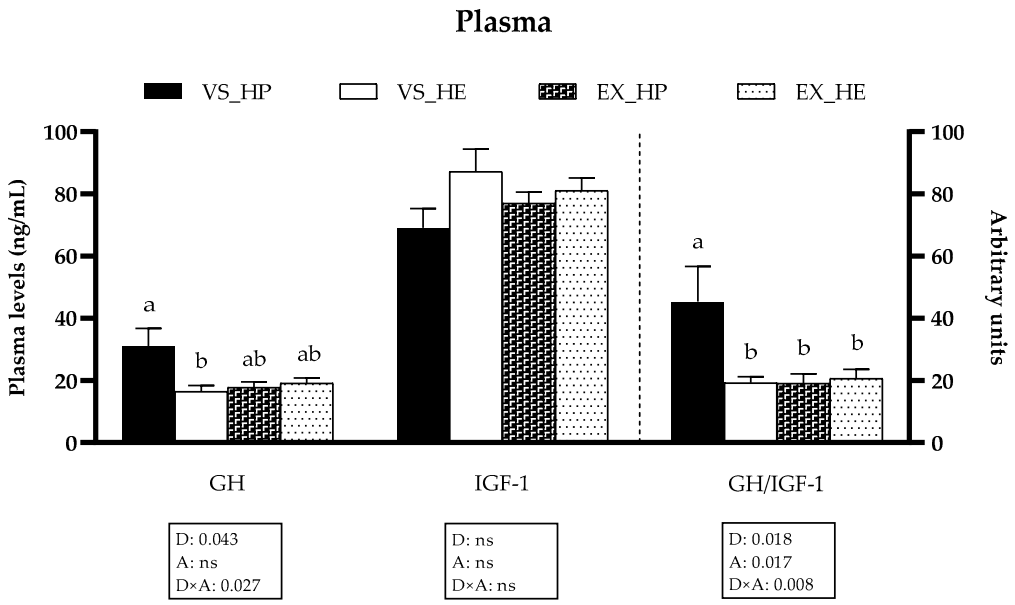

Figure 1. Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) plasma levels of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 10$; EX, $n = 22$. Factorial statistical analysis was assessed by two-way ANOVA, and p-values of factors diet (D), physical activity (A) and interaction (D×) are displayed under graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

3.2. GH-IGFs Axis Components Gene Expression in Liver

In liver, only the gene expression of *igf-1c* was significantly affected by the activity and the interaction of the two factors. Thus, under voluntary swimming, *igf-1c* showed apparent reduced expression in fish fed with HE (although not significant); while the sustained exercise caused a significant increase of *igf-1c* in fish fed with HE, equalizing the expression levels in both dietary conditions (as illustrated in Figure 2). The gene expression

of *igf-1a*, *igf-1b*, and *igfbp-1a* was also analyzed in liver but remained unaltered by any of the factors (data not shown).

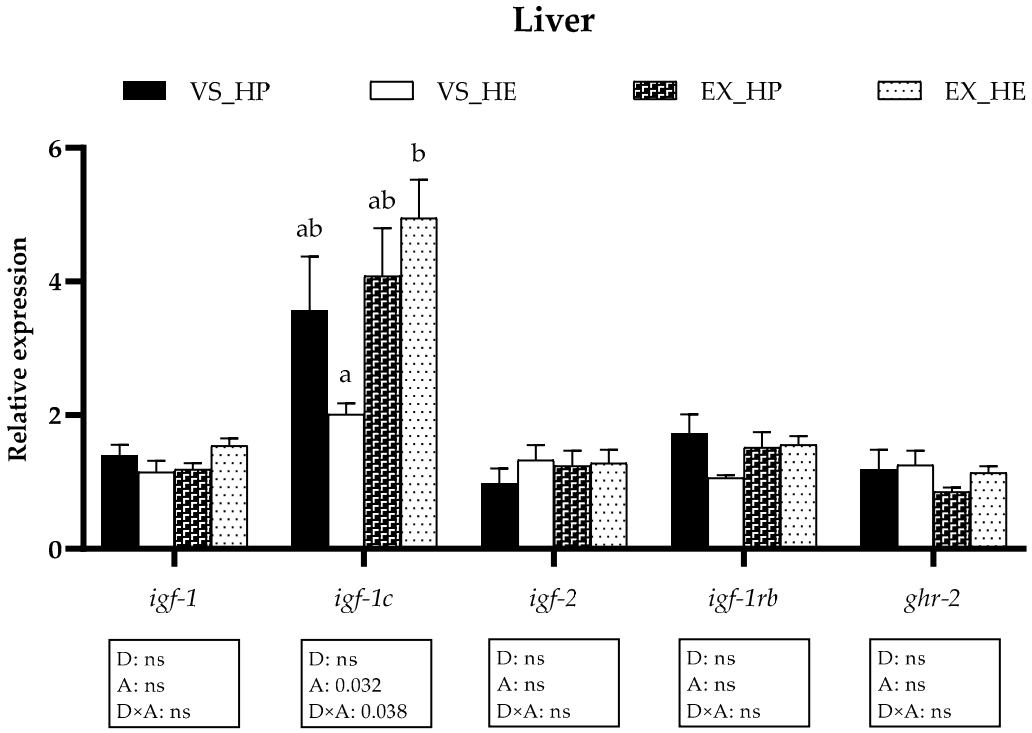

Figure 2. Relative gene expression of GH-IGFs axis members in liver of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with the high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA, and the p-values of factors diet (D), physical activity (A) and interaction (D×A) are displayed under graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

3.3. GH-IGFs Axis Components Gene Expression in Anterior and Caudal Muscle

With regards to the white skeletal muscle, in the anterior region, diet significantly modified *igfbp-1* expression; swimming activity altered the *ghrs*, while *igf-2*, *igfbp-1a*, *igf-1rb*, and *ghr-2* showed interaction of both variables. The mRNA levels of *igfbp-1a* and *ghr-2* were significantly higher in fish under voluntary swimming and fed with HP diet when compared to that of those fed with the HE. In the exercise condition, these differences disappeared due to the significant decrease of *igfbp-1a* and *ghr-2* in the HP-fed group (as illustrated in Figure 3).

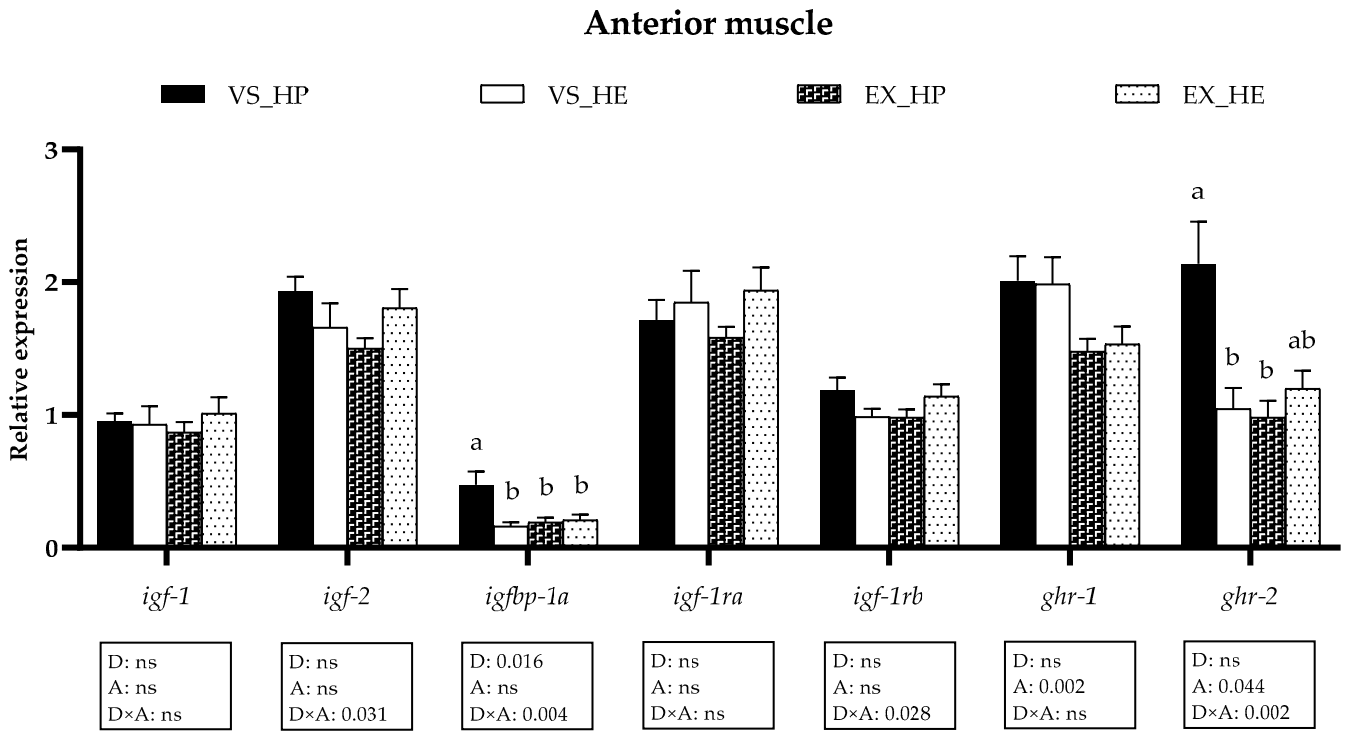

Figure 3. Relative gene expression of the GH-IGFs axis members in anterior muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with the high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA, and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under the graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

In the caudal muscle, the gene expression of the GH-IGFs axis components was similarly affected, responding *igfbp-1a* to diet, *ghr-1* to exercise, and *igf-2* and *ghr-2* to the interaction. Swimming activity significantly reduced *ghr-2* levels in the HP-fed group but not in those fish fed with HE diet (as illustrated in Figure 4). In both muscle regions, the expression of the *igf-1* splice variants was also analyzed but was not affected by any of the factors (data not shown).

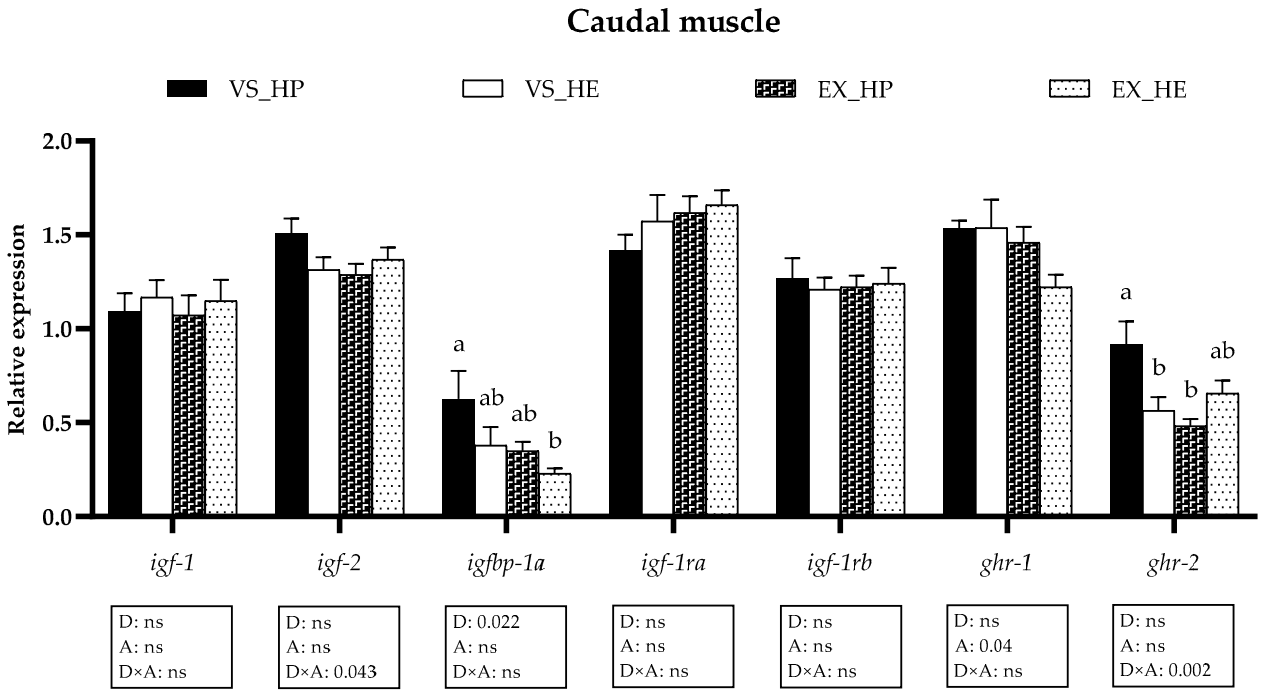

Figure 4. Relative gene expression of the GH-IGFs axis members in caudal muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, n = 12; EX, n = 16. Factorial statistical analysis was assessed by two-way ANOVA and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under the graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

3.4. Proteolytic Markers Gene and Protein Expression

In the anterior muscle the statistical analysis indicated that diet significantly affected the gene expression of *capn1*, *capn3*, *capsn1a*, *ctsl*, and *mafbx*, whereas swimming activity altered *capn3*, *capsn1a*, *ctsl*, *mafbx*, *n3 and ub*; thus, showing many of these markers interaction effect between both variables. Fish under voluntary swimming and fed with HE diet showed significantly higher mRNA levels of *capn1*, *capn3,* and *capns1a*, and lower *ctsl* and *mafbx* expression compared to that of fish fed with HP. Nevertheless, in the exercise condition, the gene expression of all the proteolytic systems in this muscle region remained stable regardless of the diet composition. These results could be explained by the significant decrease in HE-fed fish of *capn1*, *capn3,* and *capns1a*, as well as the diminution in HP-fed group of *ctsl* and *mafbx* compared to that of the voluntary swimming condition, thus reflecting the diet, activity and interaction effect. The exercise also reduced the expression of *n3* and *ub* in HE-fed fish (as illustrated in Figure 5). Western blot analysis followed a similar tendency, supporting the gene expression results, since protein expression of Mafbx was significantly downregulated in fish under voluntary swimming and fed with HE diet, while in the exercise condition, these differences were not found. Only the protein expression of Mafbx showed significant diet and activity effects (as illustrated in Figure 6).

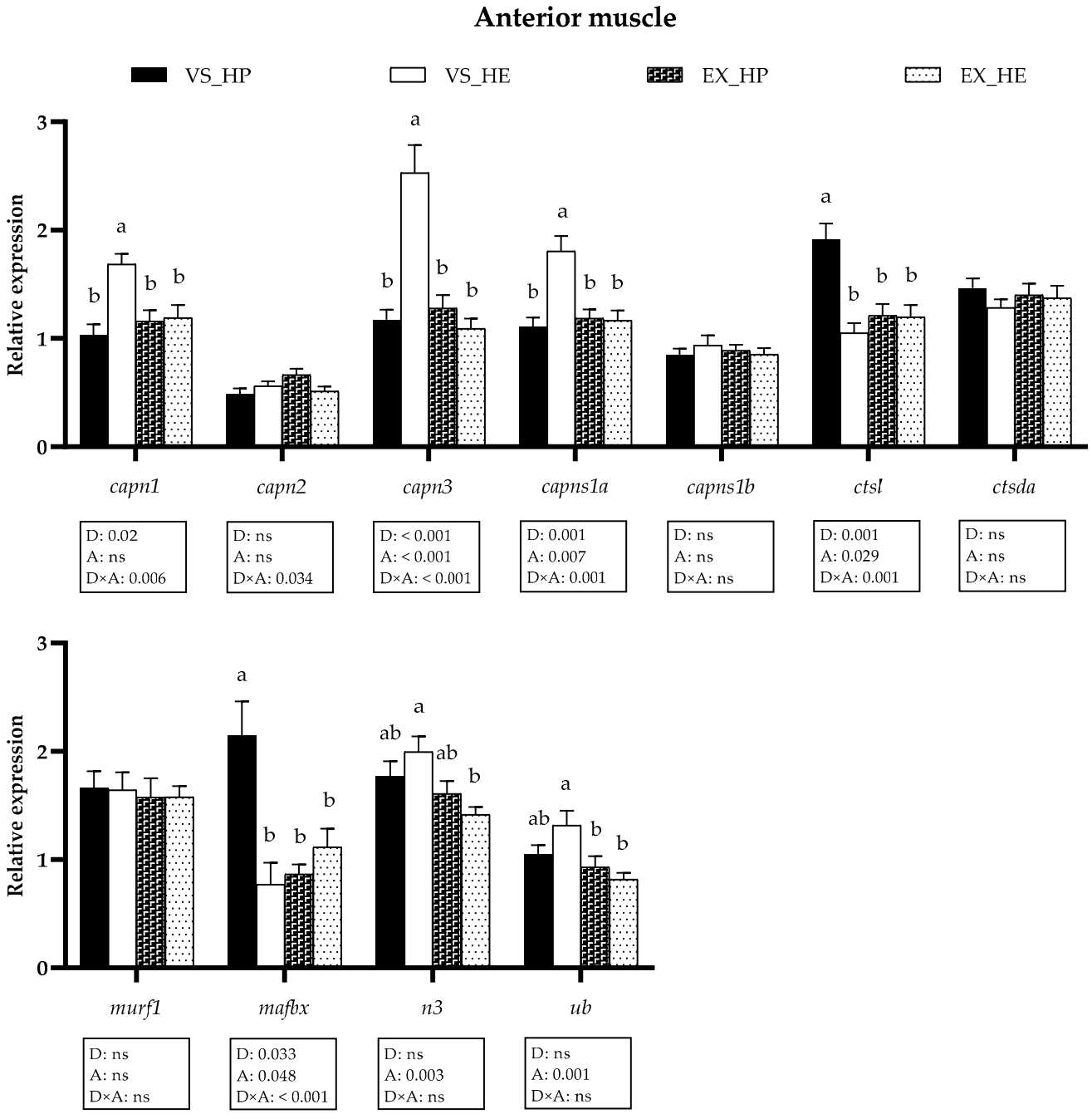

Figure 5. Relative gene expression of proteolytic markers in anterior muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

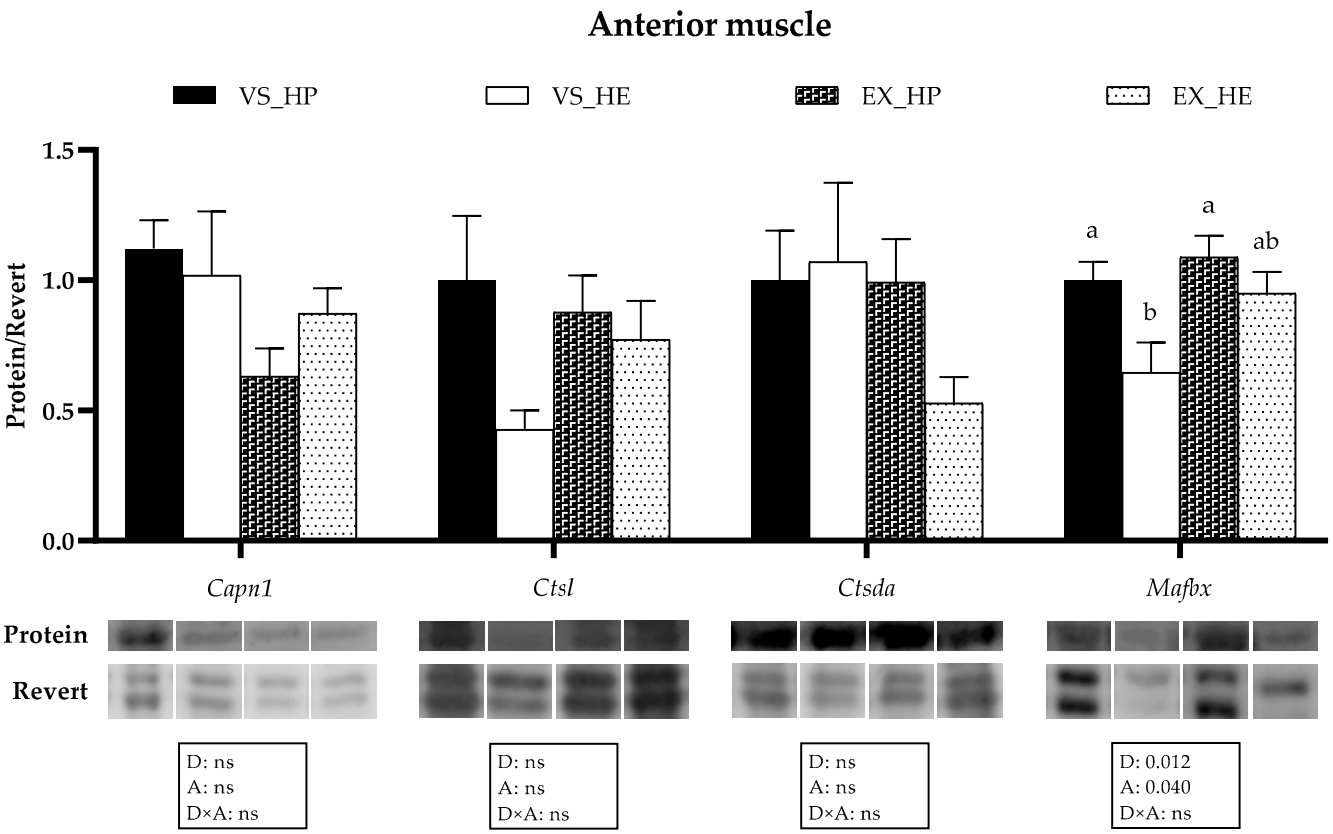

Figure 6. Representative western blot and densitometric analysis of proteolytic markers' protein levels in anterior muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Bands were normalized to their Revert^{TM} total protein staining (corresponding well is shown). To eliminate intermembrane variability, relative intensity of each specific band was normalized by geometric mean of intensity of that of VS_HP diet group of corresponding membrane. Data are shown as means + SEM. VS and EX, $n = 6$. Factorial statistical analysis was assessed by two-way ANOVA, and p-values of the factors diet (D), physical activity (A), and the interaction (D×A) are displayed under graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$). (The raw images captured for the Western Blot analysis are compiled in the supplementary Figure S1).

With respect to the transcriptional profile of the proteolytic systems in the caudal muscle, similar results were observed to those obtained in the anterior region and diet affected the same genes plus *n3* and *ub*. The activity effect was only observed in *ctsl;* while *capn1*, *capn3*, *caps1a*, *ctsl*, *mafbx*, *n3*, and *ub* showed interaction of both variables. In voluntary swimming, HE diet significantly enhanced the gene expression of *capn1*, *capn3*, and *capns1a* in fish muscle, as in the anterior region, but also that of *n3* and *ub*. Likewise, the expression of *ctsl* and *mafbx* was significantly decreased in fish fed with the HE diet in voluntary swimming. However, in the exercise condition, swimming activity attenuated these differences observed in voluntary swimming, as it provoked a significant decrease of *capns1a* and *ub* in the HE-fed group, an increase of *capn1* and *ub* in fish fed with the HP diet and a reduction of *ctsl* and *mafbx* also in HP-fed fish. In the case of *capn3*, exercise induced an inverse expression pattern, increasing its levels in fish fed with HP diet and decreasing those of fish fed with HE, as observed in anterior muscle for this gene (as illustrated in Figure 7).

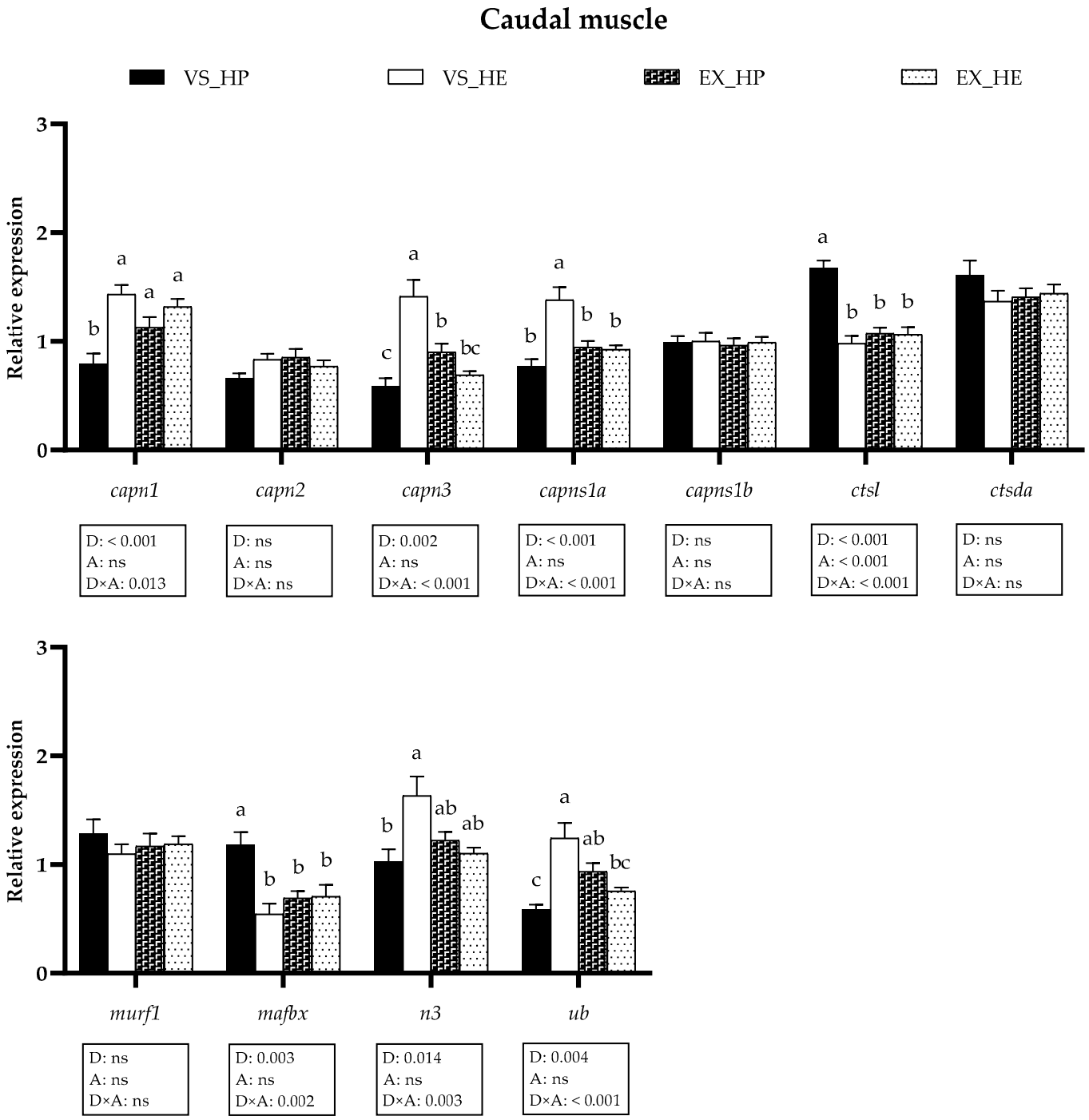

Figure 7. Relative gene expression of proteolytic markers in caudal muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA, and p-values of the factors diet (D), physical activity (A), and interaction (D×A) are displayed under the graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

3.5. Myogenic Regulatory Factors and Growth Inhibitors Gene Expression

The transcriptional profile of the *mrfs* and growth inhibitors (*mstns*) in the anterior and caudal muscle remained unaltered by diet in both voluntary swimming and sustained exercise conditions (as illustrated in Figures 8 and 9), although the expression of the *mstns* showed a tendency to increase in the anterior muscle of HE-fed fish in both swimming conditions (as illustrated in Figure 8). In the caudal muscle, the swimming activity diminished

the expression of *mrf4*, *mstn1*, and *mstn2* in both dietary conditions, being clearer the effect in the last two genes in HP-fed fish (as illustrated in Figure 9).

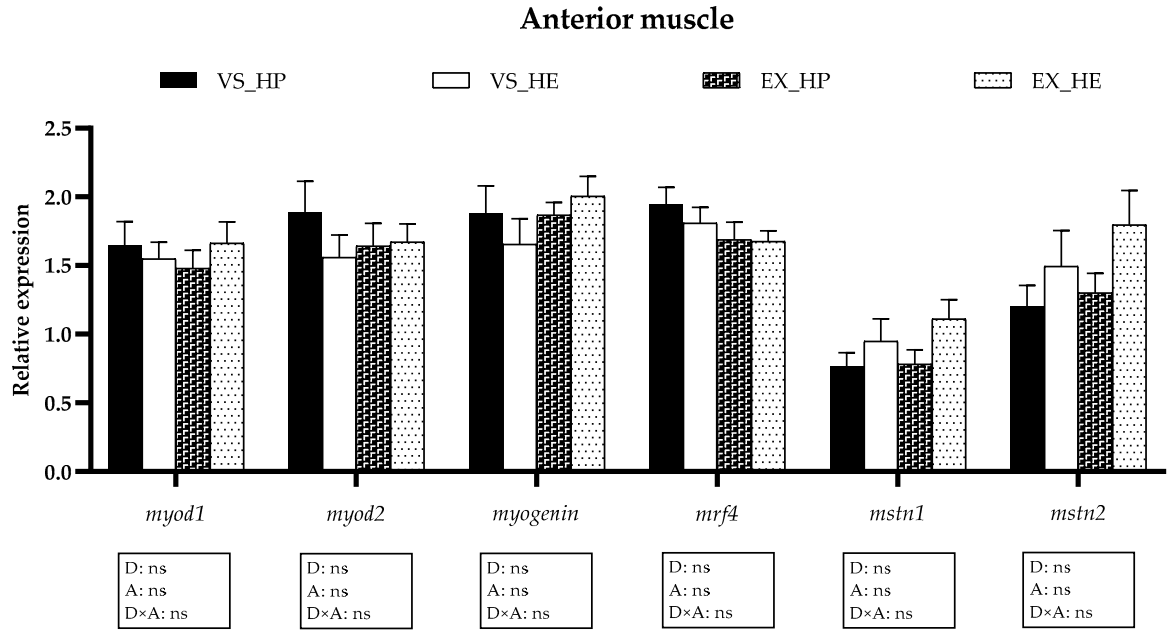

Figure 8. Relative gene expression of myogenic regulatory factors in anterior muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with the high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under graph.

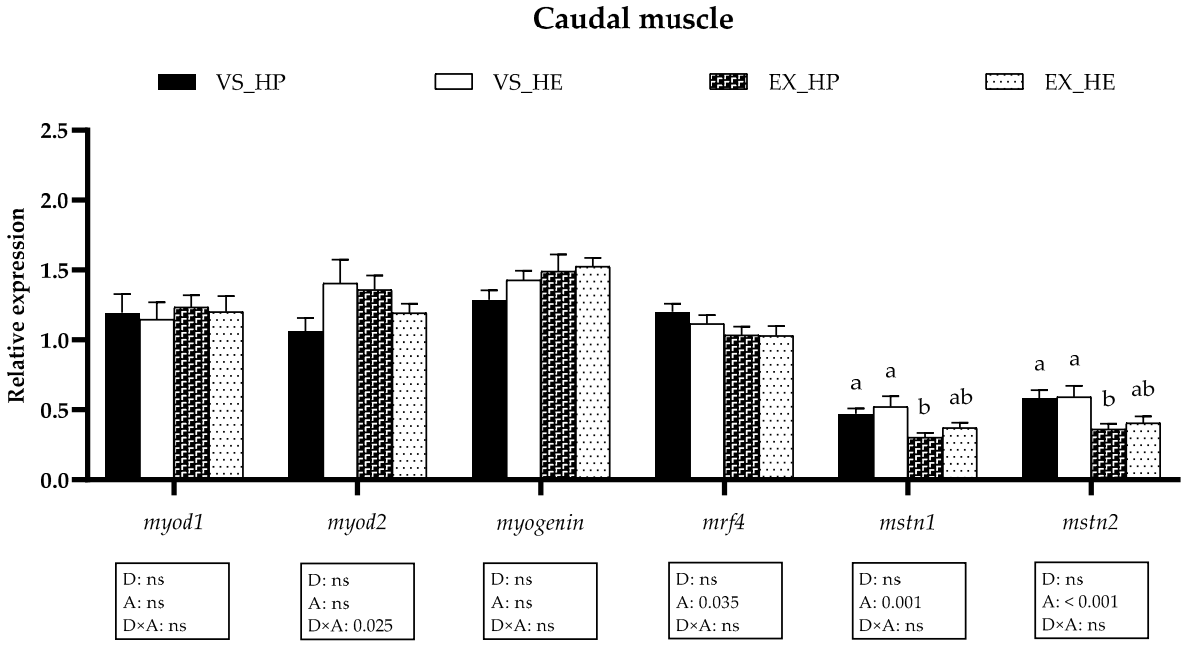

Figure 9. Relative gene expression of myogenic regulatory factors in caudal muscle of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high-protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 12$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under the graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

3.6. Muscle Texture

Diet composition and activity had significant effects on both parameters of muscle texture, maximal strength, and elasticity, and an interaction effect was also observed on maximal strength. In fish under voluntary swimming, fish fed with HE diet showed lower values of maximal strength and the same trend in elasticity compared to that of those fed with HP diet. Nevertheless, these diet-induced differences disappeared in fish subjected to sustained exercise due to the increase of these parameters in fish fed with HE (as illustrated in Figure 10).

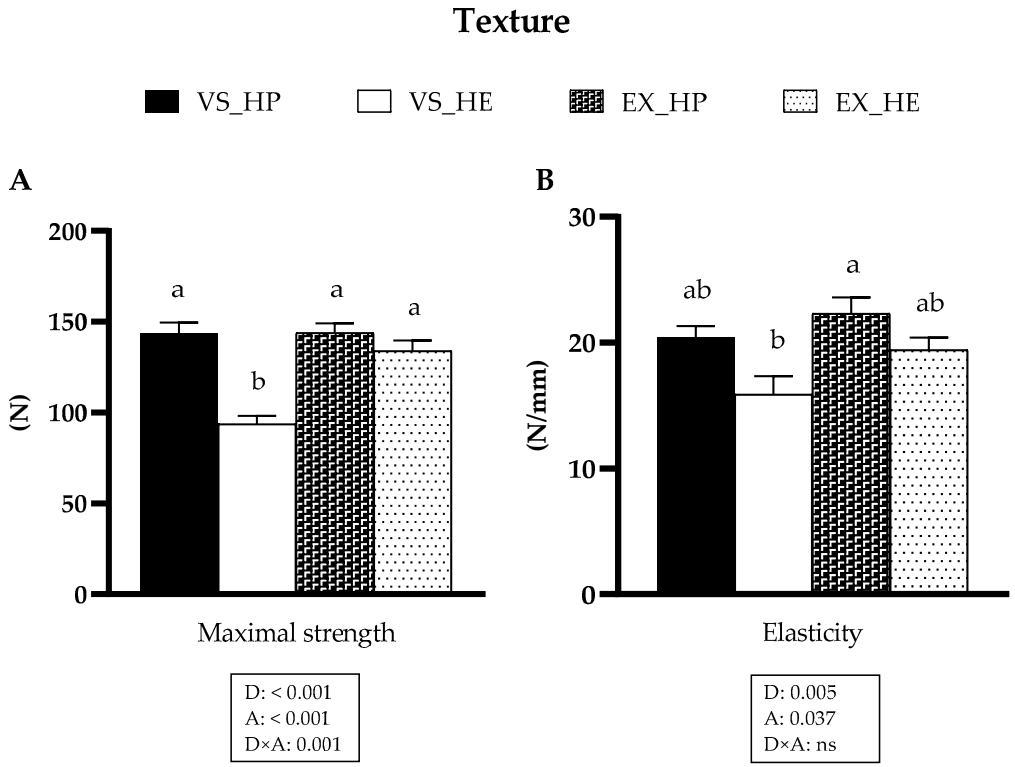

Figure 10. Muscle maximal strength (**A**) and elasticity (**B**) of fish under voluntary swimming (VS) or sustained exercise (EX) and fed with high protein diet (HP) or high-energy diet (HE). Data are shown as means + SEM. VS, $n = 10$; EX, $n = 16$. Factorial statistical analysis was assessed by two-way ANOVA, and p-values of factors diet (D), physical activity (A), and interaction (D×A) are displayed under graph. Different letters indicate significant differences (Tukey's post-hoc test, $p < 0.05$).

4. Discussion

Beneficial effects of exercise were reported in fish, including enhanced muscle growth and feed conversion efficiency [6,8,12,15,49], although the role of the GH-IGFs axis in these effects remains controversial [7]. In addition, it is still necessary to better understand how an energy demanding condition, like forced and sustained exercise, alters nutrients utilization. These insights in exercised fish would allow us to know the maximal grade of inclusion of lipids or carbohydrates in the diet, which allow the reduction of nitrogen discharges and feeding costs without compromising the metabolism, and consequently, growth. The present work is an extension of a previous one in which it was evaluated how nutrient balance affects growth, muscle composition, and mitochondrial metabolism depending on the physical activity conditions [5]. Briefly, under voluntary swimming, fish fed with HE diet showed retarded growth and higher lipid deposition in muscle compared with that of those fed with HP diet, while these differences were not present in the exercised fish. Similarly, the hepatic expression of energy metabolism and mitochondrial biogenesis markers revealed clear differences between dietary groups in nonexercised fish which

were not observed with swimming activity [5]. In the current work, we focused on the response of the GH-IGFs axis, proteolytic systems and other muscle developmental markers' expression depending on diet formulation and physical activity, aiming to provide valuable information for the applicability of exercise for a more sustainable farming of fish in aquaculture.

Many studies demonstrated that changes in fish growth rate are often followed by modifications in the GH and IGF-1 plasma levels, as these are the main muscle-accretion regulatory factors [12,14,16,50]. The results obtained in the voluntary swimming condition revealed that the greater weight gain of HP-fed fish compared to that of HE-fed group was accompanied by significantly higher plasma GH levels, which led to an increased GH/IGF-1 plasma ratio in this group. Dietary proteins and lipids can influence the homeostasis of the somatotropic axis; however, there are some discrepancies in the effects of the proportion of these nutrients on GH secretion in fish [51–53]. The protein or lipid content affected differently the plasma GH levels depending on the ration size in gilthead sea bream juveniles [53]. Fish fed *ad libitum* shows upregulated GH secretion by a high-lipid diet; while fish under fixed feeding levels appear to have increased circulating GH when fed a high-protein diet. Hence, our results could suggest that under a 5% meal ration, the higher protein content of HP diet had stronger effects on GH secretion than that of the lipids of HE diet. Furthermore, considering the saturation of the liver's mitochondrial oxidative systems found in fish fed with HE [5], the lipolytic effects of GH possibly impaired the metabolic condition in this group. In fish subjected to sustained exercise, differences in GH levels due to diet were not observed, as a consequence of a downregulation of GH production by swimming activity in those fish fed with HP diet, as we observed in different studies [12,14]. Moreover, these comparable results on circulating GH and IGF-1 levels are consistent with the similar final body weight that exercised fish showed compared to that of those under voluntary swimming, regardless of diet composition [5], pointing out that exercise caused a diet-dependent differential response of these parameters, which is clearly reflected by the significant interaction between factors.

The hepatic transcriptional profile of the GH-IGFs axis components showed few significant differences between groups. The mRNA levels of *igf-1c* in HE-fed fish showed a significant increase in the group exposed to sustained exercise in comparison to that of the values in voluntary swimming, thus reaching the levels of the HP-fed group. These results would support a possible important implication of this *igf-1* isoform in fish growth regulation, as suggested in a previous study [30]. In fish under voluntary swimming and fed with HE, the *igf-1rb* expression was lower compared to that of fish fed with HP; while in exercise, the activity caused a significant increment of its expression in HE-fed group, thus equalizing the levels of both conditions. Our results only reflect a snapshot from the time of sampling, and growth is a dynamic process in which the gene expression and synthesis of GH-IGFs axis members can experience changes over time [54,55]. In any case, of the similar *igf-1rb* hepatic expression in fish under sustained exercise is congruent with the absence of differences in their final body weight, since the growth-promoting effects of IGFs are mediated through their interaction with IGF-1Rs [17,20].

Regarding the GH-IGFs system expression in muscle, important changes were not observed in any of the experimental groups, neither in the anterior nor in the caudal muscle. The main significant differences observed were in the anterior region of fish in voluntary swimming fed with HP diet, which presented higher expression of *igfbp-1a* and *ghr-2* than those fed with HE. *igfbp-1a* and *ghr-2* are considered negative regulators of growth in fish since their protein and/or gene expression are usually upregulated in catabolic conditions (e.g., hypoxia, stress or fasting) [56–59]. However, the overexpression of muscle *igfbp-1a* and *ghr-2* that we found in fish fed with HP could indicate a specific condition produced after the period of faster growth showed in this group. In fact, the higher expression of *ghr-2* found is in concordance with the high circulating GH levels in the HP-fed group, and also with the stronger response of *ghr-2* isoform to a nutritional treatment reported by Benedito–Palos et al. [24] in the same species. In gilthead sea bream, the gene expression

of *ghrs* in liver and skeletal muscle generally increases along with GH concentration in plasma [56,59]. Hence, the positive response of *ghrs* expression to increased circulating GH might be a mechanism to prevent an excess of GH signaling, since *ghrs* can experience post-transcriptional modifications that generate truncated GHRs without the intracellular signaling domain [56,57,59]. Furthermore, the truncated forms are assumed to be the preferential substrate for proteolytic cleavage to produce the circulating binding proteins (GHBPs) [60,61], which regulate half-life and bioavailability of GH [20]. This scenario of genes expression in voluntary swimming was not observed when juveniles were subjected to sustained exercise, as the exercise reduced in the anterior muscle *igfbp-1a,* and *ghr-2* expression in the HP-fed fish, in agreement with the results of GH and GH/IGF-1 ratio in plasma. The interaction between diet and swimming activity significantly affected *igf-2* expression in the anterior and caudal muscle, but this response was not observed in *igf-1* expression, in agreement with the differential role of both IGFs already found in this species [21]. Summarizing, the changes observed in GH-IGFs system in muscle suggest that exercise tends to equalize muscle growth conditions in both dietary groups.

The transcriptional profile of the proteolytic markers in muscle showed that in voluntary swimming, HE feeding induced a significant upregulation of *capn1*, *capn3*, *capns1a*, *n3,* and *ub* mRNAs in either the anterior or caudal muscle region, while *ctsl* and *mafbx* were downregulated, also at a protein level in the case of Mafbx. Calpains appear to be more involved in the proliferation stages of the myogenesis, as observed in different species, including gilthead sea bream [31,47,62]. Regarding the cathepsins and the UbP system, they seem to have greater importance in myogenic differentiation and formation of myotubes, as suggested in gilthead sea bream [47] and Atlantic salmon (*Salmo salar*) [63]. Moreover, in gilthead sea bream fasted for 21 days, the expression of calpains is rapidly upregulated within 24 h of refeeding, whereas the cathepsins and the UbP system members respond one week later or even do not respond [25]. Altogether, in the present study, the upregulation of the calpains, along with the decrement of *ctsl* and *mafbx* in the voluntary swimming group fed with HE diet, could indicate that the myogenesis in these fish is in a less-advanced stage compared to in those fish fed with HP; in agreement with the lower body weight observed in HE-fed fish [5]. Furthermore, the saturation of the lipid oxidation systems in this group would favor the utilization of amino acids generated by the proteolytic systems [5]. Nevertheless, in fish exposed to sustained exercise, the differences among both diet groups in the proteolytic systems' expression almost disappeared, basically due to the decreased expression of those genes upregulated in voluntary swimming. This response agrees with the hypothesis that the high-energy demand induced by the sustained exercise generates a metabolic switch that promotes the optimization of nutrients use [5]. Therefore, in exercised fish fed with HE diet, lipid utilization as an energy source was improved, resulting in a protein-sparing effect. These data agree with the similar final body weight observed in exercised fish regardless of diet composition. The regulation of muscle gene and protein expression does not follow an identical pattern [25], as shown here by Capn1, Ctsl, and Ctsda; but it is interesting that in the case of Mafbx both gene and protein expression are increased in HP-fed fish under voluntary swimming, again supporting that this group was in a more advanced myogenic condition. Overall, the different responses of the proteolytic systems to distinct dietary regimes and physical activity observed in the current study open the possibility of using them as markers of nutritional status and culture conditions.

The gene expression of the *mrfs* and *mstns* was not affected by diet in any of the muscle regions of fish in voluntary swimming, suggesting that after six weeks of experiment the effects on myogenesis were still not noticeable. In gilthead sea bream fingerlings exposed to exercise for six weeks, the myogenic factors are slightly affected in the anterior muscle, while the proteolytic genes appear to be already upregulated to start muscle reorganization [13]. This response agrees with the important changes in proteolytic genes expression found in this study in fish under voluntary swimming and probably the induction of myogenesis will follow the activation of the proteolytic genes, as the importance of the proteolytic systems to facilitate the recovery of the mature muscle fiber by activating the

myogenic program was reviewed [32]. Hence, it is understandable that the main genes involved in the regulation of muscle development are not still showing significant responses after 6 weeks of treatment. In fish under exercise, myogenic genes expression presented a similar pattern to that of fish in voluntary swimming, showing no significant differences in any of the muscle regions at the time analyzed. Nevertheless, it is remarkable that only in the caudal muscle the *mstns* expression was downregulated by exercise compared to levels in voluntary swimming, significantly in fish fed HP. This decrement of *mstn1* and *mstn2* would suggest a lower growth repression in this muscle region of fish subjected to sustained exercise since Mstns are the main inhibitors of muscle development in vertebrates [40,41]. These results are in concordance with the different responses to exercise along the muscle trunk reported in gilthead sea bream [6,13].

Muscle texture was analyzed by the measurement of maximal strength and elasticity which are two well established parameters to determine the physical properties of the flesh. In general terms, fish fillets with certain degree of firmness and elasticity are preferred, while low values of these two factors are associated with product defects. The results of maximal strength and elasticity showed the same tendency as that observed with the other variables studied. In fish under voluntary swimming and fed with HP diet, both parameters followed a similar trend, being significantly higher in maximal strength in comparison with values of HE-fed fish. However, exercise in fish fed with HE caused an increase on muscle texture, reaching the levels of the HP-fed group. These results agree with the gene expression data of proteolytic markers, which in exercise condition do not present the differences observed in the fish under voluntary swimming. These findings could indicate a negative relationship between calpains' mRNA levels and flesh firmness, as fish fed with the HE diet in voluntary swimming showed higher expression of these proteases and lower texture. Similar results were described also in gilthead sea bream under fasting and refeeding conditions, thus suggesting a valuable applicability of the calpains as markers for flesh quality analysis [37]. Our results were consistent with previous reports on negative correlation between muscle texture and fat content [64,65]. Fish under voluntary swimming and fed with the HE diet presented significantly higher lipid deposition in muscle [5] and lower values of maximal strength and elasticity, but these differences were not found in the exercise condition. Altogether, these muscle texture results demonstrated the positive and interesting effects of sustained swimming to compensate or ameliorate the reduced texture parameters that are associated with high-fat feeding in fish farming.

5. Conclusions

The results obtained in this study provide convincing evidences that sustained exercise may compensate nutrient imbalances provoked by high-fat diets, improving fish growth performance and flesh texture. Furthermore, the response of the proteolytic systems to the composition of the diet and physical activity proposes them as valuable markers of fish nutritional status and muscle growth.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11082182/s1. The sequences and other relevant information about the primers used in the Real-Time quantitative PCR analysis are displayed in the supplementary Table S1, and the raw images captured for the Western Blot analysis are compiled in the supplementary Figure S1.

Author Contributions: J.B., J.F.-B. and J.G. conceived and designed the study. M.P.-A., E.J.V., I.G.-P., A.S.-M., A.I., I.A., M.P., J.C.-G., J.P.-S. performed the experiment and laboratory analysis. M.P.-A., I.G.-P. and J.G. analyzed the data and drafted the manuscript. All authors contributed to the writing and approved the submitted version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Barcelona (protocol codes CEEA 663/13 and permit number DAAM 7644).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the current article and its corresponding supplementary material.

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Conflicts of Interest: The authors declare no conflict of interest.

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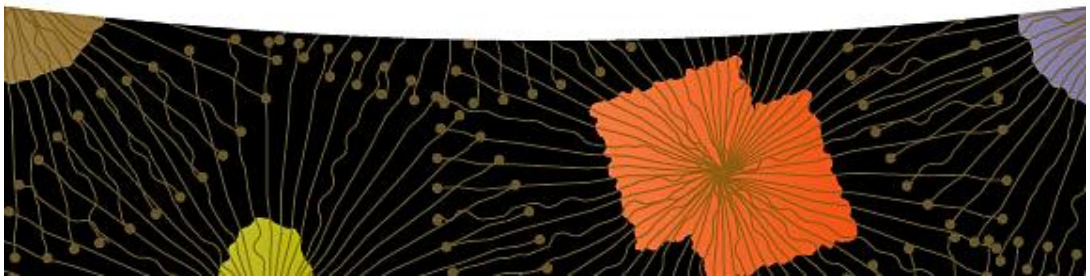
ARTICLE 5

Myomixer is expressed during embryonic and post-larval hyperplasia, muscle regeneration and differentiation of myoblats in rainbow trout (*Oncorhynchus mykiss*)



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Research paper

Myomixer is expressed during embryonic and post-larval hyperplasia, muscle regeneration and differentiation of myoblats in rainbow trout (*Oncorhynchus mykiss*)

Miquel Perello-Amoros [a], Cécile Rallière [b], Joaquim Gutiérrez [a], Jean-Charles Gabillard [b,*]

[a] Departament de Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
[b] INRAE, UR1037 Laboratory of Fish Physiology and Genomics, 35000 Rennes, France

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ABSTRACT

In contrast to mice or zebrafish, trout exhibits post-larval muscle growth through hypertrophy and formation of new myofibers (hyperplasia). The muscle fibers are formed by the fusion of mononucleated cells (myoblasts) regulated by several muscle-specific proteins such as Myomaker or Myomixer. In this work, we identified a unique gene encoding a Myomixer protein of 77 amino acids (aa) in the trout genome. Sequence analysis and phylogenetic tree showed moderate conservation of the overall protein sequence across teleost fish (61% of aa identity between trout and zebrafish Myomixer sequences). Nevertheless, the functionally essential motif, AxLyCxL is perfectly conserved in all studied sequences of vertebrates. Using *in situ* hybridization, we observed that *myomixer* was highly expressed in the embryonic myotome, particularly in the hyperplasic area. Moreover, *myomixer* remained readily expressed in white muscle of juvenile (1 and 20 g) although its expression decreased in mature fish. We also showed that *myomixer* is up-regulated during muscle regeneration and *in vitro* myoblasts differentiation. Together, these data indicate that *myomixer* expression is consistently associated with the formation of new myofibers during somitogenesis, post-larval growth and muscle regeneration in trout.

1. Introduction

Skeletal muscle consists of myofibers derived from the fusion of progenitor cells called myoblasts. In mammals, myofibers formation occurs throughout embryogenesis and during muscle regeneration in adult. Myoblasts proliferate, differentiate into myocytes that fuse to form multinucleated myotubes, and mature into functional myofibers (Dumont et al., 2015). The fusion process is highly regulated by numerous key proteins involved in distinct steps, including cell–cell recognition and adhesion, cytoskeletal reorganization and finally membrane fusion. Among those proteins, the transmembrane Myomaker protein is expressed only in skeletal muscle and is absolutely required for myoblast fusion (Millay et al., 2013). Indeed, in *myomaker* knockout mice, muscle is formed only by mononucleated myoblasts. Similarly, the muscle of *myomaker* knockout mice fails to regenerate after injury, which shows that *myomaker* is also essential for formation of new myofibers during muscle regeneration (Millay et al., 2014). Consequently, *myomaker* expression is upregulated during periods of myofiber formation (embryogenesis and muscle regeneration), and downregulated thereafter (Millay et al., 2014, 2013). In addition, ectopic expression of *myomaker* in fibroblasts promotes fusion with C2C12 myoblasts, showing its direct involvement in the fusion process (Millay et al., 2016, 2014). The mechanism of action of Myomaker remains poorly understood even though it has been shown that the C-terminal end of the protein is essential to its function (Millay et al., 2016).

Recently, another muscle-specific peptide called Myomixer with fusogenic activity was identified in mice (Bi et al., 2017; Quinn et al., 2017). The *myomixer* knockout in mice leads to muscle formation with mononucleated cells, and *in vitro,* the peptide allows the fusion of a fibroblast with a myoblast. Interestingly, the ectopic expression of *myomixer* and *myomaker* in fibroblasts promotes fibroblast-fibroblast fusion, suggesting that they should act together (Quinn et al., 2017). Nevertheless, Leikina et al. (2018) showed that Myomaker and

Abbreviations: aa, amino acid; cDNA, complementary Deoxyribonucleic acid; EST, Expressed sequence tag; ISH, in situ hybridization; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT-QPCR, reverse transcription quantitative polymerase chain reaction.

* Corresponding author.
E-mail address: jean-charles.gabillard@inrae.fr (J.-C. Gabillard).

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Myomixer are involved in distinct step of the myoblast fusion process. Whereas Myomaker is essential for hemifusion of the plasma membrane, Myomixer promotes the formation of fusion pores, and the fusogenic activities of these proteins do not require direct interaction (Leikina et al., 2018).

In zebrafish, Myomaker and Myomixer have been characterized and there are also essential for myoblast fusion (Landemaine et al., 2014; Millay et al., 2016; Shi et al., 2017; Zhang and Roy, 2017). Both proteins are expressed in embryonic myotome and their expression declines before hatching. Recently, we identified the unique *myomaker* ortholog in rainbow trout and revealed its unusual sequence. Indeed, the trout Myomaker protein contains 14 minisatellites and two sequence extensions leading to a protein of 434 aa instead of 221 in zebrafish (Landemaine et al., 2019). *In vitro*, ectopic expression of trout *myomaker* in mouse fibroblasts promotes fusion with C2C12 myoblasts. Given the original structure of trout Myomaker, we wondered whether the sequence and expression pattern of trout *myomixer* were conserved.

In this work, we showed that Myomixer protein sequence was moderately conserved across evolution and that the unique trout *myomixer* gene was highly expressed in skeletal muscle even after hatching and was upregulated during muscle regeneration and myotube formation.

2. Materials and methods

2.1. Animals

All the experiments presented in this article were developed under the current legislation that regulates the ethical handling and care procedures of experimentation animals (décret no. 2001–464, May 29, 2001) and the muscle regeneration study was approved by the INRAE PEIMA (Pisciculture Expérimentale INRAE des Monts d'Arrée) Institutional Animal Care and Use Committee (B29X777-02). The LPGP fish facility was approved by the Ministère de l'Enseignement Supérieur et de la Recherche (authorization no. C35-238–6).

2.2. Muscle regeneration experiment

As described in Landemaine et al., (2019), this experiment was carried out at the INRAE facility PEIMA (Sizun, Britany, France). Briefly, 1530 ± 279 g rainbow trout (*O. mykiss*) were anesthetized with MS-222 (50 mg/l) and using a sterile 1.2-mm needle, the left side of each fish was injured by a puncture behind to the dorsal fin and above the lateral line. The right side was used as a control for each fish. White muscle samples from both sides (within the injured region and opposite) were taken at 0, 1, 2, 4, 8, 16, and 30 days post-injury using a sterile scalpel after proper sacrifice by an MS-222 overdose. The obtained samples were properly stored in liquid nitrogen until further processing for gene expression analyses. Along the experiment, no infection was detected and the survival rate was 100%.

2.3. Trout satellite cell culture

Satellite cells from trout white muscle (15–20 g body weight) were cultured as previously described (Froehlich et al., 2013; Gabillard et al., 2010). Briefly, 40 g of tissue were mechanically and enzymatically (collagenase C9891 and trypsin T4799) digested prior to filtration (100 μm and 40 μm). The cells were seeded in poly-L-lysine and laminin precoated 6-well treated polystyrene plates at a density of 80,000 cells/cm^2 and incubated at 18 °C. The cells were cultured for 3 days in F10 medium (medium F10, Sigma, N6635) supplemented with 10% fetal bovine serum to stimulate cell proliferation. Then, the medium was changed to Dulbecco's modified Eagle's medium (Sigma, D7777) containing 2% fetal bovine serum to stimulate cell differentiation and cultured in this medium for an additional 3 days. Cells were washed twice with PBS and collected with TRI reagent solution (Sigma–Aldrich, catalog no. T9424) at 3rd (PM) and 4th (DM1), 5th (DM2) and 6th (DM3) day of culture. Samples were immediately stored at −80 °C until further processing for gene expression analysis.

2.4. Amplification and sequencing of myomixer sequence

The *O. mykiss myomixer* nucleotide sequence containing the full coding region was obtained from the Trout Genome browser of the French National Sequencing Center (Genoscope). We designed PCR (Polymerase Chain Reaction) primers in two different exons (forward, 5′-TTGGCTTTCCTTCCTCTTCAG-3′; and reverse, 5′-TGCGATCTGACTGGTGTCTCC-3′). PCR reaction was carried out from a rainbow trout muscle cDNA (complementary DNA) and the PCR product was run in agarose gel, purified and sequenced (Eurofins) and the obtained sequence was used to design primers for quantitative PCR (qPCR). The validated sequence of *myomixer* cDNA was deposited in GenBank with the accession number MN230110.

2.5. Phylogenetic analysis

Several Myomixer amino acid sequences obtained from different databases were aligned with the Mafft server software, version 7 (https://mafft.cbrc.jp/alignment/server/) using the default parameters and the G-INS-i iterative refinement method. The subsequent phylogenetic analysis was performed using the neighbour-joining method with MEGA X software in a bootstrapped method (500) to assess the robustness of the tree.

2.6. RNA extraction, cDNA synthesis, and quantitative PCR analyses

For three individual fish (~150 g), sample of white muscle, red muscle, skin, heart, brain, adipose tissue, liver, spleen, pituitary, kidney, ovary, gill, testis and intestine were collected and immediately stored in liquid nitrogen. Total RNA was extracted from cell cultures or from 100 mg of tissue (or less in the case of some small organs and tissues for the screening) using TRI reagent (Sigma–Aldrich, catalog no. T9424) and its concentration was determined using the NanoDrop ND-1000 spectrophotometer. One μg of total RNA was used for reverse transcription (Applied Biosystems kit, catalog no. 4368813). Trout *myomixer* primers for quantitative PCR (qPCR) (forward, 5′-AGACTTCCGTGACTCCTACCAG-3′; and reverse, 5′-TGCGATCTGACTGGTGTCTCC-3′) were designed in two exons to avoid genomic DNA amplification. The secondary structure formation in the predicted PCR product were determined with the mFOLD software. Quantitative PCR analyses were performed with 5 μl of cDNA using SYBR© Green fluorophore (Applied Biosystems), following the manufacturer's instructions, with a final concentration of 300 nM of each primer. The PCR program used was as follows: 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The relative expression of target cDNAs within the sample set was calculated from a serial dilution (1:4–1:256) (standard curve) of a cDNA pool using StepOneTM software V2.0.2 (Applied Bio-systems). Subsequently, qPCR data were normalized using *elongation factor-1 alpha* (*eF1a*) gene expression as previously detailed.

2.7. In situ hybridization

Trout embryos at days 10, 14 and 18 were fixed with 4% paraformaldehyde (PFA 4%) overnight at 4 °C and stored in methanol at −20 °C until use. Whole-mount *in* situ hybridization was performed using RNAscope®, an hybridization amplification-based signal system (Wang et al., 2012) according to the manufacturer's protocol (Advanced Cell Diagnostics #322360). Embryos were rehydrated in a decreasing methanol/PBS + 0.1% Tween-20 series (75% MetOH/25% PBST; 50% MetOH/50% PBST; 30% MetOH/70% PBST; 100% PBST) for 10 min each. Once rehydrated, embryos were transferred to a 2 ml Eppendorf tube. After 15 min treatment of 1x Target Retrieval (ACD #322000) at

100 °C, embryos were treated with Protease Plus solution (ACD #322331), at 40 °C for 5–45 min according to the stage. Embryos were incubated with the custom set of probes designed by ACD Biotechne (20 pairs of 18–25 nt) overnight at 40 °C in sealed Eppendorf tubes. Detection of specific probe binding sites was performed using RNAscope® 2.5 HD Detection Reagents-RED kit (ACD #322360), according to the manufacturer. Images of the embryos were obtained using a Zeiss Stemi 2000-C stereo microscope. For the histological examination of sections, the samples were embedded in 5% agarose in distilled water. Blocks were sectioned at 35 μm on a Leica vibratome (VT1000S). Images of the sections were obtained using a Nikon 90i microscope.

For the detection of *myomixer* and *myomaker* expression in 1 g and 20 g trout muscle, samples of white muscle were fixed with 4% paraformaldehyde overnight at 4 °C and embedded in paraffin. Then, cross-sections (7 μm) of muscle were cut using a microtome (HM355; Microm Microtech, Francheville, France) and *in* situ hybridization was performed using RNAscope® 2.5HD detection reagent RED kit (ACD #322360). Briefly, sections were baked at 60 °C for 1 h, dewaxed and air-dried. After 10 min in hydrogen peroxyde solution (ACD #322335), sections were treated with 1x Target Retrieval (ACD #322000) for 15 min at 100 °C, following 25 min with Protease Plus solution (ACD #322331) at 40 °C. All steps at 40 °C were performed in a ACD HybEZ II Hybridization System (#321720). Images of the sections were obtained using a Nikon 90i microscope.

For multiplex RNAscope *in situ* hybridization, trout embryos of 17 dpf (day post fertilisation) were fixed as previously described in PF4% and embedded in paraffin. Cross-sections (7 μm) were then hybridized using the RNAscope Multiplex Fluorescent Assay v2 (ACDBio #323100) according to the manufacturer's protocols. This assay allows simultaneous visualization of up to three RNA targets, with each probe assigned a different channel (C1, C2 or C3). Each channel requires its own amplification steps. *Pax7* and *myomixer* transcripts were targeted with fluorescent dyes Opal 520 (Akoya Biosciences #FP1487001KT) and Opal 620 (Akoya Biosciences #FP1495001KT) respectively. Nuclei are counter-stained with DAPI.

2.8. Statistical analyses

The data were analyzed using the nonparametric Kruskal–Wallis rank test followed by the Wilcoxon-Mann-Whitney test. All analyses were performed using the R statistical package (3.6.3 version).

3. Results

3.1. Identification of the trout myomixer gene

We performed a BLAST search in the trout genome (Berthelot et al., 2014) using the sequence of zebrafish Myomixer protein (Swiss-Prot: P0DP88.1) and we found only one locus with *myomixer* sequence similarity in the scaffold_4105 of the trout genome. We also identified two ESTs (Expressed Sequence Tag; GDKP01024145.1; GDKP01044688.1) corresponding to the *myomixer* transcript that encoded a protein of 77 aa (deposited in GenBank™ with accession number MN230110). Because both ESTs had little overlap, we performed RT-PCR with a primer on each ESTs to confirm that both ESTs belonged to the same transcript. The sequence of the PCR product obtained (599nt), validated that both ESTs belonged to a unique *myomixer* transcript. Sequence alignment between the genomic sequence and the EST sequences revealed the presence of two exons, the first containing the full coding sequence. As shown in the Fig. 1, the trout Myomixer protein was moderately conserved and shared 61% identity with zebrafish Myomixer and only 25% with the mouse one. In addition, trout Myomixer sequence shared 95% of identity with other salmonid Myomixer but only 60–65% of identity with other teleost fish. Despite this overall moderate sequence conservation, the functionally essential motif, AxLyCxL (x corresponds to leucine, isoleucine, valine and y corresponds to serine, threonine, alanine or glycine) (Shi et al., 2017) was conserved in trout Myomixer as well as several charged amino acids in the middle of the protein (arginine at position 40 and 45; lysine at position 39). The phylogenetic analysis of Myomixer proteins from several vertebrate species showed a phylogenetic tree consistent with the vertebrate evolution (Fig. 2). It was noteworthy that all the Myomixer protein sequences studied in salmonid were more divergent than the Myomixer sequences in other teleost.

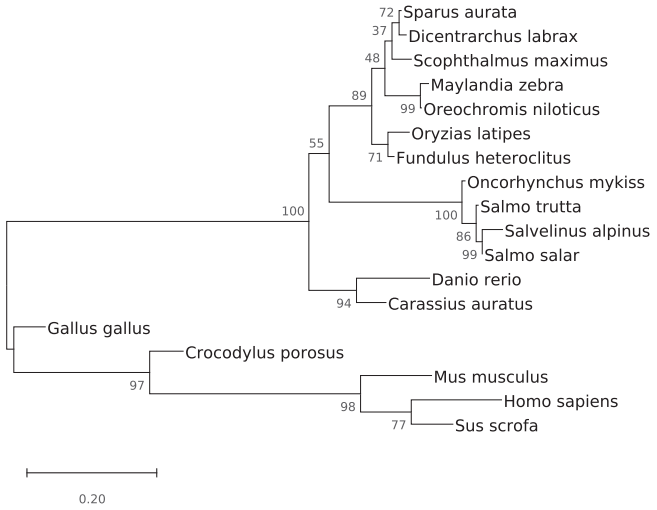

Fig. 2. Phylogenetic analysis of Myomixer in tetrapods and teleosts. The phylogenetic tree was constructed from a multiple alignment of the complete sequences of the proteins using the neighbour-joining method. The numbers at the tree nodes represent percentage of bootstrap values after 500 replicates.

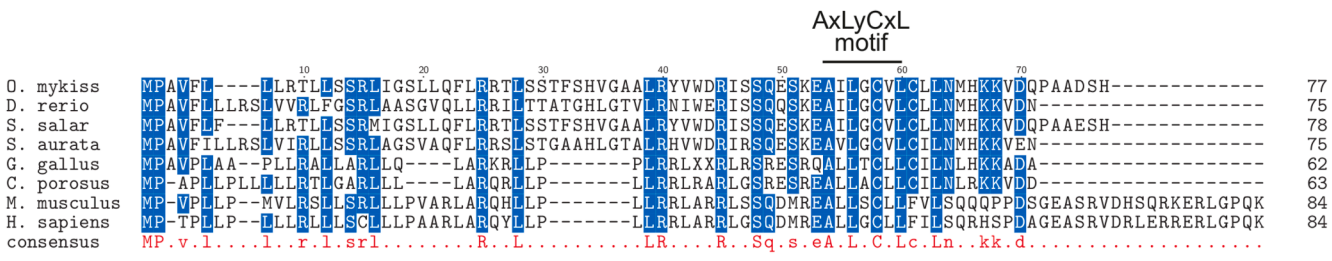

Fig. 1. The sequence alignment of vertebrate Myomixer proteins. The alignment was performed from the complete protein sequences using ClustalW multiple alignment tool. The amino acid residues present in all sequences are uppercase and lowercase when present in at least 6 sequences. The AxLyCxL motif was indicated: x corresponds to leucine, isoleucine, valine and y denotes serine, threonine, alanine or glycine. Accession numbers are as follows: *O. mykiss*, QII57370; *D. rerio*, P0DP88 ; *S. salar*, XM-014180492; *S. aurata*, ERR12611_isotig14560 (http://sea.ccmar.ualg.pt:4567/); *G. gallus*, CD218366.1; *C. porosus*, XP_019405207; *M. musculus*, Q2Q5T5 and *H. sapiens*, A0A1B0GTQ4.

3.2. Myomixer is expressed in embryonic and postlarval trout muscle

We performed whole-mount *in situ* hybridization to examine *myomixer* expression during embryonic myogenesis. *Myomixer* expression was detected as soon as the early stage of somitogenesis (10 dpf) in the deep myotome (Fig. 3A and D). Then, *myomixer* transcript was readily detected at 14 (Fig. 3B and E) and 18 (Fig. 3C and F) dpf in all somites when multinucleated fibers begin to form. *Myomixer* expression was also detected in the head muscles (18dpf) and a transient signal was observed in the otic vesicule (14dpf). In addition, cross-sections (Fig. 3F) of 18 dpf embryos have shown that *myomixer* expression was highest in the lateral part of the myotome. Double *in situ* hybridization for *pax7* and *myomixer* indicated that *myomixer* was not expressed in the undifferentiated myogenic dermomyotome-like epithelium surrounding the primary myotome (Fig. 3G–I) that was positive for *pax7*. In contrast, the myotome strongly expressed *myomixer* but contained rare *pax7* positive cells. After hatching, *myomixer* expression was still readily detected by *in situ* hybridization in the muscle of 1 g and 20 g trout (Fig. 3J and K). The signal, consisting of small red dots (1–2/fiber cross-section) adhering to myofibers was scattered throughout the muscle and was less frequent in muscle of 20 g trout than in 1 g trout. The patterns of *myomixer* and *myomaker* expression in white muscle of 20 g trout were similar (Fig. 3K and L).

The qPCR quantification of *myomixer* expression in white muscle of 15 g, 150 g and 1500 g trout (Fig. 4A) showed that *myomixer* remained clearly expressed after hatching, although its expression declined as fish weight increased. We also analyzed trout *myomixer* expression in several tissues by qRT-PCR to determine whether its expression was restricted to skeletal muscle. As shown in Fig. 4B, *myomixer* was strongly expressed in white and red skeletal muscle but not in heart. *Myomixer* expression was also detected at low level in non-muscle tissues such as skin and brain.

3.3. Myomixer is up-regulated during muscle regeneration and myotube formation in vitro

To determine whether *myomixer* is up-regulated during the muscle regeneration, we measured its expression in muscle following mechanical injury. In our previous study, we observed that the formation of new fibers and the increase of *myogenin* expression occurred 30 days following injury (Landemaine et al., 2019). Consistently, *myomixer* expression remained stable up to 16 days and was sharply up-regulated on day 30 with 6-fold higher expression in injured muscle than in the control one (Fig. 5).

We extracted satellite cells from white muscle of trout, and induced their differentiation and fusion *in vitro* (Gabillard et al., 2010). Quantitative PCR analysis showed that *myomixer* expression was significantly

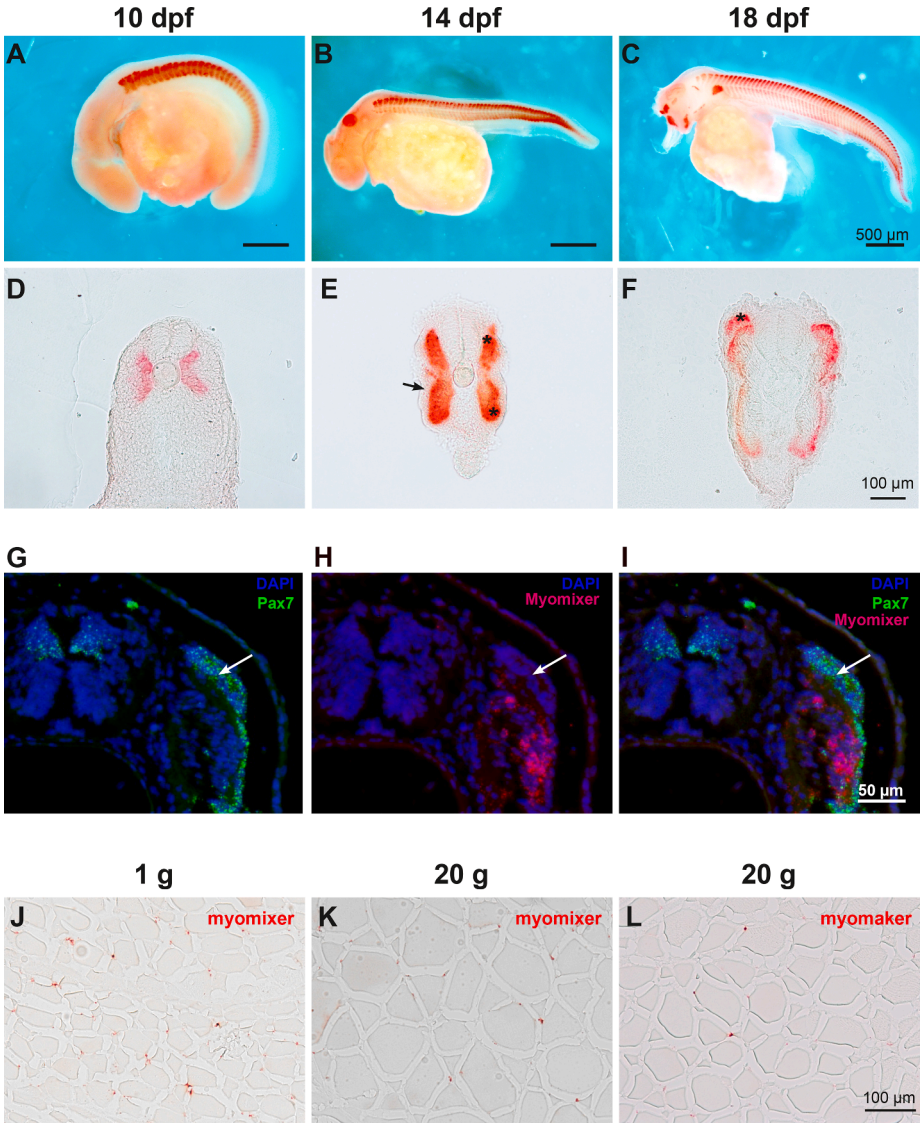

Fig. 3. Patterns of *myomixer* expression during embryonic development. (A-F) Embryos were analyzed by whole mounted *in situ* hybridization at day 10, 14 and 18 post fertilization. The corresponding vibratome section (35 µm) was presented for each stage. Asterisks indicate the dorsal and ventral domains of the myotome and arrowhead indicates the dermomyotome-like epithelium. (G-I) Double *in situ* hybridization for *pax7* and *myomixer* of 17 dpf embryo sections. The nuclei are counter-stained with DAPI and arrowhead indicates the dermomyotome-like epithelium. (J-L) The expression of *myomixer* and *myomaker* in muscle of 1 g and 20 g trout was also studied using *in situ* hybridization on cross sections (7 µm).

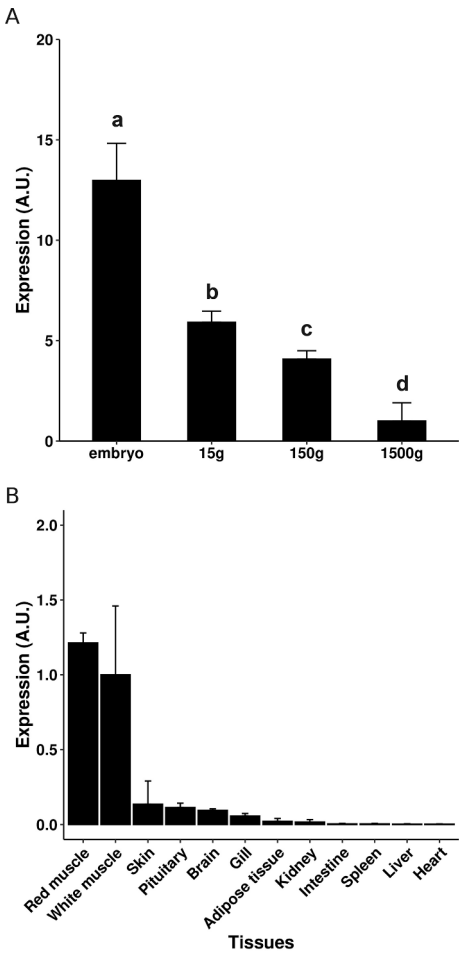

Fig. 4. Expression of *myomixer* in tissues and white muscle of different-weight trout. The quantification of *myomixer* expression was performed by qPCR analysis in muscle of different weight trout (A) and in several tissues (150 g; B). The qPCR results are presented as a ratio of *myomixer* and *eF1a* expression, and the bars represent the standard error. The letters (a-d) in A indicate the significant differences between means ($p < 0.05$; Kruskal–Wallis rank test followed by the Wilcoxon-Mann-Whitney test).

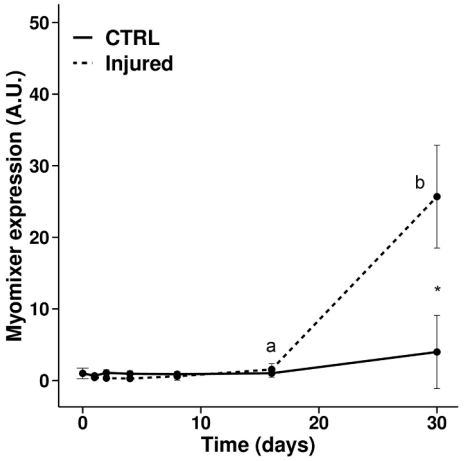

Fig. 5. Expression of *myomixer* during muscle regeneration in trout. Gene expression profile of *myomixer* during muscle regeneration in rainbow trout normalized with *eF1a* expression. Bars represent the standard error and the letters indicate the significant differences between means within the same treatment (control or injured). The asterisk indicates significant differences between treatments at a given time. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis rank test followed by the Wilcoxon-Mann-Whitney test.

up-regulated 3 days after differentiation induction and paralleled *myomaker* expression (Fig. 6A and 6B).

4. Discussion

The fusion of myocytes is highly regulated by numerous key membrane-anchored proteins such as Myomaker and Myomixer (Petrany and Millay, 2019). In the particular context of the persistence of muscle hyperplasia during post-larval growth of trout and the original structure of trout Myomaker protein, our work aimed at characterizing the sequence of *myomixer* and its expression during *in vivo* and *in vitro* myogenesis in this species.

The *in silico* analysis of the trout genome and the EST databases allowed us to identify a unique *myomixer* gene. The alignments of Myomixer protein sequences evidenced a moderate conservation of the overall amino acid sequence across vertebrate lineage. In addition, phylogenetic analysis showed a greater divergence in salmonid Myomixer sequences. This higher rate of protein sequence evolution could result from a relaxation of selection pressure or changes of the functional constraints on Myomixer protein (Zhang and Yang, 2015) although some amino acid residues are still conserved. For instance, the motif AxLyCxL, essential for Myomixer activity (Shi et al., 2017) is present in trout Myomixer protein and in all vertebrate species studied. Thus, despite overall divergence in Myomixer sequences, the key amino acids are conserved in salmonids.

Our expression analyses showed that *myomixer* is strongly expressed in the embryonic myotome during somitogenesis (10–18 dpf), when myoblasts fused to form mature myofibers (Barresi et al., 2001; Steinbacher et al., 2007). Sections of trout embryos of 10 dpf revealed that *myomixer* was expressed in the fibers of the deep myotome formed during the primary wave of myogenesis. Then, the highest expression of *myomixer* was observed in the dorsal, ventral and lateral domains of the myotome, where the secondary wave of myogenesis (stratified hyperplasia) takes place (Steinbacher et al., 2007). In addition, double *in situ* hybridization for *pax7* and *myomixer* showed mutually exclusive expression patterns. Indeed, *pax7* is expressed in undifferentiated myogenic cells present in the dermomyotome-like epithelium surrounding the primary myotome (Dumont et al., 2008). The *pax7*-positive cells spread into the myotome should correspond to the muscle stem cells (also called satellite cells) that persist in adult muscle. In contrast, *myomixer* is strongly expressed in differentiated myogenic cells in the area of muscle hyperplasia (Steinbacher et al., 2007). This expression pattern is in agreement with those obtained in zebrafish that shows a strong expression of *myomixer* from 14 hpf to 24 hpf (Shi et al., 2017). However, at the end of somitogenesis (18 dpf) of the trout embryos, *myomixer* expression is maintained in all somites, whereas in zebrafish its expression is no longer detected in the anterior somites at a comparable stage (24 dpf). Effectively, in mouse and zebrafish the expression of *myomixer* declines soon after somitogenesis (Bi et al., 2017; Shi et al., 2017), whereas in trout its expression is maintained throughout post-larval growth, *i.e.* in fry, juvenile and to a lesser extend in mature fish. Our results clearly indicate that the expression pattern of *myomixer* is similar to that of the *myomaker* in trout (Landemaine et al., 2019) during embryonic and post-larval stages. In addition, we did not observe *myomixer* and *myomaker* expression in myofibers, but only in small cells that should be fusing muscle precursors. These results are in agreement with those obtained in mouse which show that muscle overload induces *myomaker* expression in muscle precursors (myocytes) but not in myofibers, reinforcing the essential role of this protein in muscle hypertrophy and hyperplasia (Goh and Millay, 2017). Accordingly, in zebrafish, *myomixer* and *myomaker* expression is no longer detected in white muscle after hatching (Landemaine et al., 2014; Shi et al., 2017) after which post-larval muscle growth proceeds only by hypertrophy

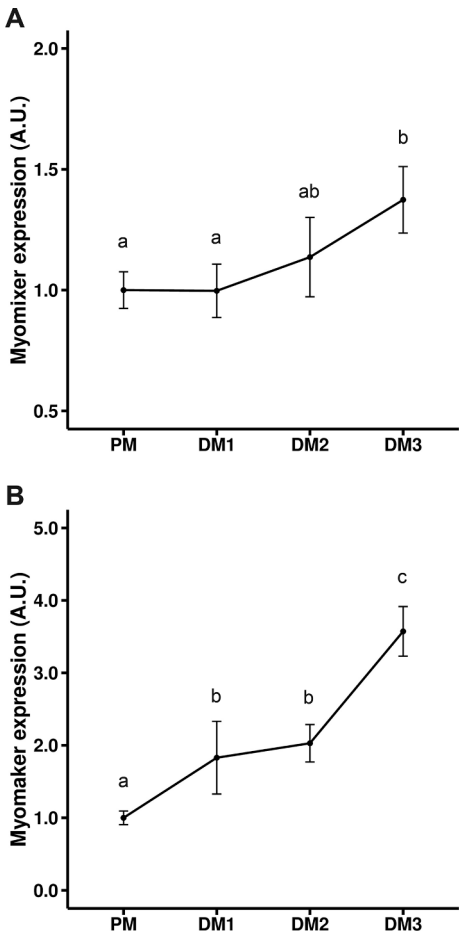

Fig. 6. Expression of *myomixer* and *myomaker* during trout satellite cell differentiation. The cells were cultivated in proliferative medium (PM) and then in differentiation medium for 1, 2, and 3 days (DM1, DM2, and DM3). The qPCR results are normalized with *eF1a* expression and bars represent the standard error. Different letters indicate significant differences between means. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis rank test followed by the Wilcoxon-Mann-Whitney test.

(Johnston et al., 2009). In contrast, in trout, muscle hyperplasia persists during post-larval growth (Steinbacher et al., 2007) and is accompanied by a maintenance of *myomixer* and *myomaker* expression indicating that they are markers of muscle hyperplasia rather than fiber hypertrophy.

Our qPCR analyses showed that *myomixer* expression was strongly stimulated in white muscle 30 days after injury, in parallel with the appearance of newly formed myofibers (Landemaine et al., 2019; Montfort et al., 2016). This kinetic of *myomixer* expression during muscle regeneration, is comparable to that one of *myomaker* and *myogenin* (Landemaine et al., 2019). Moreover, our results are in agreement with our previous transcriptomic analysis showing that numerous genes essential for hyperplastic muscle growth (*myod, myogenin, M−cadherin*, etc.) were up regulated 30 days post injury (Montfort et al., 2016). Furthermore, we showed that *myomixer* and *myomaker* were up regulated 3 days after induction of satellite cells differentiation. This latter result is reminiscent to previous data showing that *myogenin* and *myomaker* expression increase during fusion of trout myocytes (Landemaine et al., 2019). Thus, these results strongly suggest that *myomixer* is up regulated during the fusion of myocytes. It is noteworthy that recent studies using loss-of-function approaches (Zhang et al., 2020) demonstrated in human myoblasts that Myomaker and Myomixer function is very well conserved among mammals, although the regulation of these genes by other MRFs could present slight differences. The technical difficulties in performing this kind of experiments in longer-lived fish species same as the rainbow trout caused that this approach was not contemplated in the objectives of the present work, although it would be a logical continuation of the current work to study the interrelationship of Myomaker and Myomixer with other proteins that regulate the muscle growth in salmonids. Taken together, these results strongly suggest that Myomixer, like Myomaker, plays a crucial role in myoblast fusion, muscle development and muscle regeneration.

5. Conclusions

In conclusion, our work shows that despite moderate sequence conservation, *myomixer* expression is consistently associated with the formation of new myofibers during somitogenesis, post-larval growth and muscle regeneration in trout and can be considered as a good marker of hyperplasia.

CRediT authorship contribution statement

Miquel Perello-Amoros: Methodology, Visualization, Validation, Writing - review & editing. **Cécile Rallière:** Methodology, Visualization, Validation, Writing - review & editing. **Joaquim Gutiérrez:** Visualization, Supervision, Funding acquisition, Writing - review & editing. **Jean-Charles Gabillard:** Conceptualization, Visualization, Supervision, Funding acquisition, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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We particularly thank A Patinote and C. Duret for trout rearing and egg production and L Goardon from the fish facility PEIMA (Pisciculture Expérimentale INRAE des Monts d'Arrée) for muscle regeneration experiments. This work was supported by INRAE and the "Ministerio de Economía y Competitividad" (MINECO) from the Spanish Government and the fellowship of M Perello-Amoros was supported by MINECO (BES-2016–078697).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.145688.

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ARTICLE 6

Myomaker and Myomixer characterization in Gilthead Sea bream under different myogenesis conditions (In preparation draft).

Myomaker and Myomixer characterization in Gilthead Sea bream under different myogenesis conditions.

Perelló-Amorós, M., Tarrazón, A., Jorge, V., Garcia-Pérez, I., Sánchez-Moya, A., Gabillard, J.C., Moshayedi, F., Navarro, I., Capilla, E., Fernández-Borràs, J., Blasco, J., Chillaron, J., García de la Serrana, D., Gutiérrez, J.

Introduction

It is important to have a complete understanding of the myogensis to improve the animal growth and the recovery after injury as well as, to generate better flesh quality and healthy products for human consumption. Myogenesis that is the formation of skeletal muscle by the fusion of mononucleated myoblasts to form multinucleated myofibers, is a process consisting of different phases of activation, proliferation, differentiation and fusion of muscle stem cells, named satellite cells (Bentzinger, Wang, and Rudnicki 2012). Satellite cells play a central role in the maintenance of skeletal muscle in adult life. For example, after muscle damage, satellite cells are activated and migrate to the site of the injury where they quickly proliferate and differentiate into new fibers to repair the muscle (Ciciliot and Schiaffino 2010). Furthermore, in fish, in contrast to mammals, myogenesis extends beyond the adult stage due to an indeterminate and continuous growth throughout their lives, which is made possible by the mechanisms of hyperplasia and hypertrophy (Rossi and Messina 2014).

Many molecules are involved in the regulation of myogenesis, such as the myogenic regulatory factors (MRFs) and members of the Pax and Sox family. These molecules are highly conserved transcription factors in the skeletal muscle cell lineage. The MRFs control the expression pattern of structural proteins as myosin heavy chain (Mhc) or muscle-specific proteins such as the recently discovered Myomaker and Myomixer. The most important MRFs are MyoD, Myf5, Myog and Mrf4. In vertebrates, muscle differentiation is based on the sequential activation of MRFs, Pax and Sox molecules: first, Myf5, MyoD and Sox8 specify the myoblast for terminal differentiation; then, MyoG, Mrf4 and Pax7 participate in the differentiation process and trigger the expression of myotube specific genes to form the multinucleated myofibers (Vélez et al. 2016).

The muscle specific Myomaker and Myomixer proteins that participate in the myogenesis regulation play a central role in cellular fusion, development and regeneration of muscle (Chen, You, and Shan 2019). Myomaker, fist known as Tmem8c, is a highly conserved transmembrane protein in vertebrates. In mammals, myomaker encodes for a 221 amino acids (aa) protein (Millay et al. 2016), while in fish it has more variation. For instance, in trout (Oncorhynchus mykiss) myomaker encodes for 434 aa and in zebrafish (Danio rerio) for 221 aa (Landemaine et al. 2019). Although most of the experiments were done in mammals, in all vertebrates, including fish, myomaker expression is fundamental in both embryogenesis and regeneration of adult skeletal muscle. The expression pattern of myomaker is similar to myod and myog because its regulation is carried out through these MRFs.

The transcription factors MyoD and Myog bind to the two E-boxes present in the promoter of the myomaker gene to induce its expression. Several studies in mice have shown that myomaker expression was maximal during myoblast fusion and that the loss of myomaker inhibited cell fusion during myogenesis (Millay et al. 2013), all these data confirm the involvement of Myomaker in this process of myotubes formation. In fish, most of the research was done in salmonids or zebrafish. In salmonids, trout myomaker is expressed during embryogenesis and muscle regeneration after an injury, where the maximal expression is at the stage of the myoblast fusion (Landemaine et al. 2019) and in zebrafish myomaker expression is restricted to embryogenesis (Landemaine, Rescan, and Gabillard 2014). Thus, further studies in fish no salmonids are needed to understand the role of Myomaker in myogenesis and in adult muscle regeneration.

Myomixer, also called Myomerger or Minion, is a weakly conserved transmembrane protein in vertebrates. In mammals, myomixer encodes for 84 aa protein (Bi et al. 2018). In fish, Myomixer, which is only studied in zebrafish, is a 75 aa protein (Shi et al. 2017) and in trout (Perelló-Amorós et al. 2021) that is 77 aa protein. The expression of myomixer occurs during embryogenesis and myoblast formation. In mice, myomixer is also expressed throughout muscle regeneration after an injury. In the same way as in myomaker, MyoD and Myog regulate the transcription of myomixer by binding to the three E-boxes of its promoter during myogenesis (Quinn et al. 2017). A recent study observed that the lack of myomixer produced a defect in the process of cell fusion

throughout myogenesis, so this result indicates the involvement of Myomixer during myoblast fusion (Bi et al. 2018).

The expression pattern during embryogenesis and muscle regeneration of both myomaker and myomixer is very similar, therefore it has been proposed that the joint interaction between Myomaker and Myomixer could regulate myogenesis. Moreover, Myomaker and Myomixer would act in coordination to allow cell fusion and exchange of cell contents that would complete the process of myoblast fusion (Chen, You, and Shan 2019). Moreover, in vitro experiments have shown that Myomaker and Myomixer could promote the fusion of non-fusogenic cells, as fibroblasts, however, in the absence of one of these proteins, the cellular fusion was blocked (Quinn et al. 2017; Zhang and Roy 2017). Thus, Myomaker and Myomixer may act jointly to permit cellular fusion.

Myoblast fusion requires cell migration, cell recognition and adhesion, hemifusion, pore formation and expansion, and finally, syncytia formation. Different studies indicate that Myomaker and Myomixer act on hemifusion and pore formation and expansion. First, Myomaker and Myomixer are transported to the plasma membrane by vesicles, a process in which different intracellular membranous compartments, such as endosomes, endoplasmic reticulum or Golgi, are involved. Once Myomaker and Myomixer are in the plasma membrane, Myomaker induces hemifusion by bringing the two cells to be fused. In contrast, Myomixer promotes the formation and expansion of the pore between the plasma membrane of the cells by generating stress in the membranes (Fig. 3) (Chen, You, and Shan 2019).

Muscle regeneration, in vitro myogenesis or growth stages comparison offer useful models to study the functions of MRFs, Myomaker and Myomixer and their relationships in the muscle growth regulation. Thus, the objective of this study was first to characterize Myomaker and Myomixer molecules and to investigate their response to regeneration after a muscular lesion provoked, during in vitro myogenesis or at different stages of muscle growth in gilthead sea bream.

Materials and methods

Fish maintenance and distribution

In order to perform the muscle regeneration experiment, 140 gilthead sea bream (Sparus aurata) juveniles (initial body weight: 15,4 ± 3,5 g; initial length: 8,7 ± 0,6 cm) were obtained from a commercial hatchery (Piscimar, Borriana, Spain) and were placed and adapted to the fish facilities of the Faculty of Biology (University of Barcelona). Fish were randomly distributed in three 200 L seawater tanks (46-47 fish/tank). Additionally, four 200 g gilthead sea breams more were divided up in two 200 L tanks (2 fish/tank) for a tissue screening. Each tank had a constant flux of 700 L/h in a seawater semi-closed recirculation system with a weekly water renewal of 20-30%, a salinity of 35-37 ‰ at a constant temperature of 23 ± 1ºC and a photoperiod of 12 h light/12 h dark. Fish were fed ad libitum 3 times per day (9 a.m., 2 p.m. and 7 p.m.) with a commercial diet (Perla, Skretting, Burgos, Spain) and were kept in the described conditions for the acclimation period during 2 weeks before the experiments. The study was carried out following the EU recommendations and the procedures established by the Spanish and Catalan governments. The protocol was approved by the Ethics and Animal Care Committee of the University of Barcelona.

Myomaker and Myomixer characterization

The myomaker mRNA (cDNA) sequence of S. aurata (XM_030418477.1) was obtained from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the myomixer sequence was found in the CCMAR Sequence Server database (Louro et al. 2016). The myomaker and myomixer cDNA sequences were blasted against de gilthead sea bream genome deposited in the Ensembl (https://www.ensembl.org/Sparus_aurata/Info/Index) to obtain the genomic sequence of both genes for its characterization. Primers for the amplification by qPCR of myomaker and myomixer sequences (Table 1) were designed using the cDNA sequences mentioned above with the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer of myomaker was designed in the exon 1–exon 2 junction to avoid amplification of genomic DNA. The primer of myomixer was designed on its only exon, so DNase I pretreatment

of the RNA was necessary previous to the retro transcription. The quality of primers was tested by using the NetPrimer software (http://www.premierbiosoft.com/netprimer/). The collection of myomaker and myomixer sequences from different species was performed through the BLAST databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Unipro UGENE v33.0 software was used to obtain the predicted protein sequences from the nucleotide sequences. Multiple Myomaker and Myomyxer sequence alignments were performed with the MAFFT tool (https://mafft.cbrc.jp/alignment/server/). The iterative refinement method L-INS-i was used for the Myomaker sequence and the progressive method G-INS-1, for the Myomixer sequence. In both cases, a gap opening penalty of 3 was used. The alignments were confirmed with the Unipro UGENE v33.0 software. The phylogeny was developed with the Maximum Likelihood phylogenetic inference method of the MEGA X v10.1.7 program. The JTT+G protein substitution model was used with a bootstrap value of 500.

Experimental design

Tissue screening

The tissue screening allows knowing the tissues and/or organs in which myomaker and myomixer are specifically expressed and therefore, may have a physiological role. For that reason, four 200 g gilthead sea breams were used. For sampling, fish that were deprived of food over-night were anesthetized with MS222 (100 mg/l) and weighted. Then, blood was drawn from the caudal vein with a 1ml sterile syringe and a 0.4- or 0.6-mm needle previously precoated with EDTA-Li to prevent blood clotting. For tissue collection, fish were slaughtered by cutting the cervical section of the spine and the following tissues were extracted: white muscle, red muscle, skin, heart, brain, adipose tissue, liver, spleen, hypophysis, kidney, gill, intestine, bone, pyloric caeca, stomach, and gonad. The tissues were introduced in RNase-free microtubes which were stored during the sampling in liquid N2 and at the end at -80 ºC until further analysis.

Muscle regeneration experiment

The muscle regeneration experiment aimed to better understand the role of Myomaker and Myomixer in myogenesis after muscle injury. To do that, the 140 gilthead sea bream were divided into two groups: injured fish (I) and control fish (C). First of all, gilthead sea bream juveniles were all anesthetized with MS222 (100mg/l) and then measured and weighted. To identify the fish, a pit tag was inserted subcutaneously into the left anterior epaxial muscle just below the first radius.

Subsequently, the injury was performed with a 2.108 mm (14G) diameter needle inserted vertically into the left epaxial muscle below the sixth radius to a depth of 1 cm. To know exactly where the needle was introduced, the tip of the sixth radius was cut. Then, the wound was healed with iodine alcoholic solution and the fish was allowed to recover in a separated small tank before to be returned to the original tank.

Samplings were done at days 0, 1, 2, 4, 8, 16 and 30 after the injury, in which white muscle was extracted. In each period, fish were deprived of food over-night and 20 fish were randomly selected for sampling (4-5 injured fish/tank and 2 control fish/tank). To perform the sampling, fish were first anesthetized, identified reading the pig tag, weighted to note the changes on body weight and the blood was drawn. For tissue extraction, all fish were slaughtered as mentioned before. In injured fish, a section of the muscle was removed from the left side (injured) and the right side, as a self-control for each fish. The size of the muscle extracted was 0.5 cm wide and 1 cm long just below the cut radius. All tissue samples were placed in RNase-free microtube tubes which were stored in liquid N2 during sampling and then stored at -80°C until further analysis.

RNA extraction and cDNA synthesis

For RNA extraction, 1 mL of TRI Reagent Solution® (Applied Biosystems, Alcobendas, Spain) was added to the samples (around 0.04 g for liver and 0.1 g for the rest of the tissues, whenever possible). Samples were homogenized with the Precellys Evolution® (Bertin Instruments, Montigny-le-Brettoneux, France) adjusting the protocol depending on the hardness and elasticity of the tissue. Here below, RNA extraction was performed

following the manufacturer's instructions of the TRI Reagent Solution®. The final concentration of each sample was obtained using the Nanodrop 2000TM (Thermo Scientific, Alcobendas, Spain). RNA integrity was confirmed in a 1% agarose gel (m/v) stained with the SYBR-Safe DNA Gel Stain® (Life Technologies, Alcobendas, Spain). For cDNA synthesis, 1 µg of total RNA was treated with DNase I Amplification Grade® (Life Technologies, Alcobendas, Spain) to remove all genomic DNA. Reverse transcription was carried out with the First Strand cDNA synthesis Transcriptor Kit® (Roche, Sant Cugat del Valles, Spain) following the manufacturer's recommendations.2.4.2. Quantitative real-time PCR (RT-qPCR) according to the requirements of the MIQE guidelines (Bustin et al. 2009), the mRNA transcripts levels of the genes were analyzed by real-time quantitative PCR (qPCR) using the CFX384TM Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The analysis was performed in a final volume of 5 µL, containing 2.5 µL of iTaq SYBR Green Supermix® (Bio-Rad, El Prat de Llobregat, Spain), 0.125 µL of forward (250 nM) and reverse (250 nM) primers,1 µL of cDNA from each sample and 1.25 µL of DEPC water. The reaction was performed in triplicate in 384-well plates (Bio-Rad, El Prat de Llobregat, Spain) under the conditions described by (Salmerón et al. 2015). The qPCR consisted of 1) an activation phase of 3 min at 95 ºC; 2) 40 cycles of 10 s at 95 ºC and 30 s at 55-68 ºC (dependent of the melting temperature of the primer, Table 1); and 3) a melting curve from 55 ºC to 95 ºC that increases by 0.5 ºC every 30 s. Before this analysis, the adequate cDNA dilution for each gene was determined by a dilution curve with a pool of samples. With this analysis, the specificity of the amplification, the absence of primers-dimers and the efficiency of the primers were also tested.

The expression level of each gene was calculated with the Pfaffl method (Pfaffl 2001) and was analyzed relative to the geometric mean of the reference genes (RPS18, RPL27 and EF1a). The reference genes, the most stable under different conditions, were confirmed with the GrayNorm algorithm.

Table 1. Primers used for qPCR: Sequence and melting temperature of *myomaker*, *myomixer, myogenin* and reference genes (*RPS18, RPL27* and *EF1a*). Tm: melting temperature; F: forward; R: reverse

Gene	Primer sequence (5'-3')	Tm (ºC)
Myomaker	**F**: TTCACTGCGGTTTACCACGC	60
	R: CCCACATAGAGAGAGCTGTGCTG	
Myomixer	**F**: TGCCAGCAGTTTTCATCTTG	60
	R: TGAGGAACTGTGCCACTGAG	
Myogenin	**F**: CAGAGGCTGCCCAAGGTGGAG	68
	R: CAGGTGCTGCCCGAACTGGGCTCG	
RPS18	**F**: GGGTGTTGGCAGACGTTAC	60
	R: CTTCTGCCTGTTGAGGAACCA	
RPL27a	**F**: AAGAGGAACACAACTCACTGCCCCAC	68
	R: GCTTGCCTTTGCCCAGAACTTTGTAG	
EF1a	**F**:CTTCAACGCTCAGGTCATCAT	60
	R:GCACAGCAAACGACCAAGGGGA	

Statistical analyses

Data were analyzed using IBM SPSS Statistics v.25 and were presented as mean ± standard error of the media (SEM). Normal distribution was analyzed using the Shapiro-Wilk test and homogeneity of the variances (homoscedasticity) was assessed with Levene's test. If normal distribution and/or homoscedasticity do not meet, data were transformed logarithmically. Differences were tested by Student's t-test or one-way analysis of variance (ANOVA) and the post-hoc Tuckey HSD. If necessary, the nonparametric Kruskal Wallis test and the post-hoc T3 de Dunnett were used. Additionally, one-way ANOVA was performed to verify that the tank do not influence the measured parameters. Statistical differences were considered significant when $P < 0.05$.

Results

Myomaker and Myomixer characterization

A search in GenBank was performed to identify the gilthead sea bream myomaker mRNA (>XM_030418477.1). The genomic sequence of myomaker was found by a name search in the gilthead sea bream genome deposited in the Ensembl (ENSSAUG00010019449). Two paralogues of myomaker were identified, named pgap6 (ENSSAUG00010014619) and tmem8b (ENSSAUG00010020348), with a low percentage of the Query ID, 33.19

and 30.60 %, respectively. This search also revealed a single transcript (ENSSAUT00010049102.1) that apparently contained only 5 exons encoding a 232 aa protein. However, the alignment between the cDNA and the genomic sequence revealed that the automatic exon finding algorithm of the Ensembl included the exon 6 sequence inside 3' UTR. Thus, it was determined that the myomaker gene was situated in chromosome 5 and contained 6 exons encoding a protein of 285 aa (Fig. 4). The gilthead sea bream Myomaker protein (>XP_030418477.1) shared 89.50 % identity with the zebrafish Myomaker protein (NP_001002088.1) and 71.56 % identity with the mouse Myomaker protein (NP_079652.1).

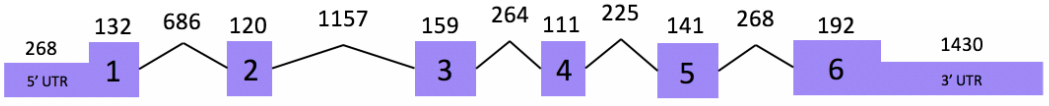

Figure 1: Structure of the *S. aurata* myomaker gene. The size of exons, UTR (purple boxes) and introns (lines) sequences are indicated in nucleotides.

The gilthead sea bream myomixer sequence was provided by the CCMAR Sequence Server. The cDNA sequence of myomixer was blasted against the gilthead sea bream genome deposited in the Ensembl to find its genomic sequence (ENSSAUG00010011859, no paralogues identified). The myomixer gene was located in chromosome 15 and had one transcript (ENSSAUT00010028952.1) containing only 1 exon encoding a 75 aa protein (data not shown). The gilthead sea bream Myomixer protein shares 70.67 % identity with the zebrafish Myomixer protein (P0DP88.1).

The phylogenetic analysis of the Myomaker and Myomixer amino acid sequences is shown in Figures 2 and 3. In both cases, a clear evolution of the proteins from the most primitive vertebrates (fish) to the most modern ones (mammals) is observed. The Myomaker and Myomixer gilthead sea bream sequences are more related to other perciformes, as D. labrax, and beloniformes. Myomixer might be slightly less conserved than Myomaker due to lower bootstrap values. The Myomaker protein sequences (Figure 2) in fish have a great disparity in length. In gilthead sea bream, it has 285 aa, while in salmoniforms, it is formed for about 430 aa. In chondrichthyans, the sequence of

Myomaker is the smallest, having 218 aa. All Myomaker protein sequences in terrestrial vertebrates (amphibians, reptiles, birds and mammals) vary between 220 and 221 aa.

In contrast, the Myomixer protein sequence (Figure 3) among the different vertebrate species has less variation in length. In most fish, Myomixer has 75 aa, as in S. aurata. However, some salmonids, such as O. Kisutch, have a sequence of 99 aa. In terrestrial vertebrates, the sequences range from 62 aa, as in birds, to 108 aa, as in the reptile P. picta. In mammals, Myomixer remains at 84 aa. The analysis of both the genomic and the mRNA sequence of myomixer determined that this mRNA also encodes for a longer protein, called Spectrin beta chain, non-erythrocytic, formed by 1708 aa.

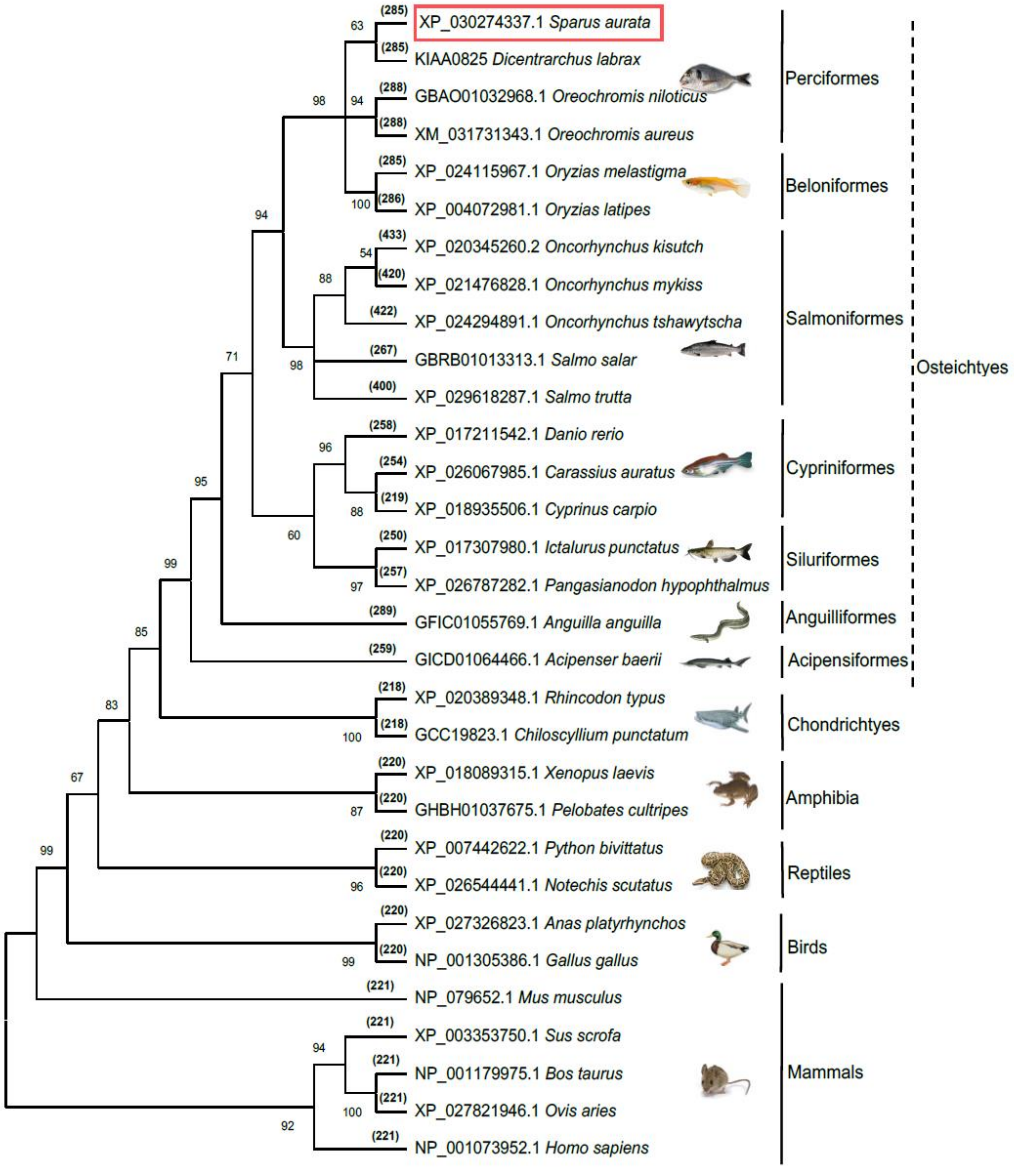

Figure 2: Phylogenetic analysis of Myomaker protein among mammals, birds, reptiles, amphibians and fish. Multiple alignment of whole protein sequences was done through the MAFFT tool (https://mafft.cbrc.jp/alignment/server/) with the iterative refinement L-INS-i method and a gap opening penalty of 3. The phylogenetic tree was developed with the Maximum Likelihood phylogeny and the JTT+G substitution model using the MEGA X v10.1.7 program. The numbers in the tree nodes represent the percentage of the bootstrap values after 500 replicates. The numbers in parentheses show the amino acids that myomaker encodes in the different species. The sequence of our studied species, S. aurata, has been marked with a red rectangle.

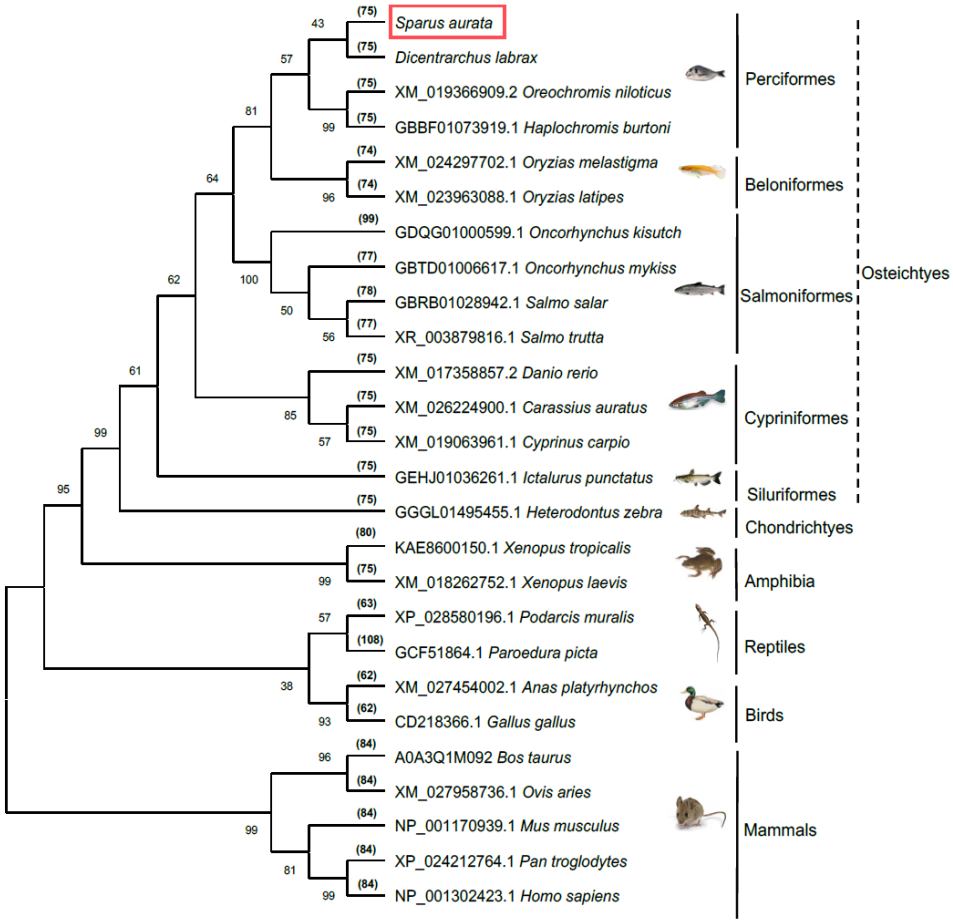

Figure 3: Phylogenetic analysis of Myomixer protein in mammals, birds, reptiles, amphibians and fish. Multiple alignment of whole protein sequences was done through the MAFFT tool (https://mafft.cbrc.jp/alignment/server/) with the progressive G-INS-1 method and a gap opening penalty of 3. The phylogenetic tree was developed with the Maximum Likelihood phylogeny and the JTT+G substitution model using the MEGA X v10.1.7 program. The numbers in the tree nodes represent the percentage of the bootstrap values after 500 replications. The numbers in parentheses indicate the number of amino acids that myomixer encodes. The accession number of the sequences of S. aurata and D. labrax lack because they were provided by CCMAR Sequence Server. The sequence of our studied species, S. aurata, was marked with a red rectangle.

Tissue screening

The Figure 4A-B shows the myomaker and myomixer expression in gilthead sea bream tissues. The myomaker gene was mostly expressed in white and red muscle at similar levels (Fig. 4A). In contrast, the myomixer gene showed expression in white and red muscle, as well as, in skin, heart, brain, bone and gonad. The myomixer transcript levels

in these tissues were also confirmed in the agarose gel after a PCR. In the other tissues, the expression of myomixer is insignificant.

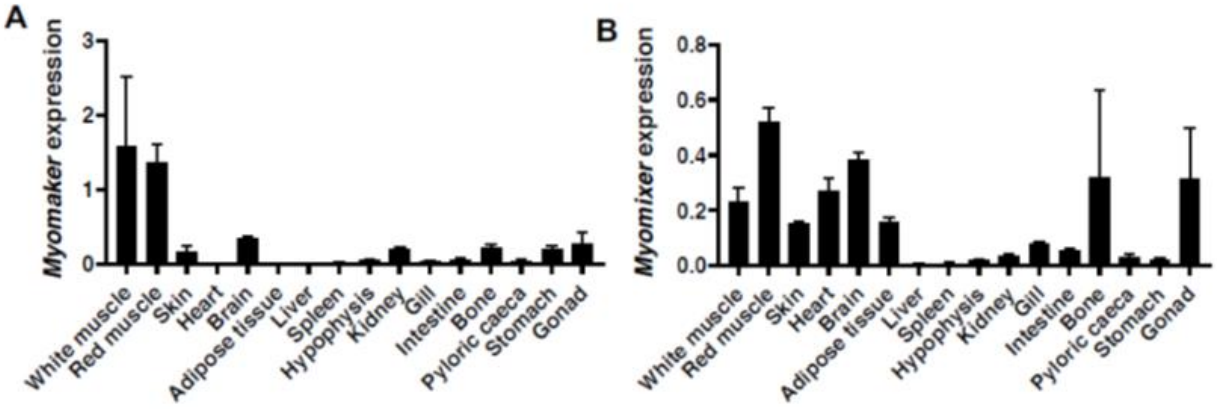

Figure 4: myomaker (A) and myomixer (B) expression in several tissues (white and red muscle,skin, heart, brain, adipose tissue, liver, spleen, hypophysis, kidney, gill, intestine, bone, pyloric caeca, stomach and gonad) of 200 g gilthead sea bream. Data are represented as mean ± SEM (n=4).

Regeneration Study

Myomaker and Myomixer

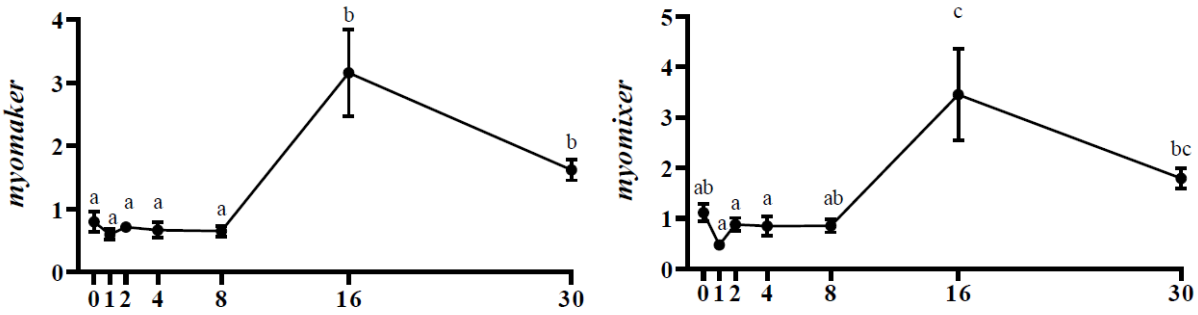

Figure 5: Relative gene expression of myomaker and myomixer along the regeneration experiment in skeletal white muscle. Data are presented as means ± SEM (n=10). Letters indicate significant differences ($p<0.05$).

The figure 5 shows de profile of both genes during the regeneration period in white muscle of gilthead sea bream, where their parallelism is clearly demonstrated. Thus, after a non-significant decease at day 1, followed a stable period with almost constant values

until day 8; since this moment a significant peak at day 16 was found where the expression of both genes triples the previous values. myomaker expression at day 30 although decreased was still significantly higher; myomixer also decreased to values not different than those at day 8.

MRFs

The different myogenic genes present a similar profile during regeneration with a maximum peak at day 16 of the experiment (Figure 6). However, Myods and Myf5 showed a profile with a more evident peak at day 6 to decrease progressively until day 30; while mrf4 and specially myogenin showed a peak at day 16 that extends quite stable until day 30.

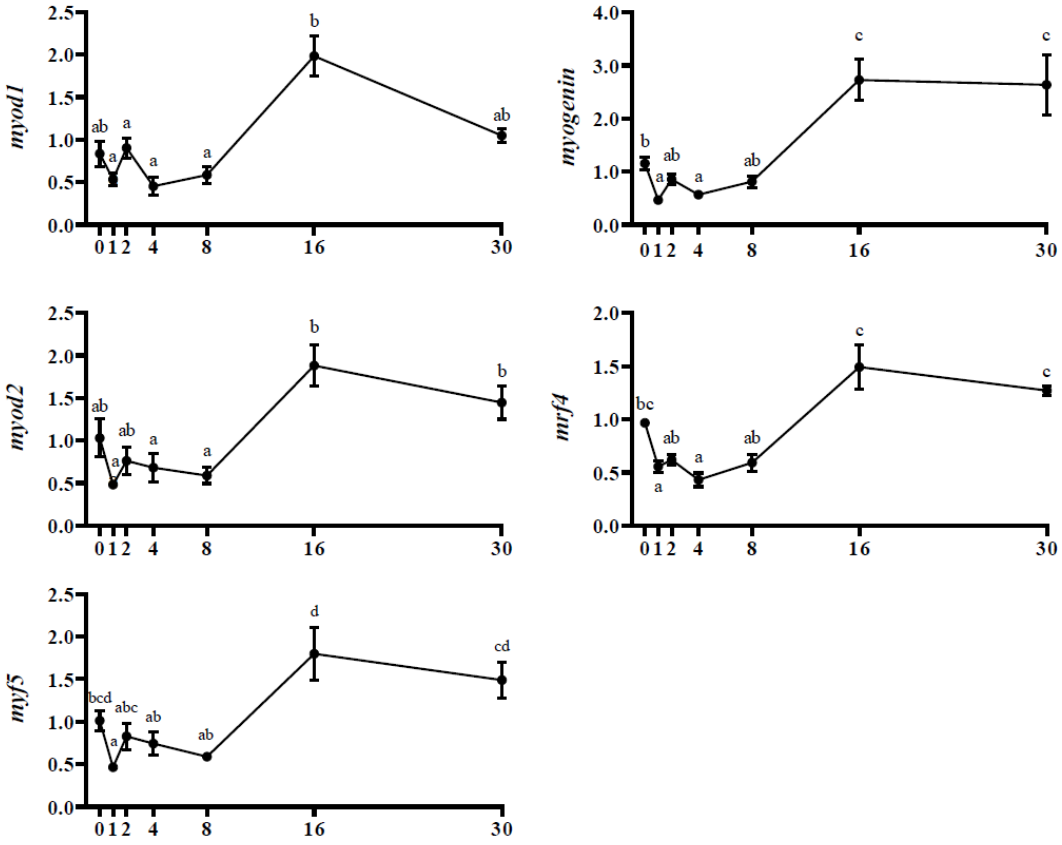

Figure 6: Relative gene expression of the myogenesis regulation factors (MRFs) along the regeneration experiment in skeletal white muscle. Data are presented as means ± SEM (n=10). Letters indicate significant differences ($p<0.05$).

In vitro myogenesis

Myomaker and Myomixer

Myomaker increased significantly already at day 4 of in vitro myogenesis of gilthead sea bream, reaching at day 6 its maxim expression, followed by a progressive decrease significant at day 12 (Figure 7). In the case of myomixer, the profile is not identical (Figure 7) and although the increase was also significant at day 4 the expression was progressively increasing to reach the maximum at day 8 to then fall dawn until day 12.

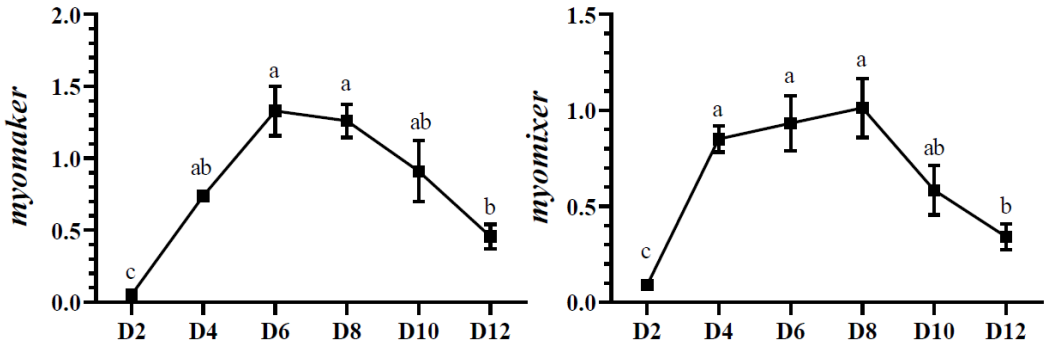

Figure 7: Relative gene expression of myomaker and myomixer along the culture of primary myoblasts. Data are presented as means ± SEM (n=6). Letters indicate significant differences ($p<0.05$).

MRFs

Myod1 and myod2 presented the earliest peak, respectively at day 4 and day 6; then, in the case of Myod1 to progressively diminish significantly at day 6 reaching lowest levels of expression at day 12; myod2 expression after its peak of expression decreased not significantly, maintaining a plateau until day 12.

Myogenin expression increased rapidly significantly at day 4, followed by its maximum at day 6 and a progressive decrease until day 12. The mrf4 expression showed a first increase, non-significant, at day 4 and a slow increase to reach the significant maximum levels at day 10 decreasing not yet significantly at day 12.

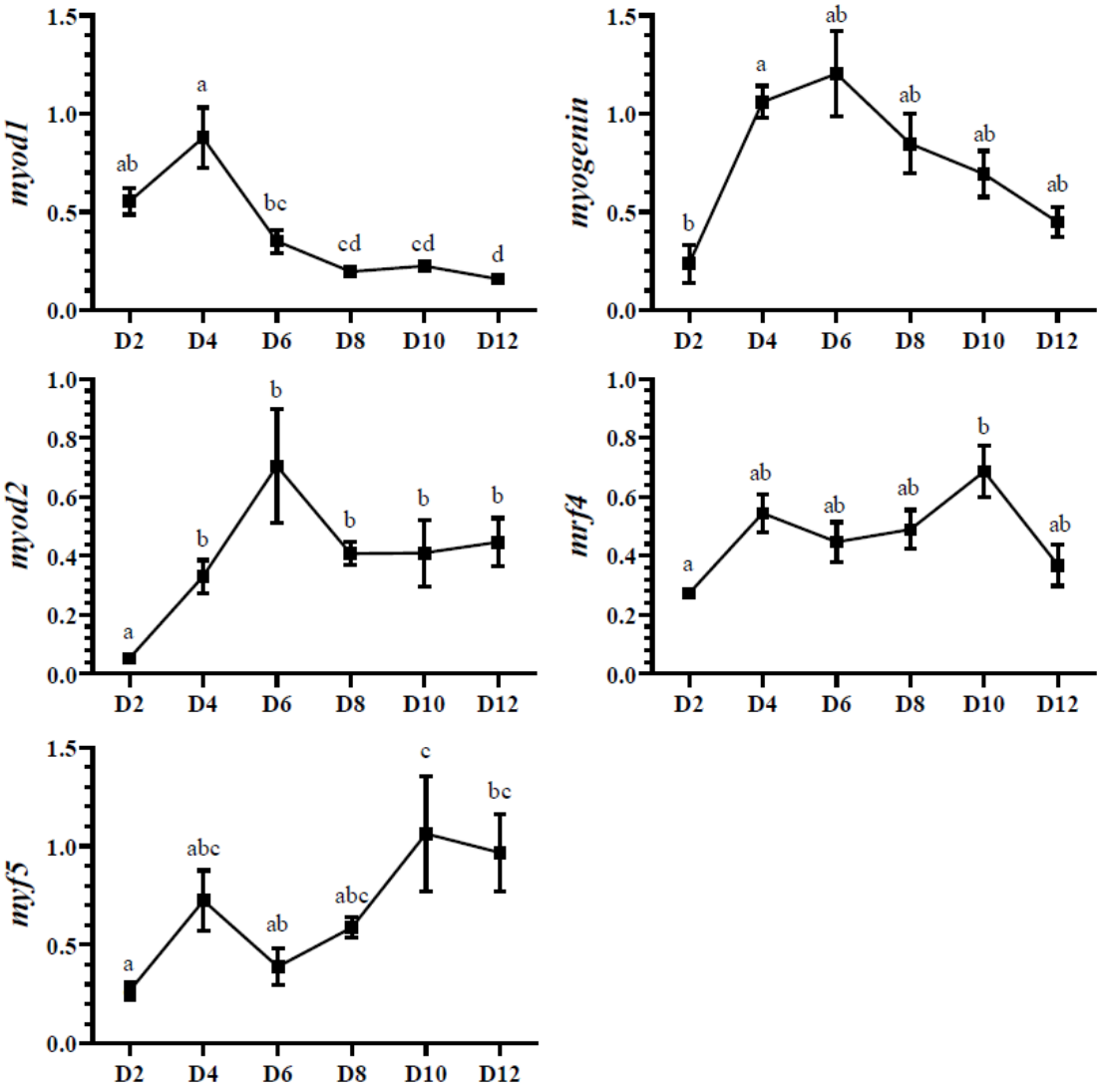

Figure 7: Relative gene expression of the myogenesis regulation factors (MRFs) along the culture of primary myoblasts. Data are presented as means ± SEM (n=6). Letters indicate significant differences ($p<0.05$).

Myomaker and Myomixer white muscle expression at different growing stages

The Figure 8 shows the expression of myomaker and myomixer in white muscle of fingerlings, juveniles and adults of gilthead sea bream. Both genes showed a progressive decrease with the age of the fish. Thus, fingerlings presented the maximum expression levels, juveniles decreased significantly and adults showed very low levels of myomaker and myomixer.

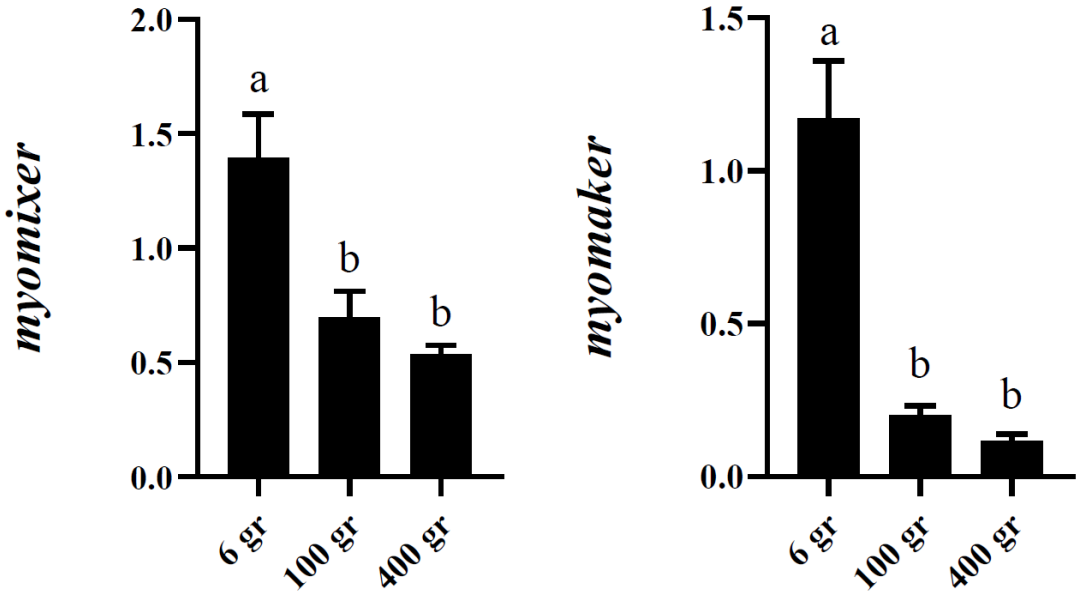

Figure 8: Relative gene expression of myomaker and in skeletal white muscle of three different sized of gilthead sea bream. Data are presented as means ± SEM (n=6). Letters indicate significant differences ($p<0.05$).

Discussion

Molecular Characterization and tissue screening

The muscle specific Myomaker protein that controls myoblast fusion was found in mice by searching in the NCBI UniGene database. In mammals, myomaker had a similar expression profile to those of myod and myog and encoded for a 221 aa protein (Millay et al. 2013), as shown in Fig. 5. In fish, Myomaker was first described in zebrafish by a BLAST search using the mouse Myomaker sequence (NP_079652.1) against the zebrafish genome (Landemaine, Rescan, and Gabillard 2014). Later on, the trout

myomaker gene (GSONMG00014531001) was identified by a BLAST search against the trout genome using the sequence of zebrafish Myomaker protein (NP_001002088). The trout myomaker gene contained six exons and encoded a 434 aa protein (Landemaine et al. 2019). In the protein sequence alignment, it was found out that the first half of the trout Myomaker protein was similar to the mice Myomaker protein, while the second half did not have homology with any known motifs (Landemaine et al. 2019). However, in the GenBank search that was performed in the present study, the trout Myomaker protein had 420 aa (Fig. 5), instead of 434 aa. This difference in the length of Myomaker could be due to the presence of different transcript variants, but still, Myomaker would be divided into two halves, as Landemaine et al. (2019) proposed. In the GenBank database, the gilthead sea bream myomaker gene (>XM_030418477.1) had 6 exons encoding a 285 aa protein (Fig. 4). Nevertheless, in the gilthead sea bream genome deposited in the Ensembl, the transcript of the myomaker gene (ENSSAUT00010049102.1) contained 5 exons encoding a 232 aa protein. This disparity in results may be because the automatic exon detection algorithm of Ensembl interpreted the last exon as part of the 3' UTR region, taking into consideration that the structure of the trout myomaker gene had 6 exons (Landemaine et al. 2019). The multiple alignment of the gilthead sea bream and trout Myomaker sequences demonstrated that the gilthead sea bream Myomaker protein corresponded to the first half of the trout Myomaker, since it is the most conserved part of the protein. Lastly, the phylogenetic analysis showed that Myomaker was a well-conserved protein across vertebrate organisms, from fish to mammals (Landemaine et al. 2019; Millay et al. 2013). For instance, the first half of the trout Myomaker protein shared 88 and 71 % identity with the zebrafish and mouse Myomaker proteins, respectively (Landemaine et al. 2019). This result was similar to the percent identity of gilthead sea bream Myomaker protein between the zebrafish (89 % identity) and mouse (71 % identity) proteins. Moreover, a clear evolution among fish orders was observed and the gilthead sea bream Myomaker resulted more closely related to other perciformes species, such as the sea bass (D. labrax), the beloniformes species, such as O. latipes, or the salmonids (Fig. 5). However, the gilthead sea bream Myomaker was more distant to cypriniformes, siluriformes, anguiliformes, acipensiformes and chondrichtyes (Fig. 5). Although the gilthead sea bream was not included in the phylogenetic analysis of (Landemaine et al. 2019), the perciform Myomaker sequences were more associated with

beloniformes species while more distant to salmoniformes or cypriniformes, a similar result to that shown in the Figure 2.

The fusogenic gene myomixer that was identified in mice, contained a single exon and encoded a 84 aa protein. Additionally, the mice myomixer gene had another transcript form, less conserved, which had 3 exons and yielded a protein of 108 aa (Bi et al. 2018; Quinn et al. 2017). Myomixer showed weak cross-species conservation because mammals and fish only shared 36 % aa identity (Shi et al. 2017). Among fish, the zebrafish Myomixer protein shared 68.5 % identity with the trout Myomixer protein (unpublished data), which was similar to the 70.76 % aa identity that shared the gilthead sea bream and the zebrafish Myomixer protein. The zebrafish Myomixer protein was formed by 75 aa (Shi et al. 2017). The rainbow trout myomixer gene, which had 2 exons, encoded a 77 aa protein (Perelló-Amorós et al. 2021). The gilthead sea bream myomixer transcript (ENSSAUT00010028952.1) that was found in the gilthead sea bream genome deposited in the Ensembl contained only one exon that encoded a 75 aa protein (Fig. 6). As is the case of Myomaker, the gilthead sea bream Myomixer was more related to other perciformes, as the sea bass, beloniformes or salmoniformes. Nevertheless, evolutionarily, the gilthead sea bream Myomixer was more distant to cypriniformes, siluriformes and chondrichtyes (Figure 3). Other phylogenetic analysis performed in gilthead sea bream Myogenin and Ghrelin proteins also shown that these molecules are evolve in the same way, as these molecules are evolutionarily close to other perciforms, while are more distant to salmoniformes or cypriniformes (Perelló-Amorós et al. 2018). Finally, an interesting aspect that could be studied in the future is the possible relationship between the Myomixer and Spectrin beta chain non-erythrocytic, as the gilthead sea bream mRNA encoded for these two proteins. It was demonstrated that Spectrin, which is a membrane skeletal protein, was required for Drosophila and mouse myoblast fusion (Cong et al. 2019; Duan et al. 2018). Although the interaction between Myomaker and Spectrin beta chain has not yet been studied, the fact that one mRNA encoded for two proteins, which are associated with myoblast fusion, suggests that these proteins could act jointly.

On one hand, the gene expression of myomaker in gilthead sea bream showed a narrow distribution among tissues, as it was expressed mainly in white and red muscle (Fig. 7A).

In other species, such as trout, myomaker gene was also expressed at similar levels in both white and red muscle (Landemaine et al. 2019). On the other hand, the myomixer expression in adult gilthead sea bream was not restricted to white and red muscle (Fig 7B). However, in the case of the trout and mice, it was detected myomixer only in white and red muscle (Quinn et al. 2017; Perelló-Amorós et al. 2021). So, further work is needed to clarify the role of myomixer in these tissues and another tissue screening should be done in juvenile gilthead sea bream to confirm if this expression pattern is similar to that observed in adults.

Regeneration study

While in mammals, myogenesis is a well-known process, in fish, some aspects remain unclear. Regeneration in gilthead sea bream after a muscle injury was first studied by Rowlerson et al. (1997). The histological analysis showed a higher cellular proliferation with a greater deposition of connective tissue and new small myofibers around the lesion site by 7–11 days after the injury was made. In trout muscle, after 20 days of a mechanical lesion, it was observed an alteration of the muscle fiber organization at the site of the damage due to a high deposit of connective tissue with new small muscle fibers (Rescan et al. 2015). Another experiment in trout after a mechanical injury showed that after 30 days, there was a replacement of the injured fibers with connective tissue containing small round and centrally nucleated cells, coinciding with a peak of myogenin expression (Landemaine et al. 2019). The differences observed between the several fish species at the moment at which the new myofibers were formed during myogenesis could be due to the greater metabolic rate that gilthead sea bream presents. Besides, the temperature at which fish live could influence the metabolic rate, as gilthead sea bream were grown at a higher temperature (21-23ºC) that trout (10-15ºC), such a higher activity could explain the faster muscle regeneration. The histological analysis of muscle regeneration in gilthead sea bream must be studied in future investigations, however, during the different days of the sampling, the changes that occurred in the muscle from day 1 to day 16 post-injury could be observed. On day 1, the injury was easily observed with the naked eye, nevertheless, on day 8 and day 16, the muscle damage was no longer noticeable (pictures shown in ANNEX I). This would mean that new myofibers would already be forming to repair the muscle injury. Another interesting aspect of myogenesis is the myoblast fusion

and the recently, the two new proteins involved in myoblast fusion, Myomaker and Myomixer, are investigated. The myomaker gene, which was first identified in mice, was expressed specifically in skeletal muscle during embryogenesis and adult muscle regeneration after cardiotoxin injury (Millay et al. 2013). In mice, myomaker expression was strongly detected in regenerating muscle after the injury, and then immediately decreased when the intact myofibers were formed, which indicated that Myomaker was essential for muscle regeneration. The regulation of myomaker was mediated by two E-boxes in the promoter, which bound MyoD and Myogenin. Furthermore, another experiment with myomaker satellite cell knockout (myomaker scKO) mice showed that these cells were able to proliferate and differentiate but could not fuse, resulting in a complete block of regeneration (Millay et al. 2014). The myomixer gene was also first described in mice, where it was expressed during development and it was induced during adult myogenesis (Quinn et al. 2017). In fish, Myomaker was studied in rainbow trout after a mechanical muscle injury and a significant increase in myomaker was detected in the injured muscle at day 30. It was also observed that Myogenin regulated Myomaker expression by binding to the E-boxes present in the promoter of myomaker, as in mammals (Landemaine et al. 2019). Myomixer was studied in zebrafish embryogenesis (Shi et al. 2017) and recently, in a regeneration experiment in trout, it was observed that myomixer expression was significantly upregulated in the late post-injury regeneration (Perelló-Amorós et al. 2021). In the present study, the muscle regeneration experiment in gilthead sea bream showed that myomaker and myomixer were upregulated from day 1 to day 8 post-injury, at the same time as myogenin (Figure 7). This response was faster than that observed in trout by Landemaine et al. (2019), which could be due to a greater metabolic rate with satellite cells more actives in gilthead sea bream. Another aspect that could affect the metabolic rate, in addition to the temperature at which fish live, might be the size of the fish. In the trout muscle regeneration performed by Landemaine et al. (2019), 1 kg trouts were used, in comparison with the 15 g gilthead sea bream that were utilized in the present study. In general, the smaller the fish shown a higher the metabolic rate and therefore, a fast muscle regeneration. Thus, this could explain the differences in the timing of increased expression of myomaker and myomixer. Moreover, these findings are in agreement with those of Rowlerson et al. (1997) by histological studies in gilthead sea bream, as the new small myofibers were deposited in the lesion site around day 8 post-injury, at the same moment that it was observed a significant increase in the

myogenin, myomaker and myomixer expression. Hence, these genes could have a role in that phase of myogenesis. Finally, as these genes have similar expression curves, they should act jointly during myogenesis as (Quinn et al. 2017) proposed. However, this hypothesis should be confirmed by new molecular and functional studies.

Myogenesis in vitro and in vivo

The in vitro study performed showed that myomaker presented an expression peak at day 6 decreasing later progressively while in myomixer the highest values was at day 8. This temporal distribution is in agreement with the described role of both molecules. Thus, the role of myomaker inducing hemifusion will be important at the beginning of this process. In contrast, Myomixer, that promotes the formation and expansion of the pore between the plasma membrane of the cells, will be involved after the hemifusion, and its function will start after that of Myomaker and will extend longer, in agreement with the profile shown in gilthead sea bream myocites. Our results coincide with those of (Perelló-Amorós et al. 2021) in trout in vitro muscle cells where the expression of both myomaker and myomixer was increasing progressively during differentiation stages.

The MRF expression in in vitro gilthead sea bream myogenesis was well described by our group (García de la serrana et al. 2014) and the results obtained here are consistent with that first report. The myod1 early peak agrees with function of this factor or early stages of myogenesis and combined with the delayed profile of myod2 that it is well know that express maximum expression after Myod1 (Tan and Jun Du 2002). The expression peak of myogenin at day 6 and the maintenance of high levels still at day 8 coincides with its role regulating the myoblast differentiation progress as well as with the maxim levels of mrf4 at day 8 more involved in the finalization and maturation of myotubes. The high parallelism between myogenin and myomixer and myomixer expression is quite clear and it is consequent with the role of these three factors on myogenesis. In fact, the interaction with Myogenin has been described and it was also determined that the Myogenin protein regulated myomaker expression by binding to E-boxes of the myomaker promoter (Landemaine et al. 2019).

The comparison of fish at different ages suggests that myomaker and myomixer plays more active work at stage of fingerlings. These results are in agreement with the findings described in trout (Perelló-Amorós et al. 2021) where the expression of myomixer is maximum at the stage of embryo, decreasing at progressively a 15, 150 and 1500 grams. All this information supports the role of both factors in somitogenesis or strong growing stages of fingerlings to decrease in juveniles or adults where the level of hyperplasia is less important. Thus, in mouse and zebrafish the expression of myomixer declines soon after somitogenesis (Bi et al. 2018; Shi et al. 2017), whereas in trout its expression is maintained throughout postlarval growth, i.e. in fry, juvenile and to a lesser extend in mature fish.

Overall, these results support that myomaker and myomixer play in gilthead seabream an important role during myogenesis, especially at the second part of the process when myocites differentiation takes place, but also during regenerative myogenesis where their appearance after first proliferative stages points out their role during differentiation. Our results then contribute to understand the role of myomaker and myomixer in a fish species of undetermined growth, normally leaving at high temperature waters and with high interest for aquaculture.

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CHAPTER 5: GENERAL DISCUSSION

4. GENERAL DISCUSSION

4.1.Hormonal regulation of systemic growth: the GH/IGF axis.

The Gh/Igf axis has long been considered the main endocrine regulator of growth in vertebrates and the role of the main molecules composing this axis in different tissues has been extensively studied in different fish species (reviewed by Fuentes *et al.* 2013 and Emilio J Vélez, Lutfi, *et al.* 2017) under different experimental conditions in both in vivo and in vitro models. However, until today, due to the variability between the experimental conditions in the existing bibliography, there are still many clues about how the molecules that conform this hormonal axis respond to different nutritional and metabolic situations in fish, as well as how they interact with other endocrine and regulatory systems. In the present thesis, the Gh/Igf axis at systemic level has been studied in two different experimental approaches in gilthead sea bream juveniles, a fasting and refeeding trial and a moderate and sustained exercise program combined with two commercial diets differing in protein and lipid content.

In the fasting and refeeding trial (Perelló-Amorós et al. 2018; **Article 1**), gilthead sea bream juveniles were fasted for 21 days and then refed for 7 days, with samplings before fasting, at the end of fasting, at 2, 5 and 24h postprandial after the first meal, and at 7 days of refeeding. Fasting provoked a marked increase in systemic Gh release reflected both in plasmatic Gh levels and *gh* mRNA expression in pituitary, being unmodified by short term refeeding, but returning to basal levels after 7 days. This response of Gh to fasting and refeeding has been observed in previous studies in various fish species, such as Chinese perch (*Siniperca chuatsi*), tilapia and black sea bream (*Spondyliosoma cantharus*) (Deng et al. 2004; Breves et al. 2014).

Contrarily to Gh, plasma Igf1 responded inversely to nutritional state, decreasing with fasting and slowly increasing with refeeding. The expression of hepatic total *igf-1* as well as its splice variants, partially recovered after 7 days of refeeding, in agreement with previous works (Pierce et al. 2005; Breves et al. 2014). These inverse profiles of Gh and Igf1 plasma levels during fasting was pointed out in gilthead sea bream previously (J Pérez-Sánchez 2000; Mingarro et al. 2002; Company et al. 2001), and has been described

in several other fish species (e.g., coho salmon, chinook salmon, channel catfish, Nile tilapia or gilthead sea bream) in diverse conditions, such as moderate sustained exercise, which will be further discussed (Vélez et al. 2016; Fox et al. 2009; Pierce et al. 2005; Duan and Plisetskaya 1993; Small and Peterson 2005; Blasco et al. 2015). These results reinforce the idea that the Gh/Igf1 ratio is a good indicator of metabolic state in gilthead sea bream and that it is clearly affected by feeding condition (J Pérez-Sánchez 2000), being usually reflected in the growth and morphometric indexes.

In the case of the sustained swimming and diet trial, the response of these two hormones to exercise was utterly dependent on the diet composition. Under voluntary activity, a greater weight gain was achieved in fish fed with a high protein diet (HP) in comparison those fish that were fed with a high-fat diet (HE). Such weight gain was accompanied by higher plasma Gh levels, which led to an increased Gh/Igf1 plasma ratio in this group. It is well known that dietary proteins and lipids can alter the response of this axis, however, because of the numerous variables that must be considered when comparing experiments, such as the age of the animals, the feeding régime and the time of sampling, there are discrepancies in the effects of the proportion of these nutrients on Gh secretion in fish. In this regard, Marti-Palanca *et al.* (1996) and Cameron *et al.* (2007) reported in gilthead sea bream and arctic charr (*Salvelinus alpinus*), respectively, an inverse correlation between circulating Gh and dietary protein/lipid ratio. On the other hand, the ration size is a factor that has to be taken into account in order to interpret the effects of nutrients over these two hormones, as in gilthead sea bream juveniles, the dietary protein to lipid ratio affected differently the plasma Gh levels depending on the ration size (Company et al. 1999). Hence, they reported that when fish were fed ad libitum with a high-protein diet, the secretion of Gh was downregulated while when the fish were fed at 1.7% of biomass/day, the Gh levels were upregulated, while contrary effects were induced by high-lipid diets. Thus, the results obtained in this study suggests that under a 5% meal ration, the higher protein content of HP diet had stronger effects on Gh secretion than the lipids of HE diet; but also it may have happened a Gh downregulation in the non-exercised fish that were fed the HE diet because of a possible saturation of the oxidative pathways (as which will be discussed later) and in such situation, the lipolytic effects of the Gh would be counterproductive. However, when the fish were subjected to sustained exercise, no differences in Gh levels were observed due to the diet, as a consequence of a

downregulation of Gh production by swimming activity in those fish fed with the HP diet. This trend of Gh to decrease under exercise in conditions in fingerlings fed with protein-rich diets, was already observed in different previous studies (Vélez et al. 2016; Blasco et al. 2015).

The hepatic transcriptional response of *igf1* in its total form and its corresponding splice variants axis components in liver presented fewer differences than expected, as only the mRNA levels of *igf1c* in HP-fed fish under voluntary swimming appear to have been slightly upregulated (although not significantly, $p < 0.07$), whereas the exercise significantly raised its expression in the fish fed with the HE diet in comparison to the values in voluntary swimming. This transcriptional response would support a possible important implication of this isoform in fish growth regulation, as the expression pattern partially reflects the plasmatic values as well as the body weight (Perelló-Amorós, Fernández-Borràs, et al. 2021; Perelló-Amorós, García-Pérez, et al. 2021, **Articles 3 and 4**). The importance of this isoform of the igf-1 in gilthead sea bream was firstly reported by Tiago, Laizé and Cancela, (2008), where they noted that igf1c is the most highly expressed splice variant in liver of gilthead sea bream, thus highly contributing to the circulating Igf1 levels and the systemic role of this hormone. Moreover, Jiménez-Amilburu et al. (2013) demonstrated the relevance of this isoform during the myogenesis in vitro in this species. Hence, these results would contribute to explain the changes in body weight in exercise in HE fed fish.

Thus, the results on circulating Gh and Igf1 in this experiment are consistent with the similar final body weight that exercised fish presented in comparison with those under voluntary swimming; then confirming the close interaction that exists between the diet composition and the energy demands towards nutrient utilization (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**).

However, the current results on plasma Gh and Igf1 obtained in the two experimental models confirms that the response of these two hormones depends on different factors that needs to be considered, if they are going to be used as markers of the growth potential of the fish. Thus, it is necessary to put in context the response of these hormones together with other information that gives a wide image of the general state of the animal, such as

markers of energy metabolism, local growth regulatory systems, proteolysis and oxidative stress.

One of the main regulation points of the Gh-Igf axis are the Ghrs present in the cell surface of all the targets of the Gh, being especially important those Ghrs present in the liver, as they will mediate both the autocrine and endocrine roles of the Gh. In the fasting and refeeding trial, the gene expression of Ghrs in the liver also reflected the nutritional status of the fish. Both receptors were dramatically downregulated after 21 days of fasting, and along with increased Gh plasma levels, it is a clear evidence of Gh liver desensitization, a common response of the liver under feeding restriction (Won and Borski 2013). With refeeding, the hepatic mRNA levels of *ghr1* rapidly increased, which is in accordance with the fact that this isoform is associated with anabolic processes in this species, while expression of the *ghr2* only increased after 7 days of refeeding, indicating that the ingested nutrients may have initiated a compensatory growth response (Won and Borski 2013).

The hepatic expression of Gh receptors was analyzed also in the sustained swimming experiment. The Ghr2 is considered a negative regulator of growth in fish since its expression is usually upregulated in catabolic conditions (e.g., hypoxia, stress or fasting) (Saera-Vila, Calduch-Giner, and Pérez-Sánchez 2007; Gahr et al. 2008; Garcia de la Serrana and Macqueen 2018; Vélez et al. 2018) and recently, a structure comparison between Ghr1 and Ghr2 sequences in hybrid grouper, highlighted a possible decrease of Gh signaling by Ghr2 that deserves further research and could explain the downregulation of the *ghr2* under growth promotion situations, such as refeeding, exercise or testosterone injection (Norbeck and Sheridan, 2011; Vélez et al. 2016; Amenyogbe, Chen and Wang, 2020; Lavajoo et al. 2020; Perelló-Amorós, García-Pérez, et al. 2021, **Articles 3 and 4**)

The next regulation point step in the Gh/Igf axis is at the level of regulating the action of the Igfs, either by altering the presence of Igf1 receptors, or by the action of Igf binding proteins, which can increase the shelf life of circulating Igfs and modulate the bioavailability of these hormone towards its receptor. In teleost fish, the role of the different Igfbps has been recently reviewed by de la Serrana and Macqueen, (2018), but curiously, the knowledge about the Igf1 receptors' regulation in fish is still scarce.

The expression of the hepatic *igf1rb* in the fasting and refeeding trial was unaltered by fasting but however, suffered a drastic post-prandial downregulation at 2h that was not recovered until the end of the trial after 7 days. Such response of the *igf1rb* was interestingly parallel to that of the *igf1b* and *igf1c* splice variants. As far as we know, this is the first time that this effect is found in refed fish (Perelló-Amorós et al. 2018, **Article 1**), but it may represent a preventive response to avoid an overresponse mediated by insulin and/or Igfs at the initial stages of refeeding. Moreover, in this species, a similar downregulation of liver *igf1rb* expression was also observed in fish subjected to moderate sustained exercise (Vélez et al. 2016), while rBGh intraperitoneal injection did not modified (Vélez, Perelló-amorós, et al. 2019) or upregulated (Vélez et al. 2018) the expression of this receptor in liver of gilthead sea bream juveniles and fingerlings, respectively; thus suggesting that the modulation of the presence of this receptor in the surface of the hepatocytes may be a crucial regulation point of the Gh/Igf axis.

In the case of the igfbps, the expression of these proteins was stable during fasting with a slight tendency to increase in the case of the *igfbp4* and *igfbp5b*. However, refeeding caused a rapid postprandial upregulation peak and downregulation of *igfbp1a* and *igfbp4*, returning to basal levels after 24h and 7 days respectively. The slight increase of *igfbp4* with fasting is in agreement with its Igf-1 conservative function and by being considered a growth-promoting igfbp in salmonids especially sensitive to nutrient deficiency (Jaume Pérez-Sánchez et al. 2018), while the increase of *igfbp1a* at 2 h post-feeding fitted well with its recognized role in mobilization conditions in this species (Garcia de la Serrana and Macqueen 2018). Similarly, in a fasting and refeeding experiment in rainbow trout, Gabillard, Kamangar and Montserrat, (2006) observed different responses of the igfbps. Hevrøy et al. (2011) described the effects of fasting on Gh/Igf1 system in Atlantic salmon, in which Igfbp1 seemed to be a marker of catabolic state. Breves et al. (2014) demonstrated different roles of Igfbps during fasting and indicated that Igfbp1b may operate to reduce Igf1 signaling during fasting in tilapia. Nevertheless, the role of the different Igfbps in gilthead sea bream has still many clues, as the *igfbp1a* and *igfbp2b* were upregulated in the liver after 6 weeks of rbGh injection in gilthead sea bream fingerlings in a similar context of high blood Gh/Igf ratio (Vélez et al. 2018), while being

downregulated in juveniles after 12 weeks of injection (Vélez, Perelló-amorós, et al. 2019).

In the present thesis, more light has been given on the response of the hepatic Igf1 receptor in exercised fish. In the sustained swimming trial, the fish under voluntary swimming and fed with the HE diet, the *igf1rb* expression was lower compared to that of fish fed with the HP diet; while as mentioned above, the circulating levels of Igf1 presented an inverse trend. Under exercise conditions, the expression of *igf1rb* in fish fed with the HE diet, significantly increased respect to the voluntary activity group and thus, equalizing the expression of this receptor in both exercised groups. It should be taken into account that our results only reflect a snapshot from the time of sampling, and that growth is a dynamic process in which the gene expression and synthesis of Gh/Igfs axis members can experience changes over time (Pierce et al. 2004; Gabillard, Kamangar, and Montserrat 2006). Then, the increase of *igf1rb* hepatic expression in untrained fish fed with HP is congruent with their higher weight gain, as well as the absence of differences between exercised groups in the expression of this gene and in their final body weight, since the growth-promoting effects of Igfs are mediated through their interaction with Igf1rs (Reindl and Sheridan 2012; Fuentes et al. 2013).

Apart to the hypophysis and liver, the Gh/Igf axis has direct actions in a large number of peripheric tissues, both at endocrine and paracrine level. In the actual thesis, the local role of this hormonal axis in the skeletal white muscle has been one of the main objectives. Fasting decreased the expression of all the *igf1* splice variants, which parallels the significant decrease of liver total *igf1* expression in the same animals (Perelló-Amorós et al. 2018; **Article 1**). Thus, both systemic and local Igf1 production, might contribute to arrest the muscle growth under unfavorable conditions that leads to a catabolic state. Similarly, responses were observed in fasted channel catfish (*Ictalurus punctatus*) by (Peterson and Waldbieser 2009) and other fish species (Fuentes et al. 2013; Gabillard, Kamangar, and Montserrat 2006). This particular response of Igf1 coupled with impaired muscle growth during a nutrient-starvation period was also observed in an in vitro muscular model of gilthead sea bream under specific amino acids limitation (i.e. lysine) (Azizi et al. 2016). The expression profile of muscular *igf2* in fish and in general, the information regarding the regulation of this growth factor in fish is still scarce. *igf2*

expression in muscle was not modified by fasting, as observed in the muscle of fasted *Cichlasoma dimerus* (Delgadin et al. 2018). While in this South American cyclid, *igf2* was upregulated in muscle after 3 weeks of refeeding, in the present study the *igf2* was acutely downregulated postprandially to recover the basal levels after 7 days of refeeding. In contrast, muscle of rainbow trout fasted during 1 week presented a downregulation of this gene, with a fast upregulation 4 and 24h postprandially with a new subsequent downregulation after 2 weeks of refeeding (Cleveland and Weber 2014). Such divergent responses of muscular *igf2* in nutritionally challenged fish could suggest different roles of the *igf2* between salmonid and non-salmonid fish species and further research is required to elucidate its physiological meaning in the context of the local muscle growth regulation.

Regarding the Igf1 receptors, in the present fasting and refeeding trial, both igfrs were affected differently. *igf1ra* did not respond to fasting but decreased at 24h postprandially, recovering the expression levels at day 7. On the other hand, the expression levels of *igf1rb* tended to decrease with fasting and rapidly decreased in early refeeding, with diminished expression levels until the end of the experiment. Such a differential response has been reported in several species using in vivo or in vitro models (Hagen et al. 2009; Núria Montserrat et al. 2007; Chauvigné et al. 2003; Azizi et al. 2016; Bower, Taylor, and Johnston 2009) and suggests a functional split between both Igfrs isoforms. Furthermore, these findings point out that during a fasting or a nutrient restriction, Igf1 production decreases, but contrarily, some of the Igfrs isoforms can be maintained, suggesting a rise in Igf sensitivity to maintain a certain basal level of anabolic activity (Hagen et al. 2009; Chauvigné et al. 2003; Hernández-Sánchez et al. 1997; Imsland et al. 2007).

The muscular Igfbps also responded differently in this feeding trial. The expression of *igfbp1a* or *igfbp4* was unaltered, while the *igfbp5* significantly decreased with fasting and recovered progressively its expression levels after 7 days of refeeding (Lavajoo *et al.* 2020, **Article 2**). Previous studies attributed to *igfbp5* the role of growth promoting marker in gilthead sea bream (Vélez et al. 2016). Similar responses were detected in some fish species (Zheng et al. 2016; Garcia de la Serrana and Macqueen 2018); but as usual with the Igfbps, in other species and perhaps other experimental environments, this

response was not conserved (Gabillard, Kamangar, and Montserrat 2006; Bower et al. 2008; Valente, Bower, and Johnston 2012). Nevertheless, this decrease is analogous to the Igfbp3 plasma levels determined in fasted coho and chinook salmon (Shimizu, Swanson, and Dickhoff 1999; Shimizu, Hara, and Dickhoff 2003; Shimizu et al. 2003) suggesting a similar response of these two Igfbps to long term food deprivation in different fish species. Thus, the Igfbps in teleost fish are differently affected by metabolic shifts (Garcia de la Serrana and Macqueen 2018) and in the case of gilthead sea bream muscle, the Igfbp5b seems to be the most sensitive form to food availability. The expression of the Gh receptors also presented a dual and inverse pattern in this study, which coincides exactly with the differential function attributed to each receptor in gilthead sea bream and other fish species. In this sense, the Ghr1 has previously attributed to the anabolic roles of the Gh, while the Ghr2 responds positively under catabolic conditions (Vélez, Perelló-Amorós, et al. 2019; Saera-Vila et al. 2009).

The expression of muscular Gh/Igf system components was also studied under moderate sustained swimming, but however and surprisingly, no important changes were observed in any of the experimental groups, neither in the anterior nor in the caudal muscle regions, although the former was more altered than the caudal region. Unver voluntary swimming and HP diet, a higher expression of the catabolic markers, *igfbp1a* and *ghr2* was observed in parallel to the increased plasmatic Gh and Gh/Igf1 ratio (Perelló-Amorós, García-Pérez, et al. 2021, **Article 4**). These genes have been reported to positively respond in fish under catabolic conditions (e.g., hypoxia, stress or fasting) (Gahr et al. 2008; Saera-Vila et al. 2009; de la Serrana and Macqueen, 2018; Perelló-Amorós et al. 2018, **Article 1**; Vélez et al. 2018)). Such response could be an indicator of a growth regulation after a period of rapid growth that could have occurred in this group. Moreover, the higher expression of *ghr2* is in concordance with the elevated circulating Gh levels in those fish and with the stronger response of *ghr2* isoform to a nutritional treatment reported by Benedito-Palos et al. (2007, 2008) in the same species. In previous studies in gilthead sea bream (Saera-Vila, Calduch-Giner, and Pérez-Sánchez 2007; Vélez et al. 2018) the levels of Ghrs transcripts in liver and skeletal muscle increased along with Gh concentration in plasma. Thus, the differential response of Ghrs expression to circulating Gh is determined by the metabolic status of the animal and in certain situations might be a mechanism to prevent an excess of Gh signaling, as *ghrs* can experience post transcriptional

modifications that generate truncated Ghrs without the intracellular signaling domain (Saera-Vila, Calduch-Giner, and Pérez-Sánchez 2007; Gahr et al. 2008; Vélez et al. 2018). Finally, the muscular expression of *igf2* was differently affected by the exercise depending on the diet composition, with a significant decrease in the anterior and caudal regions of the muscle in the fish fed with the HP diet (Perelló-Amorós, García-Pérez, et al. 2021; Article 4). These findings on the differential role of *igf1* and *igf2* in fish muscle has also been observed in previous studies in this species (Vélez et al. 2018; Rius-Francino et al. 2011; Pierce et al. 2005). Overall, the local muscular Gh/Igf axis in gilthead sea bream is proven to be under the interaction of both, the energy expenditure, and the diet composition, with special adaptations to plasma GH and IGF levels by modulating the expression of ghrs and igfbps, respectively and with a marked response of igf2 over igf1 in this tissue.

4.2.The ghrelinergic system in gilthead sea bream

Due to its potential targets and roles, the interest on the ghrelin quickly raised and in less than one decade, this hormone was starting to be characterized in several vertebrate's species, including several fish species (Kaiya, Miyazato, and Kangawa 2011). However, it was not until 2017 that the ghrelin was not started to be characterized in such an important species for the Mediterranean aquaculture as the gilthead sea bream. In the current thesis, the phylogenetic analysis of the gilthead sea bream preproghrelin peptide showed, as in other studies, a strong conservation of its most characteristic features, but with a perceptible evolution among classes and orders and with logical evolution among fish species.

The tissue screening revealed that the expression of preproghrelin was restricted to stomach and pyloric caeca, as previously described in mammals and other fish species, establishing that the main source of Ghrelin is the stomach (Surajlal Unniappan et al. 2002; Kaiya, Kojima, Hosoda, Riley, et al. 2003; Breves, Veillette, and Specker 2009; Babaei et al. 2017), but with trace expression in other tissues and organs as in different fish species (Kaiya, Kojima, Hosoda, Moriyama, et al. 2003; Kaiya, Kojima, Hosoda, Riley, et al. 2003).

Among these tissues, there were a high interest in the brain, as this hormone is well known to be act on appetite regulating areas in this organ to induce (or decrease in some species) feed intake (Kaiya, Kangawa, and Miyazato 2013b; 2013c) and moreover, there is an existing hypothesis that Ghrelin is synthetized both peripherally and centrally in this organ (Edwards and Abizaid 2017). However, the low preproghrelin mRNA levels detected in the brain of the fish in this study may be since the whole brain was taken, instead of only the hypothalamus, which is supposed to be the main production site and target in the brain. Thus, further research is needed where to elucidate the presence of *preproghrelin* expression in the different brain areas in fish exposed to different experimental conditions that may involve the ghrelinergic system.

Regarding the Ghrelin receptors, the transcriptional screening revealed that both isoforms of the receptor are widely expressed, with higher expression on pituitary, brain and liver, which support that these are the main targets of Ghrelin action in gilthead sea bream, as in many other vertebrate species (Jönsson 2013; Kaiya, Kangawa, and Miyazato 2013a; 2013b). However, until now we do not know about studies that focused on the differential expression rates of both receptors across the different tissues and organs, being the isoform a more abundant in brain and pituitary, while isoform b is more abundant in liver (Perelló-Amorós et al. 2018, **Article 1**). Such differences could suggest that Gh secretion requires the presence of the truncated isoform to achieve a better regulation, as suggested by (Kaiya, Kangawa, and Miyazato 2013a; 2013b).

In the present thesis, the functionality of the Ghrelin in gilthead sea bream was firstly studied in a fasting and refeeding trial. Gilthead sea bream, as well as many other fish species, tolerates long periods of food deprivation, as studied previously in this species (N. Montserrat et al. 2007; Company et al. 2001; Salmerón et al. 2013; Salmerón, Johansson, et al. 2015), but the obtained morphometric parameters showed a reduction after 21 days of fasting that confirmed that the fish had entered in a clear catabolic state, which was progressively reverted upon refeeding, as demonstrated by the recovery of the body indexes at the end of the experiment (Perelló-Amorós *et al.* 2018, **Article 1**).

In fish, the response of Ghrelin to fasting appears to be species-specific, as fasting has been reported to up-regulate, down-regulate or unchange the gastrointestinal tract and

brain ghrelin mRNA levels in diverse fish species (Jönsson et al. 2007; Murashita et al. 2009; Suraj Unniappan, Canosa, and Peter 2004; Hélène Volkoff 2015; Parhar, Sato, and Sakuma 2003; M. Xu and Volkoff 2009; Hélène Volkoff et al. 2009) depending on several factors, like the sex and age of individuals (Parhar, Sato, and Sakuma 2003), temperature (Nieminen, Mustonen, and Hyvärinen 2003), fasting duration (Suraj Unniappan, Canosa, and Peter 2004) or diet composition (Babaei et al. 2017). Moreover, disparities in the role of Ghrelin has been found when comparing the response of this hormone at transcriptional and plasmatic level, which also occurs in the present fasting and refeeding trial, which coincides with previous fish studies (M. Xu and Volkoff 2009; Fox et al. 2009) and suggests that post-traductional mechanisms may be taking an important place in this regulation; but also taking into account that the timings for gene and protein expression may sometimes present these unparalleled patterns. This hypothesis is supported by the fact that a rise in *preproghrelin* gene expression has been observed during the first days of fasting, to then decrease progressively to fed control values in different fish species, such as sea bass and grass carp (Terova et al. 2008; Feng et al. 2013), which reflects a very similar situation of what could have happened in the present study. However, in Atlantic salmon, plasma Ghrelin levels were significantly increased after 2, but not 14 days of fasting (Hevrøy et al. 2011), suggesting that, despite the interspecies differences, the response of Ghrelin to food deprivation occurs in the early stage of this situation.

With refeeding, it was interesting to note that the plasma Ghrelin levels remained high until 2 h after the first meal, but significantly decreased at 5 h, suggesting an inhibitory effect on Ghrelin secretion as food enters the stomach. This inhibitory response is triggered by chemoreceptors and mechanoreceptors located on the gastrointestinal vagal afferents that are activated postprandially to downregulate the expression of orexigenic peptides from the gastrointestinal tract and the involved brain feeding centers (Reviewed by Volkoff and Rønnestad, 2020). Inversely to this trend of Ghrelin, a circulating Gh decline happened, confirming a relationship between these two hormones in this species. Similar responses of Ghrelin were also observed 1 h post-prandially in tilapia (Peddu et al. 2009), and in refed striped bass (Picha et al. 2009). Moreover, the plasma Ghrelin and preproghrelin mRNA expression in stomach presented clearly inverse patterns during the postprandial stage, which could indicate possible autoregulation of this hormone by positive and negative feedback loops.

Similarly, Ghrelin is regulated periprandially, at both mRNA and circulating levels in goldfish and zebrafish (Suraj Unniappan, Canosa, and Peter 2004; Hatef, Yufa, and Unniappan 2015), with general increases premeal and decreases after meal, in accordance with the observed decrease in plasma Ghrelin and brain *preproghrelin* mRNA at 5 h post-feeding in the present experiment in gilthead sea bream.

As for almost all the hormones, the second level of regulation that they possess is by altering the presence of their receptors, which also can be functional or nonfunctional isoforms, as it happens with the Ghsra and Ghsrb respectively. The *ghsrs* responded similarly in brain and pituitary. The expression of these receptors was unmodified after 21 days of fasting but presented an acute downregulation at 5h postprandial (Perelló-Amorós et al. 2018, **Article 1**). The information about the physiological implications of the Ghsrs in fish is scarce and we are still far to understand the regulation of these important receptors in the different tissues of fish. However, in rats, brain and pituitary ghsrs were upregulated in fasting and decreased after refeeding (Kaiya, Kangawa, and Miyazato 2013a; M. S. Kim et al. 2003; Nogueiras et al. 2004). Moreover, it has been recently reviewed that Ghsrs signaling decreases in situations of satiety and/or energy surplus (Cassano et al. 2021), which partially coincides with our results and suggests that the sampling timings have to be importantly considered in the experimental designs in order to study transcient transcriptomic changes of these receptors.

Nevertheless, it must be taken into account that because Ghsrs have crucial roles in the ghrelinergic system, their expression is finely regulated by numerous hormones, such as Gh-releasing hormone (Ghrh), thyroid hormones, dexamethasone, GH, leptin, glucocorticoids and insulin-like growth factor-1 (Igf). This fact reflects that the ghrelinergic system is highly altered by nutritional condition, environmental factors, and the response of the Ghsrs may be highly variable depending on the species, especially in fish, where the genomic duplication events have provoked the appearance of new Ghsrs paralogues, as described by (Kaiya, Kangawa, and Miyazato 2013b).

In Mozambique tilapia's brain, *ghsrs* did not responded to fasting, but at feeding time, just before food administration, both receptors were upregulated to decrease within 3h

after feeding (Peddu et al. 2009). However, in a previous study in the same species, *ghsr1b* increased after 3 but not 5 fasting days (Kaiya et al. 2009). Similarly, in Atlantic salmon, 2 or 14 days of fasting did not change the expression of *ghsr1a* in brain (Hevrøy et al. 2011), neither happened after 15 days of fasting in zebrafish *ghsrs* (Eom et al. 2014).

Contrarily, in the studies of (Kaiya et al. 2010), 7 days of fasting induced a decrease in the expression of ghsr1a in the vagal lobe of goldfish brain, highlighting again, species-specific differences in the regulation of the Ghsrs and the response of these receptors may be even harder to understand given the differences in the specific feeding behavior of each species and the lack of comparable studies in terms of experimental design. Ghrelin receptors in fish pituitary have been poorly investigated, yet low basal expression levels have been found in tilapia (Riley et al. 2008), goldfish (Sánchez-Bretaño et al. 2015; Bertucci et al. 2016) or yellow catfish (J. Zhang et al. 2016). In the case of grass carp, fasting from 14 to 28 days induced an upregulation of *ghsr1a* gene expression in pituitary which correlated with increased plasma Gh and *preproghrelin* pituitary gene expression (Cai et al. 2015). Moreover, in the same study, Ghrelin administration provoked an increase in the pituitary *ghsr1a* expression. In the present study, the decrease in *ghsrs* expression in pituitary during the post-prandial stage was in accordance to the aforementioned bibliography, and correlated with the circulating Ghrelin, pointing to a slowdown of the system during food intake.

From an integrative point of view, the close interaction between Gh, Igf1 and Ghrelin in fish under different nutritional situations needs to be further investigated. The work performed in the current thesis highlights that, both, long term (21 days) and short term (24 h) fasting increased the circulating Ghrelin levels, while being strongly downregulated 5 hours after refeeding. Such negative regulation of refeeding was accompanied by a downregulation of *ghsrs* in pituitary and brain, probably to maximally diminish the Ghrelin response while controlling the Gh expression and secretion (Perelló-Amorós et al. 2018, **Article 1**).

Taken together, the data suggests that Ghrelin can be a regulator of Gh secretion in gilthead sea bream, but the metabolic state itself and other regulatory molecules may exert important effects over the Gh independently on the ghrelinergic system. Finally, in the

present thesis we also propose that, in gilthead sea bream, the Ghrelin secretion and the response of the Ghrelin receptors are tightly related to the digestive process.

4.3.The proteolytic systems in skeletal white muscle

As aforementioned, the proteolytic systems play a fundamental role in muscle growth, not only because this growth is achieved when the protein synthesis overcomes the protein degradation, but also because the presence of this proteolysis is related to protein/muscle turnover, thus having a direct beneficial effect on the quality of the muscle, but also it might be a reflection of an unappropriated metabolic state of the tissue and the animal itself. In the last 15 years, there has been a certain interest on studying the different members of the main proteolytic systems in fish and until nowadays, one of the main questions that it is still far to be fully responded is, in which situations each type of proteolytic molecule plays its function. In other words, in a relatively large group of proteins that seemingly catalyze very similar reactions, there should be a differential regulation of their action or otherwise the evolution would have reduced this group to the minimal expression, even considering the common appearance of paralogues in fish. In this regard, different studies have been performed in order to better know their responses upon different experimental challenges in different fish species (Iban Seiliez et al. 2010; Salem et al. 2005; MacQueen et al. 2010; Lepage and Bruce 2008; Preziosa et al. 2013; Cleveland and Weber 2010; Bower, de la serrana, and Johnston 2010), including gilthead sea bream (Salmerón et al. 2013; Salmerón, Navarro, et al. 2015; Vélez, Azizi, Verheyden, et al. 2017; Vélez, Azizi, Lutfi, et al. 2017). However, again due to the lack of accurate comparability between studies caused by differences in the experimental designs, it is difficult to reach a clear explanation of their respective roles. In the present thesis, we aimed to study the role of the proteolytic systems in different experimental models, such as the fasting and refeeding (food restriction and compensatory growth), the moderate sustained exercise (how exercise influences nutrient usage, muscle quality and growth) and the muscle regeneration after a physical injury. Thus, to better understand each molecule groups, it is necessary to review their responses in an integrative way over the course of the different experimental models.

In the fasting and refeeding trial, the proteolytic genes could be divided in two groups based on their response; those that were downregulated during fasting and recovered with refeeding, like the Calpains and some Cathepsins, in a similar trend to that observed for *igf1* or the MRFs; and those belonging to the ubiquitin-proteasome system, like Murf1 or Mafbx, which increased in response to fasting and decreased with refeeding.

4.3.1. The calpains

Regarding the calpains, the fasting provoked a general downregulation of the *capn1*, *capn2*, *capns1a* and specially the *capn3*. In the early postprandial stages, this downregulation was even more accused, and they gradually recovered their expression levels at different times, being the *capn1* and *capn3* the fastest, while the *capn2* did not started to recover even until 7 days of refeeding, suggesting that this calpain does not play an important role in the compensatory growth regulation of skeletal white muscle. A similar downregulation after long periods of fasting in fish was observed (Preziosa et al. 2013) in agreement with our findings, but also other studies observed no response of these genes under fasting in the same species, where the authors gave to the calpains a secondary role in the adaptation to food deprivation (Salmerón et al. 2013).

In the exercise trial, the calpains presented a different response. In fish fed with the HE diet under voluntary swimming conditions, capn1, capn2, capn3 and capns1a were upregulated in the skeletal white muscle. In *in vitro* studies with primary myoblasts in gilthead sea bream it was concluded that calpains play a significant role during the proliferation stages of the myogenesis (Vélez, Azizi, Verheyden, et al. 2017); this is in agreement with that observed in other vertebrate groups in which calpains are relevant in such early myogenic stages (Nakashima, Ishida, and Katsumata 2011; Van Ba and Inho 2013). Thus, the response of the calpains in the present study, suggests that the upregulation of the calpains in the fish fed with the HE diet under voluntary swimming, may be associated with a less advanced myogenesis than the homolog fish fed with the high protein diet, in agreement with the lower body weight observed and protein breakdown in the first group,

4.3.2. The cathepsins

The cathepsins D and L are important proteases that play important roles in the lysosomal autophagic pathway and in mammals these proteins (specially the later one) have been directly linked to the negative regulation of cellular proliferation and may also play a still understudied role in metabolism (Weiss-Sadan et al. 2019). In the present thesis, these cathepsins were analyzed in both experimental models.

The effects of fasting on the cathepsins of gilthead sea bream were firstly studied by Salmerón, Navarro, et al. (2015), where the authors observed that in skeletal white muscle, *ctsd* and *ctsl* increased significantly after 15 and 30 days of fasting. In other fish species, such as halibut (Hagen et al. 2009) fasting also upregulated *ctsb* and *ctsd* , while in arctic charr (Cassidy et al. 2018), both *ctsda* and *ctsl* transcript levels were upregulated in the early stage after 2 days of fasting to then progressively decrease until appear downregulated at 43 days in comparison to the fed control fish. Similar upregulation was obtained in *in vitro* studies with gilthead sea bream myocytes, which revealed that specific amino acids limitation caused upregulation of both cathepsins expression (Vélez, Azizi, Verheyden, et al. 2017). However, in the current fasting trial, Ctsd was clearly downregulated both at gene and protein level, while *ctsl* was unaffected by 21 days of fasting (Lavajoo et al. 2020, **Article 2**). These results suggests that Ctsl may be necessary during long-term fasting adaptation, either at basal or upregulated levels, but also the results available in the literature points out that the effects of fasting in fish muscle may be subjected to multiple variables that, if not taken into account, complicate the comparison between studies.

Contrarily to the calpains, that presented inverse responses against fasting or sustained exercise, cathepsins under an over positive energy balance, appeared to have inverse responses. Thus, in this group of proteolytic molecules, the fish fed with the HE diet under voluntary activity presented lower gene and protein expression of *ctsl* and in exercise conditions, the expression of this cathepsin in the HP fed fish decreased, eliminating the differences between diet groups. Thus, the cathepsins, and specially the Ctsl, seems to be modulated by the nutrient availability and demand of the muscle, being upregulated when the protein availability/protein requirements ratio is high, while decreasing when the

protein demand of the muscle increase, such as fasting or hyper lipidic diets in fingerlings. Hence, in the present thesis we propose that the Ctsl represents a good marker of the muscle nutritional state in terms of protein, which can be useful in the optimization of the diet composition for aquaculture production.

4.3.3. The Ubiquitin-Proteasome system

Because of the important role of the proteasome in vertebrates, the UbP system is well considered the ultimate proteolysis system in fish muscle (Bower, de la serrana, and Johnston 2010; Cleveland and Weber 2010; Salmerón, Navarro, et al. 2015; Iban Seiliez et al. 2010; Vélez, Azizi, Verheyden, et al. 2017; Vélez, Azizi, Lutfi, et al. 2017). Previous literature in fasted gilthead sea bream revealed that 15 and 30 days of fasting upregulated the expression of all the components of this gene group (*n3*, *ub*, *murf1* and *mafbx*) (Salmerón, Navarro, et al. 2015), while in the present fasting and refeeding trial only the *mafbx* presented a strong upregulation by 21-day fasting (Lavajoo et al. 2020; Article 2). Similar responses of these genes have already been observed in other fish species (Iban Seiliez et al. 2008; Valente, Bower, and Johnston 2012; Garcia de la serrana et al. 2012; Bower, de la serrana, and Johnston 2010; Zhong et al. 2012) and in gilthead sea bream myocites deprived of specific amino acids limitation (Vélez, Azizi, Verheyden, et al. 2017). These results are also supported by the finding of increased total amount of polyubiquitinated proteins in the muscle of fasted rainbow trout (Iban Seiliez et al. 2008).

The ubiquitin-proteasome system, as well as the cathepsins, has been recently associated to the latter stages of the myogenesis, where all the differentiation features of the muscle take place and the proliferation is relegated to a secondary event (Vélez, Azizi, Verheyden, et al. 2017; Vélez, Azizi, Lutfi, et al. 2017; Bower, de la serrana, and Johnston 2010). In the exercise experiment, the HE diet induced a slight upregulation of *n3* and *ub*, while *mafbx* was strongly downregulated, also at a protein level. This fact could indicate once again that the white muscle of these fish was in a less-advanced myogenic stage (Perelló-Amorós, García-Pérez, et al. 2021, **Article 4**). Furthermore, in the **Article 3** (Perelló-Amorós, Fernández-Borràs, et al. 2021), it was found in these fish that the saturation of lipid oxidation systems would favor the oxidation of amino acids, which in part could come from degraded proteins and muscle fibers. When the fish were subjected

to exercise, the differences among both diet groups in the proteolytic systems' expression disappeared by downregulating the expression of those genes upregulated in voluntary swimming. In this case, it is noteworthy that Mafbx had a calpain-like behavior, while *ub* and *n3* responded in an inverse way as the cathepsins did, suggesting differential roles of these ubiquitin proteasome pathway members in the adaptation to diet and exercise.

Overall, the different responses of the proteolytic systems to distinct dietary regimes and physical activity observed in the studies developed in the present thesis open the possibility of using them as useful markers of nutritional status and culture conditions that may have direct relation with the muscle quality. It seems that there is a clear difference between the expression of genes of the three major proteolytic pathways. Calpains seems to be protagonist in situations of high nutrient income, such as refeeding or positive energy intake/expenditure ratio and also may have important functions in the proliferative stage of the muscle development or remodeling. Cathepsins, contrarily, are important in the adaptation of the muscle subjected to nutrient deficiency and may be associated to advanced stages of the myogenesis. Finally, the ubiquitin proteasome appears to be a heterogeneous group of molecules that have similar responses to both aforementioned proteolytic groups.

4.4.The regulation of the MRFs and other molecules involved in muscle growth.

The MRFs have been one of the most covered molecular groups in the studies that focused on the muscle development in several fish species, but also in some recent studies that pursued to improve the muscle growth and quality towards an optimization of the aquaculture production. However, there is still a long way to go in order to fully understand the role of these essential molecules in muscle growth and their roles under different experimental conditions.

In the case of the nutritional restriction, the information on MRFs and other muscle growth regulators in gilthead sea bream is scarce and the results that we bring in the resent thesis contributes to understand the muscle growth regulation in such catabolic situations. In vitro studies with cultured primary gilthead sea bream myocytes under lysine deficiency (Azizi et al. 2016), showed similar responses of what observed in the current

fasting conditions, where the expression of *myod1*, *myf5*, *myogenin*, *mrf4* and *mstn2* were strongly downregulated after fasting and even in the early refeeding. However, with longer refeeding, *myods* and *myf5* recovered their basal expression levels and in this process of recovery, the sequence of MRFs upregulation during the refeeding followed the same classical order as described during the myogenesis in gilthead sea bream (García de la serrana et al. 2014), confirming that the genes that responded earlier were those implicated in the first stages of myogenesis, such as *pax7*, *pcna*, *myf5* and the *myods*. Similar results have been observed in salmonids (Bower and Johnston 2010; Chauvigné et al. 2003; Johansen and Overturf 2006; N. Montserrat et al. 2007) and non-salmonid fish species (Chauvigné et al. 2003; Nebo et al. 2013; Y. Xu et al. 2019; Zhu et al. 2016); confirming that the refeeding may cause an important muscle remodeling which involves the stimulation of the myogenic process. Moreover, *mstn2* was also strongly downregulated by fasting and was not recovered with the refeeding. This particular result agrees with previous findings in rainbow trout (N. Montserrat et al. 2007) and European sea bass (Dicentrarchus labrax) (Terova et al. 2006) which coincides with the classical function of the myostatins as proliferation inhibitors. Hence, the muscle adaptation to refeeding may be structured in similar steps as is the myogenic process, with an initial satellite cell activation, proliferation stimulation and differentiation.

Other structural proteins responded at the gene expression level to fasting and refeeding, such as the *mlc2a* and *mlc2b*, which presented inverse profiles. *mlc2a* expression not changing with fasting but showing a significant increase at 24h postprandially to return to basal levels at day 7. Bower and Johnston, (2010) described in Atlantic salmon that *mlc2* expression increase after 14 days of refeeding and previous studies in gilthead sea bream (Vélez et al. 2018; Vélez, Azizi, Lutfi, et al. 2017; Georgiou et al. 2016) associated upregulation of *mlc2a* expression to conditions favorable muscle proliferation. Thus, in the present feeding trial, the transcriptional profile of the analyzed genes supports the idea that the initial response of the muscle to refeeding starts with a strong proliferation stimulation thus highlighting the importance of the hyperplasia in the muscle compensatory growth.

In the exercise trial, the MRFs did not responded after 6 weeks of experiment in either anterior or caudal skeletal white muscle (Perelló-Amorós, García-Pérez, et al. 2021,

Article 4). In a previous exercise trial in gilthead sea bream subjected to sustained swimming at 5 $BL \cdot s^{-1}$ for 5 weeks (Vélez et al. 2016) the MRFs were little affected in the anterior muscle, while the proteolytic systems were already active to carry out the muscle reorganization and recovery of damaged mature fibers previously to the myogenic activation necessary for the adaptation to exercise (Bell, Al-Khalaf, and Megeney 2016). Thus, a similar response was observed in the current exercise trial, suggesting further experiments under exercise that include both acute and long-term samplings in order to catch the time window where the MRFs may play their role in this adaptation to exercise. Nevertheless, in the caudal muscle, the mstns expression was significantly downregulated by exercise in both diet groups compared to their counterparts in voluntary swimming. This response would suggest a *mstns* lower growth repression in this muscle region of fish subjected to forced swimming since Mstns are the main inhibitors of muscle development in vertebrates (Iban Seiliez, Sabin, and Gabillard 2012; Gabillard et al. 2013) and are in concordance with the different responses to exercise along the muscle trunk observed by Ibarz et al. (2011) and Vélez et al. (2016).

Taken together, the results obtained in the in the present thesis concerning the role of the MRFs and other muscle growth markers in these two metabolically challenging models reflects that their function has a strong spaciotemporal limitation, as most of these molecules are transcription factors that will encode for the utterly complex molecular machinery that will carry out the muscle adaptation to situations such as fasting, refeeding, excessive dietary fat and energy and sustained swimming. In this regard and considering apart the influence of the state and intrinsic nature of the animals in each experiment, as well as the sampling times, these muscle regulatory proteins seem to be more directly implicated in the adaptations to fasting and refeeding rather than moderate sustained swimming. The fasting and refeeding model, although designed with less temporally separated sampling points than the exercise trial, induced strong responses in all the growth-related systems analyzed, such as the Gh/Igf axis and the proteolytic pathways. Contrarily, long-term adaptation to moderate exercise appears to be a more progressive and smooth process that includes metabolic shift of the white myofibers which can include eventually induction of the myogenic process to promote hyperplasia and hypertrophy in an exercise intensity dependent manner. Nevertheless, the optimization of the nutrient use under exercise is also reflected in these group of muscular

growth markers, with a clear trend to eliminate differences between diet groups. Thus, both compensatory growth, diet composition and sustained exercise experimental approaches can be used, alone or in combination, to optimize the muscular growth and quality in fish as they exert complementary direct effects over the mechanisms that participates in muscle growth.

4.5. The role of Myomaker/Myomixer in the muscle development of rainbow trout and gilthead sea bream

The fusion of myocytes is a complex and highly regulated process that involves the attachment of fusogenic cells, hemi fusion, pore formation and expansion. This process requires a highly specialized molecules and among them, the current literature have put its eyes on two novelkey proteins, such as Myomaker and Myomixer (reviewed by Petrany and Millay, 2019 and Chen et al. 2020). Considering that hyperplasia persists during post-larval growth in the indeterminate growth fish muscle and that recently the Myomaker has been characterized in rainbow trout (*Oncorhynchus mykiss*), the present thesis aimed to complete the characterization of the sequences of Myomixer and/or Myomaker and its expression during *in vivo* and *in vitro* myogenesis in rainbow trout and gilthead sea bream.

The in silico analysis of the trout and gilthead sea bream genome and/or EST databases revealed that both species possesses, as for the vast majority of fish species, only one paralogue for the *myomixer* gene, revealing that for the whole genome duplication events accumulated in bony fishes and specially, in salmonids, did not lead to the appearance of paralogues with very few exceptions, such as Coho salmon (*Oncorhynchus kisutch*) and Atlantic salmon (*Salmo salar*), which seems to possess potential *myomixer* paralogues in their genomes. In the case of the gilthead sea bream, the sequences of *myomaker* and *myomixer* were obtained in silico from the NCBI GenBank and the Ensembl databases. The gilthead sea bream Myomaker appears to be a 285aa protein which is encoded by a gene structured in 6 exons, a feature that seems to be partially conserved in salmonid and non-salmonid fish species, as however in all the species of vertebrates, the 6-exon structure of the *myomaker* gene is conserved, it is not the case of the peptide length. In this regard, most of the species of vertebrates (from mammals, reptiles, aves and the vast

majority of fish, the Myomaker is a protein with a length commonly around 220aa, reaching to 285aa in some fish species and presenting unusual extended domains reaching to 434aa in rainbow trout and other salmonid species as described by (Landemaine et al. 2019).

Myomixer was first identified in mice, and its gene contained a single exon that encoded an 84aa protein (Bi et al. 2018), while in fish, Myomixer was first identified in zebrafish, as a gene encoding for a 75aa protein (Shi et al. 2017). In the present thesis, it has been confirmed that in rainbow trout, Myomixer is a 77aa protein encoded in the 1st exon of a 2-exon gene, while in gilthead sea bream, the available gene of myomixer in the Ensembl database has only one exon described that encodes a 77aa peptide.

The multiple alignment of the gilthead sea bream and trout Myomaker sequences highlights that the gilthead sea bream Myomaker protein corresponds to the N-terminal domains plus the last 15aa in the C-terminal end of the trout Myomaker. The N-terminal domain of the trout Myomaker protein shared 88 and 71 % identity with the zebrafish and mouse Myomaker proteins, respectively (Landemaine et al. 2019), and in the present thesis we report that the gilthead sea bream Myomaker also presents a good conservation, with 89%, 85% and 71% identities with its orthologues in zebrafish, rainbow trout (N-terminal part) and mouse (Perelló-Amorós et al. in preparation, **Article 6**). In the case of Myomixer, the multiple sequence alignment pointed out a moderate conservation of the overall amino acid sequence across vertebrate lineage, with noticeable low conservation across the fish species studied, where the gilthead sea bream Myomixer presents a 68.5 % identity with its orthologue in rainbow trout and 70.76% in zebrafish (Perelló-Amorós et al. in preparation, **Article 6**). However, the key amino acid residues are still conserved, including the motif AxLyCxL, essential for Myomixer activity (Shi et al. 2017), which is present in all vertebrate species studied (Perelló-Amorós, Rallière, et al. 2021, **Article 5**).

As is the case of Myomaker, the gilthead sea bream Myomixer was more related to other perciformes, as the sea bass, beloniformes or salmoniformes. Nevertheless, evolutionarily, the gilthead sea bream Myomixer was more distant to cypriniformes, siluriformes and chondrichtyes. Other phylogenetic analysis performed in gilthead sea bream Myogenin and Preproghrelin proteins also shown that these molecules are evolve

in the same way, as these molecules are evolutionarily close to other perciforms, while are more distant to salmoniformes or cypriniformes (Moutou et al. 2008; Perelló-Amorós et al. 2018, **Article 1**; Perelló-Amorós et al. in preparation, **Article 6**; Perelló-Amorós, Rallière, et al. 2021, **Article 5**).

At *in situ* hybridization level, the results obtained in rainbow trout larvae showed that *myomixer* is strongly expressed in the deep embryonic myotome during somitogenesis and primary myogenesis (10–18 dpf) (Barresi et al. 2001; Steinbacher et al. 2007). In more advanced stages, high expression of *myomixer* was observed in other regions of the myotome (dorsal, ventral and lateral), where the secondary wave of myogenesis takes place (Steinbacher et al. 2007). The double in situ hybridization for *pax7* and *myomixer* showed mutually exclusive expression patterns, as *pax7* is expressed in undifferentiated myogenic cells present in the dermomyotome-like epithelium surrounding the primary myotome (Dumont, Rallière, and Rescan 2008), or in the myotome itself as a reservoir population of undifferentiated cells in adult tissue; while *myomixer* is strongly expressed in differentiated myogenic cells in the area of muscle hyperplasia (Steinbacher et al. 2007).

When comparing this expression pattern to the one observed in determinate growth species, such as zebrafish, only strong expression of *myomixer* from 14 hpf to 24 hpf was observed (Shi et al. 2017), but at the later stages of the somitogenesis, the expression of *myomixer* was no longer detected (Landemaine, Rescan, and Gabillard 2014; Shi et al. 2017), as in this species, post-larval muscle growth proceeds only by hypertrophy (Ian A Johnston et al. 2009). On the other hand, in trout embryos, *myomixer* expression is maintained in all the somites. At cellular level, the *in situ* hybridization revealed that both *myomixer* and *myomaker* are only expressed only in in the cells that are fusing, reinforcing the essential role of these proteins in muscle hypertrophy and hyperplasia (Goh and Millay 2017). In zebrafish, *myomixer* and *myomaker* expression is no longer detected in white muscle after hatching. In contrast, in trout, muscle hyperplasia persists during post-larval growth (Steinbacher et al. 2007) and is accompanied by a maintenance of *myomixer* and *myomaker* expression indicating that they are markers of muscle hyperplasia rather than fiber hypertrophy. This fact reveals again the difference between the indeterminate growth observed in salmonids or gilthead seabream in comparison with determinate

growth fishes (zebrafish) or mammals. Moreover, the expression distribution of *myomixer* is similar to that of the *myomaker* in both trout and gilthead sea bream (Landemaine et al. 2019; Perelló-Amorós, Rallière, et al. 2021, **Article 5**) during embryonic and post-larval stages, confirming the necessary coexistence of both proteins to promote myoblast fusion.

In order to better characterize the implication of Myomaker and Myomixer in the myogenic process of both rainbow trout and gilthead sea bream, the expression of both genes was studied in combination with the MRFs and other molecules in two experimental models, the cultured primary myoblasts and the *in vivo* muscle regeneration, that will be further covered below. In primary cultured myoblasts, both *myomaker* and *myomixer* presented expression patters very similar to that of *myogenin* in both rainbow trout and gilthead sea bream, suggesting the upregulation of these three molecules as soon as the fusion myoblasts starts (Landemaine, Rescan and Gabillard, 2014; Perelló-Amorós, Rallière, et al. 2021, **Article 5**; Perelló-Amorós et al. in preparation, **Article 6**;), although further loss-of-function approaches would be required for a consistent validation (H. Zhang et al. 2020). Moreover, in gilthead sea bream cultured myocites, the rest of the MRFs were analyzed, showing that in these cultures, the initial stages of the myogenesis were governed by the *myod1,* which expression probably started to increase in the first days of culture and peaking at day 4 to then rapidly decrease with its function being relegated to the rest of the MRFs, as previously described in gilthead sea bream (García de la serrana et al. 2014).

Myomaker and Myomixer were early reported to play an essential role of in the regeneration of muscle fibers after a physical injury, first reported in mice (Millay et al. 2014; Shi et al. 2017) and more recently, the conservation of this function of Myomaker in salmonid fish was reported by Landemaine et al. (2019). In that study, a strong upregulation of the mRNA levels of this gene occurred 30 days after the injury, in parallel with the appearance of newly formed myofibers.

In the present thesis, the role of the MRFs, Myomaker and Myomixer on muscle regeneration was studied in both rainbow trout and gilthead sea bream. In trout, the gene expression analyses showed that *myomixer* expression was also strongly stimulated in white muscle 30 days after injury (Montfort et al. 2016). Thus, although considering that

the time that muscle take to fully regenerate the muscle differs among species depending on several factors, such as the metabolic rate of the animal ant the temperature, the kinetic of *myomaker* and *myomixer* expression during muscle regeneration, was parallel to that of *myogenin* in trout (Landemaine et al. 2019), gilthead sea bream (Perelló-Amorós et al. in preparation, **Article 6**) and mice (reviewed in Chen et al. 2020) and coinciding with the results *in vitro* (Perelló-Amorós, Rallière, et al. 2021, **Article 5**). Moreover, in gilthead sea bream, the rest of the MRFs were assessed in this regeneration model, showing that in this species, the myogenesis program is activated transcriptionally between 8 to 16 days post-injury, where the upregulation of all the MRFs, *myomaker* and *myomixer* occurs. This overlapping of the MRFs in muscle regeneration in vivo is not a strange pattern, as it has been also observed in mice (reviewed in Chen et al. 2020).

The obtained results in these two experimental models has confirmed that these two molecules may be also essential in fish muscle regeneration, deserving to be studied along with other genes involved in the hyperplasia (Montfort et al. 2016), inflammation and the other mechanisms affecting muscular growth such as markers of satellite cell activation, Myostatins, proteolytic systems, the Gh/Igf axis and other myokines.

4.6. Growth and mitochondrial modulation by diet and sustained exercise in gilthead sea bream

The rearing of fish in sustained moderate swimming has been extendedly studied, demonstrating that in several fish species can promote growth and improve the food conversion rate (reviewed by Davison, 1997; Davison and Herbert, 2013 and McKenzie et al. 2020). However, the growth promoting response induced by this type of exercise is closely related to the own physical activity pattern of each species, and in this regard, the pelagic fish species, which naturally are adapted to long periods of medium speed swimming, have better results in these forced swimming trials (W Davison and Herbert 2013).

Moreover, as recently reviewed, the type of effects induced by sustained exercise, highly depends on the size of the animal and the swimming speed, achieving optimal growth promotion results when the swimming speed is close to the optimal swimming speed

(U_{opt}) of each species (McKenzie et al. 2020), with the feed intake covering all the energy costs of swimming. In the case of gilthead sea bream juveniles weighing 25–45 g, recent calculations have set up the U_{opt} to be around 4.5 BL·s−1 (Palstra, Kals, et al. 2020). In a previous study with exercised gilthead sea bream fingerlings at 5 BL/s^{-1} a significant growth improvement was achieved with a diet with similar composition to that of the current HP diet (Blasco et al. 2015), hence validating the most recent calculations of the U_{opt} in this species. In the present thesis, a sustained moderate-to-low-intensity exercise at a speed near 50% of the U_{opt} was used. Such lower speed is considered to be more practical for fish farming with larger scale facilities and water volumes and this speed was used to determine how different proportions of protein and lipid in commercial diets met the increased metabolic demands of swimming activity without compromising growth (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). Thus, under voluntary swimming, the lower protein to lipid ratio of the HE diet ended up with lower body weight, increased liver size and elicited a higher percentage of muscle lipid when compared to the HP diet group. Previous literature showed that high dietary fat intake provokes a gradual deposition of lipids in several organs of this species, including the liver (Company et al. 1999). However, in the present work, sustained exercise improved almost all the growth parameters in the fish fed with the HE diet, eliminating all the differences in growth in comparison with the fish fed with the HP diet. The exercised HE group did maintain a significantly higher HSI due to a probable increased fat deposition in this organ (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). In a different previous approach, the lipid content of white muscle decreased in exercised juveniles fed a high carbohydrate/low protein diet under sustained swimming at 1.5 BL/s^{-1} (Martin-Perez et al. 2012). These results, in combination with the new ones, confirms that gilthead seabream adapts its metabolism to the type of activity they are performing and to the nutrient abundance in order to prioritize the best fuel (Magnoni, Felip, et al. 2013). The fuel prioritization under exercise conditions depends on the type of exercise in terms of duration and intensity, being the lipids the most preferred fuel at low or medium velocities (i.e., aerobic training) while the carbohydrates are the preferred fuel as the velocity (and the energy demand) increases (Richards et al. 2002b) or in burst-like exercise models. In the case of the glycogen, this energy reserve in white muscle was reduced under exercise, regardless of the diet. These results agree with the postulation by Sánchez-Gurmaches *et al.* (2013) that exercised juveniles of gilthead sea bream oxidizes carbohydrates very

efficiently to meet the high energy demands of the sustained exercise (O. Felip et al. 2013).

Another important aspect to consider in the fish muscle composition under metabolic challenges is the turnover rate of certain molecules/nutrients, and the SIA technique (Stable Isotope Abundance) allows to infer this information. In previous studies in gilthead sea bream, a markedly different isotopic profile in white muscle was observed when the fish were fed with different proportions of plant-based ingredients in the diet (Beltrán et al. 2009) or of different protein content in diet (Martín-Pérez et al. 2011). In this regard, in the present exercise trial, the fish fed with the HE diet presented lower δ15N values than in the HP diet group, both in the total muscle and in its protein fraction and regardless of the exercise. This fact reflects that the lower δ15N values of the HE diet are probably due to the higher proportion of vegetable protein in this diet. Regarding the 13C, reduced values were observed in the lipid fraction of the HE diet group due to the high lipid content in this tissue (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**), which is in agreement with the findings of previous studies (Martín-Pérez et al. 2011; Martin-Perez et al. 2013). The nitrogen dietary fractionation (δ15N) is a good marker of protein balance that reflects the protein turnover and the protein retention efficiency (Martínez Del Rio et al. 2009; Martín-Pérez et al. 2011). In the present trial, the fish fed with the HP diet exhibited a lower fractionation than those fed the HE diet, thus indicating that the protein in the HP diet had to be less transformed (less protein turnover) before being deposited into the muscle proteins (Gaye-Siessegger et al. 2003; 2007). As previous studies in gilthead sea bream stated that there is a negative correlation between the dietary protein content and nitrogen fractionation (Martin-Perez et al. 2013), the lower protein content of the HE diet with respect to the HP diet could contribute to this higher fractionation (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). Moreover, the lower growth of the HE diet group under voluntary swimming conditions could reflect also this higher fractionation, which is in agreement with that observed in gilthead seabream fed different proportions of plant proteins (Beltrán et al. 2009). Under exercise, the differences observed between the two diet groups, with respect to the isotopic composition and nitrogen fractionation of white muscle, did not changed, and then different scenarios can be described for each exercised group. In the fish fed with the HE diet, it expected a greater use of dietary lipids for energy purposes while inducing a

protein-sparing effect. On the other hand, in the HP diet group, part of the dietary protein could have been directed to energy metabolism and considering the fixed diet ration that the fish received, the exercise did not enhanced the growth of this diet group. This higher dietary amino acid oxidation in the HP group under sustained swimming conditions was reflected also in the higher nitrogen fractionation when compared to that under voluntary swimming conditions.

Understanding how changes in diet composition related to swimming activity affect mitochondria can help to clarify the molecular mechanisms that underpin growth performance and body composition. As aforementioned, mitochondria and double-membraned organelles responsible for several physiological processes including oxidative energy metabolism, ROS and oxidative stress protection, apoptosis, among other functions. Due to its endosymbiotic origin, one of the main characteristics of the mitochondria is to possess a high plasticity to undergo constant adaptive changes in response to fluctuations in energy demand and supply, by modifying increasing their abundance in the cell (biogenesis) or by altering its shape and connection in the context of a mitochondrial network through fusion/fission processes.

4.6.1. Liver

Liver is one of the main tissues implicated in the energy metabolism and in the diet and exercise trial of the present thesis, the mitochondrial proteins related to energy metabolism and fusion/fission balance responded differentially to these conditions. Under voluntary swimming conditions, the *cox4*/*cs* ratio at gene expression level was decreased in the HE group, which reflects a greater lipid oxidation. These changes induced by high-fat diets in *cox*/*cs* are supported by previous results at RNA level in goldfish and enzymatic activity in gilthead sea bream, respectively (LeMoine, Genge, and Moyes 2008; Sánchez-Nuño et al. 2018) and in both, *cs* was upregulated, but not *cox*. Although most of studies focus on the individual changes over these two enzymes, in the present study we postulate that the ratio between them is sometimes more informative. In this regard, the Cox/Cs activity ratio indicates modifications of the mitochondrial surface/volume ratio (A. Ibarz et al. 2010; Martin-Perez et al. 2012), which reflects shifts in the use of nutrients or modifications in the mitochondrial dynamics. Thus, observed

decrease in the Cox/Cs ratio in the livers of the fish fed with the HE diet can be interpreted as an increased lipid oxidation in this group (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**).

However, the high lipid content of the HE diet in these livers exceeded their oxidative capacities under low energy demanding conditions and which could conduct this organ to increased oxygen species (ROS) levels (data not shown, Sánchez-Moya et al. 2017) and *ucp2* gene expression upregulation. The Ucps have a role as redox sensors that can serve to attenuate the effects of the ROS (Rial and Zardoya 2009). The Ucp1 and Ucp2 have been characterized in gilthead sea bream (Bermejo-Nogales, Calduch-Giner, and Pérez-Sánchez 2010; Bermejo-Nogales et al. 2014). In other studies in grass carp and gilthead sea bream, the gene expression of *ucp1* increases when lipid flux to the liver increases in carp (Jastroch et al. 2005; Bermejo-Nogales, Calduch-Giner, and Pérez-Sánchez 2010). However, in the present study, the *ucp1* gene expression did not presented this kind of response, suggesting that in the liver of gilthead sea bream, the Ucp1 and the Ucp2 may have complementary or even sometimes, redundant functions that needs to be further investigated with more comparable studies (Perelló-Amorós, Fernández-Borràs, *et al.* 2021, **Article 3**).

In the present study, the high mRNA levels of *ucp2* in the fish fed with the HE diet under voluntary swimming were accompanied by a downregulation of *pgc1a*, which is consistent with that observed in mammals presenting metabolic syndrome with increased lipid accumulation and ROS production (Rius-Pérez et al. 2020). On the other hand, in the HP diet group, the higher gene expression of *pgc1a* would indicate a without signs of upregulation suggests an enhanced energy distribution in this group of fish that grew better than those fed with the HE diet. Under exercise, the energy demands increased and the cox/cs ratio was affected with an interaction between diet and activity. More specifically, exercise decreased the cox/cs ratio in the HP group. The Cox/Cs ratio has been evaluated in other energy demanding conditions such as hypothermal stress, where has been reported to decrease, usually due to the increased expression and/or activity of Cs (Lucassen et al. 2003; Eckerle et al. 2008; A. Ibarz et al. 2010; Orczewska, Hartleben, and O'Brien 2010; Sánchez-Nuño et al. 2018).

Although swimming activity did not modify *ucp2* gene expression, it caused a slight downregulation of this gene in the fish fed with the HE diet, which caused the equalization of in *ucp2* gene expression between both diet groups. This fact indicates a better balance between the TCA cycle and oxidative phosphorylation in the HE group under SS compared with VS conditions. The markers of mitochondrial dynamics were only affected by the diet, which induced changes in the expression of *mit2* and *miffb*, thus altering both antagonic processes. However, a significant interaction highlighted that the fusion processes are modulated by swimming activity depending on diet received. This fact was clearly reflected in the upregulation of both *mitofusins* and *miffb* in the fish fed HE diet under swimming activity (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). The simultaneous stimulation of mitochondrial fusion and fission processes are often associated to increased mitochondrial health turnover to restore the balance in a mitochondrial network in adaptation towards a much higher oxidative capacity (Scorrano 2013). Such stimulatory effects on the mitochondrial turnover has already been observed under exercise conditions with high gene expression of *mit1* and *mit2*, promoting the formation of a stronger network of healthy mitochondria (Liesa and Shirihai 2013; Scorrano 2013; Gonçalves et al. 2014; 2015). Moreover, this adaptative response to exercise has also been observed in rats fed a high fat diet to prevent a pathologic impairment of the mitochondrial function related to fatty deposition in liver (Gonçalves et al. 2014; 2015).

Since in mammals the mitofusins are directly regulated by pgc1a (Chandhok, Lazarou, and Neumann 2018), high expectations were pun in this master regulator of the mitochondrial biogenesis, and we found that *pgc1a* gene expression responded mainly to diet in this tissue. The lower levels of *pgc1a* in the HE group under voluntary swimming may indicate that this transcription factor was previously activated to promote *mit1*, *mit2* and *miffb* upregulation before undergoing a negative feedback. However, this trend was not noticeable in swimming conditions, hence suggesting that in fish, the pgc1a may not be the only regulator of these molecules, contributing to increase the controversy about the role of this important regulatory molecule in fish (Bremer et al. 2016).

Overall, the oxidative capacity of the liver highly depends on the diet composition, as higher lipidic content induces the respiratory uncoupling and decreased oxidation

efficiency. However, under a high energy demanding condition such as SS, the uncoupling disappeared, the lipid oxidation increased, and the mitochondria fusion process was promoted to make mitochondria more efficient.

4.6.2. Skeletal Muscle

The response of the mitochondrial proteins in white muscle reflected a higher lipid influx to this tissue in the fish fed a highly energetic diet. Thus an "oxidative overload" induced a strong *pgc1a* mRNA downregulation in response to its elevated protein levels, as well as higher Cs expression. Consequentially, such impairment situation was accompanied also with upregulated Ucp3 and altered mitochondrial dynamics markers. The increase of *Ucp3* gene expression in highly oxidative white muscle has already been described in gilthead sea bream as a prevention mechanism to the overproduction of ROS (Bermejo-Nogales, Calduch-Giner, and Pérez-Sánchez 2014; Bermejo-Nogales et al. 2014). Thus, the specific response of the Pgc1a in the present work suggests that, in the earlier weeks of the experiment, the high energy level of the HE diet might have triggered AMPK activation, inducing *pgc1a* gene expression (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). The subsequent increase in the protein levels of Pgc1a observed in the muscle would induce a strong negative feedback regulation on its gene expression, in agreement with that observed in goldfish fed with a high-fat diet (LeMoine et al. 2008). This regulation mechanism of pgc1a has been observed also in mammals fed with high-fat diets (Bonnard et al. 2008; Sparks et al. 2005).

In gilthead seabream and other fish species, sustained moderate exercise is well demonstrated to induce a more aerobic phenotype in white muscle (Martin-Perez et al. 2012; Blasco et al. 2015; Ian A. Johnston and Moon 1980; McClelland et al. 2006; Anttila, Jäntti, and Mänttäri 2010). Such adaptation is expected to be reflected in variations of the gene and protein expression of Cox and Cs enzymes, as they are the main proteins directly involved in obtaining energy.

However, in this scenario of higher energy demand, the response of these enzymes at gene and protein levels responded differently depending on the diet, which is interestingly reflected in a significant interaction of the two factors, diet and activity, in most of the

analyzed genes in this tissue. In this regard, exercise caused the downregulation of *cs* gene expression in the HP group increasing the cox/cs ratio in this group. However, at protein expression level, this Cox/Cs ratio increase is due to the increase in Cox rather than Cs in this group. This unclear response of Cox and Cs in white muscle indicates that these two enzymes may be subjected to complex regulations at transcriptional, post-transcriptional and post-translational levels.

The sustained exercise provoked a significant decrease in *pgc1a* gene expression in the HP group, thus being a possible cause of the *cs* downregulation and hence, increased protein oxidation in the HP group, while the fish fed with the HE diet oxidized predominantly lipids. Moreover, this new energy demanding condition dissipated the energy overload of the skeletal muscle in the fish fed with the HE diet without the needing of the activation of the uncoupling mechanisms (Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**). These results are in agreement with observations in endurance trained humans (Russell et al. 2003; Schrauwen-Hinderling et al. 2003). The regulation of ucp3 in fish subjected to exercise is still unknown, but in mammals an inverse relationship has been observed between this uncoupling protein and Pgc1a, where this latter is stimulated by exercise in mammals. In fish exercised, the role of Pgc1a is unclear (McClelland 2012; Bremer et al. 2016; LeMoine et al. 2010).

Concerning the mitochondrial dynamics markers in this tissue, they were mainly affected by the exercise, decreasing the gene expression of *mit1* and *mit2* in both diet groups while differentially decreasing the fission factors *fis1* and *miffb* depending on the diet. The significant downregulation of in the exercised fish fed with the HE diet could indicate that the mitochondrial machinery works more efficiently with a higher lipid content during exercise. In mammals, lipid accumulation is known to disrupt mitochondrial homeostasis (Lowell and Shulman 2005), causing the balance to move towards the fission processes in skeletal muscle (Holmström et al. 2012; Huertas et al. 2019) to direct the impaired mitochondria to mitophagy, promoting mitochondrial turnover (Chandhok, Lazarou, and Neumann 2018).

The skeletal red muscle, markedly richer in lipid and mitochondria and highly more vascularized than the white muscle, but also much scarcer in terms of muscle mass in

gilthead sea bream, responded very differently to diet composition and swimming activity in terms of mitochondrial proteins. These differences would also be reflected to the differential nutrient preferences of these two types of muscle, with red muscle being more prone to lipid oxidation while white muscle is much more glycolytic (Magnoni, Crespo, et al. 2013). In this regard, previous studies in gilthead sea bream registered activities of COX4 and CS in red muscle, 3 and 7 times higher respectively than in white muscle (Martin-Perez et al. 2012).

The higher stimulation of Cs and Cox protein expression, in parallel with a decrease in Ucp3 levels, in fish fed HE diet under voluntary swimming, would indicate that the high lipid content of the HE diet did not exceed the oxidative capacity of this tissue. Then, the downregulation of the *cs* gene expression in this group might have occurred as a response to the high increase of its protein expression. Moreover, the significantly higher Cox/Cs ratio, both at the gene and protein level in the same group indicates also increased lipid oxidation. The exercise did not affected the gene expression of *cs* and *cox*. However, an interesting increase in UCP3 levels in the fish fed with the HE diet suggests that these enzymes might be regulated to adapt to a high lipid overload, such as observed in the skeletal and cardiac muscle of mammals (Nabben et al. 2014; Perelló-Amorós, Fernández-Borràs, et al. 2021, **Article 3**).

Hence, the impact of swimming on mitochondrial proteins related to the energy metabolism was greater in the white muscle compared with the red muscle in gilthead seabream. This fact is in agreement with a previous study which showed that the red muscle workload under sustained exercise is reduced with a compensation from the white muscle (Martin-Perez et al. 2012). Moreover, the diet composition, rather than the physical activity, would modify the balance between the processes of fusion and fission in the red muscle. More research is needed to understand the meaning of the changes in these processes.

Overall, this part of the thesis contributes to give some light on how the adaptation to diet composition (in terms of protein and lipid) in combination of moderate sustained swimming can modify the energy metabolism of the fish muscle and in consequence, affected the body growth of juvenile sea bream differently. Lower protein/lipid ratio

affected negatively to growth through an unbalanced availability and use of nutrients, especially indicated by changes in key mitochondrial proteins related to energy metabolism, energy excess dissipation, mitochondrial turnover, and biogenesis in the liver and white and red and white muscles. Sustained swimming induced an important increase of the energy demand that reversed the altered mitochondrial functions by increasing the use of lipids, along with a protein sparing effect that promoted a growth enhancement. From an applied point of view, the exercise results in a valid model to optimize the feeding costs in aquaculture by reducing the protein/lipid ratio, which at the end results also in a reduced nitrogenous waste without compromising growth, even in the period of fast growth of juvenile sea bream, and to improve the welfare of fish.

CHAPTER 5: GENERAL CONCLUSIONS

1. The Preproghrelin is well conserved and markedly expressed in stomach in gilthead sea bream. Its receptors, the Ghsr1a and Ghsr1b, are well conserved too and the Ghsr1b lacks in its sequence one of the kay seven transmembrane domains. Both receptors are widely expressed, presenting abundant mRNA levels in brain (specially Ghsr1a), pituitary (both receptors) and liver (specially Ghsr1b).

2. Fasting for 21 days did not caused a growth decrease but induced an increase of the blood Gh/Igf ratio that reverted with only 7 days of refeeding in gilthead sea bream juveniles. Similarly, blood Ghrelin increased but not its transcription level and pituitary *ghsr1a* presented inverse peri prandial responses to that of blood Ghrelin.

3. The rapid post prandial inhibition of the ghrelinergic system was accompanied by an increase of blood Igf1 and a strong and rapid induction of Akt and mTor phosphorylation in skeletal white muscle.

4. The skeletal white muscle transcription of the anabolic systems was mostly downregulated by fasting, while certain catabolic markers, such as the *ghr2* and *mafbx* were activated.

5. The refeeding triggered a sequential activation of the anabolic pathways starting with the local Gh-Igf1 axis as well as the downregulation of some of the proteolytic markers to then induce a muscle recovery, remodeling and hyperplasia.

6. Bone-derived *mstn2* showed a postprandial peak that lasted at least 24h suggesting that myostatin may play an important role in the crosstalk between muscle and bone in order to achieve a well-balanced growth during refeeding.

7. Decreasing the dietary protein/lipid ratio in gilthead sea bream reduces growth, increases lipid deposition and alters the mitochondrial function by uncoupling of the respiratory chain in the liver and white muscle along with a pro-fission state. In contrast, red muscle increases lipid oxidation without signs of mitochondrial impairment.

8. Moderate swimming activity improves the mitochondrial function in gilthead seabream fed a low dietary protein/lipid ratio by reducing the uncoupling processes and adjusting the balance between mitochondrial fusion and fission in liver and white muscle. Increased oxidation of lipids by red muscle under these conditions causes ucp3 increase. Overall, the enhanced use of lipids in exercise conditions produces a protein sparing effect for growth, suggesting improved nutritional efficiency.

9. The decrease in growth of gilthead seabream fed with a lower dietary protein/lipid ratio is related to lower blood Gh, Gh/Igf1 ratio, hepatic *igf1c* expression and reduced muscle texture along with the upregulation of many proteolytic genes.

The sustained swimming reverts such detrimental effects, pointing out the benefits of exercise in this species.

10. Myomaker in rainbow trout and gilthead sea bream is well conserved while Myomixer non-essential parts of the sequence have diverged more. Both fusogens are expressed during and after the primary and secondary embryonic myogenesis, especially in the fibers ongoing hyperplasia. Their expression peaked in primary cultured myoblasts *in vitro* and in the regenerating muscle, coinciding with the main markers of muscle differentiation, suggesting that the importance of both proteins in the myogenic process is conserved in rainbow trout and gilthead sea bream.

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