# Hormonal and nutritional regulation of muscle growth and development in gilthead sea bream (*Sparus aurata*)

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A thesis submitted by **Emilio J. Vélez** for the degree of Doctor

by the University of Barcelona

Barcelona, February 2018

UNIVERSITAT DE BARCELONA

Deparmento de Biología Celular, Fisiología e Inmunología, Universidad de Barcelona

Programa de doctorado de Acuicultura

# Regulación endocrina y nutricional del crecimiento y desarrollo muscular en dorada (*Sparus aurata*)

Memoria presentada por **Emilio J. Vélez** para optar al título de

**Doctor por la Universidad de Barcelona**

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Barcelona, Febrero 2018

“Desocupado lector, sin juramento me podrás creer que quisiera que este libro, como hijo del entendimiento, fuera el más hermoso, el más gallardo y más discreto que pudiera imaginarse. Pero no he podido yo contravenir a la orden de naturaleza; que en ella cada cosa engendra su semejante.”

Prólogo de “El ingenioso hidalgo Don Quijote de la Mancha”,

compuesto por Miguel de Cervantes Saavedra.

# ACKNOWLEDGEMENTS

¿Ya ha llegado la hora de enfrentarse a esto? Posiblemente esta sea la parte más difícil de todo este proceso. Muchas veces en nuestra vida vamos sin pararnos a analizar, o pensar, en todo aquello que dejamos atrás. Olvidamos que cada detalle que hemos vivido tiene la culpa de que ahora, justo ahora, estemos donde estamos. Olvidamos que una palabra que alguien te haya podido decir en un momento clave, sin dejar de ser una palabra, te pudo servir como empuje o punto de partida a una nueva experiencia, viaje, sentimiento, o acción, que sin duda te arrastrará a otra nueva oportunidad. O no. En este punto no podría seguir sin hacer un reconocimiento a todas la entidades (me veo obligado a ello) que han contribuido con su financiación, durante años, a hacer posible mi sueño. Gracias a las becas otorgadas tuve la oportunidad de estudiar la Licenciatura de Biología, de continuar con otra nueva experiencia como fue estudiar el Máster de Fisiologia Integrativa, y a continuación, meterme en esta locura del Doctorado. En éste periodo he tenido la gran suerte de poder investigar, aprender, y crecer tanto a nivel profesional como personal. Con las estancias he ganado en seguridad, conocido a personas que me han enseñado mucho e incluso me han ofrecio su casa, su coche. Quiero agradecer a Iban Seiliez la oportunidad, y al personal del INRA de St-Pée-sur-Nivelle, especialmente a Elisabeth y Karine Dias, por sus continuas risas y gran ayuda. A mi amigo Yujie Gao, al que debo una visita a China. Por el lado oeste, en Alabama, gracias a Peggy Biga tuve la oportunidad de conocer el calor sureño, conocer New Orleans, Memphis, Atlanta y Texas. A parte de todo lo absorbido en su laboratorio, es necesesario remarcar que me traje dos grandes amigos; a Mary Latimer, y sobre todo, a ?Ross Reid, del cual espero que antes o después vuelva a cruzarse en mi vida.

Volviendo a casa, es más que obligatorio agradecer al Dr. Joaquim Gutiérrez y al Dr. Miquel Riera la gran oportunidad que me ofrecieron desde un principio, su confianza, su paciencia y sus incansables ganas de enseñar. Vuestra ilusión constante y la curiosidad por

la ciencia son unos valores que me habéis enseñado y que me esforzaré por conservar. A la Dra. Encarni Capilla, porque siempre ha sido muy especial. Sin su ayuda nada de esto sería una realidad. Muchas gracias por tu tiempo, tus ánimos y tu gran ayuda. He aprendido mucho de ti. También tengo que agradecer toda la ayuda prestada en algún momento u otro por el resto de "jefes" del grupo. Gracias a la Dra. Isabel Navarro, a la Dra. Josefina Blasco y al Dr. Jaume Fernández. Igualmente, tengo que agradecer al Dr. Jaume Pérez Sánchez y al Dr. Josep Calduch del Instituto de Acuicultura de Torre de la Sal (IATS, CSIC, Castellón) por abrirme las puertas de "su casa" y enseñarme la técnica de RIA y su forma de trabajar. Si bajo los pies al laboratorio, es imposible olvidarme de toda la gente que ha pasado por "nuestra casa", el Departamento y el laboratorio. Gracias a Marta Bou y Débora Coimbra por su tiempo con el Western blot. Gracias a Antonio Millán y a Miguel Martín Pérez por enseñarme los cultivos primarios. A Pablo Bou, que se ha convertido en un gran amigo. Gracias a Mireia Rovira, Pepe Carbó, Jonathan Matalonga y Mónica Rius por los grandes ratos que hemos compartido. A todos los TFGs y TFMs, especialmente a Claudia y Nuria, Emma y Alba, y cómo no a Jaume Vela y Julia Viñuales. Gracias a Sheida, que solamente ella sabe las horas que hemos pasado en la campana de cultivos. Te deseo mucha suerte. Gracias también a Marga. Por otro lado, y de forma muy especial, tengo que agradecer por todo el apoyo que me han dado durante estos años a Cristina Salmerón y Esmail Lutfi (alias "El negro"). A pesar de que Cristina hace más tiempo que no está por aquí, me resulta muy difícil pensar en vosotros dos por separado. Cristina nos enseñó casi todo lo que sabemos (nunca te daré las gracias lo suficiente), y nos convertimos en un equipo. Jugando en este equipo he vivivo la mejor parte del doctorado. Lo importante no era el resultado, lo importante era jugar. Jugar con tu equipo. Mil gracias a los dos. Negro, ya sabes que sólo nos falta un buen patrocinador y un terreno de juego. Tenemos un partido por jugar.

También tengo palabras de agradecimiento a todos los “nuevos”, pues son éstos los que pueden atribuirse el mérito de haberme soportado durante esta etapa final. A los inmunos Joan, Esti y Nicole, ha sido un placer compartir este tiempo con vosotros. Me encanta Pamplona, quiero conocer Chile y adoro buscar chatarra subacuática, auguro que nos volveremos a ver. A Luis, por su martes de pollo y para que vayamos muchas veces al Camp Nou. A Albert, porque antes de ser compañero de clase y de poyata siempre fuiste mi amigo. Tienes tu pequeña parte de culpa de que ahora esté aquí. Gracias. A Sari, gracias por tu sonrisa y estar siempre dispuesta a echarme una mano. Confío en que pronto saldrá el primero de tus muchos artículos. Miqui, no te enfades si me voy. Tú podrás con todo, mucho ánimo y al toro. Muchas gracias por toda tu ayuda campeón. Nunca olvides las burbujas. A Nata, por ser la primera TFG de verdad. Te debo mil favores y estoy encantado de haber conocido a alguien como tú. Gracias por tu ayuda, por tus alegrías, y por tu tiempo. Mucha suerte en Francia. Y ahora, cúrrate una buena portada, ¿no?. Gracias otra vez.

Por último, y por supuesto que no menos importante, tengo que agradecer a mi familia todos sus sacrificios para poder regalarme esta “viña”. Millones de gracias, esto nunca me lo podrán robar. Por muy lejos que haya estado estos años, ni un solo día he dejado de acordarme de vosotros. Tampoco de los que ya no están, igual que de los recién llegados.

Irene, gracias por tu comprensión durante todo este periodo, por tu paciencia y por tu apoyo constante. En definitiva, gracias por estar ahí. Siempre sabes mirar las cosas de manera vertical.

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# ABBREVIATIONS

List of relevant abbreviations used throughout the present thesis:

**4EBP1:** translation initiation factor 4E-binding protein 1

**70S6K:** p70S6 kinase

**AA:** amino acids

**AKT:** protein kinase B or PKB

**ALS:** autophagy-lysosomal system

**ARs:** adrenergic receptors or adrenoceptors

**cAMP:** cyclic AMP

**CAPN:** calpains

**CAPNS:** calpain small subunits

**CTS:** cathepsins

**FOR:** formoterol

**FOXO:** forkhead family of transcription factors

**GH:** growth hormone

**GHBPs:** GH binding proteins

**GHRs:** GH receptors

**HSL:** hormone-sensitive lipase

**IGFs:** insulin-like growth factors

**IGFBPs:** IGF-I binding proteins

**IGF-IRs:** IGF-I receptors

**MAFbx:** muscle atrophy F-box or atrogin-1

**MHC:** myosin heavy chain

**MLC:** myosin light chain

**MRF4:** myogenic regulatory factor 4

**MRFs:** myogenic regulatory factors

**MSTN:** myostatin

**MuRF1:** muscle RING-finger protein 1

**MYOD:** myogenic determination factors

**MYF5:** myogenic factor 5

**NA:** noradrenaline

**N3:** proteasome β type 4 subunit

**PAX7:** paired box protein 7

**PCNA:** proliferating cell nuclear antigen

**PI3K:** phosphoinositide 3-kinase

**PKA:** protein kinase A

**rBGH:** recombinant bovine GH

**SALM:** salmeterol

**sbGH:** sea bream GH

**TOR:** target of rapamycin

**Ub:** ubiquitin

**UbP:** ubiquitin-proteasome system

**VEGF:** vascular endothelial growth factor

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Aquaculture

Aquaculture is the set of practices, knowledge and processes necessary for farming aquatic animals and plants (from fish to aquatic plants, including crustaceans, mollusks, and algae) under controlled conditions and in either freshwater or seawater. During farming, the aquaculture producer manages these animals and controls the rearing conditions (e.g., animals stocks, feeding, or water temperature among others) in order to enhance production. This activity started many years ago, finding evidence on the cultivation of common carp (*Cyprinus carpio*) in ancient China at 3500 BC and appearing the first known treatise about fish culture in the 5$^{th}$ century BC authored by Fan Li (*The Treatise on Pisciculture*). However, until that Stephen Ludvig Jacobi achieved for the first time artificially fertilized salmonids in the year 1755 (Leitritz and Lewis, 1976), aquaculture did not represent a very important productive or economic sector. Since that moment, a great interest in aquaculture development appeared and the French government founded in 1852 the first aquaculture research center at Huningue (Nash, 2011), and soon after, in 1866 both, first fish farm and ichthyologic laboratory appeared in Spain (Nash, 2011).

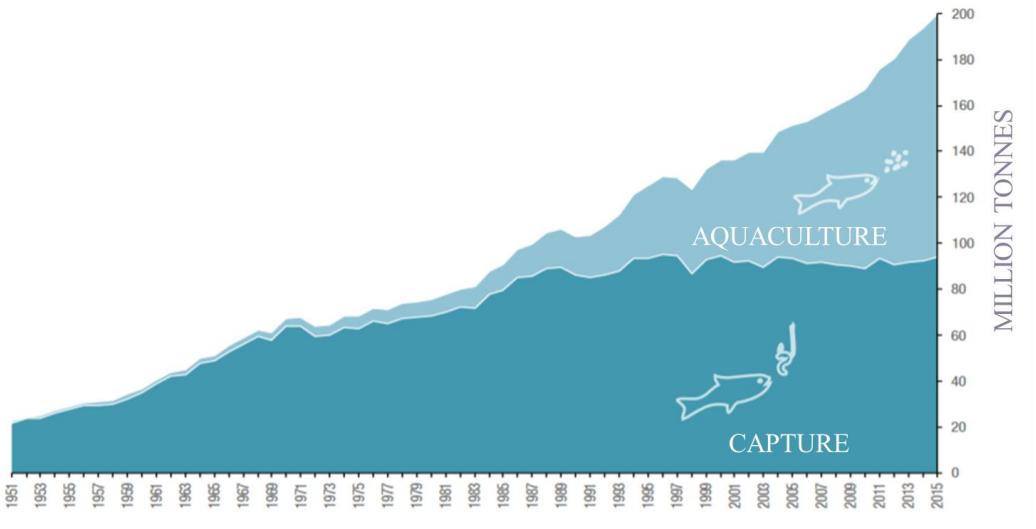



**Figure 1.** World capture fisheries and aquaculture production. (APROMAR, 2017)

Nonetheless, it is from the mid-20th century when aquaculture experienced a substantial increase in terms of production and economic value (Figure 1), and especially in the last 15 years, when the aquaculture contribution to the fish supply for human consumption overtook for the first time the wild-catch fish in 2014 (FAO, 2016). At that year, the main animals groups cultured in aquaculture for human consumption were finfish, representing up to 67.5% of the total world production (i.e., 73.783.725 tonnes), molluscs (21.8%), crustaceans (9.3%), and 0.40% corresponds to other aquatic animals (FAO, 2016). Moreover, in 2015 aquaculture production reached a 53.1% of total production, in contrast with the 46.9% obtained by traditional fishing (APROMAR, 2017).

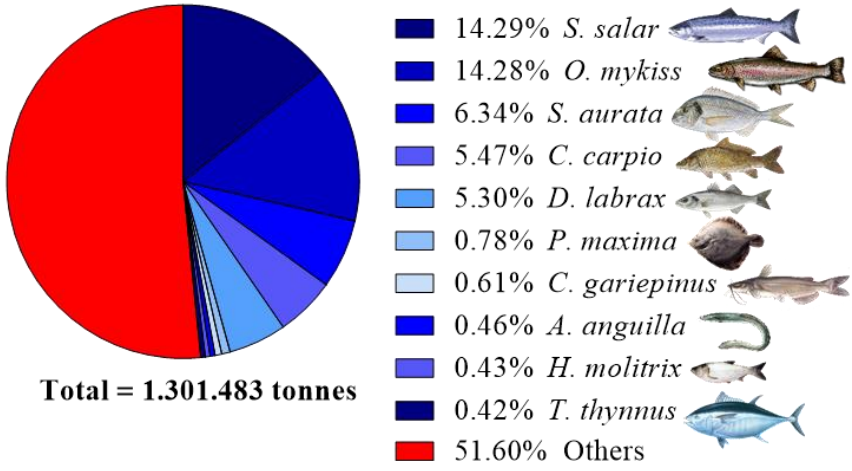



**Figure 2.** The ten most-cultured fish species in the European Union in 2015 and the percentage that their production represents in total fish produced. Data obtained from APROMAR (2017).

If European aquaculture production is analyzed in detail using the data from the Food and Agriculture Organization of the United Nations (FAO), it becomes clear that from the total of 2.930.127 tonnes obtained in 2014, an 83.7% corresponds to marine and coastal aquaculture, and the other 16.3% is due to inland practices (FAO, 2016). These data

contrast with that obtained at a global level, in which inland procedures are 63.9% of world production while marine aquaculture represents only a 36.1%. In addition, although European aquaculture represents only the 3.97% of this world production, the culture of finfish in Europe supposes the 28.8% of the total finfish production worldwide, highlighting the importance of these culture practices in Europe and their global relevance.

The most important cultured fish species in the European Union are indicated in Figure 2. The three main cultivated species are Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and gilthead sea bream (*Sparus aurata*). While most of the salmon and trout farming is done in the United Kingdom, gilthead sea bream cultivation is widely carried out in the southern area (Mediterranean region). In particular, the major countries producing gilthead sea bream are Turkey, Greece, Egypt, and Spain (Figure 3, insert.) (APROMAR, 2017). In the latter, aquaculture production of gilthead sea bream represents nowadays around 95% of total production (Figure 3).

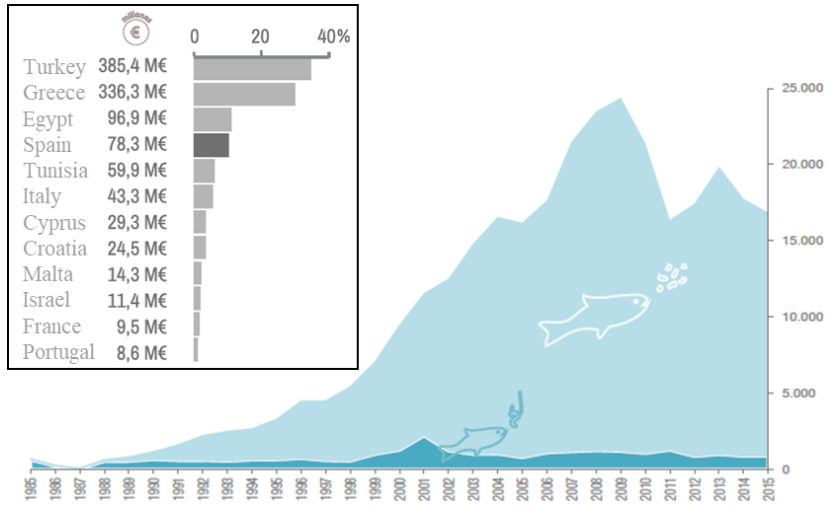



**Figure 3.** Gilthead sea bream total production in Spain through aquaculture (top) and fish capture (bottom) from 1985 to 2015. In the insert, economic value (millions of euros) of aquaculture production of gilthead sea bream in the Mediterranean region in 2016. Adapted from APROMAR (2017).

In the context of the large increase observed in aquaculture production, it is important to mention that on the contrary, wild-fish captures have remained relatively static since the latest 1980s although the stocks of some fish species are clearly below their biological limits, that is, overfished (Figure 4A). This situation, together with the expected increase in population up to more than 9 billion people by 2050 (FAO, 2016), have further fueled the need for aquaculture intensification. This is especially relevant in some regions such as the Mediterranean in Europe, where the production has been very constant in the recent years compared with the neighbors of northern Europe (Figure 4B).

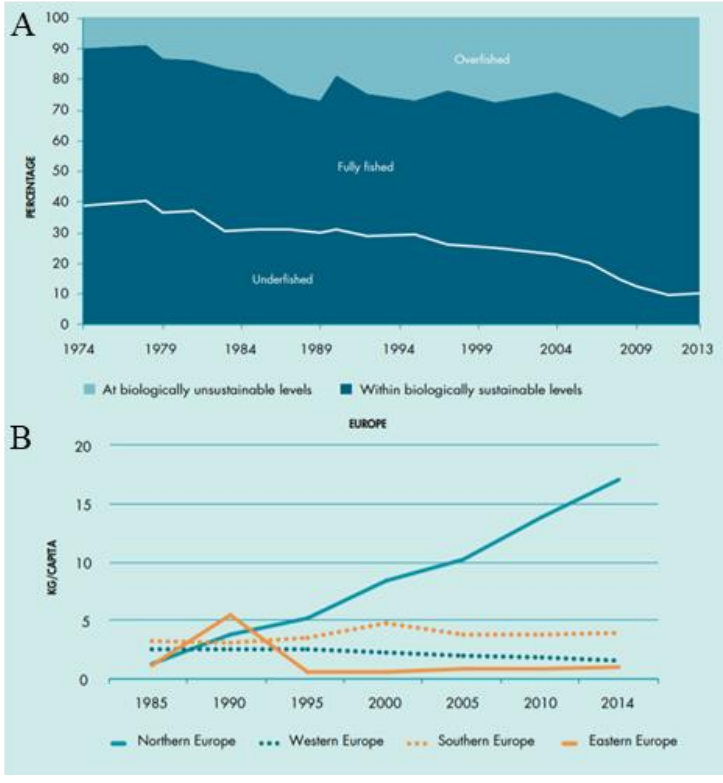



**Figure 4. A:** Global trends in the state of world marine fish stocks from 1974 to 2013. **B:** Production per capita of aquaculture (excluding aquatic plants) in different European regions. Adapted from FAO (2016).

In this line, the Spanish administration approved in 2014 “The Strategic Plan for Spanish Aquaculture 2014-2020”, in which the situation of this sector was analyzed and the priorities fixed towards a sustainable growth for the year 2030 (OESA, 2014). In this document, the importance of improving the environmental aspects of aquaculture, animal health, and welfare, and also the competitiveness through Research and Development (R&D) is highlighted. This R&D should focus on improving nutrition, reproduction, stock management, and growth, to in the last term optimize the production and quality of the species of interest.

For all this, with the ultimate goal of boosting aquaculture production and the competitiveness of the Mediterranean region, more research is necessary in Spain to improve growth, nutrition, and quality of one of the key species produced, the gilthead sea bream. To this end, white skeletal muscle represents the main target tissue to be studied in the present project.

## 1.2 Gilthead sea bream (*Sparus aurata*)

Gilthead sea bream is one of the most important species in Mediterranean aquaculture. Its natural habitat is found in brackish and marine waters of the Atlantic coast (from Great Britain to Cabo Verde) and throughout the Mediterranean Sea (APROMAR, 2017). Usually, gilthead sea bream appears in coastal zones and in depths up to 30 meters (but exceptionally, adults have been observed at 150 m), areas populated by marine phanerogams on both rocky and sandy soils. In these areas, it captures small mollusks, crustaceans, and also fish, as the main part of their carnivorous diet. In terms of reproduction it is a protandrous hermaphrodite species, thus these fish, develop first as

males by their two years of age, and then become females when they reach approximately 35 cm body length.

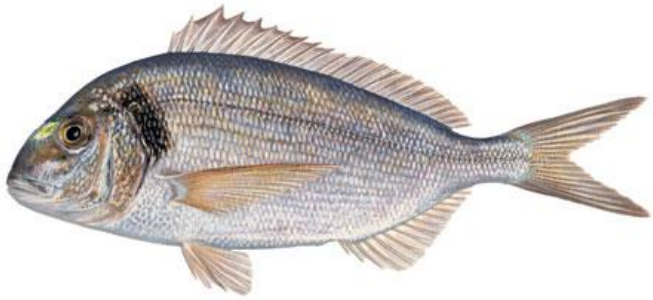


**Figure 5.** Gilthead sea bream (*Sparus aurata*).

This species is characterized by its oval and compressed body form and by the big and curved head, where a golden frontal band between eyes appears (Figure 5). The caudal fin is forked and its color is silvery grey with a large black blotch at the origin of the lateral line, and dark longitudinal lines are sometimes present on the side of the body. Wild individuals of more than one meter long, 5 kg of weight, and up to 11 years of age have been observed (Basurco et al., 2011; Campillo, 1992).

## 1.3 Growth

Growth is the process by which a quantitative and measurable parameter attributable to a body, permanently increases its value (e.g., size, weight, height, volume, surface area, etc.). In mammals, birds, and insects, the limit of somatic growth is determined by the physiology of the species itself, that is, after sexual maturity, the resources are intended to gonads development and reproduction, and thus the increase in body weight ceases, therefore are known as determinate growers (Figure 6A).

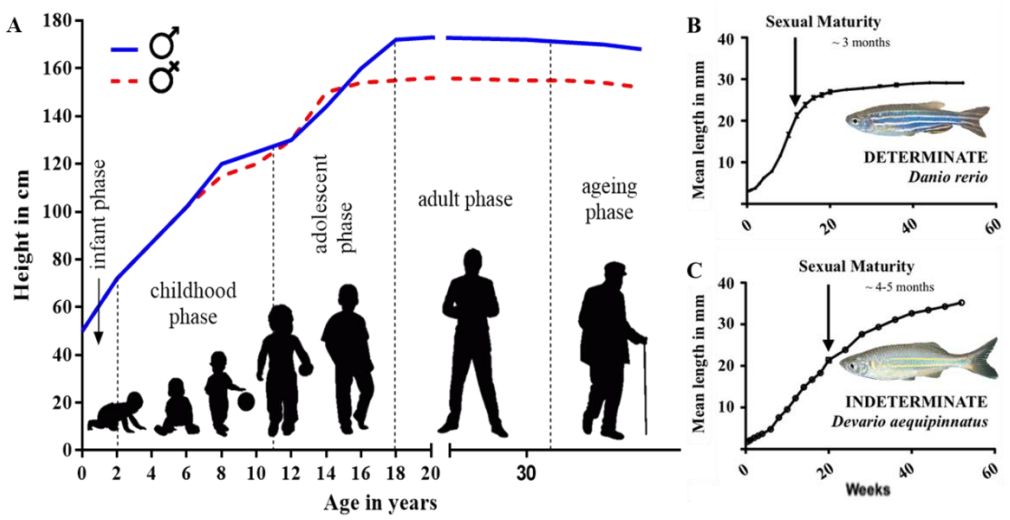



**Figure 6. A:** Human growth curve. Although some fish species such as zebrafish are determinate growers (**B**), most of the fish species follow a pattern of indeterminate growth, as is the case of giant danio (**C**). Adapted from Froehlich et al. (2013a).

Although a reduced number of fish species follow a determinate growth pattern similar to that of mammals, most teleost species, including gilthead sea bream, can grow throughout their entire lifespan in a continuous way, being known as indeterminate growers (Biga and Goetz, 2006; Johnston et al., 2011). Both types of growth are well represented in the phylogenetically close species, zebrafish, *Danio rerio*, and giant danio, *Devario aequipinnatus* (Figure 6B and 6C). After sexual maturation, the size of zebrafish reaches a *plateau*, but in the case of giant danio the growth is maintained reaching up to 13 cm in their adult phase, what it means to double or triple the size of the zebrafish (Biga and Goetz, 2006).

In fish, muscle represents up to 70% of total body mass, therefore its growth implies accretion of muscle tissue (Bone, 1978; Johnston, 2006; Mommsen, 2001). The different growth potentials among fish species described are associated with the characteristics of muscle development (Biga and Goetz, 2006; Froehlich et al., 2013a, 2013b). To better

understand this fact it is necessary first to deepen into what skeletal muscle is, how is developed, and what factors regulate its growth.

### 1.3.1 Skeletal muscle

The skeletal muscle, also known as striated muscle for its morphological aspect under the microscope, in addition to being responsible for all voluntary movements, is involved in different involuntary actions (e.g., breathe and postural maintenance), and it is also important as a metabolic tissue (e.g., storage of glycogen, proteins and lipids) (Sadava et al., 2009; Zanou and Gailly, 2013).

The skeletal muscle is under the control of the central nervous system, which can activate the contraction of the fast-twitch anaerobic fibers of the white muscle (glycolytic muscle) to scape in a stress situation, and also the activity of the slow-twitch aerobic fibers of the red muscle (oxidative muscle), which are implicated in sustained movements (Johnston et al., 2011). Moreover, the performance of the muscle depends on fibers composition and organization, and their properties in turn, depend on the process of muscle development, also called myogenesis.

- **Muscle structure**

The skeletal muscle in fish is structured very differently as it does in other vertebrates. The fish fillet is formed by different muscle units, called myomeres or myotomes, which are separated from each other by a connective tissue layer, the myocommata or myoseptum (Georgiou et al., 2016; Johnston, 1999; McClelland and Scott, 2014). As reviewed by Johnston et al. (2011), individual fibers are associated with others to form bundles that are

bound by connective tissue layers containing proliferative fibroblasts. Between the fibers, some adipocytes are usually found, mainly in red muscle, but they are especially abundant in the junctions of fibers-tendons and normally in the surface of the myosepta (Johnston et al., 2011). Once the myotomal muscle fibers are contracted, the force is transmitted through the myosepta, via tendons, to the skeleton and caudal fin to induce a body undulation and forward propulsion [Reviewed by Sänger and Stoiber (2001)]. In most fish species, approximately 90% of the myotome is composed of anaerobic white muscle, present on each side of the fish and separated in dorsal (epaxial) and ventral (hypaxial) compartments by a collagenous horizontal septum [Reviewed by Coughlin (2002) and Johnston et al. (2011)].

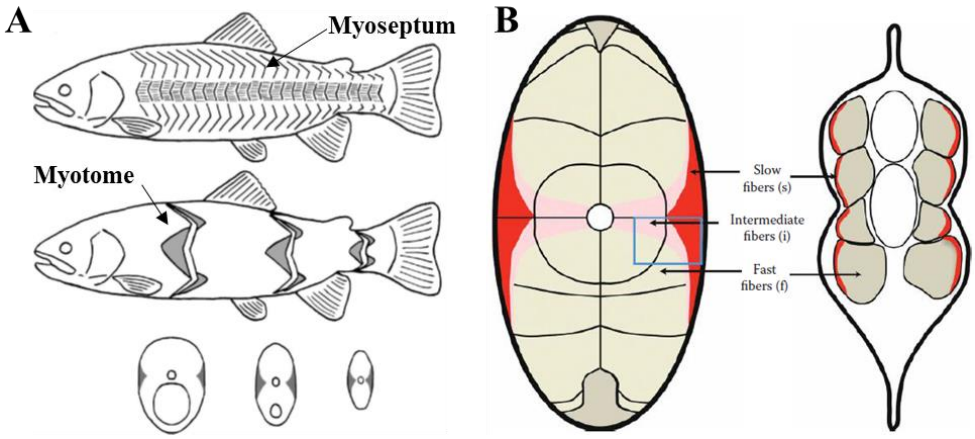



**Figure 7. A**: Diagram of the fish muscle structure. The myotomes are shown at the top. The white muscle is present in the epaxial and hypaxial region with respect to the red muscle, which appears along the lateral line. The "W" shape of individual myotomes of white muscle is shown at different body positions in the middle figure. The bottom diagram shows that red muscle has a "V" appearance when observed in a transversal cut and its cross-sectional area normally increases from anterior (head) to posterior or caudal (tail) regions, contrary to what happens with the white muscle. Extracted from Coughlin (2002). **B**: Some authors recognize a third muscle type that 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. Adapted from McClelland and Scott (2014).

The individual myotomes run in an oblique pattern perpendicular to the long axis of the fish, presenting a “W” shape in which epaxial and hypaxial anterior cones can be distinguished. Furtehrmore, two posterior cones and also a central-anterior cone, can be found. This structure facilitates a certain overlap between myotomes that facilitates the swimming movements (Figure 7). Regarding the aerobic red fibers of the myotome, they are organized forming parallel bands to the horizontal septum on each side of the fish, with a longitudinal orientation. Thus, this muscle is under the skin along the lateral line (Figure 7).

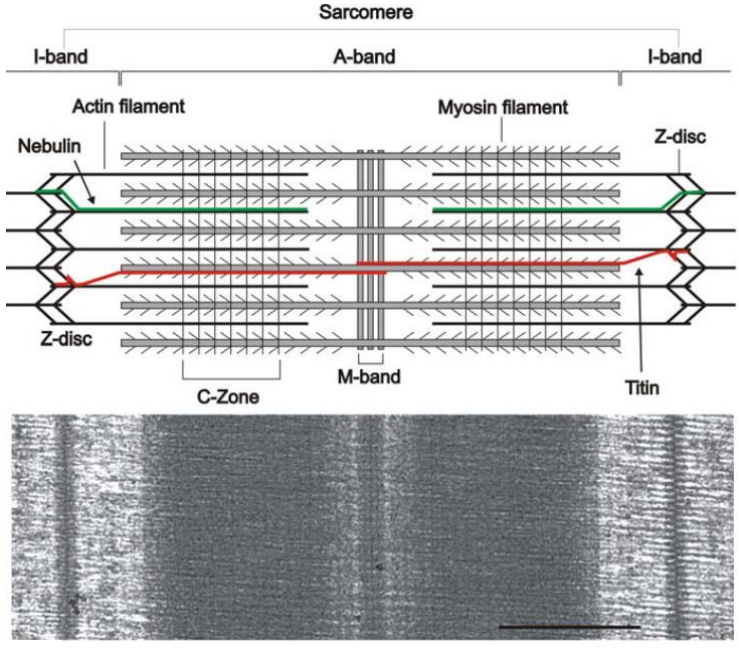



**Figure 8.** Striated muscle sarcomere structure. Top, the main components of the sarcomere are shown. Myosin filaments are linked to the center by the M-band and this set of molecules form the A-band. On the other hand, actin filaments are fixed to the Z-disc and interdigitate with the myosin filaments in the A-band. Other proteins such as nebulin or titin are important for the sarcomere structure and function. Bottom, an electron micrograph of a longitudinal section of fish white muscle showing the structure of the fish sarcomere. Scale bar = 500 nm. Adapted from Luther (2009).

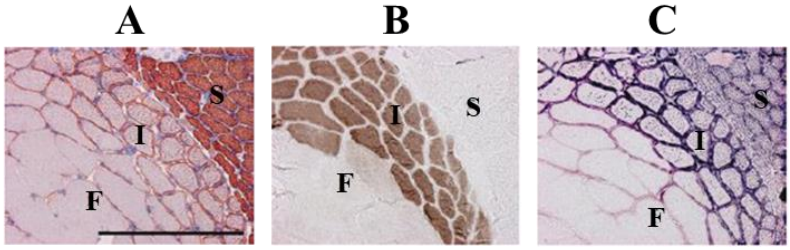
The skeletal muscle fibers are composed of many cylindrical myofibrils in which the sarcomeres are presented (Figure 8). This anatomical and functional unit of the striated

muscle is common to all vertebrates, and include both thin (composed by actin, tropomyosin and troponin, among others proteins) and thick (mainly composed by myosin) myofilaments, which are organized to form the characteristic tetragonal lattice Z-disc and the M line of the sarcomere (Ferrante et al., 2011; Luther, 2009; Squire et al., 2005).

The contraction of striated muscle consists in the interaction between actin and myosin at the sarcomere, being this fact regulated by the troponin protein complex in a calcium-dependent manner. Briefly, once a skeletal muscle fiber is activated, the electric potential of the plasma membrane is modified, and thus depolarized with the rise in cytosolic $Ca^{2+}$ coming from the sarcoplasmic reticulum to initiate contraction. The calcium activates the enzyme ATPase in the myosin filament. This enzyme catalyzes the ATP hydrolysis to produce the energy necessary for the actin filaments to slide between those of myosin and contract, in the last term, the muscle fiber. Conversely, when the calcium levels are reverted, the ATPase activity ceases and the filaments recover their original position for the final relaxation of the muscle fiber [Reviewed by Geeves and Holmes (2005)].

With regards to ATPases, differences exist depending on the type of muscle. As commented above, the red muscle is formed by slow-twitch or aerobic fibers with a high degree of vascularization and an abundant mitochondria population, being able to fuel the energy needs during sustained swimming, and the principal myosin-ATPase found is the type 1 (Figure 9). On the other hand, white muscle is composed primarily of anaerobic or fast-twitch fibers with bigger diameter (Johnston et al., 2011), and type 2b of myosin-ATPase (Figure 9). Besides these two muscle types, some authors recognize a third type of fibers with an intermediate phenotype and characteristics that it is called pink or fast-twitch aerobic muscle that also presents an intermediate myosin-ATPase of type 2a (Figure 9)

(Coughlin, 2002; McClelland and Scott, 2014; Rome et al., 2007; Sänger and Stoiber, 2001).




**D**

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

**Figure 9. A**, **B** and **C** show a representative immunohistochemistry image from histological sections of adult zebrafish. **A**: Slow fibers (S) are identified using an antibody S58 against myosin. **B**: Intermediate fibers (I) are identified by the activity of alkaline-resistant myosin-ATPase, and they are located between S and fast fibers (F). **C**: Slow and intermediate fibers present abundant mitochondrial content and both are identified by the activity of succinate dehydrogenase. **D**: Summary of the phenotypic differences among fiber types. Adapted from McClelland and Scott (2014).

The different location and orientation in the fish body of the different types of muscle fibers facilitate that, to generate the same body curvature, the white muscle needs to shorten much less than the red one (Coughlin, 2002). In fact, white muscle (anaerobic fibers) has the capacity to generate up to 10 times more power than the red muscle (Altringham and Johnston, 1990), resulting in a very useful advantage in explosive movements such as escaping a predator or hunting a prey.

In fish, similarly to other vertebrates, myosins are composed of two heavy chains (MHCs) and four light chains (MLCs). Using histochemical staining it has been possible to differentiate between fiber types since they present a characteristic composition of myosin

isoforms. Whereas the MLCs of both white and pink muscles are identical, they are different from those of red muscle fibers, as well as the MHCs are different in the three muscle types [Reviewed by Sänger and Stoiber (2001)]. During the fish life cycle the myotomal muscles present an important plasticity insomuch as, depending of swimming functional demands, both the contractile and metabolic phenotypes of the fibers that can change to adapt to new circumstances (Johnston et al., 2011). In addition to swimming demands, the fish muscle is also capable to adapt in response to other factors such as environmental changes or hormonal signals. In general, this muscle plasticity seems to be associated mainly with the transition in the expression of myosin isoforms and other proteins (e.g., ATPase, parvalbumin, troponin, etc.) (Addis et al., 2010; Campion et al., 2012; Goldspink, 1998), but also with the mitochondrial dynamics (Brijs et al., 2017).

- **Muscle development: myogenesis**

In vertebrates, during embryonic myogenesis, mesodermal precursor cells are stimulated to form part of the myogenic lineage towards myoblasts. Then, myoblasts undergo proliferation, cell cycle exit, and so differentiation to myocytes begins. Finally, the myocytes fuse to form multinucleated myofibers (Chargé and Rudnicki, 2004; Johnston, 2006). During muscle development, a different subpopulation of myoblasts fails to differentiate, and these cells remain in a quiescent undifferentiated state (quiescent muscle satellite cells) in close association with the surface of the new myofibers [Reviewed by and Almada and Wagers (2016), and Chargé and Rudnicki (2004)].

In addition, in the adult muscle, the existence of these quiescent satellite cells appears to be crucial for the regeneration capacity after injury, disease, trauma, or even exercise

(Chang et al., 2016; Bentzinger et al., 2012; Grounds et al., 2002; Sincennes et al., 2016; Zanou and Gailly, 2013). Briefly, once signals of damaged muscle fiber appear, satellite cells are activated to proliferate and then differentiate into new myoblasts that will fuse with the damaged fiber or with another myoblast to resemble the original fiber. Additionally, in order to ensure the repair capacity in the future, a subset of myoblasts returns at this point to the quiescent state (Almada and Wagers, 2016; Heslop et al., 2001; Schultz et al., 1985).

Seale et al. (2000) discovered Pax7, a transcription factor of the paired box (Pax) family, which is selectively expressed by satellite cells and supposes an excellent identification target for these cells through immunolabeling techniques (Figure 10). In addition, different studies have demonstrated that Pax7 is involved in satellite cell specification, survival, self-renewal, and thus, in the regulation of postnatal muscle development (Buckingham, 2007; Bentzinger et al, 2012; Sincennes et al., 2016; von Maltzahn et al., 2013; Yablonka-Reuveni et al., 2008).

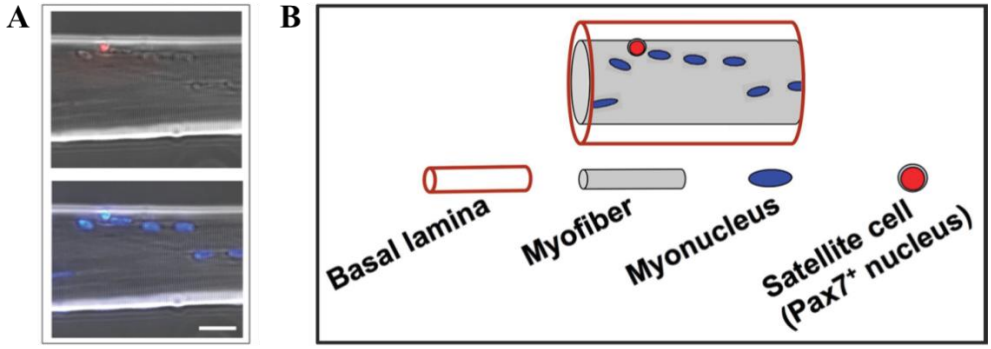



**Figure 10.** Identification of satellite cells by Pax7 expression. **A:** Images of a myofiber isolated from the extensor digitorium longus muscle of adult mouse marked with an anti-Pax7 antibody (in red, top), or myonuclei stained with DAPI (in blue, bottom). **B:** Schematic representation of the location of a satellite cell in a niche between the sarcolemma and the basal lamina of the muscle fiber. Adapted from Yablonka-Reuveni et al. (2008).

Together with the role of Pax7 regulating myogenesis, the orchestrated and well-coordinated expression of the myogenic regulatory factors (MRFs), a family of muscle-specific basic helix-loop-helix transcription factors, exert a finely control of all the processes required for muscle development [Reviewed by Bentzinger et al. (2012), Hernández-Hernández et al. (2017), Yablonka-Reuvenei et al. (2008), and Zanou and Gailly (2013)].

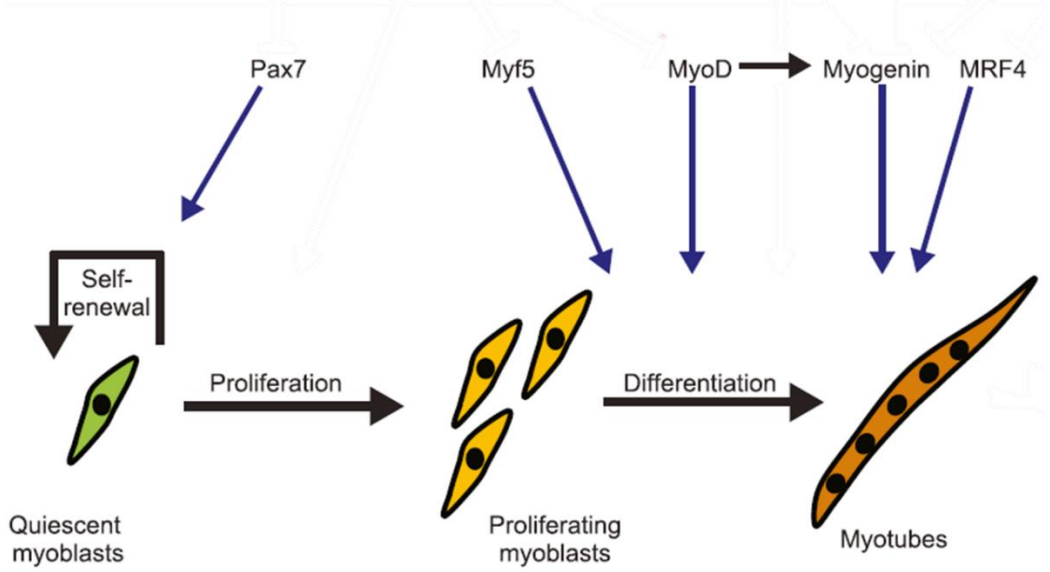



**Figure 11.** Role of Pax7 and MRFs regulating satellite cell activity and the myogenic process. Adapted from Chaillou and Lanner (2016).

Once satellite cells are activated (Figure 11), express myogenic factor 5 (Myf5) that promotes proliferation. The myogenic determination factor 1 (MyoD) expression is observed during the last part of the proliferative phase, where it induces the cells to withdraw from the cell cycle. MyoD also induces the expression of myogenin, which is the key factor leading to myoblast fusion and differentiation. Finally, for the maturation of myotubes Mrf4 expressed, which allows the structural reorganization of myofilaments and the migration of the central nuclei to the periphery (Zanou and Gailly, 2013).

Altogether, these factors that control muscle development are well conserved in vertebrates including fish, and the *in vitro* primary culture of myocytes has made possible their characterization in different fish species such as Atlantic salmon (Bower and Johnston, 2010a) and gilthead sea bream (García de la serrana et al., 2014). Figure 12 represents a diagram of their expression during *in vitro* myogenesis in the latter species.

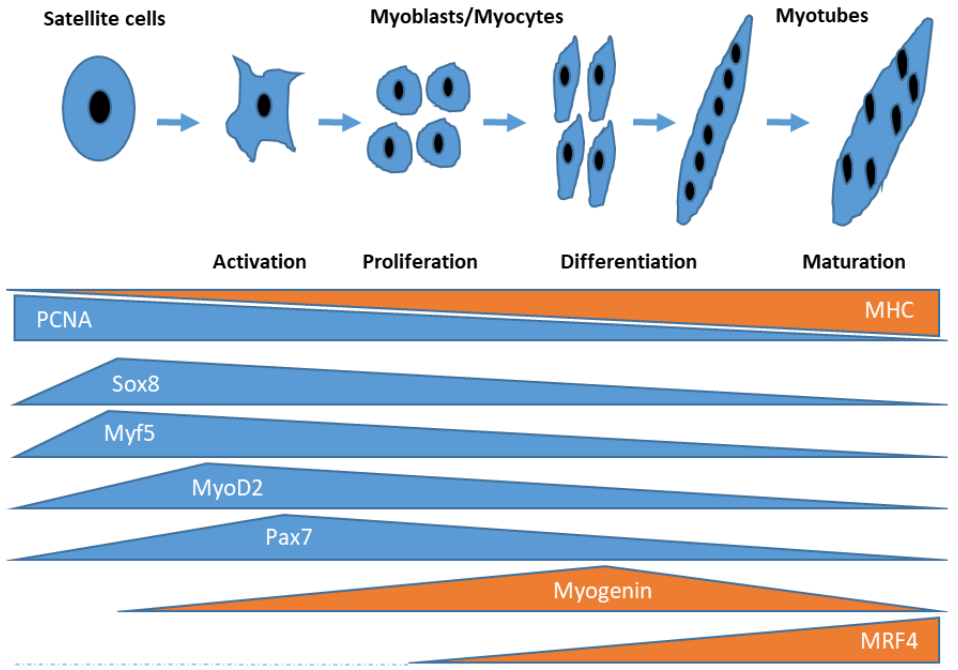



**Figure 12.** Schematic representation of myocytes development and gene expression of myogenic factors in gilthead sea bream. The proliferation process (in blue) is marked by the higher expression of the proliferating cell nuclear antigen (PCNA), and the differentiation phase (in orange) is recognized by the increased expression of myosin heavy chain (MHC). While in the proliferation phase the transcription factors expression of Sox8, Myf5, MyoD2 and Pax7 is important, in the later stages myogenin and MRF4 appear to induce the maturation of myocytes into myotubes. Extracted from Vélez et al. (2016).

The MRFs indeed control the expression of important muscle-specific proteins such as structural MHC or MLC isoforms (Schiaffino et al., 2015; Zanou and Gailly, 2013), which in fact, according to their proportion in the final fiber, can modify both the metabolic

properties of the muscle, as well as their functional capacity (e.g., strength, shortening velocity, ATPase activity, etc.) (Schiaffino et al., 2015; Siqin et al., 2017).

In addition, other factors such as myostatin (MSTN) and myocyte enhancer factors (MEFs) play specific roles regulating myogenesis, together with other less muscle-specific factors such as some members of the Pax, Sox, HGF or FGF families (Chargé and Rudnicki, 2004; Bentzinger et al., 2012; Dong et al., 2017; McPherron et al., 1997; Pawlikowski et al., 2017). In this sense, Millay and co-authors (2013) discovered a muscle-specific membrane protein, named myomaker that is essential for myoblast fusion. The expression of *myomaker* is regulated by myogenin, and in mammals, it seems to be required for muscle regeneration after injury [Reviewed by Demonbreun et al. (2015)]. This protein has been found also in fish, and Landemaine et al. (2014) demonstrated that it is essential for myocyte fusion in zebrafish. In coho salmon (*Oncorhynchus kisutch*), myomaker seems to be also involved in myotube fusion, since reduced expression has been associated with a reduced muscle fiber diameter (García de la serrana et al., 2015). In addition to all this factors, in the recent years, epigenetics has also appeared as another important regulator of myogenesis [Reviewed by Sincennes et al. (2016)].

Once commented the main factors that regulate muscle development in vertebrates including fish, it is important to take into account that myogenesis involves episodes of hypertrophy (increase in fiber size), when myoblasts are fused with pre-existing myofibers, and hyperplasia (new fibers production), when myoblasts fuse with each other forming new fibers (Rowlerson et al., 1995). In mammals, hyperplastic growth is mainly limited to the embryonic development of the muscle, to afterwards grow exclusively by hypertrophy at a postnatal stage with the only exception of some specific muscle regeneration events (Bentzinger et al., 2012; Rowe and Goldspink, 1969; Shahjahan, 2015; Zanou and Gailly,

2013). This fact produces that, as mentioned above, mammals follow a determinate growth pattern and have a finite size. However, most fish species can increase continuously the fiber number by hyperplasia throughout their entire lifespan (Biga and Goetz, 2006; Johnston et al., 2011), therefore as previously discussed, have an unlimited potential to grow.

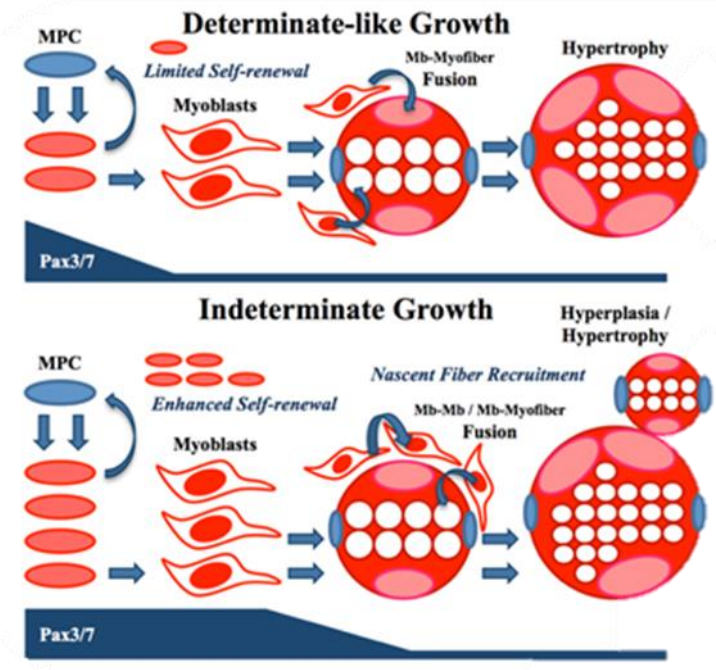



**Figure 13.** A prolonged gene expression of the transcription factors *pax3/7*, and a limited expression of *myf5* in fish muscle satellite cells, could be behind the increased self-renewal and proliferation rate of these cells and on the unlimited growth capacity of these species. Moreover, the ability to recruit new myofibers by hyperplasia could be also associated with *pax3/7* expression. Adapted from Froehlich et al. (2013a).

Froehlich and co-authors (2013a, 2013b) proposed that the main differences between these opposed growth models may be associated with a greater capacity of proliferation and self-renewal of muscle satellite cells in the indeterminate growers' species. This capacity is the result of a longer gene expression of the paired box transcription factor *pax3*, together with a limited expression of *myf5* and *pax7*, when compared with determinate growers (Figure

13). It has been proposed as well that the ability to form new fibers in the former may be also due to the different expression of *pax3/7* factors expression when compared with mammals (Froehlich et al., 2013a, 2013b).

Besides the chief myogenic stimulators of growth, Seiliez et al. (2012b) demonstrated that myostatin also plays an important role regulating negatively the proliferation of rainbow trout myocytes, whereas contrarily in mammals, myostatin inhibits both proliferation and differentiation (Joulia et al., 2003; Trendelenburg et al., 2009), and its inactivation induces muscle hypertrophy (Rodriguez et al., 2014). The overexpression of follistatin in rainbow trout, which is an inhibitor of myostatin, induces *in vivo* an increase in muscle mass that is mainly achieved by hyperplasia (Figure 14) (Medeiros et al., 2009). Therefore, myostatin appears as an important regulator of cell proliferation, and consequently, hyperplastic growth in fish, and interestingly, two paralogs of *myostatin* have been identified in teleost fish, whereas in salmonids up to four copies have been found [Reviewed by Gabillard et al. (2013)].

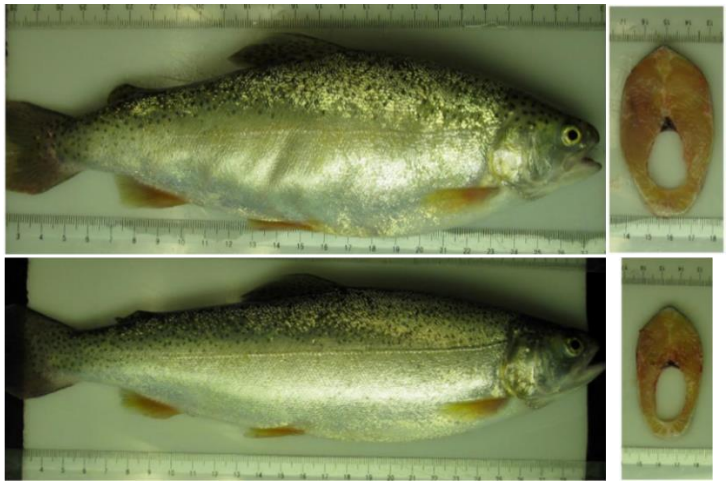


**Figure 14.** Photo of representative individuals and their whole body cross sections from transgenic trout overexpressing follistatin (top) and the control group (bottom). Adapted from Medeiros et al. (2009).

In gilthead sea bream it has been observed that, together with myostatin, the expression of the two isoforms of MLC identified in this species [i.e. *myosin light chain 2a* (*mlc2a*) and *2b* (*mlc2b*)], suppose an effective mechanism to regulate muscle development at both *in vivo* and *in vitro* levels (Georgiou et al., 2016; Moutou et al., 2001). *mlc2a* is expressed in the phases of greater new fibers formation, such as the early muscle development until metamorphosis occurs, whereas *mlc2b* expression predominates in adult muscle and it is well correlated to growth in length (Georgiou et al, 2014). More recently, these same authors have corroborated that *mlc2a* is expressed in the new fibers, and is later replaced by *mlc2b* once the fibers reach a certain size, thus, MLC2a has been postulated as a good marker of hyperplasia in gilthead sea bream (Georgiou et al., 2016). Furthermore, a similar pattern of expression among *mlc2b* and *myostatin* was found in gilthead sea bream larvae at 35 days post-hatch, which could indicate the end of muscle hyperplasia and the switch to fiber hypertrophy (Georgiou et al., 2016). Hence, the expression of both MLC2 isoforms and their association with myostatin seems to be a key factor to take into account to better understand the regulation of muscle growth and development in gilthead sea bream.

### 1.3.2 Protein synthesis and degradation

Generally when muscle growth is analyzed, it becomes clear that it mainly depends on the positive balance between protein synthesis and degradation (proteolysis). This fact is especially important in fish since they naturally suffer periods of fasting (i.e., a catabolic situation) (Vélez et al., 2017).

- **Protein synthesis: TOR and AKT signaling pathways**

In vertebrates, protein synthesis is stimulated by amino acids (AA) throughout activation of the nutrient-sensitive target of rapamycin (TOR, a.k.a. mTOR in mammals) signaling pathway (Fuentes et al., 2013; González and Hall, 2017; González and Rallis, 2017; Johnston et al., 2008; Kim, 2009; Kim and Guan, 2011; Lansard et al., 2010; Seiliez et al., 2008). Besides the effects on protein synthesis, TOR is known as the key molecule that integrates different signals from nutrients, energy status or growth factors, among others, to regulate important processes for cell growth such as the cell cycle, cytoskeleton organization, or even gene transcription (González and Hall, 2017; González and Rallis, 2017). Furthermore, also insulin and insulin-like growth factors (IGFs) activate indirectly TOR by inducing the phosphoinositide 3-kinase (PI3K)/AKT transduction pathway (Figure 15).

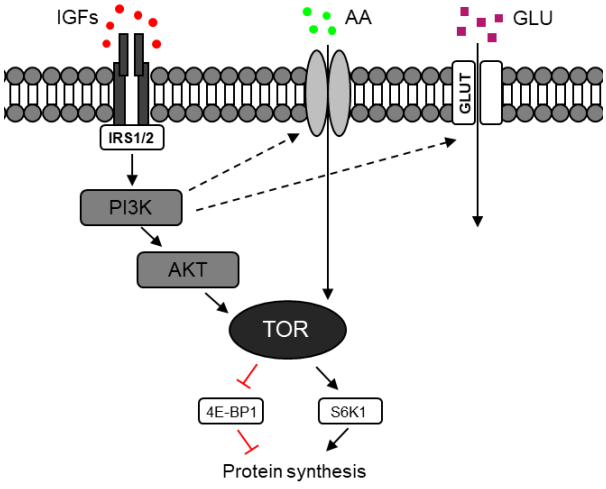



**Figure 15.** Schematic representation of TOR and PI3K/AKT signaling pathways. Amino acids (AA) uptake stimulate protein synthesis by the activation of TOR phosphorylation and the regulation of its downstream effectors 4EBP1 and S6K1. On the other hand, the binding of insulin or IGFs to their receptors regulates PI3K phosphorylation to control, among others, substrates uptake (e.g., glucose through GLUT transporters) and the phosphorylation of AKT and TOR. Adapted from Vélez et al. (2017).

TOR appears in two different multi-protein complexes: TOR complex 1 (TORC1 or Raptor), which is both nutrient and rapamycin-sensitive, and TOR complex 2 (TORC2 or

Rictor), that on the contrary is insensitive to nutrients and rapamycin [Reviewed by González and Rallis (2017)].

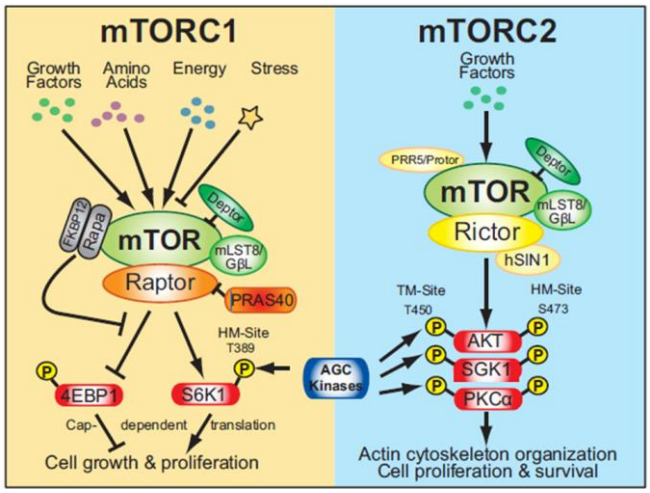



**Figure 16.** Schematic representation of the two different multi-protein complexes of TOR. Extracted from Foster and Fingar (2010).

TORC1 is involved in the regulation of protein synthesis and cell proliferation through the activation of 4EBP1 and 70S6K (Figure 16) in both, fish and mammals (González and Rallis, 2017; Seiliez et al., 2008), whereas TORC2 is more related to the regulation of glucose metabolism, cytoskeleton organization and cell survival through the activation of AKT (Foster and Fingar, 2010; González and Rallis, 2017). In fact, when AKT is phosphorylated in the residue T308 of the T-loop by the phosphoinositide-dependent kinase-1 (PDK1), its activity is low, but once TORC2 phosphorylates the residue S473, the T308 phosphorylation is stabilized and AKT reaches its maximum activity (Foster and Fingar, 2010; Kim and Guan, 2011; Manning and Toker, 2017; Vadlakonda et al., 2013).

On the other hand, AKT, which is commonly activated by the PI3K as a consequence of growth factors binding to their receptors (Figure 15), regulates cell metabolism, growth and survival, mainly through the inhibition of the forkhead family of transcription factors

(FOXO) activity (Manning and Toker, 2017; Murton et al., 2008; Vadlakonda et al., 2013). The mechanism used for AKT to reduce FOXO activity is causing the translocation of FOXO proteins out of the nucleus, and consequently, once FOXO proteins are sequestered, the expression of FOXO-induced genes is attenuated (Figure 17). Moreover, AKT has the ability to phosphorylate three conserved residues of the *foxo* promoter that cause a reduction in *foxo* transcription (Manning and Toker, 2017).

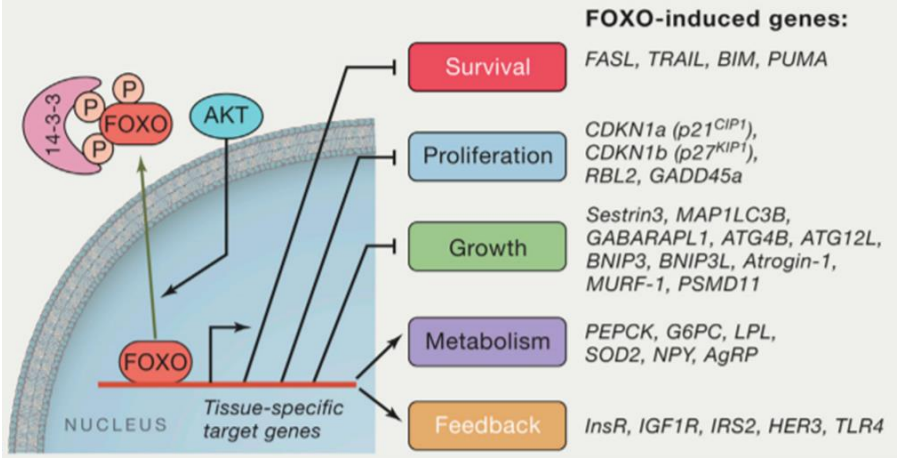



**Figure 17.** Schematic representation of FOXO phosphorylation mediated by AKT, which induces the translocation and cytosolic sequestration of FOXO proteins, and therefore, decreases the expression of the FOXO-induced genes (some of them indicated in the figure). Extracted from Manning and Toker (2017).

The bibliography in fish about these signaling pathways is limited, although some authors have demonstrated in rainbow trout the importance of nutritional factors on TOR activation at both *in vivo* and *in vitro* levels (Seiliez et al., 2008). Also, the effects of IGFs increasing *in vitro* protein synthesis, and decreasing its degradation through activating the AKT pathway has been reported (Cleveland and Weber, 2010). In the case of gilthead sea bream, as far as we know, information is not available on this matter, therefore the primary culture of myocytes represents an interesting *in vitro* model for the study of these signaling pathways in this species.

- **Protein degradation: proteolytic systems**

The proteolytic systems in vertebrates, due to their role in protein degradation, take part in many processes including protein turnover, such as AA recycling or catabolism, but also in other developmental processes as is the case of myogenesis. In vertebrates, the foremost proteolytic systems in skeletal muscle include the ubiquitin-proteasome (UbP) system, the lysosomal cathepsins that are associated with autophagy, the calpain system, and the caspases (Bell et al., 2016; Murton et al., 2008). While the first three systems are important for skeletal muscle development, the caspases are mostly linked to cellular apoptosis (Chowdhury et al., 2008), therefore are not an object of study in the present thesis.

  - **Ubiquitin-proteasome system (UbP)**

The UbP system in mammals is responsible for degrading the majority of cellular proteins (Rock et al., 1994), whereas it has been demonstrated in rainbow trout myocytes that the UbP system is only responsible for 17% of the total protein degradation in fish (Seiliez et al., 2014). In this proteolytic system, the proteins meant for destruction are several times tagged for ubiquitination (forming chains of ubiquitin) by a process involving 3 enzymes (Figure 18), and then they are recognized by the 26S proteasome complex to be degraded [Reviewed by Bell et al. (2016)]. The 26S complex consists of three parts; a 20S core and two 19S regulatory subunits. The 19S subunits recognize the ubiquitin-tagged proteins to initiate the degradation into oligopeptides with the use of ATP (Bell et al., 2016; Voges et al., 1999). In fish, both the level of polyubiquitinated proteins, as well as the expression of the β type proteasome subunit N3 (a.k.a. PSMB4) have been reported as good markers of

UbP activity (Cleveland et al., 2012; Martin et al., 2002; Salmerón et al., 2015; Seiliez et al., 2014).

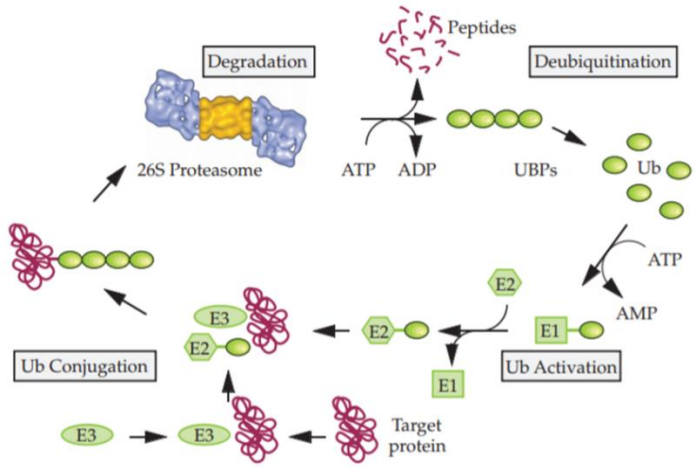



**Figure 18.** UbP system schematic representation. Target proteins are polyubiquitinated by a series of 3 enzymes that regulate Ub activation and conjugation, and then degraded into short peptides by the 26S proteasome complex. After that, the Ub-chain is released and disassembled (deubiquitination). Extracted from Voges et al. (1999).

Within the members that conform the UbP system, the muscle specific F-box protein (Atrogin1, a.k.a. MAFbx) and the muscle RING-finger protein 1 (MuRF1) are two E3 ubiquitin ligases that are expressed specifically in skeletal, cardiac and smooth muscle, where they perform different functions [Reviewed by Bodine and Baehr, (2014)]. They are both up-regulated during muscle atrophy or wasting, and its expression increases with aging in mammals (Bell et al., 2016; Bodine et al., 2001; Cai et al., 2004). Moreover, as reviewed by Murton et al. (2008), the expression of both *mafbx* and *murf1* genes, among other ways, is up-regulated by FOXO proteins. Therefore, anabolic stimuli, in addition to activating protein synthesis through AKT/TOR signaling pathways, inhibit protein degradation by the UbP system due to the cytosolic sequestration of FOXO proteins (Figure 17). However, the action of the UbP system is important for muscle growth and integrity,

especially during exercise, as resistance exercise appears to increase both protein synthesis and degradation (Bell et al., 2016). In fact, after a resistance exercise the expression of *murf1* and *mafbx* increases (Cunha et al., 2012; Murton et al., 2008), and it has been hypothesized that this increase in protein breakdown serves to restructure the myofilaments and prevent exercise-induced muscle damage (Bell et al., 2016).

Besides the proteolytic functions of the UbP system *per se* and its importance in the recovery after exercise, it has been also observed that inhibition of the proteasome induces a decrease in the differentiation and fusion of myoblasts. Due to this inhibition, some important proteins for the myogenic program cannot be degraded preventing the action of some MRFs (e.g., MyoD, Myf5 or myogenin), and therefore the normal progression of the myogenic process (Figure 19). Moreover, the UbP system seems to be a key factor for the maintenance of muscle satellite cells for its ability of Pax3/7 removal [Reviewed by Bell et al. (2016)].

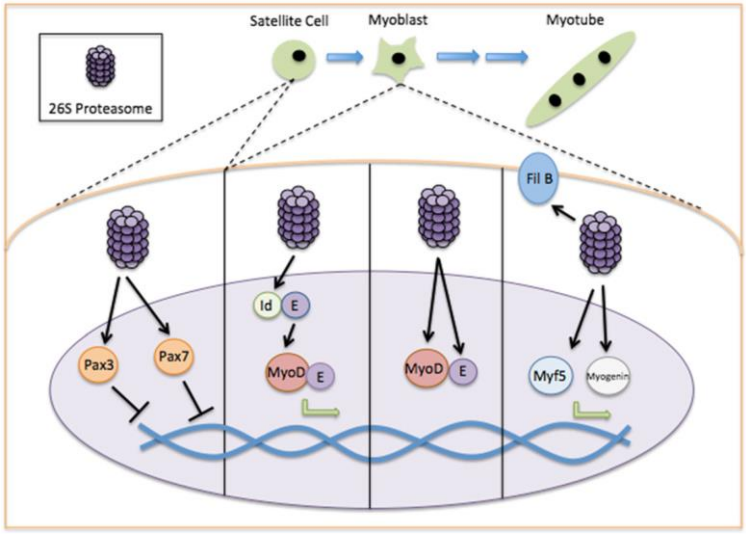



**Figure 19.** Role of the UbP system in myogenesis. The 26S proteasome can degrade Pax3/7 proteins to alter the state of muscle satellite cells. Moreover, the UbP system has also the ability to eliminate the inhibitor of MyoD (Id, inhibitor of DNA binding) to activate MyoD by the union with the E-protein, but then, the progression of the myogenic program depends on the degradation of MyoD itself, as well as of Myf5, myogenin and filamin B at the end of the differentiation phase. Extracted from Bell et al. (2016).

- **Lysosomal cathepsins**

The lysosomes are cytoplasmic organelles with a single membrane and acidic pH (3.8-5) in which more than 50 acid hydrolases are present [Reviewed by Stoka et al. (2016)]. Initially, lysosomes were considered as a non-selective system of protein degradation, but later it was discovered that this system is capable of removal of intracellular components by a highly selective manner through the autophagy-lysosomal system, ALS (Cuervo, 2004). Indeed, nowadays is considered that the lysosomal protein degradation is involved in important processes for cellular homeostasis and differentiation, since the selective degradation allows to regulate the activity and structure of some proteins, as well as different cellular functions, compared to the non-selective degradation that is more important for the general protein turnover (Stoka et al., 2016). In this sense, autophagy consists in the degradation of cytoplasmic material (e.g., damaged mitochondria) or exogenous substrates (e.g., viruses, bacteria) in the lysosomes, generally for later recycling of their components (Figure 20).

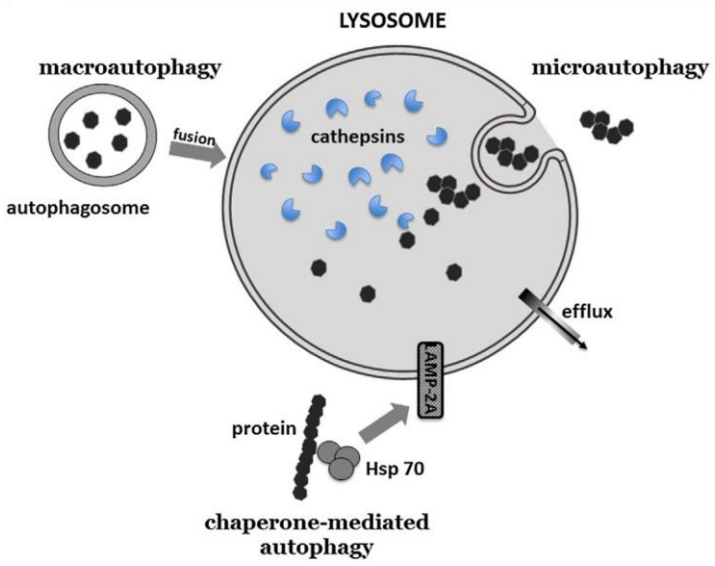



**Figure 20.** Role of lysosomal cathepsins in the final degradation stages of the three main autophagy pathways, the chaperone-mediated autophagy, the macroautophagy, and the microautophagy. Adapted from Stoka et al. (2016).

The autophagic process includes: 1) autophagy mediated by chaperones, where proteins are targeted for degradation and then translocated into the lysosome; 2) macroautophagy, which implies the formation of intermediate vesicles, the autophagosomes, containing portions of cytosol, proteins, or even complete organelles, and their posterior fusion with the lysosome membrane; and 3) microautophagy, that degrades cytosolic proteins without the necessity of autophagosomes (Cuervo, 2004; Galluzzi et al., 2017; Kaminskyy and Zhivotovsky, 2012; Stoka et al., 2016).

Within the lysosomal proteases the cathepsins are the most important ones; they are involved not only in autophagy, but also in different biological processes such as the immune response, the processing and activation of different proteins and hormones, aging, bone remodeling or angiogenesis, among others [Reviewed by Stoka et al. (2016) and Turk et al. (2012)]. As reviewed by some authors, the cathepsins family of proteases contains different classes; the cysteine proteases [cathepsins B, C, L, F, H, K, O, S, V, X, W (papain family), and the asparaginyl endopeptidase], the aspartic proteases [cathepsins D and E], and the serine proteases [cathepsins A and G].

Regarding the importance of this proteolytic system in the muscle, different studies have indicated that excessive autophagy leads to a weakening situation, although an equilibrated and basal autophagy activity is necessary for the maintenance of the muscle mass and the prevention of atrophy [Reviewed by Bell et al. (2016)]. Moreover, autophagy appears necessary to scape or delay sarcopenia by preventing the senescence of the muscle satellite cells and maintain their regeneration capacity (García-Prat et al., 2016). In addition, autophagy is involved in exercise-induced muscle growth since it supposes a way to put-out the damaged cellular components, at the same time that it can provide an alternative energy source for the muscle cells [Reviewed by Bell et al. (2016)]. For all this, and

considering that the ALS system is responsible for up to 50% of total protein degradation in rainbow trout myocytes, while in mammals appears to be proportionally less important (Seiliez et al., 2014); the fish, therefore, represent an interesting model for the study of lysosomal cathepsins.

- **Calpains**

The calpains belong to a family of $Ca^{2+}$-dependent non-lysosomal cysteine proteases that participate in a variety of cellular processes that do not correspond with protein degradation *per se* (Figure 21). For example, this is the case of different signaling pathways, proteolytic regulation of the activity/function of a substrate, apoptosis, the remodeling of the cytoskeleton/membrane attachments, or autophagy control, among others (Baudry and Bi, 2016; Goll et al., 2003; Kaminskyy and Zhivotovsky, 2012).

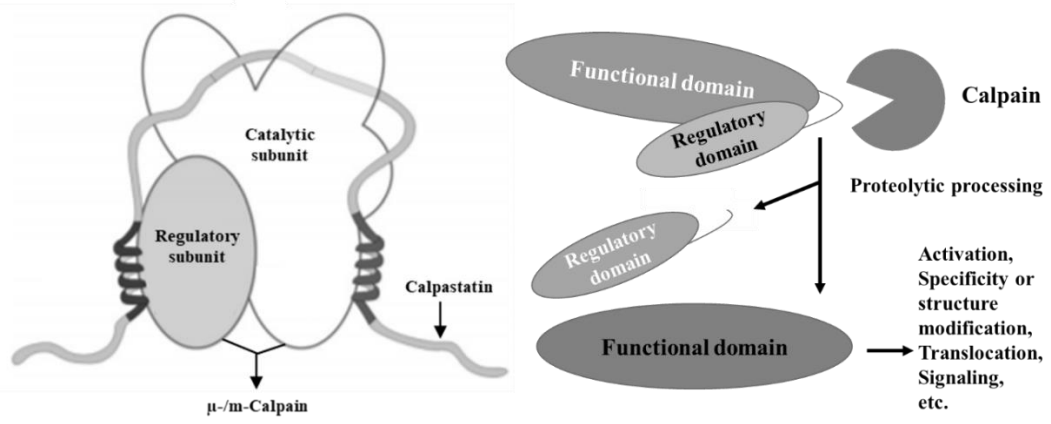



**Figure 21.** Left, schematic representation of the classical calpain structure and the interaction with the specific inhibitor calpastatin. Adapted from Kiss et al. (2008). Right, example of the proteolytic processing mediated by calpains and different possible effects.

The conventional calpain system is composed in mammals of a catalytic/large subunit, a regulatory/small subunit, and a third component, the calpastatin, which is a specific

inhibitor of calpain activity (Goll et al., 2003). In this sense, the activity of calpains depends on the association as heterodimers of catalytic and regulatory subunits; and according to the $Ca^{2+}$ requirements of the catalytic one, they are identified as micromolar (μ-) or millimolar (m-) calpains (Goll et al., 2003). Meanwhile, the small subunit is common to all calpains and it has been suggested that acts as a chaperone for the catalytic subunits, facilitating their correct folding and stability (Ono and Sorimachi, 2012).

In mammals at least 15 calpain genes have been identified (Sorimachi and Ono, 2012). In addition to the μ-/m- criterion according to the $Ca^{2+}$ requirements, calpains are divided into classical or typical calpains (calpains 1, 2, 3, 8, 9, 11-14), which exhibit an "EF-hand" type of calcium-binding domain, and into non-classical or atypical calpains (calpains 5, 6, 7, 10, 15, and 16), in which this domain is lacking [Reviewed by Baundry and Bi (2016), Ono and Sorimachi (2012), and Sorimachi and Ono (2012)], plus calpain 4 that corresponds to the regulatory/small subunit (Goll et al., 2003). Also, calpains are usually grouped depending on the tissue distribution in ubiquitous calpains (calpains 1, 2, 5, 7, 10, 13-16), or tissue-specific calpains (calpains 3, 6, 8, 9, 11, and 12), expressed only in determined tissues as is the case of calpain 3, specific of skeletal muscle (Ono and Sorimachi, 2012). In this sense, depending on the catalytic member that forms each heterodimer, the calpains exert several biological functions during skeletal muscle development (Goll et al., 2003). For example, calpain 1 may be involved in the regulation of myogenesis by its action on myogenin, ezrin, vimentin and caveolin 3 (Moyen et al., 2004); while calpain 2 participates in the fusion of myoblasts to multinucleated myotubes (Goll et al., 2003; Honda et al., 2008).

In fish, although some members of the calpain system have been characterized in different species such as gilthead sea bream, information about their functions and effects on muscle

development remains quite scarce (Vélez et al., 2017). In general, fish presents a high muscle-somatic index in which protein is the largest component (approximately 20%), and protein is the most important component in the fish diet. In addition to this, fish naturally suffer important periods of fasting in which a fine tuned control of protein turnover is essential. Moreover, most fish have the ability to increase muscle mass throughout their life by hyperplasia (i.e. myogenesis), and the activity of proteolytic systems can modify the muscle properties and affect, in the last term, the quality of the flesh. For all this, it is clear that the proteolytic systems are even more important in fish than in mammals, regulating both muscle growth and value, and thus the understanding in fish of the role of these different systems is very important. The primary culture of muscle cells supposes, again, an interesting model system to deepen into the study of these topics in fish.

### 1.3.3 Muscle quality

Considering that muscle represents up to 70% of total body mass in fish, this is the most valuable part of the farmed fish, and consequently the properties of the muscle are essential and critical for the final quality of the aquaculture product. In general, although the quality depends on many factors, the most critical parameters for the consumption and acceptability of a fish product are the color, taste, and flesh texture [Reviewed by Johnston (1999)]. In fact, the texture of the flesh seems to be the factor that may allow the consumer to differentiate in some species an aquaculture product from a wild one, rather than for the taste or the color (Fuentes et al., 2010; Periago et al., 2005). In contrast to mammalian farmed animals in which meat tenderness is desired [Reviewed by Ertbjerg and Puolanne (2017)], in fish products fillet firmness is the quality attribute (Chéret et al., 2007; Listrat et al., 2016). In this sense, different authors have postulated that muscle cellularity is an

important determinant of flesh texture, as a negative correlation between the cross-sectional diameter of the muscle fibers and the texture of the fillet has been found (Ayala et al., 2010; Johnston, 1999; Periago et al., 2005). Accordingly, while hypertrophic muscle development would be a negative trait for flesh texture, hyperplastic growth will determine an improvement of the fish fillet quality (Figure 22) (Listrat et al., 2016).

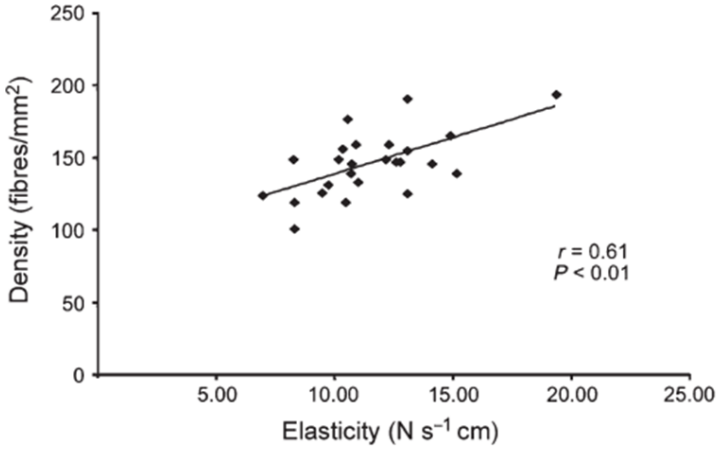



**Figure 22.** Pearson correlation between fiber density and muscle elasticity in gilthead sea bream. Extracted from García de la serrana et al. (2013).

Moreover, it is known that the texture of the fish fillet is influenced also by the contractile properties and the metabolic characteristics of the muscle (i.e., the proportion between the different types of muscle fibers and the glycogen content). These properties in turn can be altered by diet composition, feeding regime, or environmental factors (Johnston, 1999; Periago et al., 2005; Silva et al., 2012). In addition, muscle texture is also determined by the proteins present in the extracellular matrix such as collagen, as well as by the number of cross-links between these proteins (Ando et al., 1992; Li et al., 2016; Periago et al., 2005; Silva et al., 2012). For example, the enzyme lysyl oxidase is essential for the stabilization of collagen fibrils and therefore to control muscle structure (Consuegra and Johnston,

2006), therefore it has been proposed as a good marker of flesh texture (Johnston et al., 2006).

The characteristics of the muscle, besides affecting the texture directly, can alter the proteolysis and *rigor mortis* during the *post mortem* period, therefore modifying as well the quality of the final product (Ayala et al., 2010; Chéret et al., 2007; Johnston, 1999).

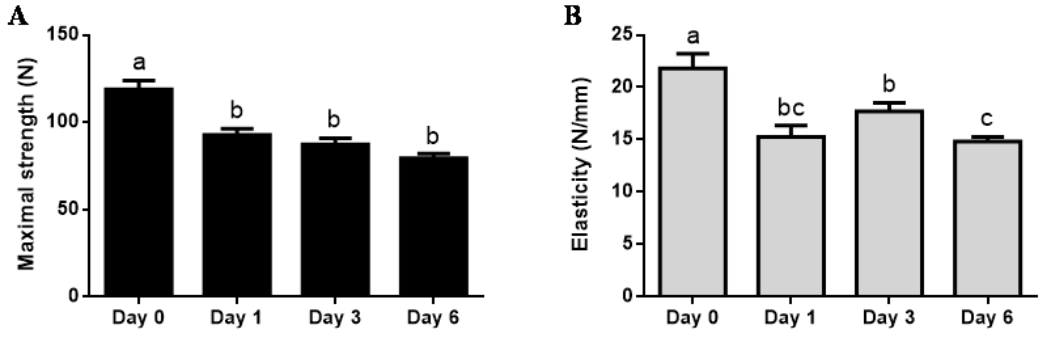



**Figure 23.** Analysis of flesh texture in gilthead sea bream at different days *post-mortem* (0, 1, 3, and 6). Maximal strength (**A**) and elasticity (**B**) values. Values are expressed as mean ± SEM (n=7-8). Different letters indicate significant differences at $p<0.05$. Extracted from Salmerón et al. (2011).

The *post-mortem* period begins very early after death and consists in the combination of chemical, biochemical, and physical processes, followed by a microbiological muscle spoilage. Initially the physio-chemical conditions (pH or osmotic pressure) modulate the action of endogenous proteases for the breakdown of different proteins, connective tissue, and the cytoskeleton, but also for modulating fat hydrolysis. All this causes the detachment of the fibers and then the decrease in the flesh firmness (Figure 23) (Ahmed et al., 2015; Ayala et al., 2010; Chéret et al., 2007; Delbarre-Ladrat et al., 2006; Periago et al., 2005).

Within the proteolytic systems involved in the *post-mortem* degradation of the muscle it is important the role of calpains and cathepsins (Ahmed et al., 2015; Ayala et al., 2010; Chéret et al., 2007; Delbarre-Ladrat et al., 2006; Johnston et al., 2008). In general, calpains are involved in the detachment of the fibers and muscle structure disorganization during the

initial stages of degradation, and cathepsins contribute to the degradation of the muscle components in later stages (Ahmed et al., 2015; Ayala et al., 2010). Delbarre-Ladrat and coauthors (2006) suggested that calpains and cathepsins probably act together in a complementary and synergic way in gilthead sea bream.

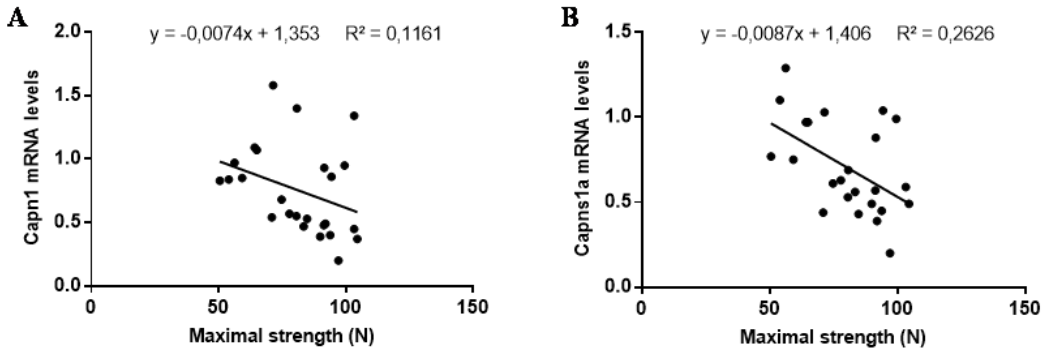



**Figure 24.** Correlation between the gene expression of (**A**) *calpain 1* (*capn1*), or (**B**) *calpain small subunit 1a* (*capns1a*), with flesh texture in gilthead sea bream. A: r = -0.409, P = 0.043; B: r = -0.449, P = 0.028. Extracted from Vélez et al. (2017).

In addition, as commented before, muscle texture could be influenced by diet composition and feeding regime (Figure 24). Salmerón et al. (2013, 2015) found in gilthead sea bream that fasting and refeeding modulate the expression of all three proteolytic systems UbP, calpains and cathepsins. Moreover, the effects of the diet on muscle texture are significantly correlated with the decreased gene expression of both *calpain 1* and the *small subunit 1a*, thus indicating that they are potential markers of flesh quality in this species [Reviewed by Vélez et al. (2017)]. Overall, this highlights the importance of studying the different systems and factors affecting flesh quality in fish in order to optimize aquaculture production.

### 1.3.4 Factors controlling growth

Fish growth is a multifactorial process influenced by nutritional, endocrine, genetic, health or environmental factors (Brett, 1979), but the main coordinator is the endocrine hypothalamic-pituitary axis of the growth hormone (GH) and IGFs system [Reviewed by Fuentes et al. (2013), Mommsen and Moon (2001), and Vélez et al. (2017)]. This axis and the other factors regulating growth of relevance for the present thesis are subsequently described.

- **Endocrine factors**

Hormones play an essential role regulating growth through a systemic or endocrine action that will affect all target tissues. However, hormones present also local (paracrine or autocrine) actions, which in the case of the GH and IGFs can exert different effects in specific tissues such as the liver or the muscle. GH/IGF axis is the most important growth regulator (Figure 25), but other hormones such as thyroid hormones, insulin, steroids or adrenergic agonists interfere in the control of muscle development, growth, and into homeostasis adaptation. Mommsen and Moon (2001) widely reviewed the effects of these groups of molecules in fish, but the present work is only focused on the GH/IGF system for its significant role, and in adrenergic agonists because, although their knowledge in fish is rather limited, they are a promising group of molecules to induce growth to boost aquaculture farming.

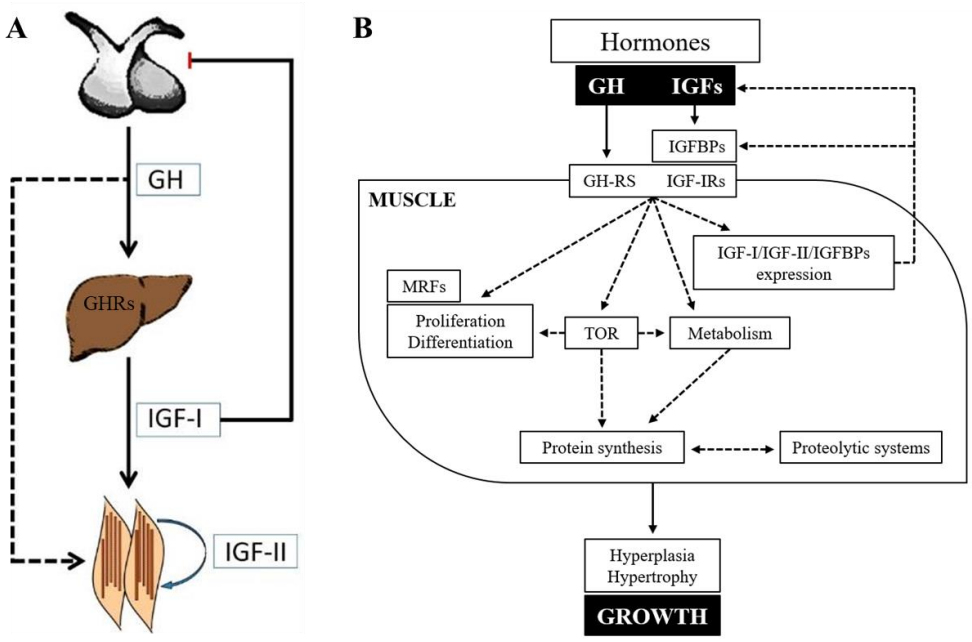



**Figure 25. A**: Proposed model of the general regulation of systemic and local components of the GH/IGF system. Adapted from Benedito-Palos et al. (2007). **B**: Proposed model of GH/IGF axis actions in muscle. Hormones regulate the expression of *igfs*, *igfbps* and *igf-1rs*, which in turn can regulate IGFs actions. Then, IGFs modulate different processes involved in the last term in the control of the hyperplastic and hypertrophic growth of muscle. Extracted from Vélez et al. (2017).

- **Growth hormone (GH)**

GH exerts different effects on growth, such as the regulation of muscle protein synthesis or the determination of growth either by hyperplasia or hypertrophy (Mommsen and Moon, 2001). Moreover, GH also exerts indirect effects on appetite control or intestinal nutrient uptake, and in addition, it is important to consider which of the GH effects are mediated directly by GH, and which ones are due to a GH-indirect way through the hepatic secretion of IGFs (Vélez et al., 2017). All these different effects of GH in fish muscle are listed in Table 1.

| GH direct actions | GH indirect actions |
|---|---|
| In muscle, via endogenous or systemic IGF-I, growth hormone increases: | Neural actions |
| Number of small diameter fibers | Dominance |
| Growth by hyperplasia | Appetite |
| Protein synthesis | Intestinal actions |
| DNA synthesis | Amino acid transport |
| RNA synthesis | Glucose transport |
| RNA/protein ratio | Transport ATPase |
| Amino acid uptake | Intestinal growth |
| Amino acid incorporation into protein | Protein synthesis |
| Lipolysis | Cell volume control |
| Transcription | |
| | Hepatic and adipose tissues |
| Support structures | Lipolysis |
| Proteoglycan synthesis | Fatty acid release |
| Sulfate uptake | IGF-I gene transcription |
| Growth | IGF-I release |
| | Amino acid transport |
| | IGF-I binding proteins |

**Table 1.** Direct and indirect actions of GH controlling muscle growth in fish. Adapted from Mommsen (2001).

Rius-Francino et al. (2011) demonstrated the direct effects of GH stimulating *in vitro* proliferation of muscle cells from gilthead sea bream. Moreover, transgenesis techniques allowed in the last decades to obtain GH-transgenic fish from different species with a significantly higher body weigth compared to age-matched controls (Devlin et al., 2001; Raven et al., 2012). These approaches resulted in valuable models to demonstrate, for example, that GH overexpression produces both muscle hyperplasia and hypertrophy in coho salmon, and also that GH increases the number of myogenic precursor cells and their proliferation rates in Atlantic salmon (Devlin et al., 1994; Levesque et al., 2008; Vélez et al., 2017). However, GH transgenic fish were unable to respond to an exogenous GH treatment (Figure 26), indicating that these fish present certain level of saturation on the stimulatory pathways inducing by GH (Devlin et al., 2001; Raven et al., 2012). Thus, being

potentially this animal model limited for the study of those growth-promoting signaling pathways.

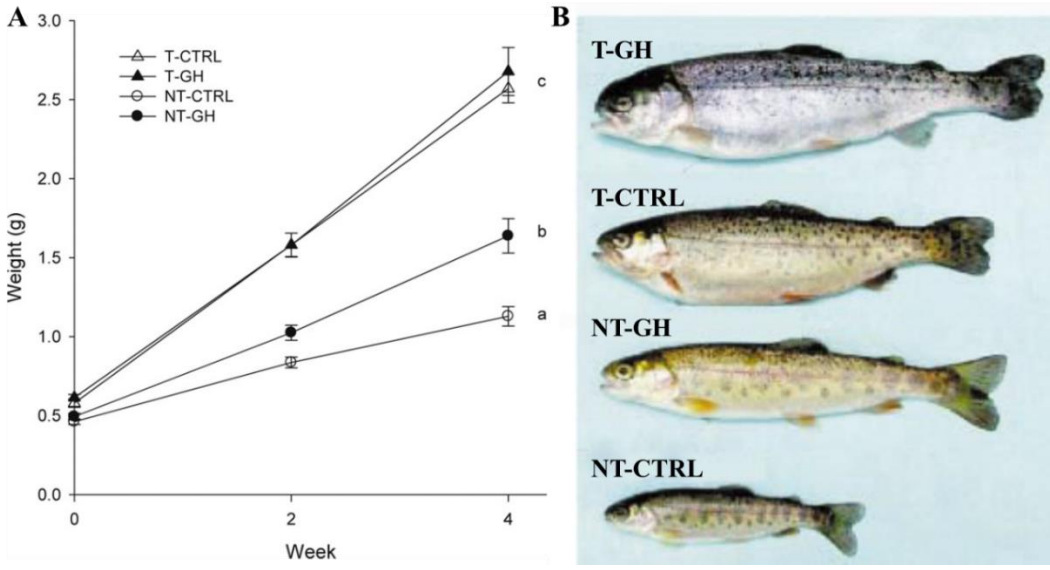



**Figure 26. A**: Mean body weight of non-transgenic (NT) and transgenic (T) coho salmon fingerlings after injection with rBGH at 4 mg/g body weight (GH), or sham-injected (CT). Extracted from Raven et al. (2012). **B**: Phenotype of T and NT rainbow trout in a similar experiment as that in A. Adapted from Devlin et al. (2001).

An alternative to transgenic fish (even cheaper and simpler) has come from terrestrial farmed animals, since a wide background exists on beef cattle treated with a sustained-release formulation of recombinant bovine GH (rBGH, Posilac). rBGH treatment induces in cattle, among other effects, milk production and protein deposition into carcass (Dalke et al., 1992; Dohoo et al., 2003). In fish, although Leedom et al. (2002) demonstrated in tilapia (*Oreochromis niloticus*) that heterologous rBGH has 100-fold less affinity to bind the GH receptors than homologous GH, McLean et al. (1997) found that rBGH remains at detectable levels in plasma up to 140 days after injection in coho salmon. Further, many other authors have reported that the use of rBGH is successful enhancing gowth in different fish species such as rainbow trout (Biga et al., 2004a, 2005, 2004b; Devlin et al., 2001;

Gahr et al., 2008; Garber et al., 1995; Kling et al., 2012), coho and chinook (*Oncorhynchus tshawytscha*) salmon (Figure 26) (McLean et al., 1997; Raven et al., 2012), tilapia (Kajimura et al., 2001; Leedom et al., 2002; Wille et al., 2002), different lines of catfish (*Ictalurus punctatus*) (Peterson et al., 2004, 2005), and more recently sturgeon (*Scaphirhynchus platorhynchus*) (Fenn and Small, 2015). rBGH treatment in these studies was reported to increase body weight, the plasma levels of IGF-I, as well as *igf-1* gene expression in different tissues such as liver or muscle, and also causes a regulation of its receptors. In parallel, this treatment also alters the expression of other genes involved in the regulation of growth and metabolism. Furthermore, the injection of rBGH in zebrafish and giant danio, two representative species of the determinate and indeterminate growth-paradigms, respectively (Figure 6B), demonstrated that growth-related genes are differentially regulated by GH treatment between them, and also suggested that myostatin could be responsible for limiting the GH-induced growth in determinate growers (Biga and Meyer, 2009).

Despite all these studies, as far as we know, information does not exist with regards to the effects of rBGH in gilthead sea bream and so, this could be an interesting strategy to expand the knowledge of growth regulation by GH in this species. Moreover, these studies can contribute to know how far we are from a maximum growth in such an important aquaculture species in order to further improve its production.

- **GH receptors**

In fish, two GH receptors (GHR-I and GHR-II) with complementary functions have been identified (Fuentes et al., 2013). Additionally, truncated variants of GHR-I (tGHR-I) have been also characterized in turbot (*Scopthalamus maximus*) and in two species of flounder flatfish (*Paralichthys olivaceus* and *P. adspersus*), and it has been hypothesized that these molecules can be responsible for the slow growth rate of those species [Reviewed by Fuentes et al. (2013) and Vélez et al. (2017)]. In gilthead sea bream only the two functional isoforms, GHR-I and GHR-II have been identified, and their different response to fasting and refeeding has been determined (Saera-Vila et al., 2005). In fact, it has been alleged that GHR-I is more involved in mediating the anabolic signal of GH, whereas GHR-II appears to be responsible for the energy mobilization that is required in a catabolic or stressful situation (Saera-Vila et al., 2007, 2009). In any case, the transduction pathways activated by the GHRs are well conserved among vertebrates, although apparently, GHR-I and GHR-II do not use the same signaling molecules; since while GHR-I utilizes the JAK2/STAT5 pathway, GHR-II uses the ERK cascade [Reviewed by Fuentes et al. (2013) and Reindl and Sheridan (2012).

- **Insulin-like growth factors (IGFs)**

The two IGFs, IGF-I and IGF-II, have an important role in fish muscle growth. Through *in vivo* approaches, it has been demonstrated that the implantation of mini-osmotic pumps that release IGF-I causes an increased growth in juveniles of coho salmon (McCormick et al., 1992). In fact, IGF-I plasma levels are significantly correlated with growth rates in salmon and it has been proposed that the amount of IGF-I is a good marker of growth (Beckman,

2011; Beckman et al., 1998; Dyer et al., 2004; Kawaguchi et al., 2013). However, although there is little information about circulating IGF-II levels (Gentil et al., 1996; Wilkinson et al., 2004), a positive correlation between IGF-I, IGF-II, and body weight was also observed in this species under a nutritional restriction situation (Wilkinson et al., 2006).

A critical point in the *in vivo* approaches to study IGFs is the difficulty to differentiate between the effects of circulating IGFs from those originated by locally secreted in the liver or the muscle tissue. For all this, the *in vitro* models offer the opportunity to analyze the effects of a specific treatment without systemic influences. In this sense, Jiménez-Amilburu et al. (2013) demonstrated in gilthead sea bream myocytes that GH treatment increases the expression of *igf-1*, and when GH treatment is combined with IGF-I, the expression of *igf-2* was also increased. More recently, it has been shown in the same model that even a treatment with both IGF-I and IGF-II stimulates the gene expression of *igf-1* but not that of *igf-2* (Azizi et al., 2016b). Moreover, in Atlantic salmon myocytes the incubation of starved cells with IGF-I and AA produces an 18-fold increase in *igf-1* gene expression (Bower and Johnston, 2010b), thus indicating a positive feedback mechanism to enhance IGF-I.

Besides the role of IGFs regulating their own expression, some of the growth-inducing effects of the IGFs on fish muscle cells have been characterized. Using BrdU or thymidine uptake techniques it was observed that IGF-I and IGF-II increase cell proliferation in rainbow trout myocytes (Castillo et al., 2004; Codina et al., 2008; Fauconneau and Paboeuf, 2000; Gabillard et al., 2010). In gilthead sea bream myocytes, Rius-Francino et al. (2011) demonstrated by immunocytochemistry against PCNA that both IGF-I and IGF-II also stimulate cell proliferation, but being stronger the effects induced by IGF-II (Figure 27).

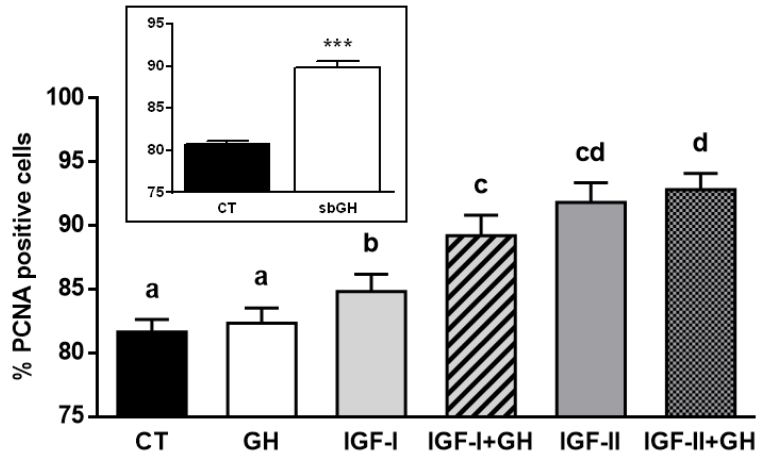



**Figure 27.** Percentage of proliferating cell nuclear antigen (PCNA)-positive cells detected by immunocytochemistry in gilthead sea bream myocytes at day 4. The treatments were performed during 6 h in the absence (control, CT) or presence of mammalian GH 10 nM, IGF-I 100 nM, IGF-II 100 nM or combined treatments of GH and IGFs. The inset figure shows the effects of sea bream GH (sbGH) in similar conditions. Different letters denote significant differences ($P < 0.05$), and *** differences with CT group ($P < 0.001$). Adapted from Rius-Francino et al. (2011).

In addition to the clear effects of IGFs on cell proliferation, their involvement in fish myogenesis has been demonstrated by different authors [Reviewed by Vélez et al. (2017)]. In Atlantic salmon myocytes it was reported that IGF-I and IGF-II, when combined with AA, stimulate the gene expression of *myod1c* (Bower and Johnston, 2010a). Moreover, both *myod2* and *myf5* increased their expression in gilthead sea bream myocytes after incubation with GH and IGF-II, whereas *myogenin* and *mrf4* were up-regulated after treatment with only IGF-I (Jiménez-Amilburu et al., 2013). Similarly, de Mello et al. (2015) showed that IGF-I increases *myogenin* expression in rainbow trout myocytes. These results suggest that both IGFs could present different functions along muscle development. In this line, taking into account the existence of three splice variants of IGF-I in gilthead sea bream (Tiago et al., 2008), Jiménez-Amilburu et al. (2013) analyzed their expression during the

*in vitro* myogenic process in this species in order to study if differences exist among them (Figure 28).

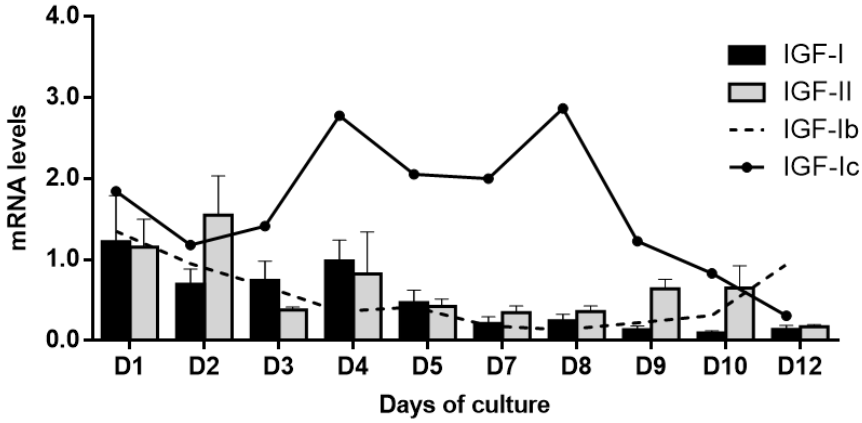



**Figure 28.** Relative gene expression of total *igf-1*, *igf-2* and the splice variants *igf-1b* and *igf-1c* throughout the gilthead sea bream myocyte culture development. Adapted from Jiménez-Amilburu et al. (2013).

During the process of myogenesis in this cellular model, the expression of *igf-2* is highest at the beginning of the culture, to decrease later when the cells start to differentiate (Jiménez-Amilburu et al., 2013). Regarding IGF-I, the expression pattern of total *igf-1* and its splice variant *igf-1b* follow a similar tendency to that of *igf-2*, whereas *igf-1c* presents a special variable profile during culture progression and *igf-1a* was undetectable (Figure 28). The expression profiles of each one of this factors can reinforce their specific roles during fish myogenesis.

On other hand, when IGF-I receptors (IGF-IRs) are considered, it is clear that whereas in homeotherms the insulin receptors are more abundant than IGF-I receptors, in fish (poikilotherms) IGF-I receptors are especially more abundant that the insulin ones in both white and red muscles, as well as in cultured myocytes and in the heart (Baños et al., 1997; Castillo et al., 2004, 2002; Moon et al., 1996; Párrizas et al., 1995). Moreover, binding characteristics and signal transduction pathways of IGF-I receptors have been also

examined in fish showing only minor differences to those in mammals (Castillo et al., 2006; Méndez et al., 2001; Montserrat et al., 2007b; Párrizas et al., 1995). Regarding IGF-II receptors there is very little information in fish, but Méndez et al. (2001) reported their presence, although not very abundant, in membranes preparation of brown trout (*Salmo trutta fario*) embryos. However, it is known that IGF-II performs its biological functions through the binding with IGF-IRs. With regards to those receptors, two copies (IGF-IRa and IGF-IRb) are usually identified in fish species [Reviewed by Fuentes et al. (2013)]. In rainbow trout muscle, *igf-1ra* remained stable in fasting situations but increased in refeeding, whereas the *igf-1rb* expression was regulated in an inverse manner (Montserrat et al., 2007a). In gilthead sea bream, it has been recently demonstrated that IGFs reduce the gene expression of *igf-1ra*, whereas *igf-1rb* regulation appears to be more complex (i.e., IGF-I treatment reduce its expression, but incubation with IGF-II increases the *igf-1rb* mRNA levels) (Azizi et al., 2016b). Overall, it seems that there are functional distribution and regulation differences between both IGF-IRs isoforms, thus more investigation in fish is necessary.

Nonetheless, the largest number of IGF-I receptors (IGF-IRs) give rise to postulate that IGFs in fish perform some other functions (i.e., metabolic functions) that would correspond in mammals to insulin, and such a theory was confirmed with different studies. For example, Castillo et al. (2004) demonstrated that IGF-I increases glucose uptake in rainbow trout myocytes at a higher level than insulin, as well as it occurs in the same model with IGF-II (Codina et al., 2008), or in the studies of Montserrat et al. (2012, 2007b) in myocytes extracted from gilthead sea bream (Figure 29A). Moreover, IGF-I treatment also increases the expression of the glucose transporter 4 (Glut4) in both, rainbow trout myocytes and gilthead sea bream small myotubes (Díaz et al., 2009; Montserrat et al., 2012). In these

works, especially in the case of gilthead sea bream, IGF-I exerts a greater effect than insulin on the expression of Glut4.

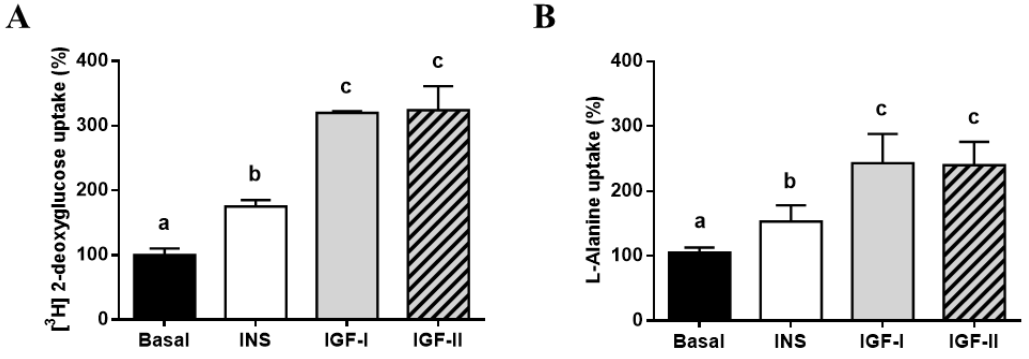



**Figure 29.** Endocrine regulation of nutrients uptake on day 4 myocytes of gilthead sea bream. Effects of insulin (INS) 1 µM, IGF-I 100 nM or IGF-II 100 nM on 2-deoxyglucose (**A**) and L-alanine (**B**) uptake. Treatments were performed for 1 h for (**A**) and 2 h for (**B**). Data are normalized to the basal levels (mean ± SEM). Different letters indicate significant differences ($P < 0.05$). Adapted from Montserrat et al. (2012).

In addition, IGFs also stimulate alanine uptake in myocytes of these two species (Castillo et al., 2004; Codina et al., 2008; Montserrat et al., 2012), being the effects of IGFs again stronger than those of insulin (Figure 29B). Further, other authors found in rainbow trout myocytes that IGF-I increases the uptake of free fatty acids more than insulin (Sánchez-Gurmaches et al., 2010).

These anabolic effects of IGFs in muscle cells, together with their actions stimulating cell proliferation and differentiation, and also regulating myogenesis, point out the important role of IGFs in the control of metabolism and muscle growth in fish. However, to better understand the actions of IGFs it is necessary to consider also the role of the IGF-I binding proteins (IGFBPs). IGFBPs represent another level of regulation of IGFs; they can promote or limit growth. In addition to the control of *igf-1rs* expression to enhance or reduce the binding capacity of IGFs and their downstream signaling, IGFBPs suppose a fine control

of IGFs actions. Most of the information about IGFBPs in fish is based in zebrafish, salmon species or cell-line culture models [Reviewed by Duan et al. (2010), Fuentes et al. (2013), Shimizu and Dickhoff (2017) and Vélez et al. (2017)]. Similar to those in mammals, at least six different IGFBPs have been described in fish, although they can have paralogs with specific functions (Johnston et al., 2011; Shimizu and Dickhoff, 2007). For example, IGFBP-1 is considered a negative growth regulator that is induced under catabolic conditions (e.g., stress or fasting) (Amaral and Johnston, 2011; Duan et al., 2010; Shimizu and Dickhoff, 2017). A similar inhibitory role was assumed for IGFBP-2 (Gabillard et al., 2006; Safian et al., 2012), although now there is evidence that in Chinook salmon the variant IGFBP-2b could exert stimulating effects such as those corresponding to IGFBP-3 in mammals (Shimizu and Dickhoff, 2017). In the case of IGFBP-6, a similar expression profile to that of *igfbp-2* was found during fasting and refeeding experiments in rainbow trout and fine flounder (*Paralichthys adspersus*), suggesting inhibitory actions of IGFBP-6 as well (Gabillard et al., 2006; Safian et al., 2012). In the case of IGFBP-3, although apparently in salmon, circulating IGFBP-3 plays only a small role regulating endocrine IGFs, local actions have been suggested for this binding protein in fine flounder (Safian et al., 2012; Shimizu and Dickhoff, 2017). Hence, IGFBP-3 has been proposed as a growth stimulator in fish. Furthemore, IGFBP-4 and IGFBP-5 seem to play also anabolic functions and can be considered pro-myogenic molecules (Azizi et al., 2016b; Fuentes et al., 2013). In fact, Ren et al. (2008) demonstrated that IGFBP-5 stimulates myogenesis through enhancing the binging between IGF-II and IGF-IRs, thus maximizing the PI3K/AKT signaling pathway and increasing, in turn, the expression of *igf-2* and some MRFs such as *myogenin* (Figure 30). Therefore, IGFBPs can be good species-specific markers to indicate the growth condition in fish.

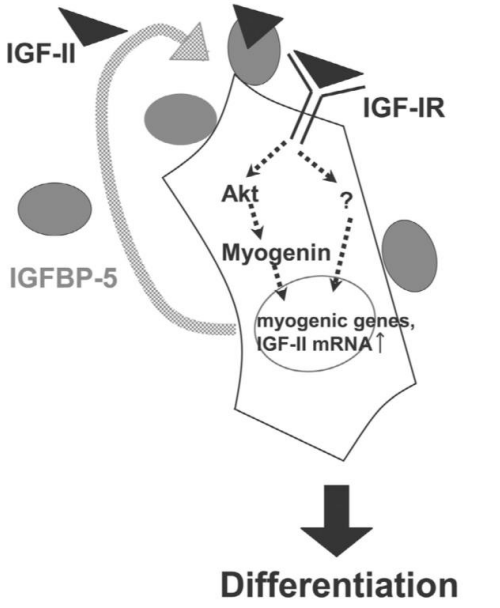



**Figure 30.** Proposed model of IGFBP-5 regulation of IGF-II action. IGFBP-5 binds to IGF-II to bring it closer to IGF-IRs, thereby enhancing the activation of these receptors and corresponding signaling pathways to ultimately induce cell differentiation. Extracted from Ren et al. (2008).

- **Adrenergic agonists**

In vertebrates, it is commonly known that catecholamines (e.g., adrenaline and noradrenaline, NA) make possible a "fight-or-flight" response when a critical situation arises, but these molecules have also other roles such as the regulation of energy metabolism in normal situations (Fabbri and Moon, 2016). Catecholamines can activate different signaling pathways through binging to a wide variety of adrenergic receptors types (ARs, or adrenoceptors). Six α-ARs and at least three β-ARs subtypes included in the family of guanine nucleotide-binding G-protein coupled receptors have been identified in different tissues and distinct proportions [Reviewed by Ahles and Engelhardt (2014), and Lynch and Ryall (2008)]. G proteins appear located in the cellular cytoplasm and are formed by three subunits: the Gα and the dimer Gβγ (Bockaert and Pin, 1999). After union of the agonist with the receptor, the Gα subunit binds with GTP and dissociates from the Gβγ complex. At this point, the Gα subunit has the ability to activate some transduction pathways and different effectors such as the enzyme adenylyl cyclase. This enzyme

produces cAMP that will activate, in turn, the PKA transduction pathway (Lynch and Ryall, 2008; Sato et al, 2011). Then, activated PKA can phosphorylate different proteins to regulate their activity, as well as modulate the expression of some genes involved in muscle development (Chen at al., 2005).

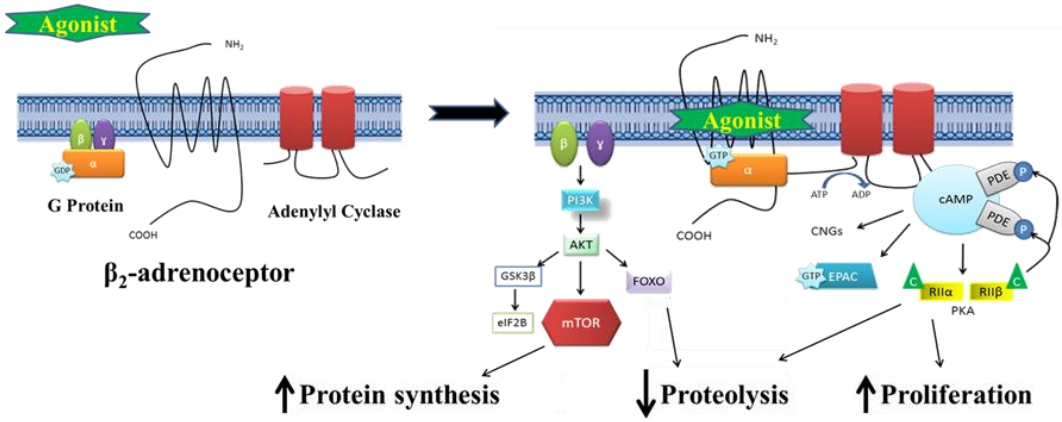



**Figure 31.** $\beta_2$-adrenoceptor structure (left) and signaling pathways activated and functions regulated after binding of the agonists (right). Adapted from Lynch and Ryall (2008).

In skeletal muscle, the $\beta_2$-ARs subtypes represent up to 99% of total ARs abundance (Johnson et al., 2014; Kim et al., 1991; Williams et al., 1984). The $\beta_2$-ARs have special relevance because their Gβγ complex can activate the PI3K/AKT signaling pathway independently of the Gα subunit action (Figure 31) (Yamamoto et al., 2007). As explained before, the PI3K/AKT pathway is involved in different processes such as protein synthesis, or cell proliferation partly by means of regulating gene transcription (Figure 15). Hence, the modulation of the cellular cycle, the enhancement of protein synthesis and the reduction of protein degradation, induced by the activation of both PKA and PI3K/AKT transduction pathways after the activation of $\beta_2$-ARs, is an interesting option to consider, to in the last term, improve muscle growth. Even more, at the same time, $\beta_2$-ARs activation induces the mobilization of energy by increasing the activity of the hormone-sensitive lipase (HSL)

initiating lipolysis (Johnson et al., 2014), an ability that has caused $\beta_2$-agonists to be referred as "repartitioning agonists". Although $\beta_2$-agonists (i.e., the conventional ligands, like NA) have been traditionally used for the treatment of respiratory diseases (e.g., asthma) (Cheung et al., 1992), a new generation of synthetic $\beta_2$-agonists including formoterol (FOR) and salmeterol (SALM) (Figure 32), have been used in a wider array of applications.

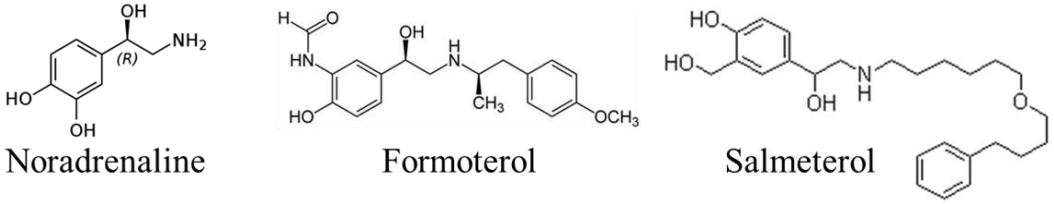



**Figure 32.** Chemical structure of noradrenaline and two synthetic $\beta_2$-agonists of the new generation (e.g., formoterol and salmeterol). The addition of a second benzene ring and the end of the carbon chain characterize these new agonists, being these traits responsible for a longer binding with the ARs. Adapted from Lynch and Ryall (2008).

Within the properties of these new $\beta_2$-agonists it is included that they present a second benzene ring bounded to a long carbon chain that gives them the ability to induce longer activations of $\beta_2$-ARs, therefore allowing reducing the doses to obtain the same effect [Reviewed by Lynch and Ryall (2008), and Waldeck (1996)].

All the properties reported for $\beta_2$-agonists are very interesting for the livestock industry, as they can reduce the costs of production in parallel to improving the quality of the final product (Fiems, 1987; Sillence, 2004). The effectiveness of this strategy to increase growth and reduce fat depots has been demonstrated in pigs (Figure 33), beef cattle, and lamb, among others (Baker et al., 1984; Ricks et al., 1984; Sillence et al., 2004).

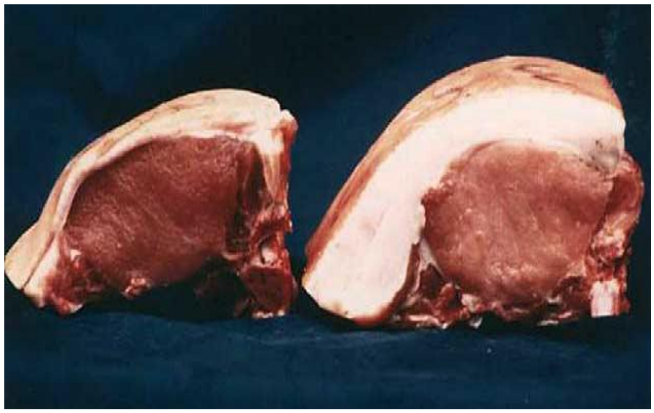


**Figure 33.** Cross-section of pig carcasses showing the longissimus muscle and the typical backfat from a control animal (right) or from an animal treated during a finishing phase of four weeks with the agonist clenbuterol (included in the diet) in combination with daily injections of porcine GH for 3 weeks (left). Extracted from Sillence (2004).

The attractiveness of such approach has also been perceived by the aquaculture sector, and in this sense, both α- and β-ARs have been identified in fish (Aris-Brosou et al., 2009; Fabbri and Moon, 2016; Lortie and Moon, 2003). Moreover, some authors have demonstrated that *in vivo* administration of $\beta_2$-agonists increases also body weight in fish, enhancing protein synthesis while decreasing visceral fat deposition (Mustin and Lovell, 1993; Oliveira et al., 2014; Salem et al., 2006; Satpathy et al., 2001; Vandenberg and Moccia, 1998; Webster et al., 1995). However, data on the effects of $\beta_2$-agonists in cultured fish muscle cells is unavailable, as well as specifically with regards to the muscle sensitivity of these molecules and their effects in gilthead sea bream.

- **Nutritional factors**

In addition to endocrine factors, nutrition is an essential aspect of growth control. It is well-known that each species presents particular nutritional needs, as well as they have different capacities to obtain energy from different ingredients (De Silva and Anderson, 1995). For example, some species are inefficient using carbohydrates and thus, they obtain the energy mainly from proteins, while for others the greatest source of energy are actually, the

carbohydrates. This is due to the nature of each species; carnivorous species such as salmonids are included in the first group, while the herbivorous like the grass carp (*Ctenopharyngodon idella*) are part of the second one. However, others species such as gilthead sea bream are omnivorous, although they prefer proteins as energy source. For this reason, to optimize aquaculture production it is crucial that diets are adjusted to the requirements and characteristics of each specific fish species, and through this, to improve growth and quality. At the same time it is also important to reduce costs and waste generation during the production process. In this sense, although fish feed has been traditionally based on fish meal, this is a limited resource and in the last years a great effort has been made to replace totally or partially this ingredient for plant protein formulations (Gaylord and Barrows, 2009; Gómez-Requeni et al., 2004). Initially, some authors achieved a complete replacement of fish meal by using vegetable protein in rainbow trout (Kaushik et al., 1995; Watanabe et al., 1997), but it was later observed that some of these vegetable sources could contain anti-nutritional factors, besides a correct balance of AA was necessary to avoid physiological problems (Cowey, 1994; Gómez-Requeni et al., 2004; NRC, 2011; Wacyk et al., 2012). Nonetheless, in the last decade it was achieved the milestone of net production of Atlantic salmon by the total replacement of fish ingredients for plant proteins and vegetable oils (Liland et al., 2013; Torstensen et al., 2008), reaching to produce 1 kg of fish product with only 500 to 800 g of wild fish resources.

The AA profile between plant proteins and fish meal is different. Consequently, when the fish meal is substituted by plant protein, the supplementation with some crystalline AA is often required to guarantee the adequate AA equilibrium, and also to cover the necessities of essential and non-essential AA (Azizi et al., 2016a; Gaylord and Barrows, 2009; Gómez-Requeni et al., 2004; NRC, 2011; Wacyk et al., 2012). For example, the supplementation

with lysine or methionine increases growth in rainbow trout (Belghit et al., 2014; Gaylord and Barrows, 2009; Rolland et al., 2015). On the other hand, an excess of leucine seems prejudicial for the growth of this species (Choo et al., 1991).

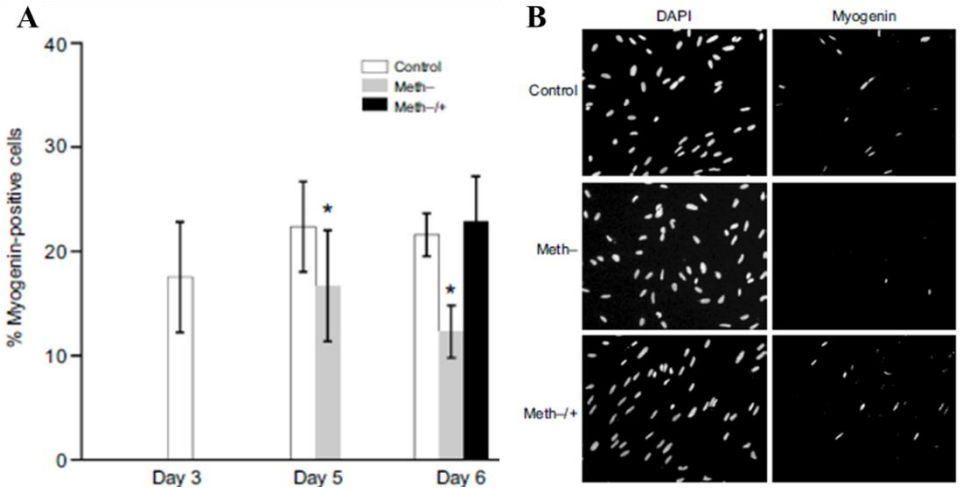



**Figure 34.** Effects of methionine restriction (from day 3 to day 6), or restriction (day 3 to day 5) and then replenishment (day 5 to day 6), in the protein expression of myogenin in rainbow trout myocytes. **A**: percentage of myogenin-positive cells. **B**: representative images of the immunofluorescence analysis of myogenin. Adapted from Latimer et al. (2017).

Notwithstanding, the *in vitro* model of myocytes has facilitated to study in fish the effects of AA in muscle [Reviewed by Vélez et al. (2016)]. In this sense, AA activate TOR signaling pathway stimulating protein synthesis in mammals (Figure 16), and Seiliez et al. (2008) confirmed that this effect also occurs in rainbow trout myocytes. Other authors have found that AA treatment increases the expression of *igfs* in Atlantic salmon myocytes (Bower and Johnston 2010b). Additionally, AA starvation modifies the expression of autophagy-related genes in rainbow trout myocytes (Seiliez et al., 2012a). Besides the effects of AA on protein turnover and in the control of growth factors expression, they also play an important role regulating myogenesis (Figure 34) (Latimer et al., 2017). In this case, methionine restriction blocks cell differentiation by decreasing the expression of

*myod* and *myogenin*, and when methionine levels are recovered, the expression of these genes and the differentiation program is restored.

Despite all this knowledge, in gilthead sea bream there is limited information about the role of AA regulating muscular protein synthesis and proteolytic systems, as well as on the signaling pathways activated by AA and its effects on *in vitro* myogenesis.

- **Others factors: Swimming activity**

In teleost fish, swimming activity (exercise) stimulates growth (Ibarz et al., 2011; Palstra and Planas, 2013); however, the effects of exercise depend on the typical behavior of each species (e.g., pelagic *vs.* benthic) (Davison and Herbert, 2013; Huntingford and Kadri, 2013), but also on the type of exercise. In general, swimming is characterized as aerobic (sustained swimming) when is mainly supported by the slow-twitch red muscle and aerobic metabolism (Davison, 1997), and anaerobic (e.g., predator-prey interactions), when the swimming speed increases and the capacity of red muscle is exceeded. In this last case, the recruitment of intermediate (pink) and finally fast-twitch (white) muscle fibers occurs, and the energy is obtained mainly by anaerobic glycolysis (Johnston et al., 2011). Additionally, the recruitment of distinct types of muscle fibers is also different across the axis length, depending on the speed and the species (Altringham et al., 1993; Ellerby et al., 2001). In the *Sparidae* fish family, it is known that in the red muscle the power is generated mainly in the posterior (caudal) region (Coughlin et al., 1996; Gerry and Ellerby, 2014; Syme et al., 2008), whereas in the white muscle the power comes mainly from the anterior muscle region (Altringham et al., 1993; van Leeuwen et al, 1990) and usually such area presents faster contractile properties for both white and red muscle types (Coughlin et al., 2007). Another difference between species is the capillary density, which in fact directly affects

the swimming performance of the fish (Egginton and Cordiner, 1997). Furthermore, muscle activity generates a biochemical and mechanical environment around blood vessels that induces a vascular remodeling, being angiogenesis an integrated response to optimize the aerobic performance in which the vascular endothelial growth factor (VEGF) plays an important role [Reviewed by Egginton (2009)]. For example in zebrafish, swimming adaptation induces a muscle remodeling that includes increased capillarity and mitochondrial biogenesis (Hasumura and Meguro, 2016; LeMoine et al., 2010; McClelland et al., 2006; Palstra et al., 2014). Similarly, Ibarz et al. (2011) demonstrated in gilthead sea bream juveniles that moderate and sustained swimming produces an adaptative process that differs between anterior and caudal regions of white muscle (Figure 35).

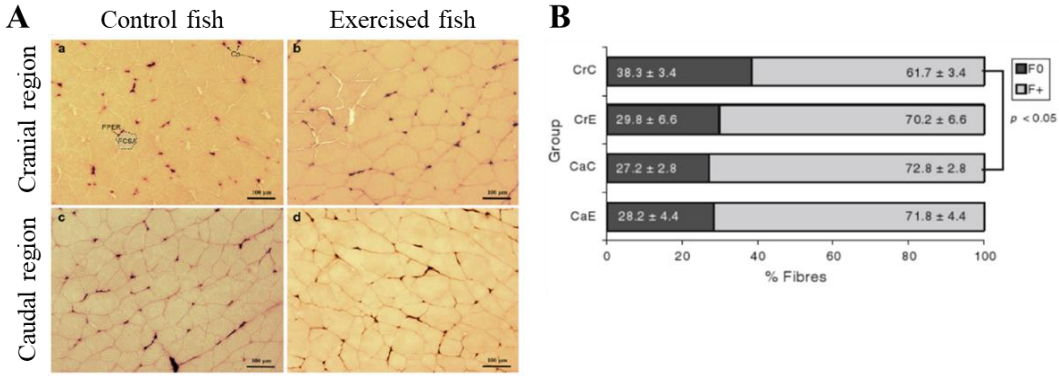



**Figure 35. A**: Representative images of cross-sectional muscle from the cranial (anterior) and caudal region of control and exercised gilthead sea bream juveniles, revealed by an ATPase staining. **B**: Surrounding capillarization of white muscle fibers in CrC (Cranial region, control), CrE (Cranial, Exercise), CaC (Caudal region, control) and CaE (Caudal, exercise). The percentage of fibers with no surrounding capillaries is indicated in black (F0), and the percentage of fibers contacting with at least one capillary appears in grey (F+). The difference between groups is indicated. Adapted from Ibarz et al. (2011).

In that study, Ibarz et al. (2011) also demonstrated that swimming induces capillarization and a hypertrophic condition in the anterior muscle, but not in the caudal one. Whereas in the control group the capillarization is different between the two regions, being higher in the caudal area, exercise stimulates angiogenesis in the anterior region, and thus this

difference disappears (Figure 35B). In any case, creating a current is possible to force fish to swim against it, and by adjusting the protocol (current flux, speed, time of activity, etc.) to the characteristics of each species, it seems that positive growth effects can be achieved. Among these effects, some authors have demonstrated that equilibrated exercise training modifies muscle morphology (increasing fiber size), enzymatic activity and the aerobic capacity (increasing the number of red fibers and tissue capillarization) [Reviewed by Davison (1997) and Palstra and Planas (2011)]. Moreover, exercise improves feed conversion efficiency and specific growth rates (Davison ant Herbert, 2013), consequently, enhancing growth. In addition, swimming has been shown to increase flesh texture in some species such as Atlantic salmon and brown trout (Bugeon et al., 2003; Totland et al., 1987). Therefore, the use of forced swimming to improve muscle growth and quality to in last term, optimize aquaculture production is very promising.

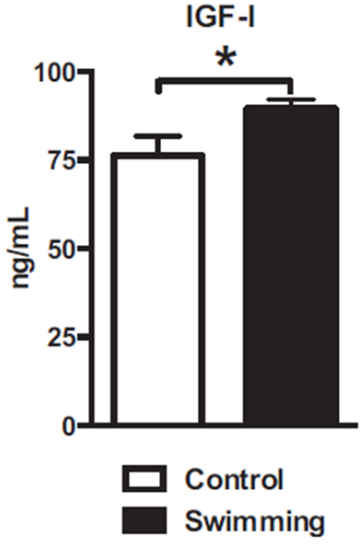



**Figure 36.** Plasmatic levels of IGF-I in control and exercised fish during 21 days. Mean ± SEM. Significant differences between groups are indicated with * at $P < 0.05$. Adapted from Sánchez-Gurmaches et al. (2013).

In gilthead sea bream juveniles, besides increasing hypertrophy and capillarization in the anterior region of the white muscle, sustained swimming also increased growth by improving feed conversion efficiency (Felip et al., 2013; Ibarz et al., 2011; Martín-Pérez et

al., 2012; Sánchez-Gurmaches et al., 2013). Moreover, Sánchez-Gurmaches et al. (2013) found that the observed enhanced growth mediated by the swimming condition occurs through an increase in the plasma levels of IGF-I (Figure 36). Moreover, it has been recently observed in fingerlings of the same species that 5 weeks of sustained and moderate exercise (5 body lengths (BL)/s) enhances growth and increases the plasma levels of IGF-I, with a concomitant decrease in GH (Figure 37) (Blasco et al., 2015); therefore corroborating that the GH/IGF axis plays an important role medating the effects of exercise in gilthead sea bream. Nevertheless, differences in food intake and hepatosomatic index were not found in this work, although a decrease in mesenteric fat index was observed indicating a reduction of fat depots (Blasco et al., 2015).

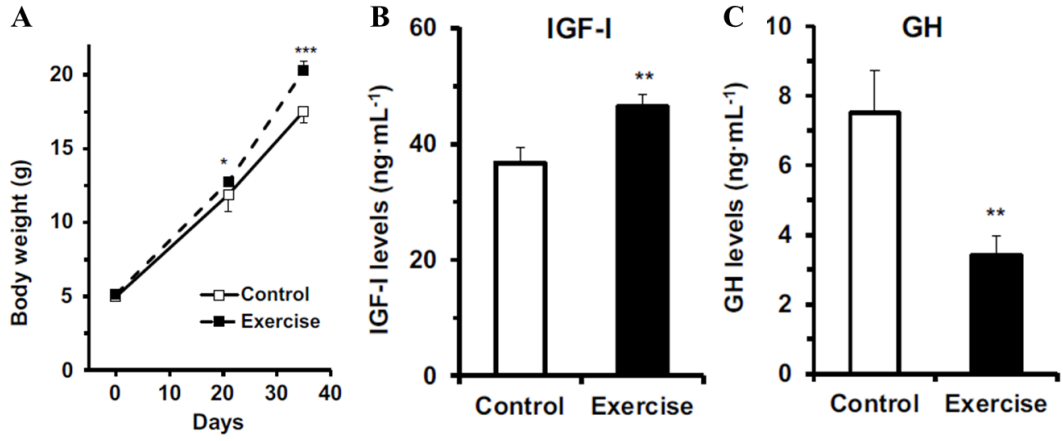



**Figure 37. A**: Body weight of gilthead sea bream fingerlings forced to swim at 5 BL/s, or maintained under control conditions during 5 weeks. **B**: Plasma levels of IGF-I after 5 weeks of exercise. **C**: Plasma levels of GH. Adapted from Blasco et al. (2015).

Despite all this knowledge, information on the local regulation of the GH/IGF axis during exercise is not known (e.g., *igfs, igf-bps, igf-1rs* and *ghrs* expression in muscle and liver tissues). Also, it is completely unknown the role of IGF-I splice variants during exercise in gilthead sea bream. Further, there is little information about the effects of exercise on the

gene and protein expression of the different signaling molecules (e.g., TOR, AKT), myogenic regulators (e.g., MRFs, myostatin, PCNA, of myosin isoforms), proteolytic systems (e.g., calpains, cathepsins, and the UbP system), or the angiogenesis marker VEGF. Therefore, this will be also object of study within the present thesis.

# CHAPTER 2: OBJECTIVES

With the ultimate purpose of optimizing the aquaculture production of gilthead sea bream, the overall goal of the present thesis is to increase the knowledge on the regulation of growth and flesh quality in this species. This work includes both *in vitro* and *in vivo* approaches to achieve the following specific aims:

***In vitro***:

- To investigate the role of amino acids (AA) and growth factors, and the signaling pathways involved, on the regulation of muscle growth and development in gilthead sea bream using cultured myocytes.
- To characterize the main proteolytic systems (cathepsins, calpains and the ubiquitin-proteasome) during myogenesis in gilthead sea bream, and the transcriptional modulation of its members by AA.
- To study in primary cultured gilthead sea bream myocytes the effects of $\beta_2$-agonists on adrenergic receptors activation, myogenesis, protein synthesis and lipolysis.

***In vivo***:

- To analyze the role of GH/IGF axis' molecules mediating the positive growth effects of sustained and moderate swimming in fingerlings of gilhead sea bream.
- To characterize the gene and protein expression of myogenic, angiogenic, proliferation, and proteolytic markers, in the muscular adaptation to exercise of gilthead sea bream fingerlings.
- To analyze in fingerlings the effects of a single injection of rBGH on somatic growth, GH/IGF axis, myogenic- and osteogenic-related molecules expression, and muscle histology.

# CHAPTER 3: SUPERVISORS' REPORT

**Department of Cell Biology, Physiology and Immunology**
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El Dr. Joaquim Gutiérrez Fruitós y el Dr. Miquel Riera-Codina, como codirectores, y la Dra. Encarnación Capilla Campos, como tutora de la tesis doctoral presentada por Emilio J. Vélez titulada "Hormonal and nutritional regulation of muscle growth and development in gilthead sea bream (*Sparus aurata*)", 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.

Emilio J. Vélez ha participado de forma muy activa en la escritura de los artículos en todos sus aspectos, como se refleja en la relación de autores donde consta como primer autor en todos ellos. Emilio ha realizado todos los experimentos, la posterior analítica, la elaboración e interpretación de los resultados y la preparación de los manuscritos; y ha contribuido de modo muy significativo al diseño de los experimentos. El primer artículo lo realizó en estrecha colaboración con Esmail Lutfi (por ello la coautoría), que en aquel período realizaba la tesis en el grupo. No obstante, el ahora Dr. Lutfi no utilizó dicho trabajo en su tesi doctoral, ya que la temática encajaba con los objetivos de esta tesis, y Emilio siempre fue el principal autor del trabajo.

Además de los artículos presentados en esta tesis, como puede observarse en el apartado de Publicaciones adicionales, Emilio ha participado en otros trabajos colaborando tanto con otros doctorandos del grupo como equipos de investigación externos.

**Artículo I: IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes.**

**Autores**: Emilio J. Vélez*, Esmail Lutfi*, Vanesa Jiménez-Amilburu, Miquel Riera-Codina, Encarnación Capilla, Isabel Navarro, and Joaquim Gutiérrez. (*equal contribution).

**Revista**: General and Comparative Endocrinology 205: 296-304 (2014).

**Factor de impacto**: 2.470 JCR 2014 (Q3).

**Estado**: Publicado.

**Artículo II: Proteolytic systems' expression during myogenesis and transcriptional regulation by amino acids in gilthead sea bream cultured muscle cells.**

**Autores**: Emilio J. Vélez, Sheida Azizi, Dorothy Verheyden, Cristina Salmerón, Esmail Lutfi, Albert Sánchez-Moya, Isabel Navarro, Joaquim Gutiérrez, and Encarnación Capilla.

**Revista**: PLoS ONE 12(12): e0187339 (2017).

**Factor de impacto**: 2.806 JCR 2016 (Q1).

**Estado**: Publicado.

**Artículo III: $\beta_2$-adrenoceptor agonists' effects in gilthead sea bream (*Sparus aurata*) cultured muscle cells.**

**Autores**: Emilio J. Vélez, Sara Balbuena-Pecino, Encarnación Capilla, Isabel Navarro, Joaquim Gutiérrez, and Miquel Riera-Codina.

**Revista**: Frontiers in Endocrinology.

**Factor de impacto**: 3.675 JCR 2016 (Q2).

**Estado**: En revisión.

**Artículo IV: Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*).**

**Autores**: Emilio J. Vélez, Sheida Azizi, Antonio Millán-Cubillo, Jaume Fernández-Borràs, Josefina Blasco, Shu Jin Chan, Josep A. Calduch-Giner, Jaume Pérez-Sánchez, Isabel Navarro, Encarnación Capilla, and Joaquim Gutiérrez.

**Revista**: American Journal of Physiology: Reg, Integr, Comp Physiol 310: R313-R322 (2016).

**Factor de impacto**: 2.982 JCR 2016 (Q2).

**Estado**: Publicado.

**Artículo V: Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*).**

**Autores**: Emilio J. Vélez, Sheida Azizi, Esmail Lutfi, Encarnación Capilla, Alberto Moya, Isabel Navarro, Jaume Fernández-Borràs, Josefina Blasco, and Joaquim Gutiérrez.

**Revista**: American Journal of Physiology: Reg, Integr, Comp Physiol. 312: R643-R653 (2017).

**Factor de impacto**: 2.982 JCR 2016 (Q2).

**Estado**: Publicado.

**Artículo VI: Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*).**

**Autores**: Emilio J. Vélez, Miquel Perelló, Sheida Azizi, Alberto Moya, Esmail Lutfi, Jaume Pérez-Sánchez, Josep A. Calduch-Giner, Isabel Navarro, Josefina Blasco, Jaume Fernández-Borràs, Encarnación Capilla, and Joaquim Gutiérrez.

**Revista:** General and Comparative Endocrinology 257: 192-202 (2018).

**Factor de impacto**: 2.585 JCR 2016 (Q3).

**Estado**: Publicado.

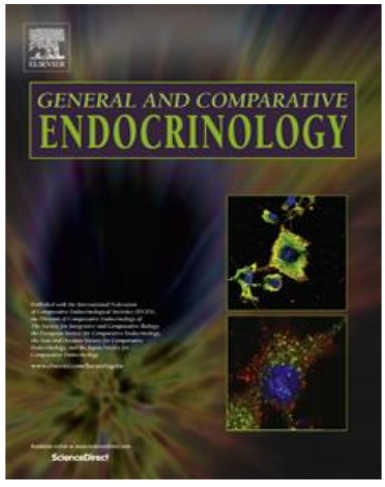
Barcelona, Febrero 2018

Dr. Joaquim Gutiérrez Dr. Miquel Riera-Codina Dra. Encarnación Capilla

# CHAPTER 4: ARTICLES

# ARTICLE I

## IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes




General and Comparative Endocrinology 205 (2014) 296–304

# IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes

Emilio J. Vélez*, Esmail Lutfi*, Vanesa Jiménez-Amilburu, Miquel Riera-Codina, Encarnación Capilla, Isabel Navarro and Joaquim Gutiérrez†

*Equal contribution

Departament de Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, 08028, Barcelona, Spain

**Running title**: IGF-I and amino acids effects in sea bream myogenesis

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**Abstract**

Skeletal muscle growth and development is controlled by nutritional (amino acids, AA) as well as hormonal factors (insulin-like growth factor, IGF-I); however, how its interaction modulates muscle mass in fish is not clearly elucidated. The purpose of this study was to analyze the development of gilthead sea bream cultured myocytes to describe the effects of AA and IGF-I on proliferating cell nuclear antigen (PCNA) and myogenic regulatory factors (MRFs) expression, as well as on the transduction pathways involved in its signaling (TOR/AKT). Our results showed that AA and IGF-I separately increased the number of PCNA-positive cells and, together produced a synergistic effect. Furthermore, AA and IGF-I, combined or separately, increased significantly Myogenin protein expression, whereas MyoD was not affected. These results indicate a role for these factors in myocyte proliferation and differentiation. At the mRNA level, AA significantly enhanced PCNA expression, but no effects were observed on the expression of the MRFs or AKT2 and FOXO3 upon treatment. Nonetheless, we demonstrated for the first time in gilthead sea bream that AA significantly increased the gene expression of TOR and its downstream effectors 4EBP1 and 70S6K, with IGF-I having a supporting role on 4EBP1 up-regulation. Moreover, AA and IGF-I also activated TOR and AKT by phosphorylation, respectively, being this activation decreased by specific inhibitors. In summary, the present study demonstrates the importance of TOR signaling on the stimulatory role of AA and IGF-I in gilthead sea bream myogenesis and contributes to better understand the potential regulation of muscle growth and development in fish.

**Keyword**s: *Sparus aurata*, growth and myogenic factors, 4EBP1, 70S6K, AKT, myocytes

## 1. Introduction

The gilthead sea bream (*Sparus aurata* L.) has become one of the most important species for Mediterranean aquaculture over the last 30 years, overcoming the capture fisheries production (FAO, 2012). However, despite its commercial interest, more research is needed to better understand its growth and development.

The growth pattern in fish differs from other vertebrates, since most fish can grow in length and weight until they die (Johnston et al., 2011; Talbot, 1993). The majority of this growth is due to accretion of muscle tissue, mostly the white skeletal muscle (Johnston, 2006; Mommsen, 2001). Contrary to most vertebrates, this muscle growth implies not only muscle hypertrophy (increase in fiber size), but also hyperplasia (new muscle fibers formation), which is mediated by muscle satellite cells (Stoiber and Sänger, 1996). During skeletal muscle development, precursor cells become myoblasts, which undergo proliferation, cell cycle exit, differentiation and then, fusion to form multinucleated myofibers (Chargé and Rudnicki, 2004; Johnston, 2006).

Myogenesis in fish is regulated by several growth and transcription factors expressed in a sequential manner (García de la serrana et al., 2014; Johnston, 2006). Some of these myogenic regulatory factors (MRFs) are essential for muscle lineage determination and cell proliferation (Myf5 and MyoD) while others contribute to the initiation and maintenance of the differentiation program, which turns myoblasts into myotubes (MRF4 and Myogenin). In addition to MRFs, growth is hormonally regulated mainly by the hypothalamic-pituitary axis through the growth hormone (GH) and the insulin-like growth factors (IGFs) system (Fuentes et al., 2013; Le Roith et al., 2001; Montserrat et al., 2007a; Reindl and Sheridan, 2012; Reinecke et al., 2005; Wood et al., 2005). Interestingly, it has been demonstrated that IGFs (IGF-I and IGF-II) stimulate *in vitro* nutrients uptake and

protein synthesis (Castillo et al., 2004; Codina et al., 2008; Montserrat et al., 2012), as well as myoblast proliferation, which has been observed in gilthead sea bream myocytes using a proliferating cell nuclear antigen (PCNA) immunocytochemical technique (Rius-Francino et al., 2011), and in rainbow trout myocytes by BrdU labeling (Gabillard et al., 2010).

In addition to the endocrine factors, fish growth is a multifactorial process influenced by nutritional, genetic and environmental factors (Garcia de la serrana et al., 2012). Amino acids (AA) are important precursors that stimulate protein synthesis mainly activating the target of rapamycin (TOR) nutrient-sensitive signaling pathway (Kim, 2009; Meijer, 2003). Insulin and growth factors (e.g. IGFs) also activate the TOR signaling pathway through its ability to induce the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (Glass, 2010), resulting in an increase in protein synthesis via indirect activation of TOR (Vander Haar et al., 2007). In vertebrates, it is commonly known that TOR integrates signals from nutrients, energy status and growth factors, and that is an essential regulator of cell growth by controlling cell cycle, gene transcription, cytoskeleton organization and protein synthesis among other functions (Destefano and Jacinto, 2013; Dowling et al., 2010; Edinger and Thompson, 2002; Gough, 2012; van Dam et al., 2011; Yang et al., 2008). TOR is present in two distinct multi-protein complexes: TORC1 (Raptor) that is rapamycin and nutrient-sensitive, and TORC2 (Rictor), which is rapamycin and nutrient-insensitive. TORC1 promotes cellular growth, proliferation and metabolism by stimulating protein synthesis through 4EBP1 and 70S6K either in fish as in mammals (Sarbassov et al., 2005; Seiliez et al., 2008). Moreover, TORC2 controls various metabolic processes and promotes cell proliferation and survival by facilitating the phosphorylation of the active loop of AKT by the phosphoinositide-dependent kinase-1 (PDK1), which is necessary for AKT

activation (Destefano and Jacinto, 2013; Foster and Fingar, 2010; Liao et al., 2008; Manning and Cantley, 2007; Sarbassov et al., 2005). Hence, AKT also regulates cell metabolism, growth and survival by inhibiting the forkhead family of transcription factors, FOXO (Calnan and Brunet, 2008; Héron-Milhavet et al., 2006; Manning and Cantley, 2007; Vadlakonda et al., 2013). Nevertheless, studies exploring the role of AA on fish myogenesis as well as the signaling pathways involved are very scarce. Seiliez and co-workers (2008) showed in rainbow trout *in vivo* and *in vitro* the importance of nutritional factors on the activation of TOR and its downstream effectors. Moreover, a study in Atlantic salmon using cultured myocytes has demonstrated the effects of AA alone or combined with IGF-I up-regulating the expression of different members of the IGF system (Bower and Johnston, 2010b); thus supporting a nutritional stimulatory role on muscle growth.

In this framework, the main aim of the present study was to investigate the role of AA and growth factors in muscle growth and development in gilthead sea bream *in vitro* using a primary cell culture system. Thus, we analyzed the effects of IGF-I and AA on protein expression of PCNA and the myogenic factors, MyoD and Myogenin, as well as the gene expression of PCNA and MRFs (MyoD1, MyoD2, Myf5, Myogenin). Furthermore, we investigated the action of these molecules on the mRNA expression of TOR, AKT2 and its downstream effectors (4EBP1, 70S6K and FOXO3, respectively), as well as the activation by phosphorylation of these signaling pathways.

## 2. Material and methods

### 2.1. Animals

Fish were obtained from a commercial fishery located in the north of Spain and maintained at the facilities of the School of Biology at the University of Barcelona (Spain) in 0.4 $m^3$ tanks with a temperature-controlled seawater recirculation system (21 ± 1 ºC) and 12L:12D photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (Skretting, Burgos, Spain) and fasted for 24 h previously to the isolation of muscle cells. All animal handling procedures were conducted with the Ethics and Animal Care Committee of the University of Barcelona approval, following the EU, Spanish and Catalan Government-established norms and procedures.

### 2.2. Myocyte cell culture

For the different studies a total of twenty independent primary cultures of muscle satellite cells were performed as described by Montserrat et al. (2007b). We used approximately 40 gilthead sea bream (*S. aurata* L.) juveniles from 4 to 24 g body mass per culture. Although the size of the fish influenced the recovery efficiency, since a lower number of cells is obtained per gram of tissue as the fish increases in body size, all the cells extracted were muscle satellite cells and the cultures were very homogeneous. Cells were cultured in 6 well-plates (9.6 $cm^2$/well) for Western blot and quantitative real-time PCR (qPCR) studies and in 12 well-plates containing glass cover slips (2.55 $cm^2$) for immunofluorescence and immunocitochemistry analyses. Cells were counted, diluted and plated to a final density of 0.2-0.25•$10^6$ cells/$cm^2$ for both types of plates. The cultures were maintained at 23 ºC in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.11% NaCl, 10% fetal

bovine serum (FBS) and 1% antibiotic/antimycotic solution (A/A). The development of the cells was first analyzed throughout the culture (days 2, 4, 8 and 12) in order to describe the proliferation and differentiation patterns using immunocytochemical and immunofluorescence assays as described below.

### 2.3. Experimental treatments

For the different studies, myocytes at day 4 were used. This day of culture development was chosen because at this point, the cells retain the ability to proliferate and have also the capacity to start fusing and differentiating (Montserrat et al., 2007b). The cells were held for 12 h with DMEM containing 0.02% FBS and 1% A/A, then starved for 5 h with medium B (without amino acids, AA) containing 10% Earle's Balanced Salt Solution (EBSS, E7510) + 1% MEM vitamins (M6895) + 0.9% NaCl + 0.13% BSA. Subsequently, the cells were maintained in medium B alone (Control) or were treated with human IGF-I at 100 nM and/or an AA cocktail, which contained the concentration of AA of a standard cell culture medium, previously reported by Lansard et al., (2010) (1% MEM Amino Acids Solution (M5550) + 1% MEM Non-essentials Amino Acids Solution (M7145)). The incubations lasted for 3 h in the case of Western blot experiments and for 6 h for the rest of analyses. When using for Western blot studies the specific inhibitors of TOR, rapamycin (R8781) at 100 nM, and AKT, wortmannin (W3144) at 1 μM, these were added for the last 30 min of starvation and during subsequent incubation with the different treatments. Once the incubation time passed, wells were washed twice with cold phosphate buffered saline (PBS) and the samples were recovered accordingly depending on the assay type to be performed. All reagents were obtained from Sigma-Aldrich (Tres Cantos, Spain) except

human IGF-I that was from Bachem (Weil am Rhein, Germany) and all plastic ware and glass cover slips were from Nunc (LabClinics, Barcelona, Spain).

### 2.4. Immunocytochemistry

Cell proliferation was analyzed by immunostaining using a commercial PCNA staining kit (Cat. No. 93-1143. Life Technologies, Alcobendas, Spain). After washing, cells were fixed at room temperature in 4% paraformaldehyde (PFA, Sigma-Aldrich, Spain) for 15 min, washed, and postfixed for 5 min in 50% and 70% ethanol. Briefly, coverslips were incubated in PCNA staining reagents following the suggested manufacturer's protocol. Coverslips were incubated in a blocking solution to prevent non-specific binding before incubation with anti-PCNA primary antibody for 1 h and a biotinylated secondary antibody for 5 min, both at room temperature. Finally, cells were dehydrated in a graded alcohol series and mounted with histomount. The amount of PCNA-positive cells was calculated by dividing the PCNA-positive stained cells by the total number of nuclei in 6-8 images per coverslip using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Digital images were acquired with a CC2 camera coupled to a microscope at 40x using analySIS (Soft Imaging System) software. All images were analyzed by the same researcher.

### 2.5. Immunofluorescence

Immunofluorescence was performed based on the protocol described by Gabillard et al. (2010) with minor modifications. After washing with PBS, cells were fixed in 4% PFA for 30 min, and permeabilized in 0.2% Triton X-100/PBS for 3 min. Then, cells were rinsed

three times in PBS and blocked for 1 h with 3% BSA, 0.1% Tween20 in PBS (PBST) at room temperature. Cells were incubated with primary antibodies diluted in blocking solution (3% BSA in TBST) for 24 h at 4°C. Polyclonal rabbit anti-Myogenin (M-225; 1:100 dilution) and polyclonal rabbit anti-MyoD (M-318; 1:100 dilution) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody incubation (Goat anti-rabbit 568, A21069, Life Technologies, Alcobendas, Spain) was carried out in PBST for 1 h at room temperature. Nuclei were counterstained with Hoescht (H1399, Life Technologies, Alcobendas, Spain). Images were obtained at 36x magnification on a Leica TCS SP2 confocal microscope. Protein expression was evaluated by nuclei fluorescence intensity quantification normalized to the total number of nuclei (Hoescht stained) in each field by using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and 5-10 images per coverslip were analyzed.

### 2.6. Gene expression

#### 2.6.1. RNA extraction and cDNA synthesis

RNA samples were collected and processed with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using the procedure previously described (Jiménez-Amilburu et al., 2013). Concentration and RNA purity was determined using a NanoDrop ND2000 (Thermo Scientific, Alcobendas, Spain) and integrity of the samples was confirmed in a 1% agarose gel and staining with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). Afterwards, 500 ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain) following the manufacturer's recommendation to remove all genomic DNA, and further the RNA was reverse transcribed

with the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain).

### 2.6.2. Quantitative real-time PCR (qPCR)

The mRNA transcript levels of PCNA, MyoD1, MyoD2, Myf5, Myogenin, TOR, 4EBP1, 70S6K, AKT2 and FOXO3, plus three reference genes (ribosomal protein S18 (RPS18), elongation factor 1 alpha (EFI-α) and β-actin) were examined according to the requirements of the MIQE guidelines (Bustin et al., 2009) in a CFX384$^{TM}$ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The analyses were performed using 2.5 μL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1) and 1 μL of cDNA for each sample in a final volume of 5 μL. Reactions were performed in triplicate in 384-well plates in the same conditions previously described by Salmerón et al. (2013). Shortly: initial activation for 3 min at 95 ºC and 40 cycles of 10 s at 95 ºC, 30 s at 58-68 ºC (primer dependent, Table 1) followed by an amplicon dissociation analysis from 55 to 95 ºC at 0.5 ºC increase each 30 s. Prior to analyses, a dilution curve with a pool of samples was run to confirm specificity of the reaction, absence of primer-dimers and to determine the appropriate cDNA dilution. The expression level of each gene analyzed was calculated relative to the reference genes RPS18 and β-actin, the two most stable of the genes analyzed, using the Pfaffl method (Pfaffl, 2001).

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

| Gene | Primer sequences (5'–3') | Tm °C | Accession number | References |
|---|---|---|---|---|
| *EF1α* | **F**: CTTCAACGCTCAGGTCATCAT<br>**R**: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | Salmerón et al. (2013) |
| *RPS18* | **F**: GGGTGTTGGCAGACGTTAC<br>**R**: CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 | Vieira et al. (2012) |
| *β-Actin* | **F**: TCCTGCGGAATCCATGAGA<br>**R**: GACGTCGCACTTCATGATGCT | 60 | X89920 | Salmerón et al. (2013) |
| *PCNA* | **F**: TGTTTGAGGCACGTCTGGTT<br>**R**: TGGCTAGGTTTCTGTCGC | 58 | AY550963.1 | García de la serrana et al. (2014) |
| *MyoD1* | **F**: TTTGAGGACCTGGACCC<br>**R**: CTTCTGCGTGGTGATGGA | 60 | AF478568.1 | Present study |
| *MyoD2* | **F**: CACTACAGCGGGGATTCAGAC<br>**R**: CGTTTGCTTCTCCTGGACTC | 60 | AF478569 | Jiménez-Amilburu et al. (2013) |
| *Myf5* | **F**: CTACGAGAGCAGGTGGAGAACT<br>**R**: TGTCTTATCGCCCAAAGTGTC | 64 | JN034420 | Jiménez-Amilburu et al. (2013) |
| *Myogenin* | **F**: CAGAGGCTGCCCAAGGTCGAG<br>**R**: CAGGTGCTGCCCGAACTGGGCTCG | 68 | EF462191 | Jiménez-Amilburu et al. (2013) |
| *TOR* | **F**: CAGACTGACGAGGATGCTGA<br>**R**: AGTTGAGCAGCGGGTCATAG | 60 | - | García de la serrana unpublished |
| *70S6K* | **F**: GCACCAGAAAGGCATCATCT<br>**R**: AAGGTGTGGGTCACTGTTCC | 60 | - | García de la serrana unpublished |
| *E4EBP1* | **F**: CCAACCTGCGACTCATCTCT<br>**R**: GTTCCTCTCATCCTCCCACA | 60 | - | García de la serrana unpublished |
| *AKT2* | **F**: GCTCACCCCACTCTTCAGAC<br>**R**: AAATTGGGAAATGTGCTTGC | 60 | ERA047531 | García de la serrana et al. (2012b) |
| *FOXO3* | **F**: CAGCAGCCTGGAGTGTGATA<br>**R**: CCAGCTCTGAGAGGTCTGCT | 60 | - | García de la serrana unpublished |

### 2.7. Western blot analysis

Protein homogenates from cells were obtained as described by Codina et al. (2008). The amount of protein from each sample was measured (Bradford, 1976) and 10-20 μg of protein were separated by electrophoresis (SDS-PAGE) on 10% polyacrylamide gel (125 V for 1 h 30 min) in electrophoresis buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS, pH 8.3). Then, samples were transferred to a PVDF membrane overnight at 100 mA in transfer buffer (25 mM Tris-HCl, 192 mM Glycine, 20% Methanol, pH 8.3). After transfer, the membrane was blocked in non-fat milk 5% buffer at room temperature for 2 h and then incubated overnight at 4 ºC with the respective primary antibodies diluted in washing buffer (20 mM Tris•HCl, 150 mM NaCl, 0.05 % Tween 20, pH 7.6). The primary antibodies used were as follows: rabbit polyclonal anti-phospho AKT (Cat. No. 9271); rabbit polyclonal anti-total AKT (Cat. No. 9272); rabbit polyclonal anti-phospho TOR (Cat. No. 2971), all from Cell Signaling Technology (Beverly, MA); and rabbit polyclonal anti-total TOR (Cat. No. T2949. Sigma-Aldrich, Spain). For the phosphorylated forms 1:200 dilution was used and a 1:500 for the total forms. After washing (3 times for 15 min), the membranes were incubated with the peroxidase-conjugated secondary antibody (Cat. No. 31460. Thermo Scientific, Alcobendas, Spain) for 1 h at room temperature. The membranes were rewashed and the different immunoreactive bands were developed by using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain). Once the phosphorylated forms were developed, primary and secondary antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Alcobendas, Spain) incubating 10 min at room temperature and then, the membranes were blotted again following the same procedure with the total forms for both AKT and TOR.

Finally, the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### 2.8. Statistical analyses

Data was analyzed using IBM SPSS Statistics v.20 and presented as means ± SEM. Normal distribution was first analyzed using Shapiro-Wilk test followed by Levene's to test homogeneity of variances. Treatments effects between groups were tested by T-test or one-way analysis of variance (ANOVA), followed by the Tukey test. When data did not follow a normal distribution, non-parametric Kruskal-Wallis ANOVA and Mann-Whitney test were applied. Statistical differences were considered significant when P-value $<0.05$ or $<0.01$.

## 3. Results

### 3.1. Cell culture development characterization

In order to determine the developmental patterns of myocyte culture in gilthead sea bream we examined cell proliferation throughout the culture at days 2, 4, 8 and 12 and, the protein expression of two MRFs, MyoD and Myogenin, at days 4 and 8. The amount of PCNA-positive cells was significantly higher during the first days of culture (days 2 and 4) and progressively decreased over time (Fig. 1A). On the other hand, the measurement of MRFs protein expression by immunofluorescence showed that MyoD was similar at both days (4 and 8), whereas Myogenin abundance was significantly higher during the differentiation phase of the culture (day 8) than at day 4 (Fig. 1B).

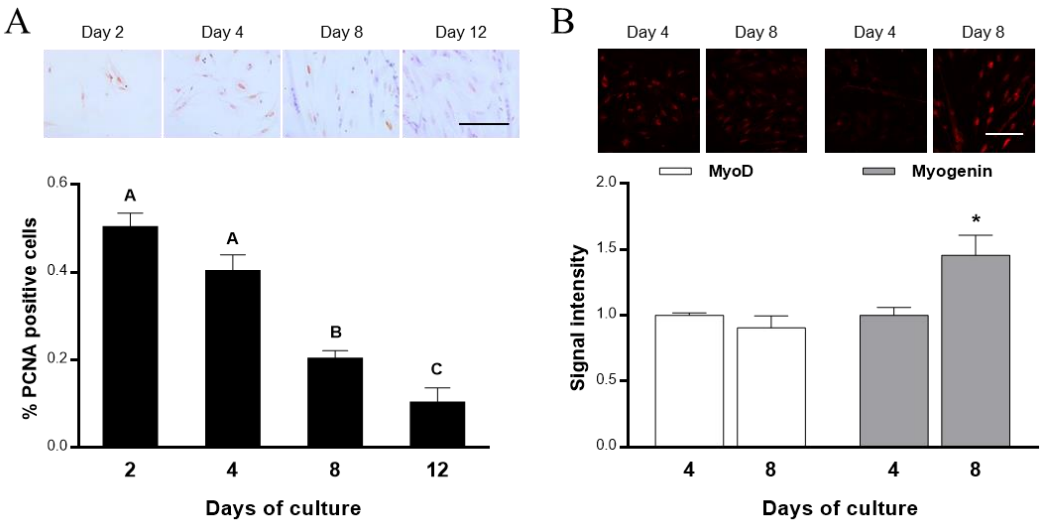


**Fig. 1.** Characterization of gilthead sea bream myocyte culture development. (**A**) Representative images and quantification of PCNA-positive cells from gilthead sea bream muscle cells at different days in culture (2, 4, 8 and 12). Slides were immunostained for PCNA as described in material and methods showing the different stages of development from myoblasts to myotubes (40x). (**B**) Representative images and quantification of MyoD and Myogenin protein expression at days 4 and 8 of culture. MyoD and Myogenin were detected by immunofluorescence and the expression intensity in the nuclei quantified as described in Section 2. Data are shown as mean values ± SEM (n = 3). Significant differences are shown by different letters or an asterisk ($P < 0.05$). Scale bar = 100 μm.

### 3.2. Effects of AA and IGF-I incubation on cultured myocytes

#### 3.2.1. Myogenic developmental markers protein and gene expression

The percentage of PCNA-positive cells significantly increased after 6 h of treatment with IGF-I alone or in combination with AA, whereas it was not altered by only AA (Fig. 2). Furthermore, the immunofluorescence analyses showed that MyoD showed a tendency to increase upon treatment, while both AA and IGF-I treatments, in conjunction or separately, significantly increased Myogenin protein expression over the Control (Fig. 3).

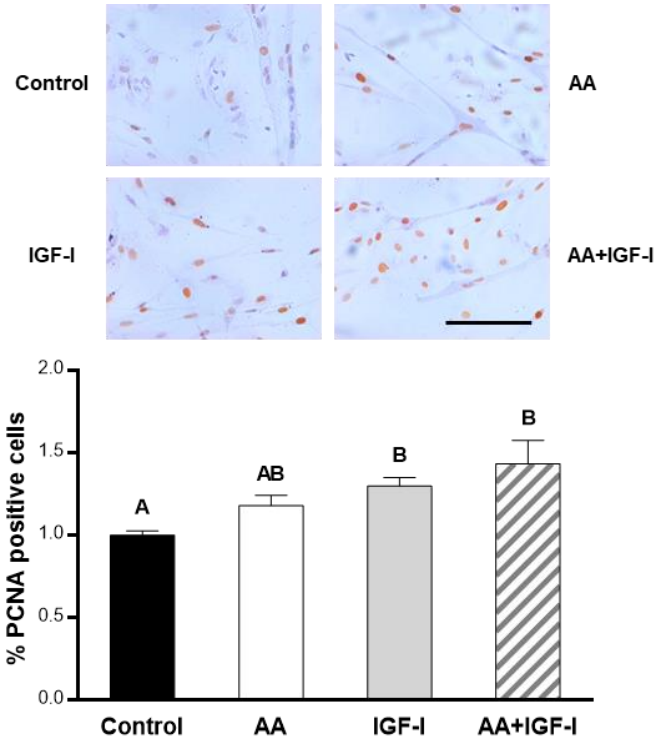


**Fig. 2.** Effects of amino acids (AA) and IGF-I on PCNA protein expression in gilthead sea bream cultured muscle cells. Representative images and quantification of PCNA-positive cells in gilthead sea bream muscle cells at day 4 incubated with AA, IGF-I and AA + IGF-I. PCNA was detected by immunostaining as described in Section 2 (40x). Results are presented as fold change over Control. Mean ± SEM (n = 3). Different letters indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

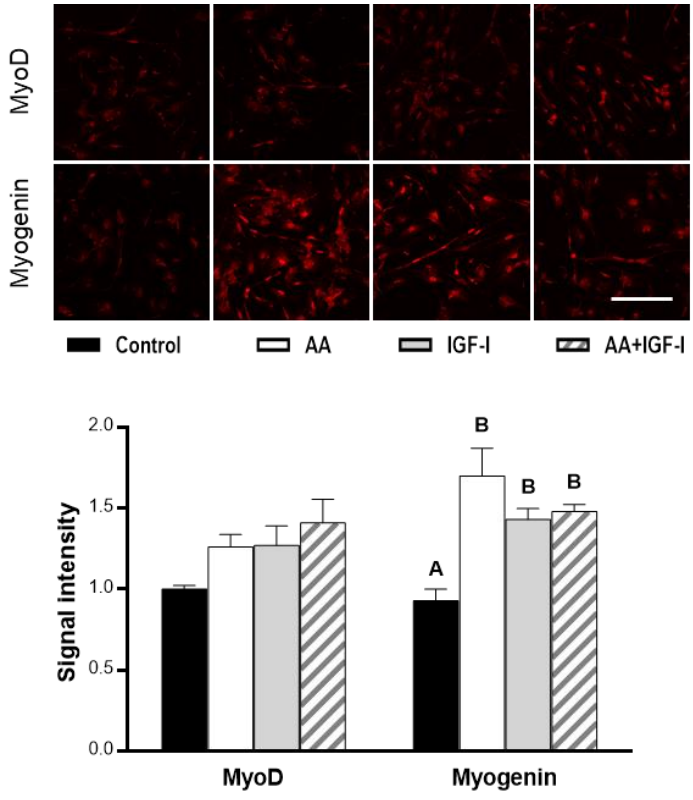


**Fig. 3.** Effects of amino acids (AA) and IGF-I on MyoD and Myogenin protein expression in gilthead sea bream cultured muscle cells. Representative images and quantification of nuclei MyoD and Myogenin protein expression in gilthead sea bream cultured muscle cells at day 4 incubated with AA, IGF-I and AA + IGF-I. MyoD and Myogenin were detected by immunofluorescence as described in Section 2 (40x). Results are presented as fold change over Control. Mean ± SEM (n = 3). Different letters indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

Regarding the gene expression of proliferation and progression markers of culture development, AA and the combined treatment of AA + IGF-I significantly increased PCNA mRNA levels over the Control, whereas the expression levels remained unaltered in the case of IGF-I treatment (Fig. 4A). Moreover, the mRNA levels of the different MRFs, MyoD1, MyoD2, Myf5 and Myogenin appeared unaltered by any treatment (Fig. 4B).

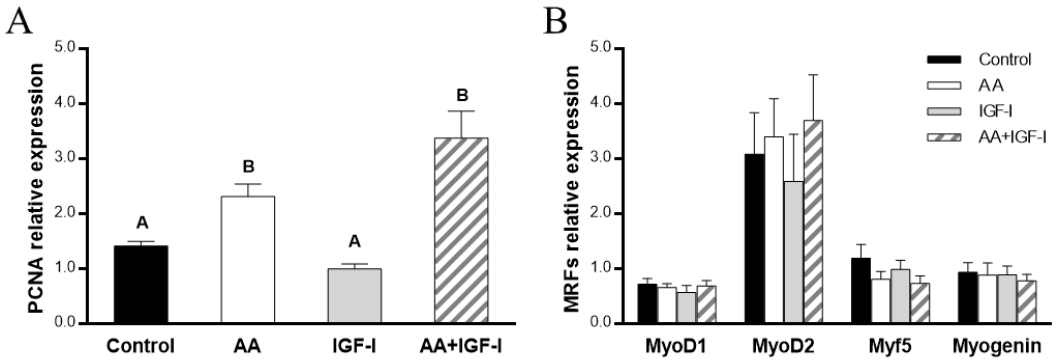


**Fig. 4.** Effects of amino acids (AA) and IGF-I on the gene expression of PCNA and MRFs in gilthead sea bream cultured muscle cells. (**A**) PCNA and (**B**) MRFs (MyoD1, MyoD2, Myf5 and Myogenin) expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM (n = 4-7). Different letters indicate significant differences ($P < 0.05$).

### 3.2.2. AKT and TOR signaling pathways

Next, we examined the gene expression of two key signal transduction molecules; TOR and AKT2, and its principal substrates. The results showed that AA significantly increased TOR gene expression while IGF-I or AA combined with IGF-I caused no effects (Fig. 5A). Then, both AA and AA + IGF-I treatments significantly increased the expression of 4EBP1 (Fig. 5B). In the case of 70S6K, AA treatment significantly increased its gene expression, whereas AA + IGF-I only caused a slight increase (Fig. 5C).

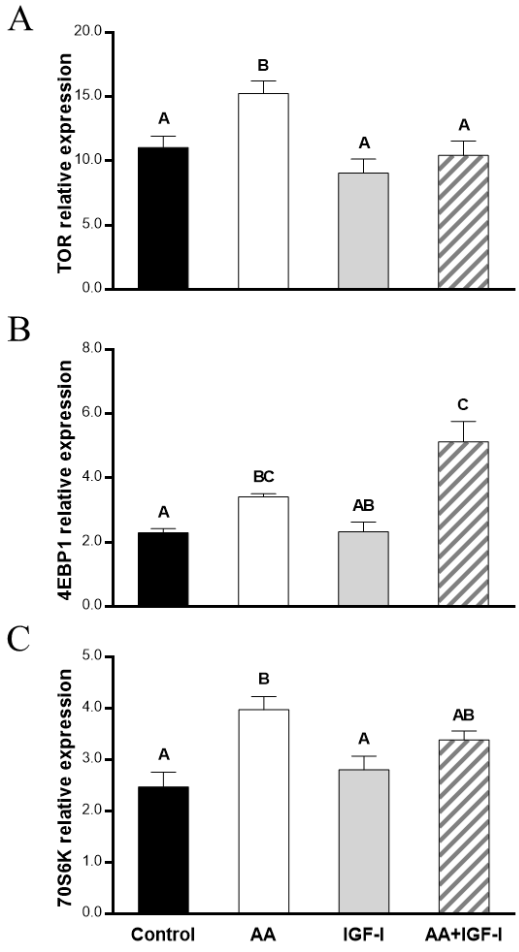


**Fig. 5.** Effects of amino acids (AA) and IGF-I on the gene expression of key molecules in the TOR signaling pathway in gilthead sea bream cultured muscle cells. (**A**) TOR, (**B**) 4EBP1 and (**C**) 70S6K expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM (n = 4-7). Different letters indicate significant differences ($P < 0.05$).

On the other hand, neither AA nor IGF-I, in combination or separately, altered the expression of AKT2 and its downstream effector FOXO3 (Fig. 6A and 6B).

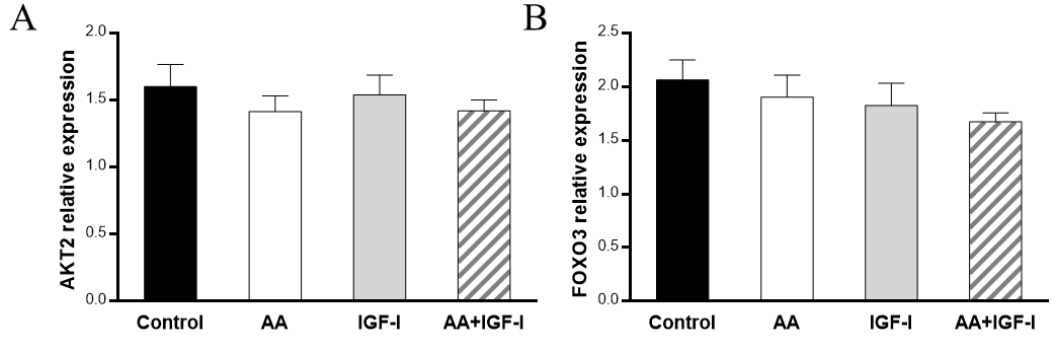


**Fig. 6.** Effects of amino acids (AA) and IGF-I on the gene expression of key molecules in the AKT signaling pathway in gilthead sea bream cultured muscle cells. (**A**) AKT2 and (**B**) FOXO3 expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM (n = 4-7). Different letters indicate significant differences ($P < 0.05$).

Additionally, Western blot results showed that AA, alone or combined with IGF-I, significantly increased the phosphorylation of TOR over the Control (Fig. 7A) whereas IGF-I had no effects. Moreover, the treatment with the TOR specific inhibitor, rapamycin (R), decreased significantly TOR phosphorylation versus the samples without inhibitor (N. I.) in the Control group, although not in the other groups; whereas TOR was not altered by wortmannin (W) treatment in any case (Fig. 7A).

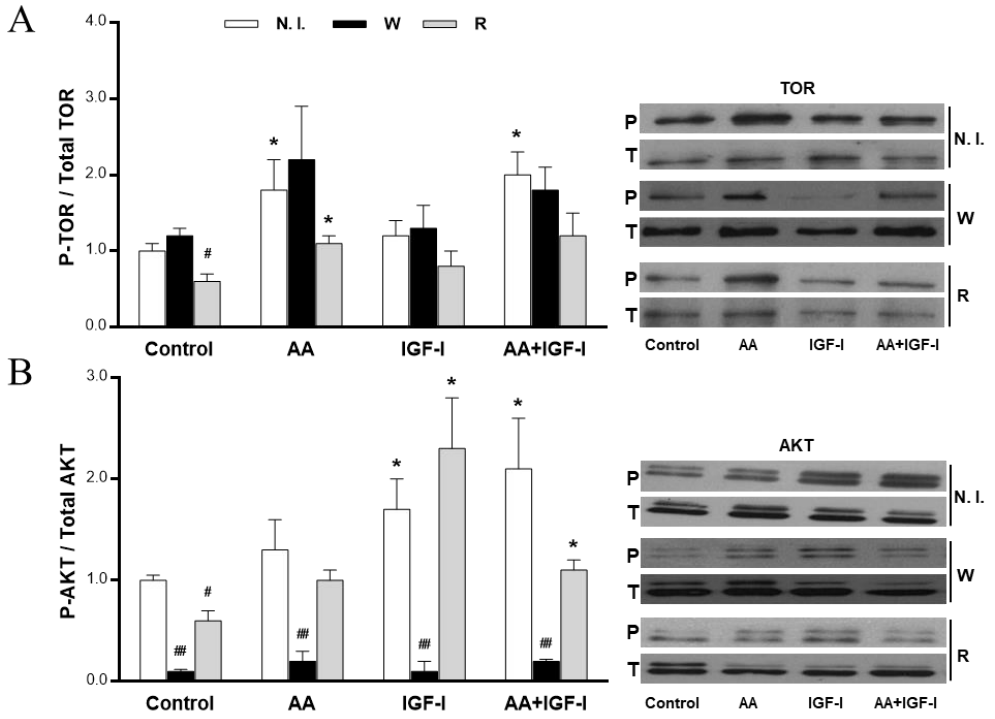


**Fig. 7.** Effects of amino acids (AA) and IGF-I on TOR and AKT signaling pathways activation in gilthead sea bream cultured muscle cells. Representative Western blots and quantification of TOR (**A**) and AKT (**B**) signaling pathways evaluated in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I, in the absence (N.I.) or presence of rapamycin (R) or wortmannin (W). Phosphorylated forms (P) band intensity was normalized to total forms (T). Results are presented as a fold change over Control. Mean ± SEM (n = 3-15). Asterisks indicate significant differences with the Control for each treatment and # indicates significant inhibition over N.I. within each treatment. Significant differences were considered at $P < 0.05$ (*/#) or $P < 0.01$ (##).

Regarding AKT, incubation with IGF-I alone or in combination with AA significantly increased AKT activation, whereas AA although induced a minor stimulation did not change significantly the level of phosphorylation (Fig. 7B). These effects disappeared upon incubation with the AKT's inhibitor, wortmannin (W), whereas rapamycin (R) did not produce any changes in AKT activation except in the case of the Control group, where it caused also decrease in AKT phosphorylation (Fig. 7B).

## 4. Discussion

Skeletal muscle biology is studied in many fields, from physiology to immunology through genetic diseases, tissue regeneration and even meat production, which is the most interesting from the view point of the industry (Muñoz-Cánoves and Michele, 2013). Skeletal muscle development has a well-ordered structure with high plasticity, being able to adapt to different conditions (Pisconti et al., 2012; Relaix and Zammit, 2012; Rescan, 2001). In this sense, the nutritional status has been shown to affect protein synthesis, and thus, muscle mass, both in mammals and teleost fish (Martin-Perez et al., 2013; Tirapegui et al., 2012).

In this study, we used as a model system a primary culture of muscle cells from gilthead sea bream to investigate the effects of AA and IGF-I on the gene and protein expression of proliferative and myogenic regulatory factors. Moreover, we analyzed the activation of transduction pathways involved in its signaling to better understand the process of myogenesis and its regulation by these nutritional and hormonal factors in this species.

PCNA is an auxiliary protein of DNA polymerase that helps to increase the processivity of the leading strand synthesis during DNA replication (Strzalka and Ziemienowicz, 2011).

Specific PCNA-staining is observed in proliferating cells nuclei during normal muscle growth (Veggetti et al., 1999). Our results showed a progressive decrease in the percentage of PCNA-positive cells in culture, indicating that proliferation occurs mainly at early stages of development, in agreement with that observed in gene expression (García de la serrana et al., 2014). Nevertheless, the protein expression of the MRF involved in satellite cell specification, MyoD showed no differences. The fact that the polyclonal antibody used does not distinguish between the two MyoD isoforms and that they present different gene expression patterns throughout the culture (Vélez et al. unpublished data), could explain the little changes in MyoD signal intensity as suggested by Froehlich et al. (2013) in zebrafish. On the other hand, Myogenin protein expression was higher at day 8 than at day 4, confirming that myoblast differentiation takes place when proliferation decreases, supporting a role for this MRF in the late phases of culture development when formation of myotubes occurs. It is important to note that these results are consistent with the common knowledge about the process of myogenesis in teleosts (Johnston et al., 2008; Rescan, 2001) and specifically, in agreement with the results reported recently in gilthead sea bream (García de la serrana et al., 2014; Montserrat et al., 2007b) and rainbow trout (Gabillard et al., 2010).

With regards to the hormonal and nutritional regulation of myocyte development, our data indicated that a treatment with IGF-I alone or in combination with AA increased the amount of PCNA-positive cells as previously demonstrated for IGFs in gilthead sea bream and rainbow trout (Gabillard et al., 2010; Rius-Francino et al., 2011), supporting a role for IGF-I stimulating proliferation. Furthermore, the immunofluorescence results revealed that although MyoD did not change, both AA and IGF-I, in conjunction or separately, enhanced Myogenin protein expression, which is related to cell differentiation (Gabillard et al., 2010;

García de la serrana et al., 2014). At the level of gene expression, only AA and the AA + IGF-I combination increased PCNA gene expression, meanwhile IGF-I alone had no effects; suggesting that the regulatory role of IGF-I on proliferation seems to occurs primarily at a post-transcriptional level. Moreover, none of the treatments altered the expression of MyoD1, MyoD2 or Myf5 as it was also observed previously (Jiménez-Amilburu et al., 2013). However, in that study it was shown that IGF-I, and mainly IGF-I plus GH increased Myogenin expression, effect not observed here. Nevertheless, different responses in MRFs expression after IGF-I treatment were also observed in rainbow trout myocytes depending on the incubation time (Garikipati and Rodgers, 2012a, 2012b).

Next, we analyzed the gene expression of TOR and AKT and its downstream effectors, as well as the activation of TOR and AKT by phosphorylation in response to AA and IGF-I. Our results indicated that AA have the ability to regulate the mRNA expression of TOR and its effectors while IGF-I did not cause any changes in the expression of these molecules. Nevertheless, the combination of AA with IGF-I also increased 4EBP1 expression, suggesting that IGF-I may have a supporting regulatory role in TOR pathway. Interestingly AA also enhanced TOR phosphorylation as previously reported for TOR and its downstream effectors in different fish species (Garcia de la serrana and Johnston, 2013; Seiliez et al., 2008), therefore showing a double effect on TOR simultaneously increasing its expression and activity. Although IGF-I alone had no effects activating TOR, the combination of AA and IGF-I stimulated also TOR phosphorylation as in recent reports in mammals (Destefano and Jacinto, 2013; Kim and Guan, 2011). Similarly, in previous studies in Atlantic salmon myocytes and rainbow trout hepatocytes, IGF-I or insulin alone, respectively, only slightly increased TOR signaling but when combined with AA caused a greater stimulation (Garcia de la serrana and Johnston, 2013; Lansard et al., 2010). The

lack of effects on TOR phosphorylation by IGF-I alone could be due to species or cell type differences or to the incubation time, 3 h in our study versus 24 h or 15 min in those studies. Nevertheless, overall our results are in agreement with the effects of AA and growth factors on TOR activation, although little information exists at the level of gene expression, therefore the present study permits to demonstrate the important effect of AA not only on protein activation but also on gene expression of this signaling pathway.

This study was completed with a rapamycin treatment, the TORC1 specific inhibitor (Nobukuni et al., 2005). Rapamycin decreased TOR phosphorylation in all conditions although only significantly in the Control group, in agreement with previous results (Lansard et al., 2010), confirming the specificity of the pathway, but also indicating that perhaps the sensitivity of the TORC1 complex in gilthead sea bream seems to be lower than that of mammals or other fish species. In this sense, the understanding of the complete regulation of TOR complexes in this species deserves more investigation.

Concerning AKT results, we have proved that IGF-I stimulates the PI3K/AKT pathway in myocytes by increasing AKT phosphorylation, in agreement with the results related by our group in gilthead sea bream (Montserrat et al., 2012) and rainbow trout (Codina et al., 2008), or in mammals by others (Laviola et al., 2007; Philippou et al., 2007). Although there was no significant effect of AA, the combined treatment with AA + IGF-I maintained the activation of this signaling pathway. However, since effects were not found on AKT2 or FOXO3 gene expression in response to any treatment, we hypothesize that the regulation of AKT takes place at a post-traductional level.

Moreover, wortmannin the specific inhibitor of PI3K, did not affect TOR activation, but dramatically decreased AKT phosphorylation in all conditions, as it has been previously shown (Montserrat et al., 2012). Contrariwise, rapamycin treatment did not affect AKT

activity, except in the case of the Control group, indicating that AKT phosphorylation is mostly independent of AA action through TORC1, as in mammals (Kim and Guan, 2011). Although more studies are needed to confirm it, the significant suppressive effects of rapamycin in Control myocytes in our study could suggest mechanistic regulatory differences in the activation of this signaling pathway between fish and mammals.

In summary, this work shows the important role of IGF-I and AA in muscle growth regulation including the stimulation of the processes of proliferation and differentiation and the conservation of the main signaling pathways involved through vertebrates. However, it should be taken into consideration that some particularities among fish species could be related to the physiological and metabolic differences, as well as to the importance of proteins in the diet. Altogether, it contributes to improve the knowledge on the regulation of myogenesis in gilthead sea bream and provides potential nutritional treatments to optimize muscle growth in this cultured species.

**Acknowledgments**

The authors would like to thank Carlos Mazorra from Tinamenor S.L and the personnel from the facilities at the School of Biology for the maintenance of the fish. E.C. is a "Ramón y Cajal" researcher fellow from the "Ministerio de Ciencia e Innovación" (MICINN). E.J.V. and E.L. are supported by a predoctoral fellowship from the MICINN. This study was supported by the projects from the MICINN AGL2009-12427 and AGL2012-39768 to J.G. and AGL2010-17324 to E.C., the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and SGR2009-00402 from the "Generalitat de Catalunya", and by funds from the European Union through the project LIFECYCLE (EU-FP7 222719).

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# ARTICLE II

## Proteolytic systems' expression during myogenesis and transcriptional regulation by amino acids in gilthead sea bream cultured muscle cells

PLoS ONE 2017 12(12): e0187339

Article II

# Proteolytic systems' expression during myogenesis and transcriptional regulation by amino acids in gilthead sea bream cultured muscle cells

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**Running title:** Proteolytic markers characterization in gilthead sea bream myocytes

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**Abstract**

Proteolytic systems exert an important role in vertebrate muscle controlling protein turnover, recycling of amino acids (AA) or its use for energy production, as well as other functions like myogenesis. In fish, proteolytic systems are crucial for the relatively high muscle somatic index they possess, and because protein is the most important dietary component. Thus in this study, the molecular profile of proteolytic markers (calpains, cathepsins and ubiquitin-proteasome system (UbP) members) were analyzed during gilthead sea bream (*Sparus aurata*) myogenesis *in vitro* and under different AA treatments. The gene expression of calpains (*capn1*, *capn3* and *capns1b*) decreased progressively during myogenesis together with the proteasome member *n3*; whereas *capn2*, *capns1a*, *capns1b* and ubiquitin (*ub*) remained stable. Contrarily, the cathepsin D (*ctsd*) paralogs and E3 ubiquitin ligases *mafbx* and *murf1*, showed a significant peak in gene expression at day 8 of culture that slightly decreased afterwards. Moreover, the protein expression analyzed for selected molecules presented in general the same profile of the mRNA levels, which was confirmed by correlation analysis. These data suggest that calpains seem to be more important during proliferation, while cathepsins and the UbP system appear to be required for myogenic differentiation. Concerning the transcriptional regulation by AA, the recovery of their levels after a short starvation period did not show effects on cathepsins expression, whereas it down-regulated the expression of *capn3*, *capns1b*, *mafbx*, *murf1* and up-regulated *n3*. With regards to AA deficiencies, the major changes occurred at day 2, when leucine limitation suppressed *ctsb* and ctsl expression. Besides at the same time, both leucine and lysine deficiencies increased the expression of *mafbx* and *murf1* and decreased that of *n3*. Overall, the opposite nutritional regulation observed, especially for the UbP

members, points out an efficient and complementary role of these factors that could be useful in gilthead sea bream diets optimization.

**Keywords:** Proteolytic molecules; cathepsins; calpains; ubiquitin-proteasome; myocytes; *Sparus aurata*

## Introduction

Muscle growth regulation in vertebrates requires an equilibrium among protein synthesis and degradation (proteolysis). This balance acquires special interest in fish, since they mostly present indeterminate growth, and thus increase their muscle mass throughout their life, as well as they are naturally exposed to periods of low food availability, when metabolic mobilization becomes important to survive fasting.

Fish muscle growth differs in many species from other vertebrates in so most mammals can develop muscle mass only by hypertrophy after sexual maturation, whereas fish still continue to increase their muscle mass with important rates of muscle hyperplasia (myogenesis). Understanding on the regulation of fish myogenesis has increased significantly in the last years (reviewed by Fuentes et al. [1]; Johnston et al. [2]; Vélez et al. [3]), as well as the importance of proteolysis on muscle growth *in vivo*; however, the investigation involving the proteolytic systems *in vitro* has been only limited [4–8]. Furthermore, muscle proteolysis research is important in aquaculture because the proteolytic systems play a key role in determining the fish flesh quality. During the post mortem period, the muscle tissue is subjected to changes caused by many factors such as temperature, pH or microbial activity, which in conjunction with the action of endogenous

proteases can modify muscle properties [5]. Therefore, due to its significance in regulating both, muscle growth and value, to fully elucidate the role of the different proteolytic systems in fish is of utmost importance.

The four chief endogenous proteolytic systems in vertebrates include: cathepsins, calpains, the ubiquitin-proteasome (UbP) and caspases [9, 10]; although the caspases will not be considered in this study as they are mostly linked to cellular apoptosis [11].

The cathepsin family contains several classes of proteases comprising: 1) cysteine proteases (CTSB, L, H, K, S and O), 2) aspartyl proteases (CTSD and E) and, 3) serine proteases (CTSG). Most cathepsins are lysosomal enzymes and part of the autophagic-lysosome system (ALS) involved in cellular degradation. In fact, they are characterized as regulators of an enormous number of biological processes like bone remodeling or angiogenesis, and have been implicated in the development of different pathological conditions (e.g. inflammation and cancer) [12]. In fish, Seiliez and co-workers have recently demonstrated in rainbow trout (*Oncorhynchus mykiss*) myotubes that the ALS is responsible for up to 50% of total protein degradation in contrast to mammals, in which this system appears to be proportionally less important [8].

The calpain system is composed by intracellular proteases that are $Ca^{2+}$-dependent and belong to the papain superfamily of cysteine proteases. The catalytic CAPN1 and CAPN2 subunits bind a common regulatory member, CAPN4 or calpain small subunit (CAPNS) to form an active heterodimer, which has different biological functions during myogenesis depending on the catalytic member [13]. While CAPN1 may be involved in the myogenic regulation via its action on myogenin, ezrin, vimentin and caveolin 3 [14], CAPN2 participates in the fusion of mononuclear myoblasts to multinucleated myotubes in muscle

cell cultures [15]. To date, members of this proteolytic system have been characterized in several teleost fish including gilthead sea bream (*Sparus aurata*). In addition in this species, the expression of *capn1* and *capns1a*, was shown to be inversely correlated with muscle texture, indicating they may serve as potential genetic markers of flesh quality [16].

In the UbP system, a large proportion of the proteins intended for degradation in the cell (representing up to 50% in mammals) are tagged by ubiquitination, and then recognized by the 26S proteasome complex, where they are degraded to oligopeptides [9]. Nonetheless, in fish, the UbP system is only responsible for 17% of the protein degradation as demonstrated in rainbow trout myotubes [8]. Among the members that conform this system, the muscle specific F-box protein (MAFbx, a.k.a. Atrogin1/Fbox-32) and the muscle RING-finger protein 1 (MuRF1) are key E3 ubiquitin ligases specifically expressed in skeletal, cardiac and smooth muscle that perform multiple functions [17], and have been found up-regulated in situations of muscular atrophy [18]. Similarly, although ubiquitin has multiple functions either proteolytic or non-proteolytic, its expression has been found to increase with age in mammals, which has been related with the poorer healing capacity of the muscle in the elderly [19]. Furthermore, N3 (a.k.a. PSMB4) is a β type proteasome subunit that has been previously used as a proteolysis marker of this degradation system in fish [16, 20-22].

To study skeletal muscle in vitro development in mammals, several cell lines have been characterized (e.g. C2C12, L6 or HSkM), but equivalent models are not available in farmed fish, turning primary cultures essential. Therefore, during the last decade primary cultures of myocytes derived from isolated white muscle satellite cells have been established for some economically important fish species, like rainbow trout [23], gilthead sea bream [24], Atlantic salmon (*Salmo salar*) [25], giant danio (*Devario cf. aequipinnatus*) [26] and even,

zebrafish (*Danio rerio*) [27]. These fish models represent a useful tool to study not only the conserved mechanisms taking place during myogenesis, but also can facilitate the identification of specific-critical factors involved in this process. In this sense, for example the regulation of myogenic development by nutritional factors such as amino acids (AA) has been investigated in several fish species including gilthead sea bream [28, 29]. These studies demonstrated the stimulatory effect of AA on myocytes proliferation and differentiation, as well as the critical negative effect on such processes of lysine limitation. Notwithstanding, information regarding the function of cathepsins, calpains and UbP members on fish myogenesis and how these catabolic systems respond to either AA supplementation or limitation is scarce, and most of the studies reported to date have been performed in salmonids [8, 30, 31].

Thus, the aim of this study was to characterize these 3 main proteolytic systems in gilthead sea bream during *in vitro* myogenesis and the transcriptional modulation of its members by AA to better understand the overall regulation of muscle development and growth in this important farmed species.

## Material and methods

### Experimental animals and ethical statement

The gilthead sea bream were provided by a commercial hatchery in northern Spain (Tinamenor S.L., Pesués, Cantabria). The fish were kept in tanks of 0.4 $m^3$ with a closed-water flow circuit at the facilities of the Faculty of Biology at the University of Barcelona. Conditions in the tanks, such as temperature of the sea water (21 ± 1 °C), photoperiod (12

h light: 12 h dark) and pH (7.5-8), were kept stable at all times. Twice a day fish were fed *ad libitum* with a commercial diet (Skretting, Burgos, Spain). The animal handling procedures were carried out with the specific approval of the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 168/14 and DAAM 7749), following the EU, Spanish and Catalan Government-assigned principles and legislations.

**Myocyte cell culture**

A total of fifteen independent white muscle satellite cell cultures were performed following the method described previously by Montserrat et al. [24]. Around 40 juvenile fish weighing 5 to 15 g were used for each culture. The fish were sacrificed by a blow to the head, weighed and immediately, their external surfaces were sterilized by immersion in 70% ethanol during 0.5 to 1 min. Then, fish were dissected and the epaxial white muscle tissue was collected in cold Dulbecco's Modified Eagle's Medium (DMEM), containing 9 mM NaHCO3, 20 mM HEPES, 0.11% NaCl, and 1% (v/v) antibiotic/antimycotic solution, and in this case supplemented with 15% (v/v) horse serum (HS) at a rate of 5 mL/g of tissue. Subsequently, muscle was minced to small fragments and centrifuged (3000 xg, 5 min), washed twice in DMEM and afterwards, the muscle shreds were enzymatically digested with 0.2% collagenase type IA dissolved in DMEM with gentle agitation during 80 min at 21 ºC. The obtained suspension was centrifuged and the pellet washed with DMEM medium (300 xg, 5 min), resuspended again and triturated by repeated pipetting. After centrifuged once more (300 xg, 5 min), the tissue fragments were digested twice during 20 min at 21 ºC, with 0.1% trypsin solution prepared in DMEM and gentle agitation. After each digestion the remained fragments were pelleted (300 xg, 1 min) to collect the

supernatants, which were pooled and diluted in complete medium (DMEM supplemented with 15% of HS) to block trypsin activity. Then, the supernatant was centrifuged (300 xg, 20 min) and the obtained pellet resuspended, forced to trituration by pipetting and then, the suspension was filtered first on a 100 µm, and subsequently on a 40 µm nylon cell strainer, and finally centrifuged one last time (300 xg, 20 min). Later, the obtained cells were diluted in growth media (DMEM supplemented with 10% fetal bovine serum (FBS) and seeded in six well-plates (9.6 $cm^2$/well) at a final density of 1-2 x $10^6$ cells per well. Cultures were kept at 23 °C in growth medium with medium change every 2-4 days. To characterize the role of the different proteolytic systems during myogenesis, cell samples for gene and protein expression were taken at days 2, 4, 8 and 12 of culture. These days were chosen because they represent well the different stages of myogenesis, which can be followed according to cell morphology, and are supported by data reported in previous publications [24, 28, 32, 33].

**Experimental treatments**

To study the effects of AA recovery, as described previously by Vélez et al. [28], cells at day 4 were first maintained for 12 h with DMEM with 0.02% FBS, and then, starved during 5 h with a medium deficient in AA (medium B: 10% Earle's Balanced Salt Solution (EBSS, E7510) with 1% MEM vitamins (M6895), 0.9% NaCl and 0.13% bovine serum albumin (BSA)). Next, cells were held 6 h in medium B alone (Control) or supplemented with an AA cocktail (1% MEM Amino Acids Solution (M5550) and 1% MEM Non-essentials Amino Acids Solution (M7145)) before samples were collected. In the case of the leucine or lysine deficiency experiments, as described before by Azizi et al. [29] other 3 different

media were prepared (control, without leucine or without lysine) using DMEM/F12HAM (D9785) devoid of leucine and lysine as a base media, and adding 10% FBS, and the missing AA. The concentration of either leucine (24.2 µM) or lysine (24.7 µM) provided by the FBS in each corresponding deficient medium was reduced in a 93.8% respect to the control condition (where total concentration was 389.6 µM and 398.0 µM for leucine and lysine, respectively). In this experiment, the growth medium was replaced with the corresponding media at day 1 of culture for samplings at days 2 and 4, and at day 7 for the sampling at day 8.

All plastic ware were obtained from Nunc (LabClinics, Barcelona, Spain) and all reagents were from Sigma-Aldrich (Tres Cantos, Spain) unless stated otherwise.

**Gene expression**

**RNA extraction and cDNA synthesis**

Cell samples for RNA extraction from each independent culture were collected from 3 replicate wells pooled together per sampling point during myogenesis characterization and from 2 replicate wells pooled together per condition in both AA experiments using 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) and processed following the manufacturer's instructions. A NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain) was used to determine total RNA concentration and purity. Confirmation of RNA integrity was performed in a 1% m/v) agarose gel stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). In order to obtain cDNA, 500 ng of the total RNA was first exposed to a DNase I enzyme (Life Technologies, Alcobendas, Spain) to remove all genomic DNA and after reversely transcribed by using a

Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendation.

**Quantitative real-time PCR (qPCR)**

Levels of mRNA transcripts of different cathepsins (*ctsda*, *ctsdb*, *ctsb* and *ctsl*), calpains (*capn1*, *capn2*, *capn3*, *capns1a* and *capns1b*) and UbP members (*mafbx*, *murf*, *n3* and *ub*), as well as the reference genes ribosomal protein S18 (*rps18*), elongation factor 1 alpha (*ef1α*) and beta-actin (*β-actin*) were analyzed according to the MIQE guidelines requirements [34] in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The qPCR reactions were performed using 2.5 µL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1) and 1 µL cDNA of each sample at the corresponding dilution for an efficient measurement in a final volume of 5 µL. Each run was performed in triplicate using 384-well plates and conditions were the same as those described previously [16]. Briefly, a short initial activation of 3 min at 95 °C was followed by 40 cycles of 10 sec at 95 °C, 30 sec at 54-61 °C (primer dependent, Table 1) and ended with an amplification dissociation analysis from 55 up to 95 °C with a 0.5 °C increase every 30 sec. Although all the primers have been previously validated [16, 22], a dilution curve with a pooled sample was made before the analyses to confirm reaction specificity, absence of primer-dimers, efficiency of the primers pairs (Table 1) and to determine the appropriate cDNA dilution to work with. Transcript abundance of each studied gene was calculated relative to the geometric mean of the three reference genes (*rps18*, *β-actin* and *ef1α*) since they were all stable (confirmed

by the geNorm algorithm) using the method described by Pfaffl [35] with the Bio-Rad CFX Manager 3.1 software.

**Table 1. Primer sequences used for qPCR**.

| Gene | Primer sequences (5'-3') | Accession No. | Ta (ºC) | Amplicon (bp) | E (%) |
|---|---|---|---|---|---|
| *ef1a* | **F:**CTTCAACGCTCAGGTCATCAT<br>**R:**GCACAGCGAAACGACCAAGGGGA | AF184170 | 60 | 263 | 96.6 |
| *β-actin* | **F:**TCCTGCGGAATCCATGAGA<br>**R:**GACGTCGCACTTCATGATGCT | X89920 | 68 | 50 | 98.3 |
| *rps18* | **F:**GGGTGTTGGCAGACGTTAC<br>**R:**CTTCTGCCTGTTGAGGAACCA | AM490061.1 | 60 | 160 | 97.7 |
| *ctsda* | **F:**CCTCCATTCACTGCTCCTTC<br>**R:**ACCGGATGGAAAACTCTGTG | AF036319 | 56 | 107 | 102.1 |
| *ctsdb* | **F:**AAATTCCGTTCCATCAGACG<br>**R:**CTTCAGGGTTTCTGGAGTGG | KJ524456 | 56 | 131 | 95.6 |
| *ctsb* | **F:**GCAGCCTTCCTGTTATTGG<br>**R:**AGGTCCCTTCAGCATCGTA | KJ524457 | 57 | 185 | 95.0 |
| *ctsl* | **F:**ACTCCTTGGGCAAACACA<br>**R:**CCTTGAACTTCCTCTCCGT | DQ875329 | 54 | 116 | 94.5 |
| *capn1* | **F:**CCTACGAGATGAGGATGGCT<br>**R:**AGTTGTCAAAGTCGGCGGT | KF444899 | 56 | 114 | 103.2 |
| *capn2* | **F:**ACCCACGCTCAGACGGCAAA<br>**R:**CGTTCCCGCTGTCATCCATCA | KF444900 | 61 | 405 | 91.3 |
| *capn3* | **F:**AGAGGGTTTCAGCCTTGAGA<br>**R:**CGCTTTGATCTTTCTCCACA | ERP000874 | 56 | 113 | 97.2 |
| *capns1a* | **F:**CGCAGATACAGCGATGAAAA<br>**R:**GTTTTGAAGGAACGGCACAT | KF444901 | 56 | 92 | 100.2 |
| *capns1b* | **F:**ATGGACAGCGACAGCACA<br>**R:**AGAGGTATTTGAACTCGTGGAAG | ERP000874 | 56 | 51 | 99.7 |
| *mafbx* | **F:**GGTCACCTGGAGTGGAAGAA<br>**R:**GGTGCAACTTTCTGGGTTGT | ERA047531 | 60 | 158 | 94.3 |
| *murf1* | **F:**GTGACGGCGAGGATGTGC<br>**R:**CTTCGGCTCCTTGGTGTCTT | FM145056 | 60 | 50 | 98.5 |
| *n3* | **F:**AGACACACACTGAACCCGA<br>**R:**TTCCTGAAGCGAACCAGA | KJ524458 | 54 | 118 | 99.1 |
| *ub* | **F:**ACTGGCAAGACCATTACCTT<br>**R:**TGGATGTTGTAGTCGGAAAG | KJ524459 | 54 | 160 | 97.2 |

F: forward; R: reverse; Accession No.: GenBank accession numbers; Ta: annealing temperature; Amplicon: product size (base pairs); E: qPCR efficiency.

**Protein expression**

**Protein extraction**

Protein isolation was carried out from the same samples taken at different time points during myogenesis and obtained from triplicate wells pooled together from each independent culture using the interphase and organic phase produced during the RNA isolation and following the TRI Reagent Solution manufacturer protocol. The protein pellets were resuspended with 60 µL of RIPA buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 2 mM, NP-40 1%, SDS 0.1% and Na-deoxycholate 0.5% plus protease inhibitor cocktail P8340) and homogenated with a Pellet pestle (Sigma-Aldrich). Then, the samples were kept for 1 h in an orbital at 4 °C and centrifuged at 15,000 xg for 30 min at 4 °C. Finally, the supernatant was transferred to a new tube and stored at -80 ºC until further analysis.

**Western blot analysis**

Protein concentration of each sample was measured using the Bradford assay, with BSA as reference protein. Four to ten µg of soluble fraction protein were subjected to a SDS-PAGE gel electrophoresis on a 12% acrylamide gel (1 h 30 min at 125 V) following the procedure previously described [28]. After blocking with 5% non-fat milk in washing buffer (20 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6), the membranes were incubated with the primary antibodies diluted in washing buffer overnight at 4°C. The primary polyclonal antibodies used were CAPN1 (sc-7530), CTSD (sc-6486), CTSL (sc-6501) and MAFbx (sc-33782), all from Santa Cruz Biotechnology (Santa Cruz, CA, US). CAPN1, CTSD and

CTSL were used at 1:200 and MAFbx at 1:400 final concentration. Afterwards, the membranes were washed and incubated with the respective secondary antibodies (sc-2020 and sc-2004, also provided by Santa Cruz Biotechnology) in a 5% non-fat milk washing buffer solution at a final concentration of 1:10000. After washing, an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain) was used to develop the bands. When required, the membranes were stripped for 15 min at 65 °C and 30 min at 37 °C on a roller with a commercial stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific). The software ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the obtained bands by densitometry. Since the band corresponding to the immature form of cathepsin D (CTSD imm) was stable during myogenesis, the expression of this protein was used as a loading control to normalize the expression of all the other proteins analyzed.

**Statistical analyses**

IBM SPSS Statistics v.20 was used to analyze the data. The results are presented as means ± 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. When normality existed, data was subjected to a one-way ANOVA followed by a Tukey or Dunnet T3 *post-hoc* test depending if respectively there was homogeneity of variances or not. Nevertheless, when normality was not assumed, the non-parametric Kruskal-Wallis test was used followed by a Mann-Whitney U test. Similarly, correlations between mRNA and protein levels were established either with a Spearman's rank correlation coefficient ($\rho$) or a Pearson correlation (PC). Differences were considered significant at $p<0.05$.

## Results

### Gene and protein expression profiles of proteolytic markers during myogenesis

The transcriptional profile of several members of the three endogenous proteolytic systems was studied in gilthead sea bream myocytes at days 2, 4, 8 and 12 of culture. On day 2, activated mononucleated myoblasts cells are undergoing active proliferation to become myocytes (day 4). Then, cells subsequently differentiate and fuse to form small myotubes (day 8), and later on (day 12) some large myotubes can be observed (Fig 1).

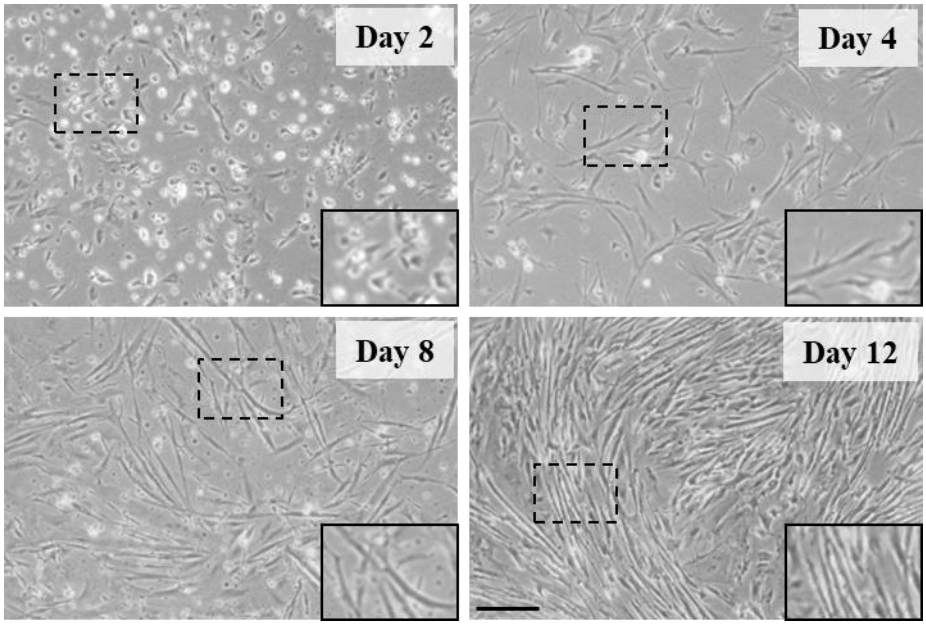



**Fig 1. Representative images of gilthead sea bream cultured myocytes at days 2, 4, 8 and 12 of development.** Images were taken with an EOS 1000D Canon digital camera coupled to an Axiovert 40C inverted microscope (Carl Zeiss, Germany). Objective: 10x. Scale bar: 50 μm. Insets in each image are enlarged views of cells from each panel.

Concerning the cathepsins gene expression, although the profile of the two *ctsd* paralogs throughout the culture was quite similar, *ctsda* showed increased mRNA expression at day 8 compared to the other days, while the changes observed in the expression of *ctsdb* were

not significantly different (Fig 2A). *ctsb* and *ctsl* showed as well a similar profile to that of *ctsdb*, with *ctsl* being significantly down-regulated in the last stage of myocyte differentiation (Fig 2B). Regarding calpains gene expression, a significant decrease during myogenesis was observed for *capn1*, *capn3* and *capns1b*, while *capn2* and *capns1a* remained stable (Figs 2C and 2D). In the case of the UbP members, the gene expression data showed that while ubiquitin E3 ligases (*mafbx* and *murf*) significantly increased up to day 8 to decrease afterwards (Fig 2E), *ub* remained stable and the proteasome beta-type subunit *n3* was significantly decreased along with myogenesis (Fig 2F).

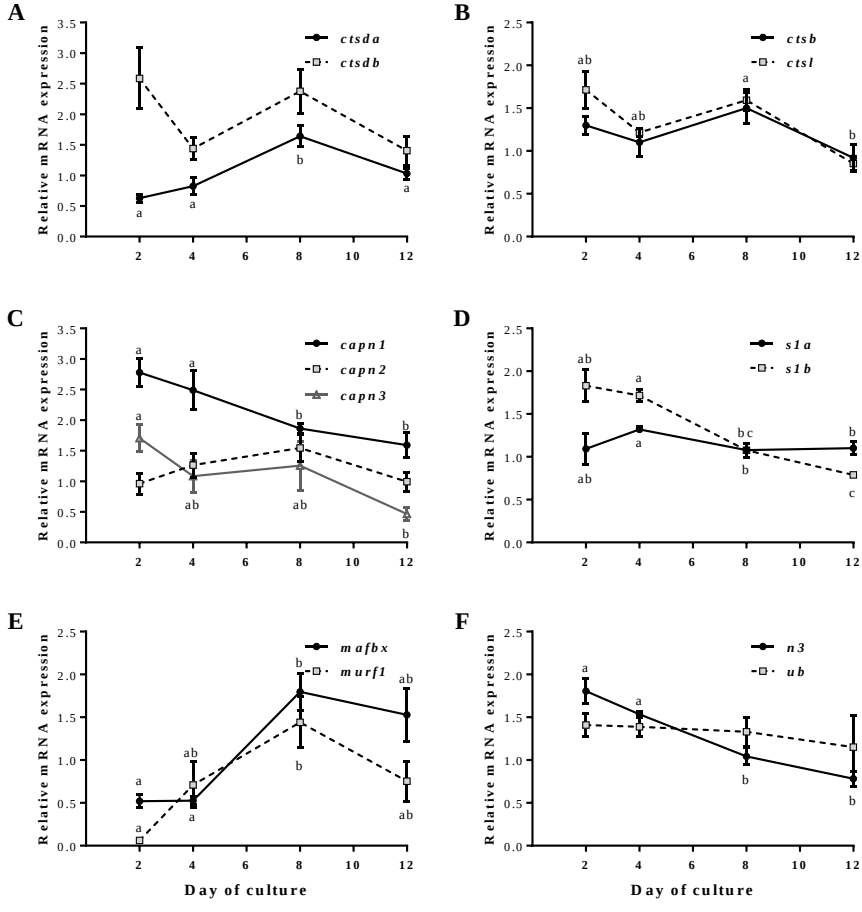



**Fig 2. Cathepsins, calpains and ubiquitin-proteasome members mRNA levels during *in vitro* myogenesis in gilthead sea bream.** Quantitative gene expression relative to the geometric mean of *β-actin*, *rps18* and *ef1α* of (**A**) *ctsda* and *ctsdb*, (**B**) *ctsb* and *ctsl*, (**C**) *capn1*, *capn2* and *capn3*, (**D**) *capns1a* (*s1a*) and *capns1b* (*s1b*), (**E**) *mafbx* and *murf1*, and (**F**) *n3* and *ub*. Results are shown as means ± SEM (n = 4 independent cultures). Different letters indicate significant differences at $p<0.05$.

In contrast to the gene expression results, the immunoblotting data did not show any significant differences. In this sense, the protein levels of the immature form of CTSD remained very stable throughout myocyte differentiation, although both the mature and intermediate enzymes showed a tendency to increase at day 8 (Fig 3). MAFbx presented as well a peak on protein expression at day 8 of culture (Fig 4C), whereas both CTSL and CAPN1 were gradually increased reaching a maximum of expression at day 12 (Figs 4A and 4B).

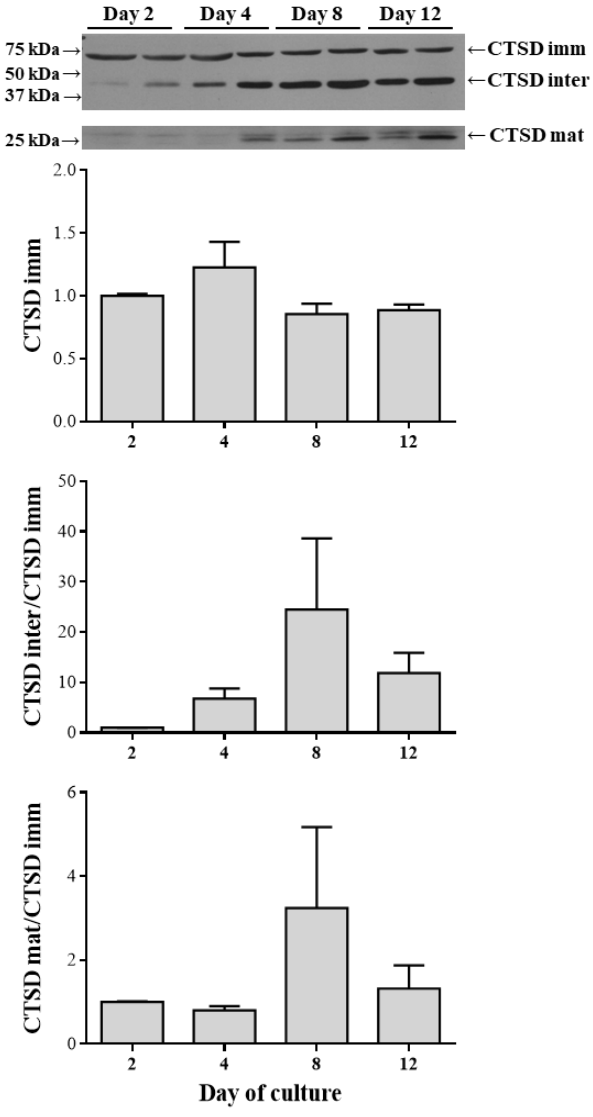



**Fig 3. Cathepsin D protein abundance in gilthead sea bream during *in vitro* myogenesis.** Representative Western blot showing the immature (top, CTSD imm), intermediate (middle, CTSD inter) and mature (bottom, CTSD mat) forms at days 2, 4, 8 and 12 of myocytes culture. The densitometric data for CTSD inter and CTSD mat was normalized to the corresponding CTSD imm band. Results are shown as means ± SEM (n = 3 independent cultures). Note: Although all three bands are from the same Western blot, the mature form is shown separated because the image comes from a longer exposed film for better visualization.

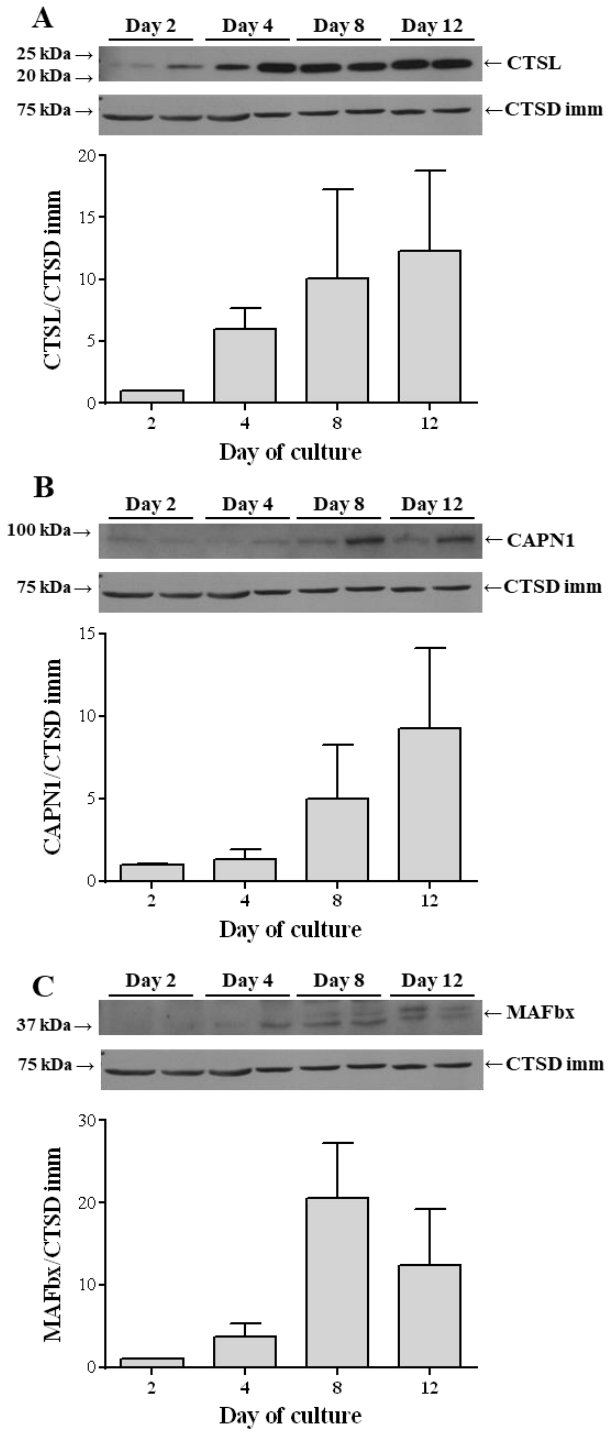



**Fig 4. Cathepsin L, calpain 1 and MAFbx protein abundance in gilthead sea bream cultured myocytes.** Representative Western blots from (**A**) CTSL, (**B**) CAPN1 and (**C**) MAFbx at days 2, 4, 8 and 12 of myocytes culture development. The densitometric data was normalized relative to the corresponding cathepsin D immature form (CTSD imm). Results are shown as means ± SEM (n = 3 independent cultures).

**Correlation between gene and protein expression of proteolytic markers during myogenesis**

Despite the absence of significant changes on protein expression during myogenesis, when the data were plotted against the corresponding gene expression levels, a significant positive correlation was found between *ctsda* and mature CTSD (Fig 5A), *ctsda* and intermediate CTSD ($R^2$=0.4399, PC=0.662, p=0.019; S1A Fig) and *mafbx* with MAFbx (Fig 5C). On the other hand, a significant negative correlation was found between *ctsl* mRNA and CTSL protein levels (Fig 5B), whereas also a negative but non-statistically different correlation was found between *capn1* and CAPN1 ($R^2$=0.1116, $\rho$=-0.500, p=0.098; S1B).

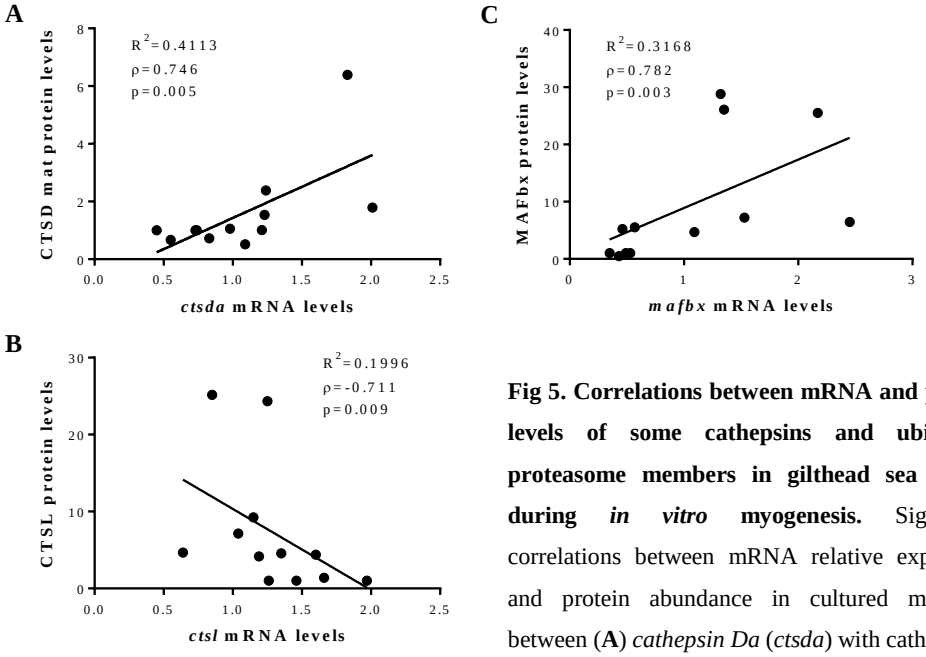
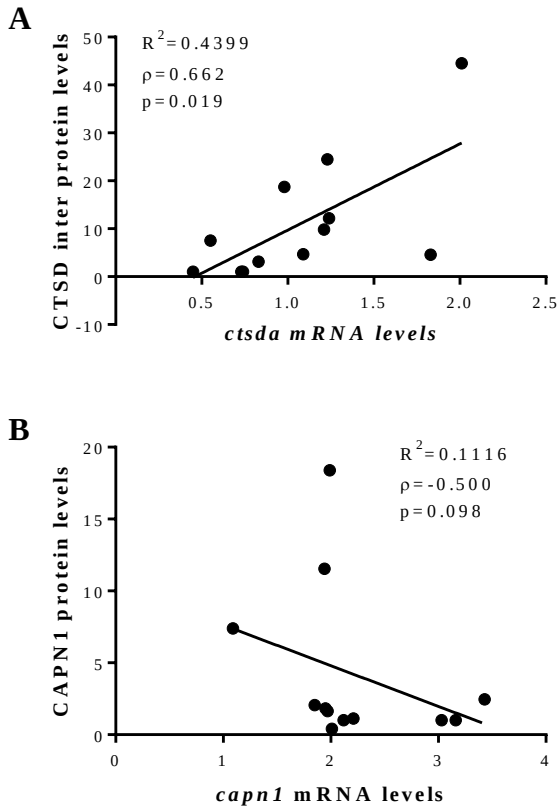



**Fig 5. Correlations between mRNA and protein levels of some cathepsins and ubiquitin-proteasome members in gilthead sea bream during *in vitro* myogenesis.** Significant correlations between mRNA relative expression and protein abundance in cultured myocytes between (**A**) *cathepsin Da* (*ctsda*) with cathepsin D mature form (CTSD mat), (**B**) *cathepsin L* (*ctsl*) with CTSL and (**C**) *muscle atrophy F-box* (*mafbx*) with MAFbx. Data are from n = 3 independent cultures. The $R^2$ of the linear regression, the Spearman's rank correlation coefficient ($\rho$) and the p-value are shown.




**S1 Figure. Correlations between mRNA and protein levels of some cathepsins and calpains in gilthead sea bream during in vitro myogenesis.** (**A**) *cathepsin Da* (*ctsda*) with cathepsin D intermediate form (CTSD inter), and (**B**) *calpain 1* (*capn1*) with CAPN1. Data are from n = 3 independent cultures. The $R^2$ of the linear regression, the Spearman's rank correlation coefficient ($\rho$) and the p-value are shown.

## Proteolytic genes expression regulation by recovery or deficiency in selected amino acids

The expression of all cathepsin genes studied remained unchanged when the culture medium was supplemented with a cocktail to recover the AA levels at day 4 (Fig 6A). Similarly, differences were not observed for the calpains *capn1*, *capn2* and *capns1a* (Fig 6B). Contrarily, AA recovery caused a significant decrease on *capn3* and *capns1b* gene expression (Fig 6B) and the same effect was found for *mafbx* and *murf1* (Fig 6C).

Nevertheless, this response to recovered AA was not general to all the UbP genes because *ub* was not affected and *n3* was significantly increased.

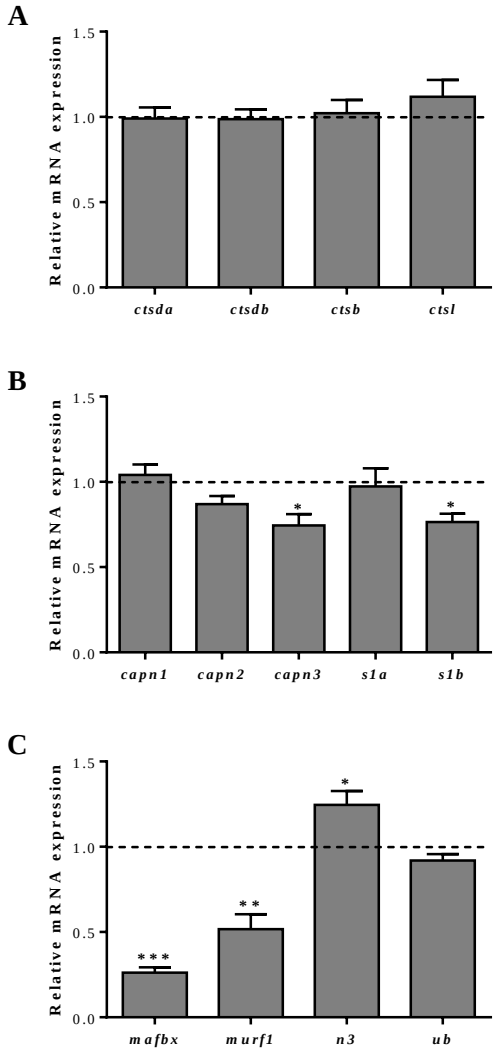



**Fig 6. Effects of amino acids (AA) recovery on proteolytic molecules gene expression in gilthead sea bream cultured myocytes.** Quantitative gene expression of (**A**) cathepsins (*ctsda*, *ctsdb*, *ctsb*, *ctsl*), (**B**) calpains [*capn1*, *capn2*, *capn3*, *capns1a* (*s1a*), *capns1b* (*s1b*)] and, (**C**) UbP members (*mafbx*, *murf1*, *n3*, *ub*) relative to the geometric mean of *ef1α* and *rps18* in day 4 cultured myocytes supplemented with a cocktail of AA for 6 h after a 12 h starvation period. Results are shown as fold change relative to the control condition (cells maintained without AA for the 18 h period including starvation and treatment), represented by the dotted line. Means ± SEM (n = 4-7 independent cultures). Asterisks indicate significant differences compared to the control (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Next, the deficiency of leucine or lysine on the proteolytic gene markers expression through *in vitro* myocytes development was examined (Fig 7). Deficiency in leucine significantly decreased *ctsb* and *ctsl* gene expression in day 2 myocytes (Fig 7A). Otherwise, lysine deficiency did not provoke such an inhibitory effect and contrarily at day 8 *ctsb* expression resulted significantly increased.

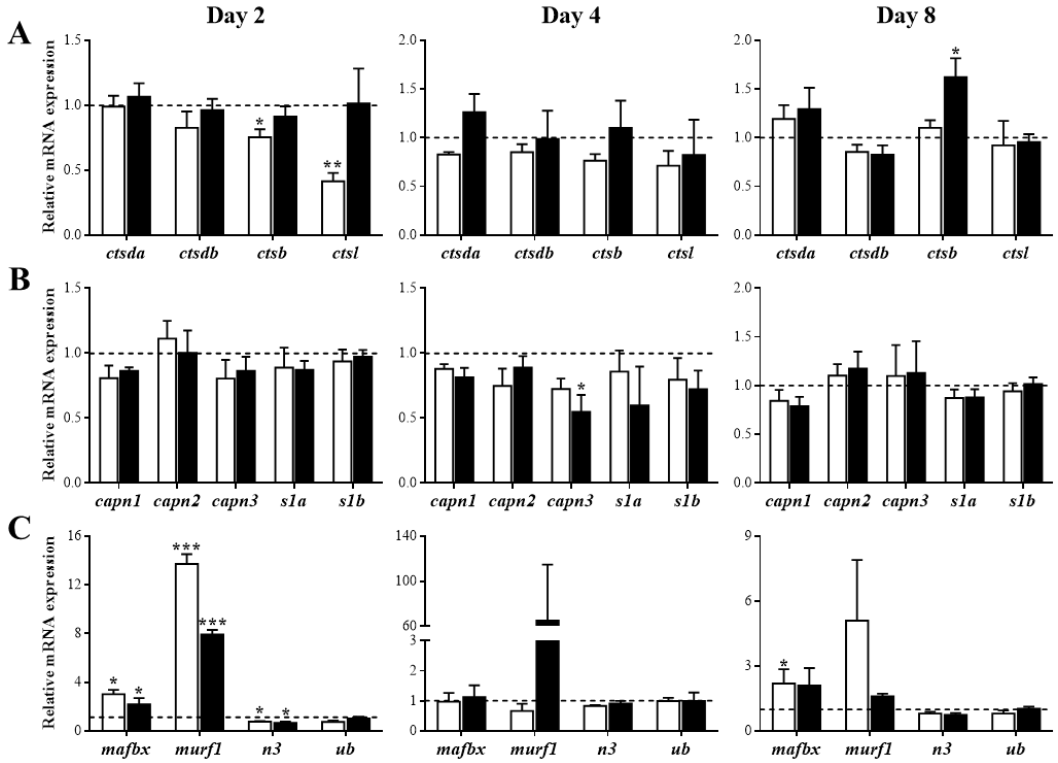



**Fig 7. Effects of leucine or lysine deficient media on proteolytic molecules gene expression in gilthead sea bream cultured myocytes.** Quantitative gene expression of (**A**) cathepsins (*ctsda*, *ctsdb*, *ctsb*, *ctsl*), (**B**) calpains [*capn1*, *capn2*, *capn3*, *capns1a* (*s1a*), *capns1b* (*s1b*)] and (**C**), UbP members (*mafbx*, *murf1*, *n3*, *ub*) relative to the geometric mean of *ef1α* and *rps18* in myocytes at days 2, 4 or 8 of culture after incubation from day 1 (for samples at days 2 and 4) or day 7 (for samples at day 8) with a growth medium deficient in leucine (open bars) or lysine (filled bars). Data are shown as fold change relative to control condition (growth medium without AA deficiencies), represented by the dotted line. Means ± SEM (n= 3-4 independent cultures). Asterisks indicate significant differences compared to the control at each time (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Furthermore, AA limitation provoked little effects in the gene expression of calpains and only *capn3* was significantly decreased at day 4 in lysine deficient medium (Fig 7B). Among the UbP genes, *mafbx* and *murf1* were the most affected (Fig 7C) with significant up-regulation at day 2 in response to both deficiencies, and at day 8 for *mafbx* when incubated in a medium deficient in leucine. Moreover, *ub* gene expression was not affected at any time upon any condition, while *n3* was significantly decreased after two days in both AA deficiencies.

## Discussion

The first objective of this study was to analyze the mRNA and protein levels of various cathepsins, calpains and UbP members throughout *in vitro* myogenesis in gilthead sea bream in order to unravel the phase in which those systems are required for the adequate progression of the process. Second, we assessed the involvement of AA regulating the gene expression of these three catabolic systems' members to define how crucial they are and to consider that for fish feeds formulation.

### Characterization of proteolytic markers gene and protein expression during gilthead sea bream myogenesis

The comparison of protein and gene expression of selected proteolytic members revealed the intricate control of these factors. In this sense, correlation analysis for cathepsin D and MAFbx confirmed a parallelism between gene and protein levels, while in the case of cathepsin L and calpain 1 the results indicated an opposite pattern. Such negative

correlation could indicate a complex network of mRNA regulation at different levels: transcription, translation and degradation. The same opposite pattern for calpain 1 was observed during L8 rat myoblast fusion [36, 37] with increase of CAPN1 protein abundance in the maturation phase as in this study. Besides, it is interesting to note that even without showing significant differences, most of the molecules studied presented their highest protein levels at the end of myogenesis, suggesting an active role for these enzymes in muscle stabilization/consolidation. This means that these proteolytic molecules might be essential for the remodeling that occurs during muscle formation (i.e. breaking of the cytoskeletal/plasma membranes linkages necessary to create points for myoblast fusion [13, 38]).

Concerning gene expression, the data suggest that cathepsins would have greater importance during the early differentiation phase of myogenesis. In agreement with that, Colella et al. [37] found that *ctsb* gene expression decreased after fusion of myotubes in the L6 rat myogenic line, and Ebisui et al. [39] that the differentiation of C2C12 myoblasts involved up-regulation of lysosomal cathepsins. Contrarily, in chick myoblasts, *ctsb* showed the highest mRNA levels at the proliferative phase [40]. In fish, little information exists, but during salmon myocytes development, Bower and Johnston [41] described the increase of *ctsl1* expression with a peak at day 8, followed by a decrease at day 11 as in our study, and a new increase later at days 17 or 20, suggesting overall a relevant role for CTSL1 in differentiation and formation of myotubes.

Regarding calpains, in the present study expression of *capn1*, *capn3* and *capns1b* decreased progressively during myogenesis but *capn2* and *capns1a* remained stable. Similarly, Nakashima et al. [40] found in chick myoblasts a progressive decrease of *capn1* gene expression through *in vitro* development; whereas in rat muscle primary culture, Stockholm

et al. [42] found that *capn1* and *capn2* increased while *capn3* decreased, indicating that the various calpains can be regulated in an opposite way as it occurs in our model. Moreover, Van Ba and Inho [43] also demonstrated that CAPN1 is involved in proliferation and survival during myogenesis in bovine muscle cells. Overall these data indicate that although differences exist among the different vertebrate groups, the main trend is to observe higher gene expression of calpains at the early myogenic stages.

The E3 ubiquitin ligases, MAFbx and MuRF1, are important members of the UbP system. There is evidence that MuRF1 is necessary for the initiation and stabilization of myogenesis [44], being its actions located mainly in the cytoplasm of muscle cells, where it recognizes myofibrillar proteins, such as myosin heavy chain (MHC), and targets them for breakdown [10, 45]. In gilthead sea bream, *mhc* gene expression increased up to day 9 in cultured myocytes and became stable afterwards [32], which is parallel to *murf1* expression and supports also in this species the functional relationship of these molecules. On the other hand, MAFbx is essential for myogenic stem cell function in adult skeletal muscle, as it identifies and targets for ubiquitination several transcription factors with key roles in the control of skeletal muscle development (i.e. myogenic differentiation 1 (MyoD1) or myogenin) [45–47]. García de la serrana et al. [32] found for *myod2* expression in gilthead sea bream myocytes a profile opposite to *mafbx*. This opposed relationship can be explained by the fact that at the start of development the stem cells have to determine their fate and so *myod* levels have to be high, while contrarily, when muscle cells become differentiated, MyoD is no longer needed, and its expression decreases probably due to the up-regulation of *mafbx*. Overall, the profiles of both E3 ligases are similar, which agrees with the findings of Spencer et al. [44] and Perera et al. [48] in mouse skeletal muscle and C2C12 cells, respectively demonstrating that *murf1* is required for myoblast differentiation and myotube

fusion, pointing out very well the conserved role of this UbP molecule as well in muscle development.

Concerning the other members of the UbP system, it is well accepted that *n3* is a good marker of proliferation [49, 50], which is in agreement with it showing the same pattern of gene expression as that reported for the proliferation marker *pcna* in gilthead sea bream [29, 32]. Finally, Nakashima et al. [40] observed a significant reduction on *ub* gene expression during chicken myoblast differentiation, although we found it unaltered in gilthead sea bream myocytes. In support of this absence of changes in gene expression, Kimura and Tanaka [51] suggested that ubiquitin plays multiple roles controlled by complex regulatory mechanisms to actually maintain its levels stable.

In summary, as far as we know, the present study shows for the first time in cultured fish myocytes the expression of several proteolytic members that seems to be in agreement with a more relevant role of calpains during the proliferative phase of myogenesis and of cathepsins and the UbP system in muscle cells differentiation. This in concordance with the more anabolic aspect of calpains in comparison to cathepsins and the UbP system, since they do not degrade proteins up to small peptides or AA, but only disassemble the sarcomeric structure of the muscle [10, 13, 15, 44]. Moreover, the expression of *ctsb*, *ctsl*, *ctsdb*, *mafbx* and *n3* was reported greater in the muscle of fingerlings than in juvenile or adult gilthead sea bream, pointing out a major role for these two endogenous systems (cathepsins and UbP) when the myogenic process is more active [22].

**Regulatory effects of recovery or deficiency of selected AA in proteolytic markers gene expression in gilthead sea bream myocytes**

Previous studies have demonstrated in gilthead sea bream that almost all cathepsins and UbP system-related genes are up- and down-regulated during fasting and refeeding, respectively [22], and similar results were observed, although to a lesser extent, with regards to calpains [16]. Besides, it has been shown that forced swimming provokes in gilthead sea bream up-regulation of cathepsins and UbP members [38], supporting that muscle remodeling is taking place under both catabolic and anabolic conditions. In this sense, we have found now in myocytes of the same species that specifically the AA seem to have an important role controlling proteolytic systems, although mostly the expression of UbP members.

In agreement with that, Cleveland and Weber [4] found that *ctsd* and *ctsl* expression was not affected by a leucine treatment in rainbow trout myocytes; while contrarily, lysine supplementation had an inhibitory effect on ALS activity in C2C12 myotubes [52]. With regards to calpains, response to AA in this study was observed only for *capn3* and *capns1b*, the same genes modified in response to fasting and refeeding in the same species [16]. In the case of halibut (*Hippoglossus hippoglossus*) and channel catfish (*Ictalurus punctatus*), skeletal muscle *capn3* mRNA was at its lowest level during fasting, and highest in refeeding [53, 54], providing overall these data an evidence for species-specific differences concerning the activity of this gene. Notwithstanding, considering that calpain 3 is a muscle specific regulator of other calpains' expression and activity, as well as its levels have been correlated with bovine and ovine muscle tenderness [55, 56], these variable responses in fish deserve to be further investigated.

Moving to the expression of UbP genes affected by AA, it is interesting to emphasize that in our study, AA levels recovery decreased the expression of *mafbx* and *murf1* but increased *n3* whereas contrarily, leucine and lysine deficiencies stimulated, mainly at day 2, *mafbx* and *murf1* expression while inhibiting *n3*. These results suggest that both MAFbx and MuRF1 could be increasing the amount of proteins sent to the proteasome when AA are lacking; however, the opposite response of *n3* and the stable *ub* expression might be indicating that the flux of ubiquitinated proteins through the proteasome is constrained (or slowed down). Then, these proteins would be probably degraded by autophagy, as it has been observed in mammals, demonstrating that there is an important cross-talk regulation within the proteolytic systems [56]. This hypothesis makes even more sense in fish, in which in contrast to mammals, the ALS is responsible for around two to three times more protein degradation than the UbP system [8].

Furthermore in *in vitro* models, AA limitation increases proteolysis in an UbP-dependent manner in C2C12 myotubes, although an increase in AA or leucine alone down-regulates protein degradation and the expression of components of the UbP pathway [57]. However, also in C2C12 cells, the expression of *murf1* was not affected after incubation with lysine [52]. Similarly, in rainbow trout myocytes, leucine supplementation did not affect *murf1* while serum deprivation increased the expression of the ubiquitin ligases *mafbx*, *fbx25* and *murf1* [4]. In the case of salmon muscle cells, an starving of AA caused down-regulation of *mafbx* [31], whereas in a previous study analyzing two different splice variants in the same cell model, it was demonstrated that serum and AA starvation resulted in a 6-fold increase in the expression of *mafbx-α*. This isoform expression declined subsequently in response to an AA treatment [58], but *mafbx-β* appeared to be less sensitive to AA since its expression remained similar to the control, and only was altered when insulin or insulin-

like growth factors (IGFs) were present in the culture media. Probably, this differential response between isoforms is due to their specific roles during salmon *in vitro* myogenesis, where *mafbx-α* gene expression is highest in differentiated myotubes (similarly to our data), and *mafbx-β* mRNA is more abundant at myoblast stage [58]. Moreover, serum depletion and specifically AA withdrawal in rainbow trout myocytes induced the expression of the autophagy-proteasome genes (*lc3b*, *gabarapl1*, and *atg4b*) [7, 30], suggesting an important role for the AA released by muscle mobilization during fasting, to regulate proteolytic genes.

In this sense, considering our experimental model, Vélez et al. [28] after AA recovery found increases on proliferation, differentiation and protein synthesis markers such as *pcna*, *myogenin*, *tor*, *4ebp1* and *70s6k*, while the expression of *foxo*, a factor involved in the activation of the proteolytic pathway, remained unaffected. After 2 days of leucine limitation, Azizi et al. [29] found that the expression of the AA deficiency indicator *chop* was increased, whereas *4ebp1* and *foxo* diminished. Furthermore, after 8 days of lysine deficiency, an increased expression of other two AA-limitation markers (i.e. *atf4* and *as*) was observed; and also, a decrease in important proteogenic/anabolic pathways' molecules including members of the IGF system (i.e. *pcna*, *igf-1*, *igf-2*, *igf-1rb*, *akt*, *erk* and *70s6k*) [29]. These data confirm an overall negative effect of the reduced AA levels, especially lysine, on protein turnover and thus, muscle growth in gilthead sea bream, which is supported by our results.

The present study provides new information about the potential role of key members of the endogenous proteolytic systems (cathepsins, calpains and UbP) in gilthead sea bream cultured muscle cells. We can suggest that there is a functional distribution between the

different proteolytic system molecules throughout the *in vitro* development of muscle cells at least until the phases of myocyte differentiation and small myotube formation (day 8). Besides, it is interesting to note the up-regulation of *mafbx* and *murf1* in response to AA deficiencies and their down-regulation with AA recovery and the reverse response of *n3*, pointing out to an efficient and complementary role of these UbP system members to AA supply.

In summary, the research on the function of proteolytic systems in fish offers interesting information on the evolution of myogenesis regulation and the effects of AA on such process that can have valuable application in aquaculture in order to optimize diet composition for this species.

**Acknowledgments**

The authors would like to thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the Faculty of Biology for the supply and maintenance of the fish, as well as José Rives for his help with the cell cultures and laboratory analyses.

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# ARTICLE III

## $\beta_2$-adrenoceptor agonists' effects in gilthead sea bream (*Sparus aurata*) cultured muscle cells


Frontiers in Endocrinology (Under review)

Article III

# $\beta_2$-adrenoceptor agonists' effects in gilthead sea bream (*Sparus aurata*) cultured muscle cells

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**Running head:** $\beta_2$-agonists effects in fish myocytes

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**Keywords:** Fish, *in vitro* myocytes, cell proliferation, IGFs, lipolysis, salmeterol, formoterol, adrenoceptors

**Abstract**

$\beta_2$-adrenoceptors are a subtype of G-protein coupled receptors whose activation leads to increased protein synthesis and decreased degradation in mammalian skeletal muscle, causing hypertrophy. In different species such as pigs, beef cattle, or lambs, the positive effects of $\beta_2$-agonists increasing growth gain and reducing fat depots has been demonstrated. Hence, the incorporation of such agonists in the diet as additives has been successfully used as means to optimize livestock production, but information is scarce with regards to fish species. This work evaluates *in vitro* the effects of the $\beta_2$-agonists noradrenaline (NA), formoterol (FOR) and salmeterol (SALM) in primary cultured muscle cells of gilthead sea bream in order to see its potential use in the aquaculture industry. For that, activation of signaling pathways, cell development and expression of the most important growth-related genes were analyzed in day 4 myocytes. The three agonists increased cAMP levels, TOR phosphorylation, and the proportion of proliferating cell nuclear antigen (PCNA)-positive cells, in agreement with raised *pcna* mRNA levels. Thus, demonstrating that these cells are $\beta_2$-adrenergic-sensitive in fish, and supporting enhanced cell proliferation and hyperplasia. Besides, the expression of the myogenic factor *myf5* was significantly down-regulated, suggesting that the cells were already destined to the muscular linage. With regards to insulin-like growth factors, the incubation with $\beta_2$-agonists up-regulated *igf-1* and *igf-2* mRNA levels, proposing an additional anabolic effect through their local production. Furthermore, SALM treatment up-regulated expression of the lipases *hsl* and *lipa* and the β-oxidation marker *cpt1a*, and all three agonists increased mitochondrial dehydrogenase *hadh* mRNA levels. These data correspond with a situation of enhanced lipolytic and β-oxidation capacity, a fact supported by the higher glycerol released into the media in response to the agonists. Overall, these results demonstrate a

hyperplastic growth condition and a favorable protein/fat ratio profile upon these treatments; suggesting that $\beta_2$-agonists (especially SALM) may be considered good candidates to optimize the growth and flesh quality in aquaculture species such as gilthead sea bream.

## 1. Introduction

In vertebrates, most actions of the sympathetic nervous system are mediated by catecholamines, the most important molecules to make a "fight-or-flight" response if a critical situation or stimulus arises, and can also modulate other processes as energy metabolism in a non-stressful situation (1). In fact, catecholamines can bind to a variety of adrenergic receptor types (or adrenoceptors, ARs) to induce diverse responses on target cells through activating different signaling pathways. Nowadays, six α-ARs and at least three β-ARs subtypes have been identified in various tissues, where they are present in different proportions [Reviewed by Lynch and Ryall (2), and Ahles and Engelhardt (3)]. ARs are included within the guanine nucleotide-binding G-protein coupled receptor family of rhodopsin receptors. G-proteins are located in the cytoplasm and consist of three subunits, the Gα and the dimer Gβγ (4). Briefly, once the receptor is activated by the union with the ligand, the Gα subunit binds GTP and then it separates from the dimer Gβγ, acquiring the ability to activate, for example, the protein kinase A (PKA) transduction pathway by mediating the action of the enzyme adenylyl cyclase (2, 5). When PKA is activated by increased cAMP levels, it can phosphorylate some proteins to regulate their activity and the expression of several genes involved in myogenesis (e.g., the myogenic regulatory factors, MRFs), proliferation and differentiation (6).

In skeletal muscle the most abundant ARs subtype is the $\beta_2$, representing up to 99% of the total ARs in this tissue (7-9). This subtype has special relevance considering that the Gβγ dimer can activate the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway independently of the Gα action (10). It is commonly known that the PI3K/AKT pathway, which indirectly activates the target of rapamycin (TOR) transduction cascade, is involved in modulating different processes like protein synthesis, cell proliferation and even gene transcription. Therefore, due to the ability to activate both PKA and PI3K/AKT signaling pathways, it has been recognized that $\beta_2$-ARs activation by corresponding agonists can be an option to regulate the cell cycle, increase protein synthesis and decrease degradation, improve muscle regeneration and repair after injury, and thus overall, to increase skeletal muscle mass by hypertrophy [Reviewed by Lynch and Ryall (2)]. Besides the effects of $\beta_2$-ARs activation controlling muscle growth and development, this is also modulated by the growth hormone /insulin-like growth factors (IGFs) axis in vertebrates, including fish. In fact, it has been well demonstrated in fish that the IGFs (I and II) can modulate *in vitro* myogenesis and proliferation (11, 12), stimulate protein synthesis (13), and also play an important role regulating cell metabolism processes (e.g. nutrients uptake), to in the last term increase muscle growth [reviewed by Fuentes et al. (14), Johnston et al., (15), and Vélez et al. (12)]. Overall, this endocrine system is of special importance considering that in fish the IGFs perform functions that in mammals are carried out mainly by insulin [reviewed by Vélez et al. (12)]. In addition, the main endogenous proteolytic systems (i.e., ubiquitin-proteasome (UbP), calpains, and cathepsins), besides being involved in protein degradation, play an important role in muscle regeneration and other essential processes for muscle growth, such as the fusion of myofibers (16-20).

In mammalian reserve tissues, such as the adipose tissue, it has been observed that $\beta_2$-ARs activation induces the activity of the hormone-sensitive lipase (HSL) to initiate lipolysis in order to mobilize energy to the muscle (7, 21, 22), and for that reason, $\beta_2$-agonists have also been named as "repartitioning agonists". These muscle anabolic effects are very interesting for the livestock industry, since production costs can be reduced and the quality of the final product improved (23, 24). In this sense, many authors have demonstrated that the incorporation as additives in the diet of $\beta_2$-agonists induces positive effects increasing growth gain and reducing fat depots in different species, such as pigs, beef cattle, or lambs (24-26). Notwithstanding, $\beta_2$-agonists have been traditionally used as bronchodilators for the treatment of human respiratory diseases such as asthma (27). However, the discovery by chance that a new generation of $\beta_2$-agonists (e.g. formoterol and salmeterol), characterized by a second benzene ring bounded with a long carbon chain, produce a longer duration effect of the $\beta_2$-ARs, made them even more interesting than conventional ligands (i.e. noradrenaline) [Reviewed by Waldeck (28)]. In this sense, it has been shown that when these new $\beta_2$-agonists were administered at lower doses preserve their anabolic properties in comparison with the classical ones (2).

In fish, in a similar way than in mammals, both α- and β-ARs have been characterized (1, 29, 30). Some authors have demonstrated *in vivo* that $\beta_2$-agonists administration in fish increases body weight, potentiates protein synthesis, and reduces visceral fat deposition (31-36). These effects are also of potential importance for the aquaculture industry; however, to our knowledge, data is not available concerning muscle sensitivity and the effects of $\beta_2$-agonists in gilthead sea bream (*Sparus aurata* L.), one of the most important farmed species in the Mediterranean. In this sense, our group has developed a primary culture of muscle cells from this species (37), and nowadays a wide knowledge on the

regulation of the myogenic and metabolic processes of this *in vitro* model exist, making of it a good tool for the study of specific $\beta_2$-agonists effects in fish muscle [Reviewed by Vélez et al. (12)].

The aim of the present study was to investigate, for the first time in primary cultured fish myocytes, the hypothesis that $\beta_2$-agonists treatment activates ARs, to then promote protein synthesis and lipolysis, through similar pathways than those in mammals. To this end, activation of the signaling pathways by $\beta_2$-agonists, cell proliferation and expression of the most important growth, proteolysis and lipid metabolism-related genes were analyzed in gilthead sea bream myocytes.

## 2. Material and methods

### 2.1. Animals

Gilthead sea bream (*Sparus aurata* L.) juveniles from 5 to 20 g body mass were obtained from a commercial fishery located in the Spanish East coast (Piscimar, Andromeda Group, Burriana) and maintained in 0.4 $m^3$ tanks at the facilities of the School of Biology at the University of Barcelona (Spain), with a temperature-controlled seawater recirculation system (23 ± 1 ºC) and a 12 h light: 12 h dark photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (Skretting, Burgos, Spain) and before the isolation of muscle cells, fish were fasted for 24 h, sacrificed by a blow to the head, weighed and sterilized by immersion in 70% ethanol for 0.5 to 1 min.

### 2.2. Myocyte cell culture and experimental treatments

A total of twenty independent primary cultures of muscle satellite cells were performed as described by Montserrat et al. (37). Briefly, epaxial white muscle tissue collected in cold Dulbecco's Modified Eagle's Medium (DMEM), with 9 mM $NaHCO_3$, 20 mM HEPES, 0.11 % NaCl, and 1 % (v/v) antibiotic/antimycotic solution, supplemented with 15 % (v/v) horse serum was first minced and then enzymatically digested once with 0.2 % collagenase type IA and twice with 0.1 % trypsin solution. Subsequently, the suspension was filtered through a 100 µm, and a 40 µm nylon cell strainer, and the obtained cells counted, diluted in growth media (DMEM supplemented with 10 % fetal bovine serum), plated to a final density of $0.2\text{-}0.25 \cdot 10^6$ cells/$cm^2$ in all cases and maintained at 23 ºC. Cells were cultured in 24 well-plates for cAMP analysis (1.8 $cm^2$/well), 6 well-plates (9.6 $cm^2$/well) in the case of Western blot, quantitative real-time PCR (qPCR) and glycerol assays, and in 12 well-plates containing glass coverslips (2.55 $cm^2$ surface) for immunocytochemistry analyses.

Myocytes at day 4 of culture development were used for the different studies and treatments, since at this day, the cells retain the ability to proliferate but also have the capacity to start fusing and differentiating (37). Due to the short duration of the cultures, analyses to discard mycoplasma contamination were not performed.

Before the treatments with the agonists, cells were incubated for 2 h with DMEM containing 0.02 % fetal bovine serum and 1 % antibiotic/antimycotic solution, with the objective of minimizing the proliferative effects of growth factors present in the serum (38, 39). Next, medium was changed, and the cells were maintained during the corresponding times with media alone (Control, CT), or supplemented with 1 µM of noradrenaline hydrochloride (Cat. No. A7256, NA), formoterol fumarate dihydrate (Cat. No. F9552, FOR), or salmeterol xinafoate (Cat. No. S5068, SALM). The incubations lasted for 1 or 3

min in the case of cAMP analysis, 15 or 60 min in the case of Western blot experiments, 6 h for immunocytochemistry, 4 or 18 h for gene expression analyses and finally, 18 h for the glycerol assay. In all cases, after the incubation time passed, cells were washed twice with cold phosphate buffered saline and the samples collected according to the assay to be performed. All reagents were obtained from Sigma-Aldrich (Tres Cantos, Spain), and all plastic ware and glass coverslips were from Nunc (LabClinics, Barcelona, Spain).

### 2.3. cAMP levels assay

Activation of $\beta_2$-ARs was tested through the analysis of cAMP levels as described in Gao et al. (40) using a commercial kit (cAMP Biotrak Enzymeimmunoassay (EIA) RPN2251, GE Healthcare, distributed by Sigma-Aldrich). Once the treatments were stopped by washing, the intracellular content was collected in 200 µL/well of lysis buffer after checking membrane integrity by a microscopic evaluation. The cAMP levels were analyzed in duplicate for each experimental condition and culture (n = 3).

### 2.4. Western blot analysis

Protein homogenates from cells were obtained as described by Codina et al. (41) and quantified (42). Subsequently, 10 µg of protein from each sample were separated by electrophoresis (SDS-PAGE) on 10 % polyacrylamide gel and transferred to a PVDF-FL membrane in transfer buffer as in Vélez et al. (39). After the transfer, the total protein amount was tested with Revert Total Protein Stain solution (Cat. No. 926-11011, Odyssey reagents, Servicios Hospitalarios, Barcelona, Spain) and the membrane was blocked at

room temperature for 1 h (Cat. No. 927-40000, Servicios Hospitalarios). Then, the membranes were incubated overnight at 4 ºC with the primary antibodies of phosphorylated forms diluted in the same blocking buffer. The primary antibodies used [previously validated for gilthead sea bream (39)] were: rabbit polyclonal anti-phospho PKA C (Thr197; Cat. No. 4781), rabbit polyclonal anti-total PKA C-α (Cat. No. 4782), rabbit polyclonal anti-phospho TOR (Cat. No. 2971), all from Cell Signaling Technology (Beverly, MA); and rabbit polyclonal anti-total TOR (Cat. No. T2949. Sigma-Aldrich, Spain). For phosphorylated and total forms of PKA, 1:350 and 1:500 dilutions were used respectively. In the case of TOR, a 1:1000 dilution was used for both forms. After washing (4 times for 5 min), the membranes were incubated for 1 h at room temperature with a goat anti-rabbit fluorescence-conjugated secondary antibody (Cat. No. 925-32211, Servicios Hospitalarios) at 1:10000 dilution. The membranes were rewashed and the bands signal detected at 800 nm with the Odyssey® FC Imaging System (Li-Cor, Alcobendas, Spain). Once the phosphorylated forms were developed, primary and secondary antibodies were removed with stripping buffer (Cat. No. 928-40032) for 20 min at room temperature and then, the membranes were blotted again following the same procedure with the corresponding total forms. Finally, the bands were quantified by Odissey software Image Studio ver. 5.2.5. The Western blot data were analyzed in 4 independent cultures (n = 4).

**2.5. Immunocytochemistry**

Cell proliferation was analyzed using a commercial PCNA staining kit (Cat. No. 93-1143. Life Technologies, Alcobendas, Spain) following the procedures previously reported (39). Briefly, cells were fixed in 4 % paraformaldehyde (Sigma-Aldrich, Spain) for 15 min at room temperature, washed, and post-fixed for 5 min in an ascending series of ethanol (50-

70 %). Next, cells were incubated with the PCNA staining reagents, dehydrated in a graded alcohol series and mounted with histomount. Digital images were acquired using a CC2 camera coupled to a microscope at 40x with analySIS software (Soft Imaging System). The percentage of PCNA-positive cells was calculated by dividing the PCNA-positive stained cells by the total number of nuclei in 10-15 images per coverslip (2 coverslips/culture for each treatment, with 4 independent cultures; n = 4) using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). All images were analyzed by the same researcher in a blinded manner.

### 2.6. Gene expression analyses

#### 2.6.1. RNA extraction and cDNA synthesis

RNA samples of each experimental condition and incubation time were collected in duplicate wells for 5-7 independent cultures (n = 5-7) with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using cell-scrappers, and processed following the manufacturer's protocol. Then, RNA concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain) and the integrity of the samples confirmed in a 1 % (w/v) agarose gel stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). Afterwards, 500 ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain) following the manufacturer's guidelines in order to remove all genomic DNA, and finally the RNA was reverse transcribed using the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain).

### 2.6.2. Quantitative real-time PCR (qPCR)

qPCR gene expression (mRNA) analyses were carried out according to the requirements of the MIQE guidelines (43) using the CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain) and following the procedures and conditions previously described (44, 45). Briefly, reactions were performed in triplicate with 2.5 µL of iTaq SYBR Green Supermix (Bio-Rad), 250 nM (final concentration) of forward and reverse primers (Table 1A and 1B) and 1 µL of diluted cDNA for each sample in a final volume of 5 µL in 384-well plates. Prior to analyses, a dilution curve with a pool of samples was run to confirm reaction specificity and the absence of primer-dimers through the analysis of the melting curve, and to determine the appropriate cDNA dilution. The expression levels of each gene analyzed were calculated by the Pfaffl method (46) relative to the geometric mean expression of the most stable reference genes analyzed (*rps18* and *ef1α*) as determined by the GeNorm algorithm using the Bio-Rad CFX Manager 3.1 software.

**Table 1A. Sequences, annealing temperatures (Ta), GenBank accession numbers and efficiency of the primers used for qPCR.** F: forward. R: reverse.

| Gene | Primer sequences (5'–3') | Ta (ºC) | Accession number | PCR efficiency (%) |
|---|---|---|---|---|
| *rps18* | **F**: GGGTGTTGGCAGACGTTAC<br>**R**: CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 | 95.5 |
| *ef1α* | **F**: CTTCAACGCTCAGGTCATCAT<br>**R**: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | 91.2 |
| *β-actin* | **F**: TCCTGCGGAATCCATGAGA<br>**R**: GACGTCGCACTTCATGATGCT | 60 | X89920 | 97.6 |
| *tor* | **F**: CAGACTGACGAGGATGCTGA<br>**R**: AGTTGAGCAGCGGGTCATAG | 60 | - | 90.2 |
| *4ebp1* | **F**: CCAACCTGCGACTCATCTCT<br>**R**: GTTCCTCTCATCCTCCCACA | 60 | - | 98.5 |
| *70s6k* | **F**: GCACCAGAAAGGCATCATCT<br>**R**: AAGGTGTGGGTCACTGTTCC | 60 | - | 94.4 |
| *akt* | **F**:GCTCACCCCACTCTTCAGAC<br>**R**:AAATTGGGAAATGTGCTTGC | 60 | ERA047531 | 98.3 |
| *pcna* | **F**: TGTTTGAGGCACGTCTGGTT<br>**R**: TGGCTAGGTTTCTGTCGC | 58 | NM_131404.2 | 90.9 |
| *myf5* | **F**: CTACGAGAGCAGGTGGAGAACT<br>**R**: TGTCTTATCGCCCAAAGTGTC | 64 | JN034420 | 96.6 |
| *myod1* | **F**: TTTGAGGACCTGGACCC<br>**R**: CTTCTGCGTGGTGATGGA | 60 | AF478568.1 | 98.3 |
| *myod2* | **F**: CACTACAGCGGGGATTCAGAC<br>**R**: CGTTTGCTTCTCCTGGACTC | 60 | AF478569 | 92.6 |
| *myogenin* | **F**: CAGAGGCTGCCCAAGGTCGAG<br>**R**: CAGGTGCTGCCCGAACTGGGCTCG | 68 | EF462191 | 90.1 |
| *mrf4* | **F**: CATCCCACAGCTTTAAAGGCA<br>**R**: GAGGACGCCGAAGATTCACT | 60 | JN034421 | 104.9 |
| *mstn1* | **F**: GTACGACGTGCTGGGAGACG<br>**R**: CGTACGATTCGATTCGCTTG | 60 | AF258448.1 | 100.2 |
| *mlc2a* | **F**: GCCCCATCAACTTCACCGTCTTT<br>**R**: GGTTGGTCATCTCCTCAGCGG | 60 | AF150904 | 98.7 |
| *mlc2b* | **F**: TCCCTTTGCTATTCTGCCTTC<br>**R**: AAATCAGCCCTATTCCCCATA | 60 | FG618631 | 93.7 |
| *mhc* | **F**: AGCAGATCAAGAGGAACAGCC<br>**R**: GACTCAGAAGCCTGGCGATT | 58 | AY550963.1 | 102.9 |
| *igf-1* | **F**:ACAGAATGTAGGGACGGAGCGAATGGAC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | EF688016 | 105.1 |
| *igf-2* | **F**:TGGGATCGTAGAGGAGTGTTGT<br>**R**:CTGTAGAGAGGTGGCCGACA | 60 | AY996778 | 100.3 |

**Table 1B. Sequences, annealing temperatures (Ta), GenBank accession numbers and efficiency of the primers used for qPCR.** F: forward. R: reverse.

| Gene | Primer sequences (5'–3') | Ta (ºC) | Accession number | PCR efficiency (%) |
|---|---|---|---|---|
| *capn1* | **F**: CCTACGAGATGAGGATGGCT<br>**R**: AGTTGTCAAAGTCGGCGGT | 56 | KF444899 | 106.6 |
| *capns1a* | **F**: CGCAGATACAGCGATGAAAA<br>**R**: GTTTTGAAGGAACGGCACAT | 56 | KF444901 | 97.4 |
| *capns1b* | **F**: ATGGACAGCGACAGCACA<br>**R**: AGAGGTATTTGAACTCGTGGAAG | 56 | ERP000874 | 91.6 |
| *ctsda* | **F**: CCTCCATTCACTGCTCCTTC<br>**R**: ACCGGATGGAAAACTCTGTG | 56 | AF036319 | 93.5 |
| *ctsdb* | **F**: AAATTCCGTTCCATCAGACG<br>**R**: CTTCAGGGTTTCTGGAGTGG | 56 | KJ524456 | 93.6 |
| *mafbx* | **F**: GGTCACCTGGAGTGGAAGAA<br>**R**: GGTGCAACTTTCTGGGTTGT | 60 | ERA047531 | 102.0 |
| *murf1* | **F**: GTGACGGCGAGGATGTGC<br>**R**: CTTCGGCTCCTTGGTGTCTT | 60 | FM145056 | 98.7 |
| *n3* | **F**: AGACACACACTGAACCCGA<br>**R**: TTCCTGAAGCGAACCAGA | 54 | KJ524458 | 93.5 |
| *hsl* | **F**: GCTTTGCTTCAGTTTACCACCATTTC<br>**R**:GATGTAGCGACCCTTCTGGATGATGTG | 60 | EU254478 | 104.9 |
| *lipa* | **F**: ACTACATCGGACACTCTCAAGGAAC<br>**R**: GTGGAGAACGCTATGAATGCTATCG | 60 | JQ308831 | 92.7 |
| *cpt1a* | **F**: GTGCCTTCGTTCGTTCCATGATC<br>**R**: TGATGCTTATCTGCTGCCTGTTTG | 60 | JQ308822 | 92.8 |
| *cpt1b* | **F**: CCACCAGCCAGACTCCACAG<br>**R**: CACCACCAGCACCCACATATTTAG | 60 | DQ866821 | 101.8 |
| *hadh* | **F**: GAACCTCAGCAACAAGCCAAGAG<br>**R**: CTAAGAGGCGGTTGACAATGAATC | 60 | JQ308829 | 92.1 |
| *cs* | **F**: TCCAGGAGGTGACGAGCC<br>**R**: GTGACCAGCAGCCAGAAGAG | 60 | JX975229.1 | 98.1 |
| *cox4* | **F**: ACCCTGAGTCCAGAGCAGAAGTCC<br>**R**: GCCAGTGAAGCCGATGAGAAAGAAC | 60 | JQ308835 | 91.0 |

### 2.7. Glycerol release assay

Glycerol release into the media was analyzed using a commercial enzyme kit (Serum Triglyceride Determination Kit, Cat. No. TR0100, from Sigma-Aldrich, Tres Cantos, Spain) following the manufacturer's recommendations. Briefly, after treatment 100 μL of media were analyzed in duplicate for each experimental condition and culture (n = 5-7).

### 2.8. Statistical analyses

The software IBM SPSS Statistics v.22 (IBM, Armonk, USA) was used to analyze the data, whilst all the figures were prepared with GraphPad Prism v.6.01 (GraphPad Software, La Jolla California USA, www.graphpad.com). First, normal distribution was tested by Shapiro-Wilk analysis and homogeneity of variances was analyzed by Levene's test. Treatments effects among groups and incubation times were tested by two-way ANOVA, followed by Tukey's *post hoc* test when differences for agonist group were found. Moreover, when the interaction between factors was significantly different, the analysis of simple main effects was performed. In addition, when the ANOVA indicated time effects, the difference between the two incubation times tested within each treatment was analyzed using a Student's *t*-test. As an exception, both the percentage of PCNA-positive cells and also the quantification of glycerol release were analyzed by one-way ANOVA due to the fact that in these analyses only one incubation time was tested. Data is presented as mean ± SEM and statistical differences were considered significant when $p$-value < 0.05.

## 3. Results

### 3.1. Receptors and signaling pathways activation

The activation of the ARs-downstream signaling pathway through the Gα subunit was tested by analyzing both group and time effects on the cAMP levels and phosphorylation of PKA. cAMP levels were significantly increased by NA and FOR treatments in gilthead sea bream myocytes (Fig. 1A). Regarding PKA, its phosphorylation was significantly up-regulated after SALM treatment when compared with CT group, although a time-response difference was also found, with higher PKA phosphorylation after 60 min treatment (Fig.

1B). The activation induced by the Gβγ dimer was tested through the analysis of TOR phosphorylation (Fig. 1C), and results demonstrated a significant stimulatory group effect with the three agonists, and also a significant interaction between group and time in the case of SALM ($F$ (3, 52) = 3.205, $p$ = 0.031). The simple main effects analysis revealed that this interaction caused a significantly higher TOR phosphorylation at 15 min than at 60 ($p$ = 0.006). In addition, the gene expression of several important molecules of the AKT-TOR signaling pathway were analyzed after 4 and 18 h of incubation with the three agonists (Fig. 2). mRNA levels of *tor*, *70s6k* and *akt* were significantly modified with regards to incubation time, being significantly higher at 18 h in the SALM group, as well as *70s6k* was affected upon NA treatment. Moreover, the *akt* gene expression was also significantly different between NA and SALM groups; whereas *4ebp1* expression was not changed by the different treatments.

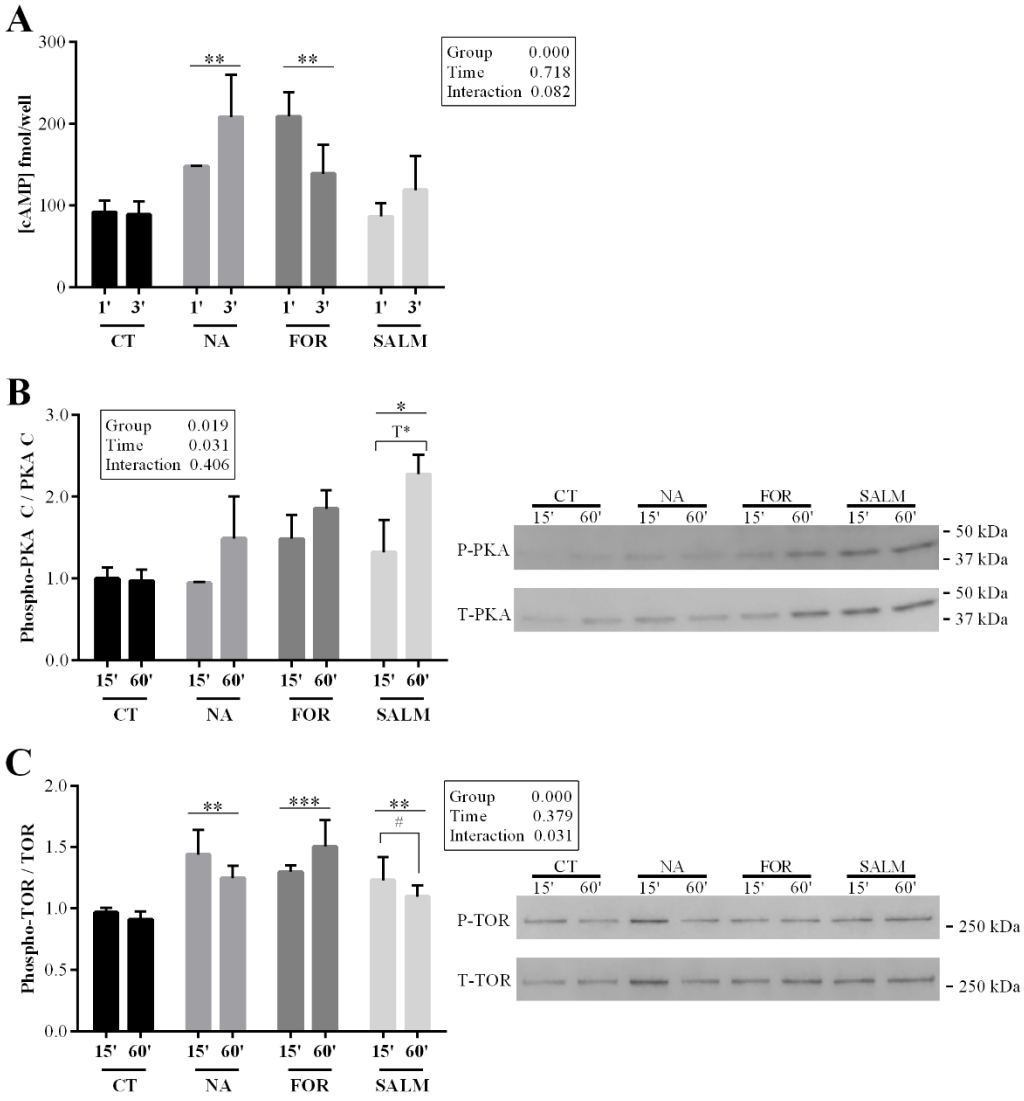



**Fig. 1. β2-adrenoceptors activation in gilthead sea bream cultured muscle cells.** (**A**) cAMP levels in myocytes at day 4 after incubation with the $\beta_2$-agonists noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) for 1 or 3 min. Mean ± SEM (n = 3). Representative Western blot and quantification of (**B**) PKA and (**C**) TOR as effect of $\beta_2$-agonists treatment for 15 or 60 min. Phosphorylated forms (P) band intensity was normalized to total forms (T). Results are presented as fold change over Control group (CT). Mean ± SEM (n = 4). Asterisks indicate a significant Group-factor effect with the CT tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's *t*-test, and are indicated with a T. Moreover, # indicates a significant interaction between the two factors. [$p < 0.05$ (*/#), $p < 0.01$ (**) or $p < 0.001$ (***)].

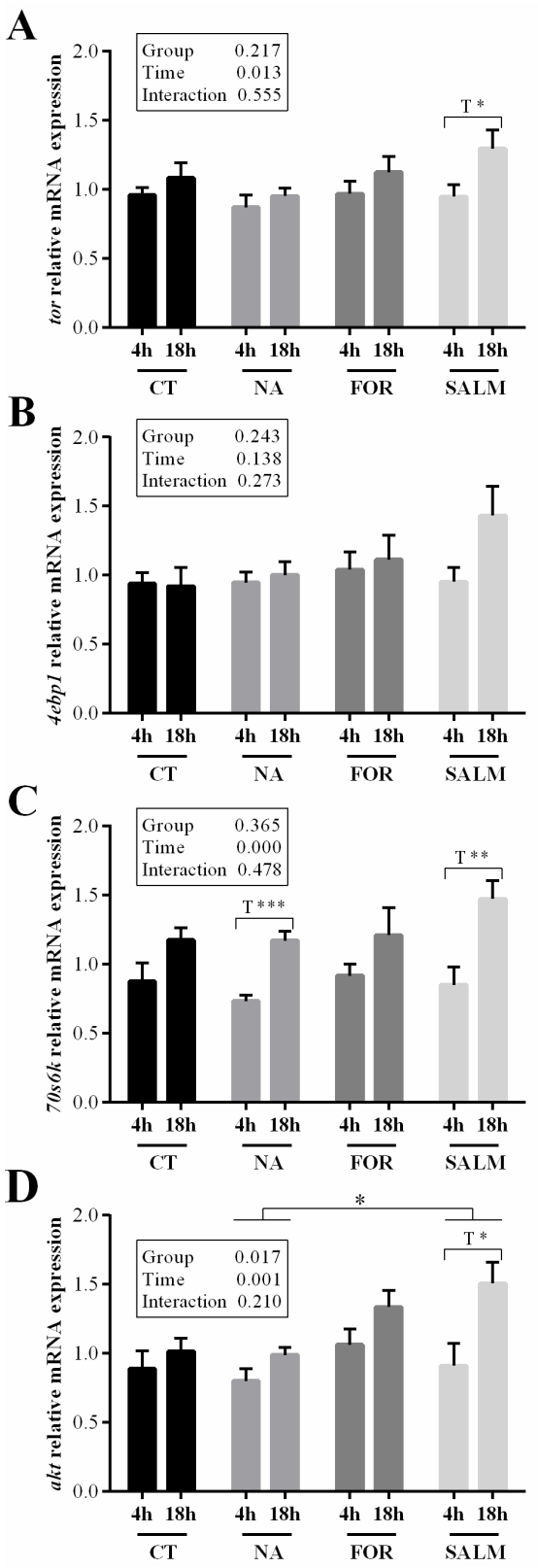



**Fig. 2. β2-agonists effects in the expression of signaling-related genes in gilthead sea bream cultured muscle cells.** mRNA levels of (**A**) *tor*, (**B**) *4ebp1*, (**C**) *70s6k* and (**D**) *akt* in myocytes at day 4 incubated with noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). Asterisk indicates a Group-factor effect between NA and SALM groups at $p < 0.05$ (*) tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's *t*-test and are indicated with a T. [$p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)]. CT: Control group.

### 3.2. ARs agonists' effects on *in vitro* muscle development and growth-related factors expression

The immunostaining images revealed increased protein expression of the proliferation marker PCNA (brown nuclei) after 6 h of incubation with either NA, FOR or SALM (Fig. 3A). This result was confirmed by the quantification of the percentage of PCNA positive cells that was significantly higher for the three experimental treatments compared to the CT group (Fig. 3B). Furthermore, FOR and SALM treatments significantly increased *pcna* gene expression compared with the CT group, although these mRNA levels decreased after 18 h of incubation (Fig. 3C). On the other hand, mRNA levels of *igf-1* were significantly up-regulated by SALM treatment compared with CT group (Fig. 3D). Longer incubation time increased the gene expression of *igf-1*, but it was only significantly different in the NA group. In the case of *igf-2*, incubation with either NA or SALM significantly increased its mRNA levels when compared with the FOR group (Fig. 3E). Moreover, the expression of *igf-2* was enhanced after 18 h of incubation with SALM.

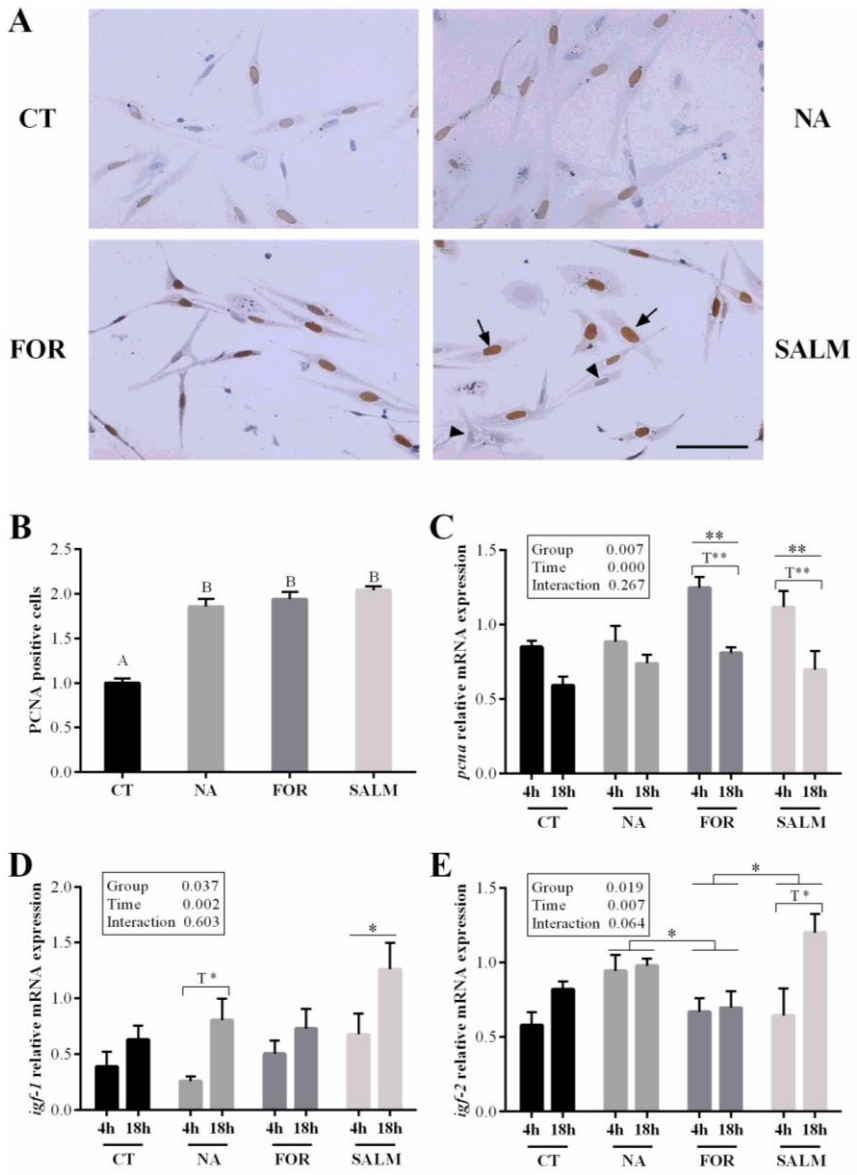



**Fig. 3. Effects of β$_2$-agonists on proliferation and growth factors gene expression in gilthead sea bream cultured muscle cells.** Representative images (**A**) and quantification (**B**) of PCNA-positive cells detected by immunostaining in day 4 myocytes after incubation with the β$_2$-agonists noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) for 6 h. Arrows indicate PCNA-positive cells and arrowheads negative ones. Results of the percentage of PCNA-positive cells with respect to the number of total cells are presented as fold change over the Control group (CT). Mean ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$), tested by One-Way ANOVA. Scale bar = 100 µm. mRNA levels of (**C**) *pcna*, (**D**) *igf-1* and (**E**) *igf-2* in myocytes at day 4 after incubation with the β$_2$-agonists during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). Asterisks indicate a Group-factor effect with CT or FOR groups tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's t-test and are indicated with a T. [$p < 0.05$ (*) or $p < 0.01$ (**)].

The myogenic process was tested by measuring the mRNA levels of the MRFs, the growth-inhibitor MSTN1 and some structural molecules (Fig. 4 and Fig. 5). The expression of *myf5* was significantly decreased by the three agonists' treatments when compared with the CT group (Fig. 4A). Expression of *myod2*, *myogenin* and *mrf4* was only different in some conditions with regards to time (Fig. 4C, 4D and 4E, respectively). In contrast, *myod1* and *mstn1* were not altered neither by treatment with the agonists nor by incubation time (Fig. 4B, 4F). In addition, the gene expression analysis of three muscle-structural molecules, myosin light chain 2A and 2B (*mlc2a, mlc2b*), and myosin heavy chain (*mhc*), did not revealed any differences among the experimental treatments, and those were only observed by incubation time in *mhc* expression in CT and NA groups (Fig. 5).

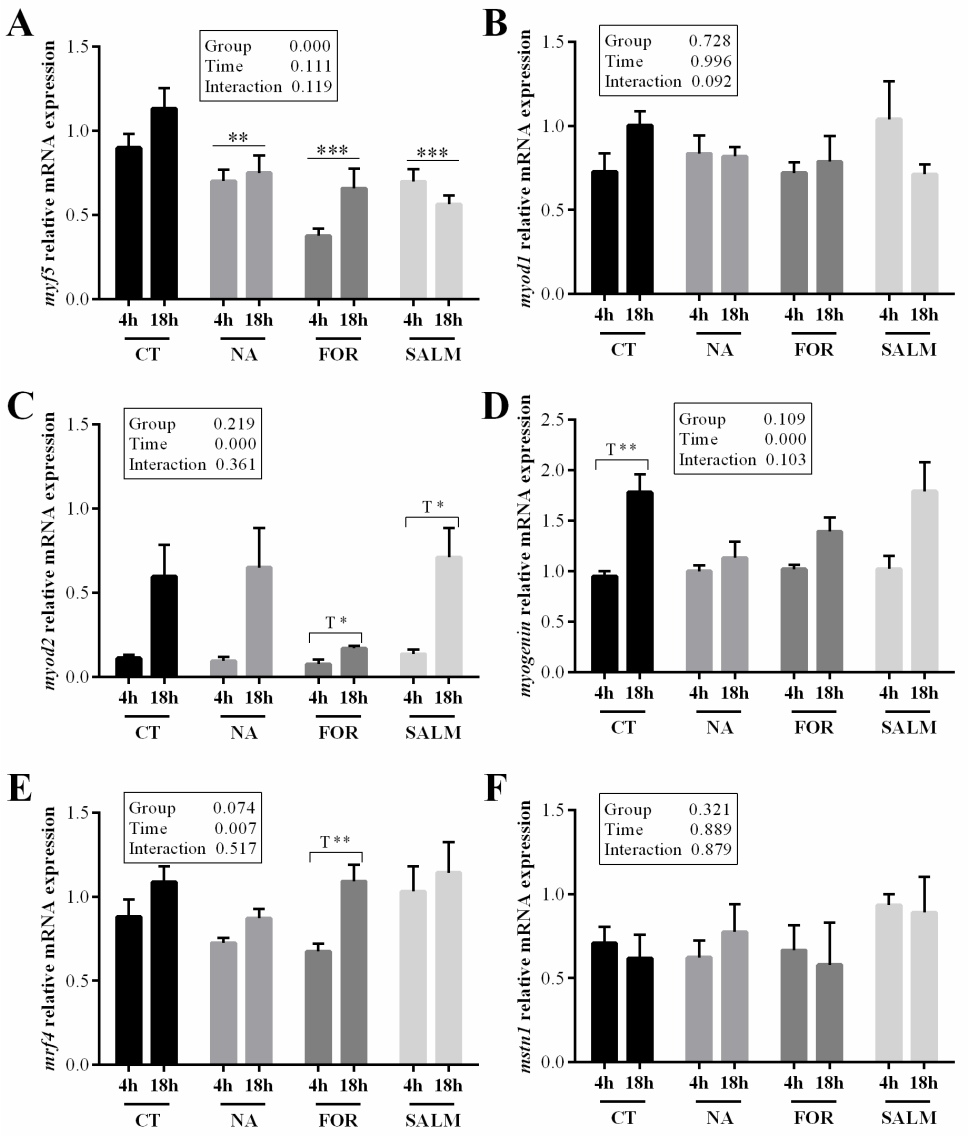



**Fig. 4. β2-agonists effects in the gene expression of myogenic regulatory factors (MRFs), and *mstn1* in gilthead sea bream cultured muscle cells.** mRNA levels of (**A**) *myf5*, (**B**) *myod1*, (**C**) *myod2*, (**D**) *myogenin*, (**E**) *mrf4*, and (**F**) *mstn1* in myocytes at day 4 incubated with noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). Asterisks indicate a significant Group-factor effect with the Control group (CT) tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's t-test and are indicated with a T. [$p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)].

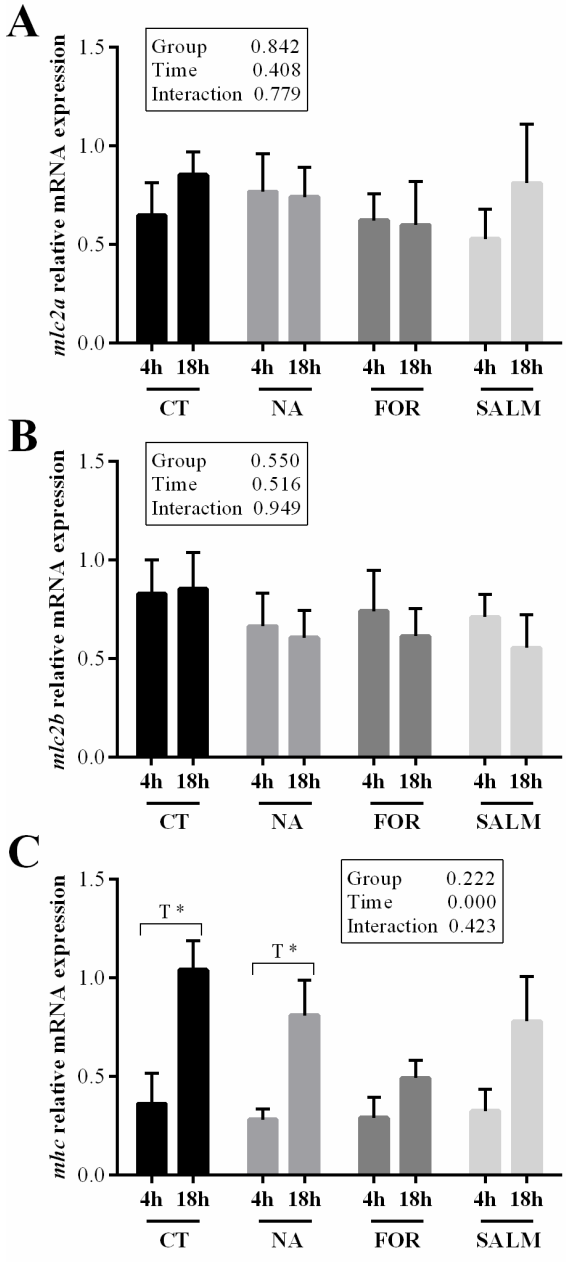



**Fig. 5. $\beta_2$-agonists effects in the gene expression of structural muscular markers in gilthead sea bream cultured muscle cells.** mRNA levels of (**A**) *mlc2a*, (**B**) *mlc2b* and (**C**) *mhc* in day 4 myocytes incubated with noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). When the Two-Way ANOVA analysis revealed a Time-factor effect, differences between incubation times within each experimental group were tested by Student's *t*-test and are indicated with a T. [$p < 0.05$ (*)]. CT: Control group.

### 3.3. ARs agonists' effects on proteolytic markers expression

The gene expression analyses of diverse proteolytic markers showed that among the UbP members, the mRNA levels of *mafbx* were higher in the SALM group than in the other experimental conditions, since a significant group effect was found (Fig. 6A). Moreover, also for *mafbx* an incubation time effect was observed in CT, NA and SALM groups. In the case of *murf1* and *n3*, their expression remained unchanged upon the different experimental conditions (Fig. 6B and 6C, respectively), and only a time effect was observed for *murf1* expression in the CT group. With respect to cathepsins, *ctsda* mRNA levels were significantly increased after 18 h of incubation in all groups (Fig. 6D), whereas for *ctsdb* this increase was only observed in the CT and SALM conditions (Fig. 6E). In addition, the gene expression of *ctsdb* was significantly up-regulated in response to FOR or SALM treatments when compared with the CT group. Regarding calpains, *capn1*, as well as the *capn small subunits 1a* and 1*b*, were not affected upon agonists treatments (Fig. 6F, 6G and 6H). However, the incubation time increased the expression of *capns1a* in the case of SALM and caused a down-regulation of *capns1b* expression in the FOR group.

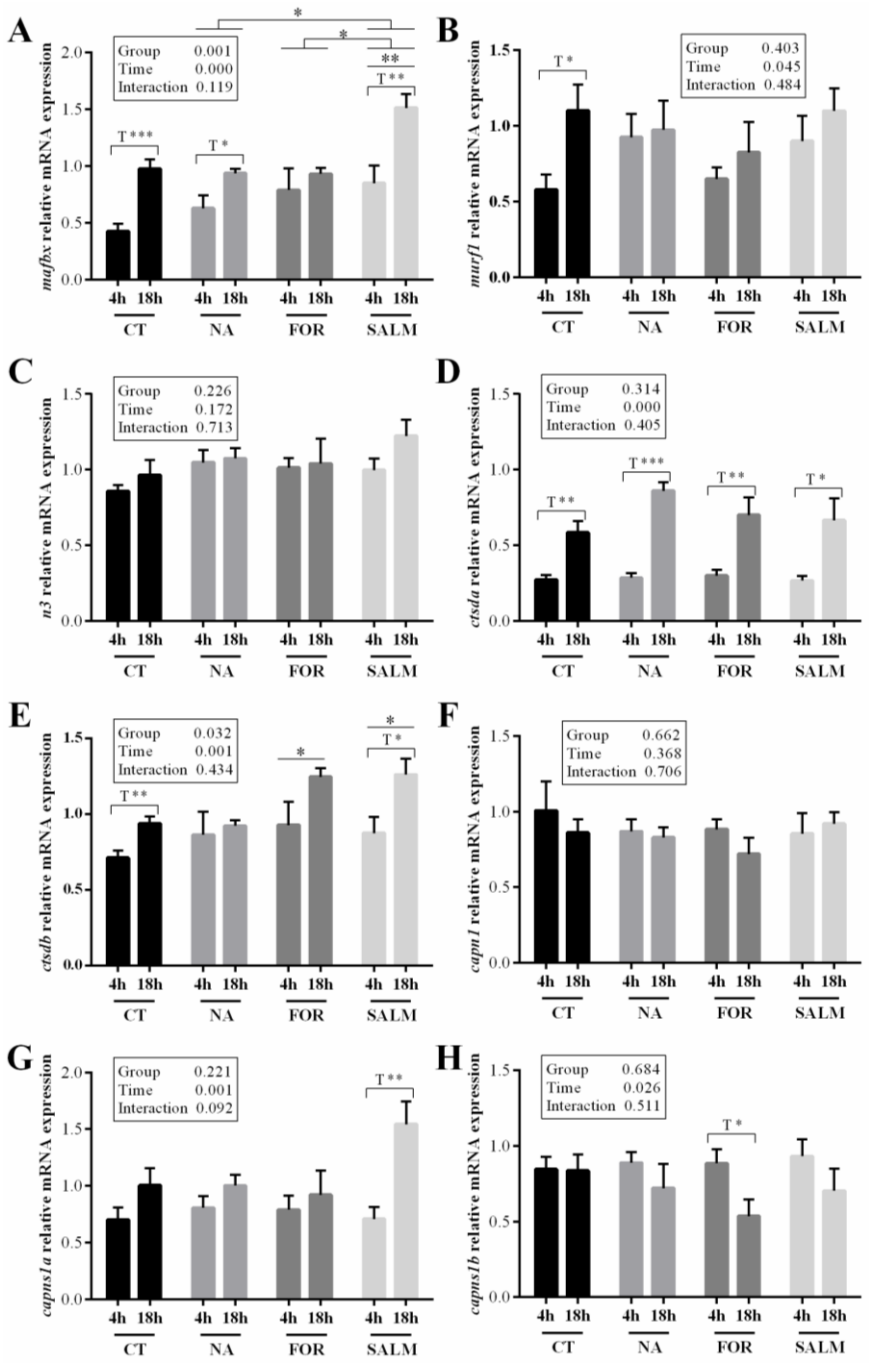



**Fig. 6. $\beta_2$-agonists effects in the gene expression of some proteolytic markers in cultured muscle cells from gilthead sea bream.** mRNA levels of (**A**) *mafbx*, (**B**) *murf1*, (**C**) *n3*, (**D**) *ctsda*, (**E**) *ctsdb*, (**F**) *capn1*, (**G**) *capns1a* and (**H**) *capns1b* in day 4 myocytes incubated with noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). Asterisks indicate a significant Group-factor effect with the Control (CT) or SALM groups tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's t-test and are indicated with a T. [$p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)].

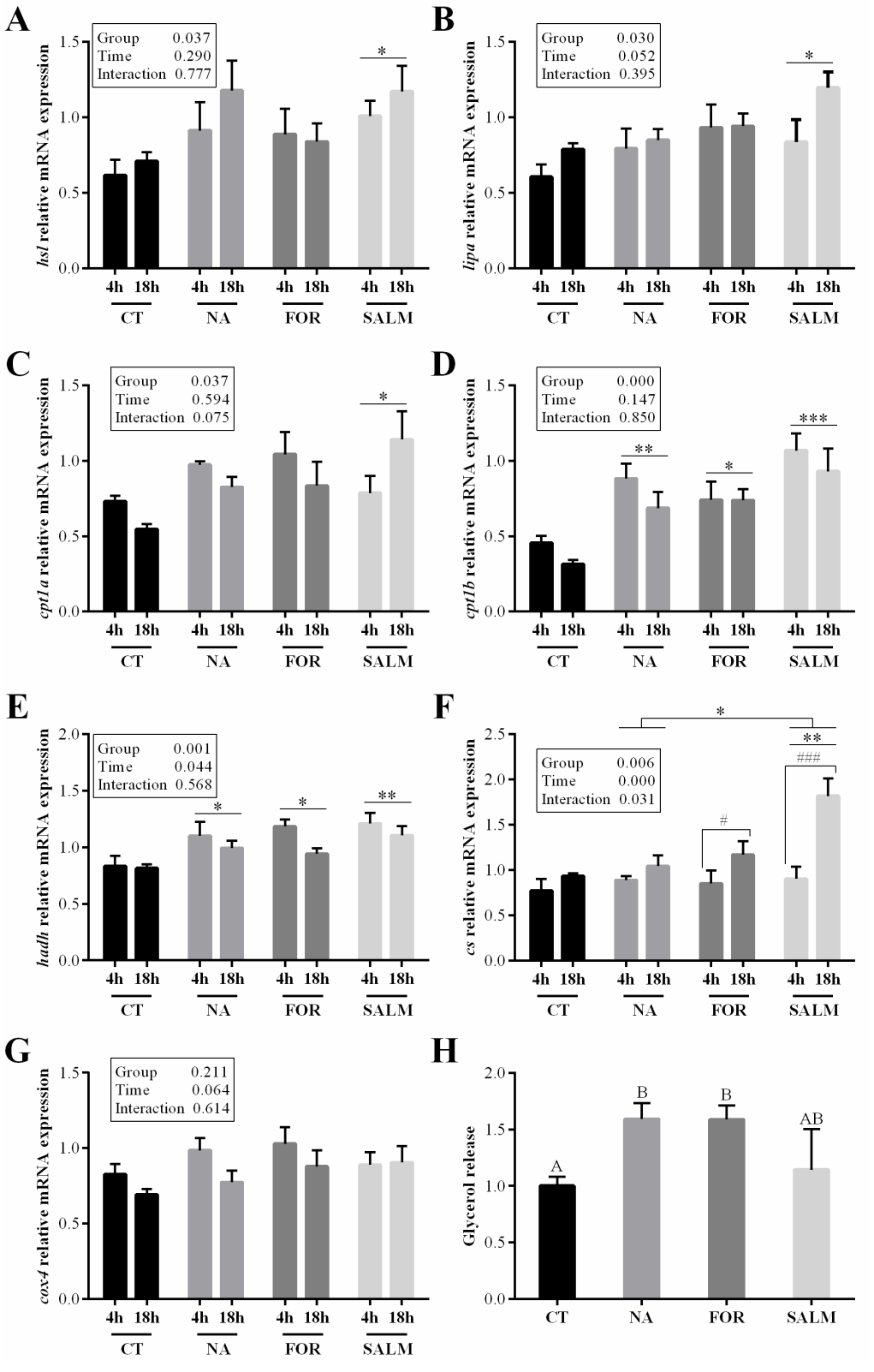
### 3.4. ARs agonists' effects on *in vitro* lipolysis and lipid metabolism-related genes expression

Concerning the ARs agonists' role on *in vitro* lipolysis, the mRNA levels of the two lipases analyzed, *hsl* and lipase a (*lipa*), were significantly up-regulated as an effect of the incubation with SALM (Fig. 7A and 7B, respectively), whereas no differences were caused by NA or FOR treatments. Similarly to that observed with the lipases, SALM treatment significantly increased the mRNA levels of the carnitine transferases *cpt1a* (Fig. 7C) and *cpt1b*, although in this case all three agonists provoked an increased expression that was independent of the incubation time (Fig. 7D).

On the other hand, in a similar way to that found for *cpt1b*, the gene expression of hydroxyacyl-CoA dehydrogenase (*hadh*), a key enzyme in fatty acid β-oxidation, was significantly up-regulated upon treatment with the three agonists, although this effect decreased with incubation time (Fig. 7E). Furthermore, the mRNA levels of citrate synthase (*cs*), a key enzyme of the Krebs cycle, were significantly enhanced after SALM treatment when compared with CT and NA groups, but not with FOR (Fig. 7F). Nevertheless, a significant interaction between agonist group and incubation time was found for both FOR and SALM treatments ($F$ (3, 33) = 3.354, $p$ = 0.031). The analysis of simple main effects demonstrated that the interaction effect induced an increase in *cs* gene expression due to incubation time (FOR $p$ = 0.029; SALM $p$ = 0.000).

In the case of the subunit 4 of the cytochrome c oxidase (*cox4*), its mRNA levels remained stable after the incubation with the different agonists at all times (Fig. 7G). Finally, NA and FOR, but not SALM, significantly increased the glycerol released into the media compared to the CT group (Fig. 7H).

**Fig. 7. Effects of $\beta_2$-agonists in the gene expression of lipid metabolism markers and glycerol release in cultured muscle cells from gilthead sea bream.** mRNA levels of (**A**) *hsl*, (**B**) *lipa*, (**C**) *cpt1a*, (**D**) *cpt1b*, (**E**) *hadh*, (**F**) *cs* and (**G**) *cox4* in myocytes at day 4 incubated with noradrenaline (NA), formoterol (FOR) or salmeterol (SALM) during 4 or 18 h. Data are shown as mean ± SEM (n = 5-7). Asterisks indicate a significant Group-factor effect with the Control (CT) or SALM groups tested by Two-Way ANOVA. When the factor Time was significant, differences between incubation times within each experimental group were tested by Student's t-test, and are indicated with a T. Moreover, # indicates a significant interaction between the two factors. [$p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***/###)]. (**H**) Quantification of the glycerol released into the media in day 4 myocytes after incubation with the agonists for 18 h. Results are presented as fold change over Control group (CT). Mean ± SEM (n = 5-7). Different letters indicate significant differences ($p < 0.05$), tested by One-Way ANOVA. **(In next page)**


A
hsl relative mRNA expression
Group 0.037
Time 0.290
Interaction 0.777
B
lipa relative mRNA expression
Group 0.030
Time 0.052
Interaction 0.395
C
cpt1a relative mRNA expression
Group 0.037
Time 0.594
Interaction 0.075
D
cpt1b relative mRNA expression
Group 0.000
Time 0.147
Interaction 0.850
E
hadh relative mRNA expression
Group 0.001
Time 0.044
Interaction 0.568
F
cs relative mRNA expression
Group 0.006
Time 0.000
Interaction 0.031
G
cox4 relative mRNA expression
Group 0.211
Time 0.064
Interaction 0.614
H
Glycerol release
4h 18h
CT NA FOR SALM

## 4. Discussion

In humans, β-ARs act regulating cardiovascular, metabolic and reproductive functions, but they can also induce the relaxation of airway smooth muscle and for this reason, their agonists are commonly used in the treatment of bronchospasm and asthma (2, 47). Furthermore, it has been shown that some $\beta_2$-agonists have the capacity to modulate protein turnover in order to increase skeletal muscle mass and its repair potential, both *in vivo* and *in vitro* (2, 47), reducing at the same time the body fat (48). These effects result very interesting for the livestock industry in general, but especially for aquaculture (30, 31); however, as far as we know, there is very little data available about the likely species-specific responses to $\beta_2$-agonists administration in fish. Therefore, with the aim of expanding our knowledge on β-ARs and the potential use of its agonists in fish production, using a primary culture of gilthead sea bream muscle cells as a model, we have investigated the effects of NA, FOR and SALM on PKA and AKT-TOR signaling pathways activation, cAMP production, cell proliferation and lipolytic rate, as well as on the gene expression of some IGFs, MRFs, proteolytic systems' members and lipid metabolism markers.

### 4.1. Signaling pathways activation.

The results revealed that both NA and FOR increased the cAMP levels as a result of $\beta_2$-ARs activation in gilthead sea bream myocytes, similarly to that found *in vivo* by Lortie and Moon (30) in both red and white muscle of rainbow trout. In addition, the enhanced phosphorylation of PKA by SALM treatment observed in the present study indicates that this agonist also activates the $\beta_2$-ARs. Nevertheless, SALM seems to function with a

different dynamic activating the Gα signaling pathway. Meanwhile, the incubation with all three agonists significantly increased the phosphorylation of TOR, which is indirectly activated by the PI3K/AKT pathway. Similar results were found as well in rat skeletal muscle by Joassard et al. (49), in which FOR administration increased the phosphorylation of both, AKT and TOR downstream effectors (i.e. 4EBP1 and S6). Moreover, the gene expression of *tor*, *70s6k* and *akt*, was up-regulated after a long incubation with SALM in the present study. An enhanced gene expression of these signaling molecules was also previously observed in the same model system when cells were exposed to characteristic activators of TOR and AKT pathways (i.e. amino acid cocktail or growth factors) (11, 39). Therefore, the activation of PKA and TOR suggests increased cell proliferation and survival, leading protein turnover towards an anabolic direction (50-52). These results demonstrated that gilthead sea bream myocytes are quite sensitive to $\beta_2$-adrenergic stimulation, and once agonist-ARs binding occurs, both the Gα subunit and the Gβγ dimer are activated, inducing the corresponding transduction cascades as in mammals (2), altogether suggesting that $\beta_2$-ARs signaling is well-conserved across vertebrates.

### 4.2. ARs agonists' effects on *in vitro* muscle development and growth-related factors expression

The proportion of PCNA-positive cells was increased after the incubation with all the $\beta_2$-agonists tested suggesting new cells formation (i.e. hyperplasia), and this was in parallel with the higher expression of *pcna* induced by FOR and SALM treatments. However, with time *pcna* expression decreased, indicating that the cells were then in a post-proliferative stage. This fact could be a consequence of the reduced levels of growth factors in the culture

media, which could be promoting cell differentiation instead of proliferation. Thus, it is possible that in a medium richer in growth factors, the proliferative effects of $\beta_2$-agonists could have been even stronger, since increased cell proliferation has been previously seen in the same cellular model after incubation with either amino acids, IGFs, growth hormone, or their combinations (38, 39). In this sense, the increased gene expression of *igf-1* by SALM and *igf2* by NA and SALM, although it could be partially due to the reduced levels of growth factors within the medium, suggests an additional effect for these $\beta_2$-agonists on cell proliferation and muscle development through the regulation of these growth factors (11, 39). In support of these data, Spurlock et al. (53) also found the expression of *igf-1* up-regulated in skeletal muscle of mice after 24 h of intraperitoneal injection with clenbuterol, another $\beta_2$-agonist. In any case, the synthesis of both IGF peptides would multiply the positive effects of $\beta_2$-agonists on muscle growth also in gilthead sea bream, corroborating the proliferative effects of these compounds in our cellular model. Moreover, among the agonists tested within the analyzed incubation times, it seems that SALM has the greatest effect inducing the expression of growth factors.

Furthermore, present results indicate that the analyzed $\beta_2$-agonists stimulate cell proliferation without affecting the expression of the proliferation inhibitor *mstn-1*, and this is usually associated with hyperplastic growth. Nonetheless, these data do not coincide with the observed effects in mammals, in which β-agonists treatment promotes muscle growth by hypertrophy (increase in fiber size) through regulation of *myostatin* expression (2, 49). Moreover, whereas in mammals such treatments induce changes in muscle fibers composition that correspond with switching from slow to fast-twitch skeletal fiber-type and in structural proteins (5, 22), in the present work the mRNA levels of the structural markers analyzed (i.e., *mhc*, *mlc2a*, and *mlc2b*) remained stable after incubation with the different

$\beta_2$-agonists. Similarly, most of the MRFs analyzed were also unaffected by the experimental treatments, although this is in agreement with that found in C2C12 cells after incubation with FOR or SALM (47). In contrast, *myf5* was down-regulated in our study in response to all three $\beta_2$-agonists. This factor, which plays a crucial role controlling the muscular linage, reaches its maximum expression at day 2 in gilthead sea bream myocytes, to decrease later with culture progression (54). A similar reduced *myf5* expression was previously observed when myocytes differentiation was activated by IGF-I treatment (11). Therefore, the obtained results, besides confirming cell proliferation effects, demonstrated that $\beta_2$-agonists enhance the myogenic process. Since the reduction in MYF5 has been considered to promote hyperplasia in developing fish muscle (55), these data suggest that stimulation of $\beta_2$-agonists in fish could induce skeletal muscle growth mainly through formation of new fibers. Similarly, increased TOR phosphorylation and PCNA expression induced by sustained swimming activity were observed during hyperplastic muscle growth in gilthead sea bream (45, 56). The different response observed in our cellular model compared with mammals can be understood considering that indeterminate grower fish species such as gilthead sea bream, besides increasing their muscle mass by hypertrophy, are also able to grow by hyperplasia throughout their lifetime (57). Thus, the hypertrophy induced by adrenergic stimulation would be in these species less significant than in determinate growers, like mammals.

### 4.3. ARs agonists' effects on proteolytic markers expression

The mRNA levels of selected proteolytic members of the UbP, cathepsins and calpains systems revealed that, in general, the $\beta_2$-agonists exert little influence in their gene expression in gilthead sea bream myocytes. In rats, *in vivo* activation of β-ARs with NA or

clenbuterol not only increases the AKT-TOR signaling pathway, which was proposed to be a mechanism to enhance muscle growth and to diminish atrophy, but also reduces the expression of both *murf1* and *mafbx* UbP system genes (58, 59). Similarly, studies on C2C12 cultured cells showed that the expression of these two genes is also reduced by NA and clenbuterol, but not in response to FOR or SALM (47). In contrast, Spurlock et al. (53) reported that *in vivo* administration of clenbuterol in mice increases the mRNA levels of other three ubiquitin ligases (i.e. *Ubr1*, *Siah2* and *Psmb1*). In this sense, MAFBx that recognizes and targets for ubiquitination some MRFs involved in muscle differentiation (i.e. MyoD or myogenin) (60-62), is considered a good differentiation marker. In addition, *mafbx* expression is regulated in opposition to *myod* or *myogenin* (63-64), being more expressed in the muscle of fingerlings than in juveniles or adult fish (65), in which the myogenic process remains slackened. Hence, the increased expression of *mafbx* found after the longer incubation with SALM, is in agreement with the PCNA results, supporting at that time the post-proliferative state of the cells.

Moreover, the increase observed in the present study in *ctsdb* gene expression in response to FOR and SALM seems to be also a consequence of this post-proliferative condition, since recent studies in gilthead sea bream have indicated that cathepsins act mainly during the early differentiation phase of *in vitro* myogenesis (64). Specifically, also in support of this, and similarly to *mafbx*, the *in vivo* expression of some cathepsins (i.e. *ctsdb*) is higher in the stages where myogenesis is more active in this species (65).

Regarding the calpains system, Douillard et al. (66) found that a clenbuterol treatment increases in rats calpain activity, but not its gene expression. This higher calpain activity, together with the increased expression of UbP system genes, has been related to a fiber-

type transition or remodeling situation in skeletal muscle, more than with an enhancement in protein degradation, especially when myogenic genes are up-regulated (53, 66). In gilthead sea bream, increased calpains gene expression and PCNA protein levels have been also associated with the positive effects of sustained swimming in muscle tissue renovation and hyperplastic growth (56). In the present study, *capns1a* mRNA levels were up-regulated in response to SALM, suggesting that this $\beta_2$-agonist participates in fish in the control of the structural remodeling that is essential for muscle development. This result, in agreement with the increased PCNA protein and mRNA levels observed, reinforces the idea stated above that $\beta_2$-agonists might be inducing in fish a hyperplastic muscle growth. Additionally, these results reinforce the idea that SALM appears to act at a different timeframe and with major intensity than the other agonists analyzed.

Summarizing all these aspects, the present results point out that $\beta_2$-agonists in gilthead sea bream, besides increasing cell proliferation, also regulate myocytes differentiation and the formation of myotubes by controlling the expression of some members of the different endogenous proteolytic systems.

### 4.4. ARs agonists' effects on *in vitro* lipolysis

Concerning the *in vitro* lipolysis determination, the treatment with SALM significantly increased the gene expression of both lipases, *hsl* and *lipa*, showing again the greatest effect for SALM in our cellular model. In human skeletal muscle, β-adrenergic signaling induces HSL phosphorylation by PKA, resulting in increased activity of this enzyme (67). According to this, the enhanced phosphorylation of PKA by SALM treatment we found is in parallel with the increased expression of *hsl*. The metabolic consequence of these results is an increase in the lipolytic machinery for energy purposes by enhancing free fatty acid release, similarly to that observed *in vivo* in mammals (7, 22, 24). In this sense, the

treatments also increased the expression of *cpt1a* and *1b*, involved in the incorporation of fatty acids into the mitochondria, where different oxidative enzymes such as HADH metabolize them through β-oxidation. Our results support these functions, since all the $\beta_2$-agonists tested stimulated the gene expression of *hadh*.

Besides the apparent increase in lipolysis and β-oxidation, the Krebs cycle appeared to be also potentiated by $\beta_2$-agonists, since both FOR and SALM significantly increased the gene expression of the main regulatory enzyme of this cycle, the CS. Furthermore, although the gene expression of *cox4* remained unchanged upon $\beta_2$-agonists treatment, a decrease in the ratio CS/COX4 has been previously recognized as a good marker of an oxidative condition in gilthead sea bream (68). Thus, in this work, the results support enhanced oxidative capacity of the cells and the use of fatty acids for ATP production. In addition, the culture media's analysis showed that $\beta_2$-agonists increased the release of glycerol in gilthead sea bream myocytes. Although in the present study it cannot be discarded that part of the glycerol produced is being used by the cells to synthesize glycogen, as it has been reported in the muscle of lamprey during spawning migration (69), or in gilthead sea bream directly or indirectly after glycerol inclusion on the diet (70), the observed glycerol levels demonstrate the ability of the tested $\beta_2$-agonists to increase the lipolytic metabolism in our *in vitro* model.

Overall, the stated effects are usually related to an important decrease in total body fat composition in vertebrates including fish (7, 21, 24, 31, 34-36), as well as have been also associated with a decrease in the fatty composition of the fish fillet (31, 32, 36, 71), or an increase in the muscular glycogen deposition, both affecting textural parameters (70). These attributes are desirable in aquaculture production. Thus, the effects of $\beta_2$-agonists on

lipid metabolism and cell proliferation should be considered as an interesting strategy in order to optimize growth and flesh quality in fish.

### 4.5. Conclusions

The present study demonstrates that gilthead sea bream myocytes are quite sensitive to $\beta_2$-agonists, and that the signaling pathways involved are well conserved between fish and mammals. However, in contrast to mammals in which such agonists' treatments activate hypertrophy, in fish myocytes appear to stimulate cell proliferation, suggesting a hyperplastic growth condition. Moreover, $\beta_2$-agonists up-regulate lipid metabolism leading to a favorable protein to fat ratio. Overall, the molecular mechanisms highlighted in this work can be considered good means to optimize, in the last term, the growth and flesh quality in gilthead sea bream. Therefore, in the next years, the research for alternative natural candidates able to mimic these signaling pathways and effects in fish to be used as dietary additives will be a promising research topic in experimental endocrinology.

### Abbreviations

AKT: protein kinase B; ARs: adrenoceptors; CS: citrate synthase; CT: control; DMEM: Dulbecco's Modified Eagle's Medium; FOR: formoterol; HADH: hydroxyacyl-CoA dehydrogenase; HSL: hormone-sensitive lipase; IGFs: insulin-like growth factors; LIPA: lipase A; MRFs: myogenic regulatory factors; NA: noradrenaline; PCNA: Proliferating Cell Nuclear Antigen; PI3K: phosphoinositide 3-kinase; PKA: protein kinase A; qPCR: quantitative real-time PCR; SALM: salmeterol; TOR: target of rapamycin; UbP: ubiquitin-proteasome.

**Acknowledgments**

The authors would like to thank the personnel from the facilities at the Faculty of Biology for the maintenance of the fish. E.J.V. is supported by a predoctoral fellowship from the "Ministerio de Economía y Competitividad" (MINECO). This study was supported by the projects from the MINECO AGL2012-39768 and AGL2015-70679-R to J.G., AGL2014-57974-R to I.N. and E.C., the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and 2014SGR-01371 from the "Generalitat de Catalunya", and by funds from the European Union through the project LIFECYCLE (EU-FP7 222719).

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# ARTICLE IV

## Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*)


American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 310 (2016) R313-R322

Article IV

# Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*)

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**Running title:** GH-IGFs axis modulation by sustained exercise

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**Abstract**

The endocrine system regulates growth mainly through the growth hormone (GH)/insulin-like growth factors (IGFs) axis and, although exercise promotes growth, little is known about its modulation of these factors. The aim of this work was to characterize the effects of 5 wk of moderate sustained swimming on the GH-IGFs axis in gilthead sea bream fingerlings. Plasma IGF-I/GH ratio and tissue gene expression of total IGF-I and three splice variants, IGF-II, three IGF binding proteins, two GH receptors, two IGF-I receptors, and the downstream molecules were analyzed. Fish under exercise (EX) grew more than control fish (CT), had a higher plasma IGF-I/GH ratio, and showed increased hepatic IGF-I expression (mainly IGF-Ia). Total IGF-I expression levels were similar in the anterior and caudal muscles; however, IGF-Ic expression increased with exercise, suggesting that this splice variant may be the most sensitive to mechanical action. Moreover, IGFBP-5b and IGF-II increased in the anterior and caudal muscles, respectively, supporting enhanced muscle growth. Furthermore, in EX fish, hepatic IGF-IRb was reduced together with both GHRs; GHR-II was also reduced in anterior muscle, while GHR-I showed higher expression in the two muscle regions, indicating tissue-dependent differences and responses to exercise. Exercise also increased gene and protein expression of TOR, suggesting enhanced muscle protein synthesis. Altogether, these data demonstrate that moderate sustained activity may be used to increase the plasma IGF-I/GH ratio and to potentiate growth in farmed gilthead sea bream, modulating the gene expression of different members of the GH-IGFs axis (i.e. IGF-Ic, IGF-II, IGFBP-5b, GHR-I, TOR).

**Keywords:** Growth hormone and GH receptors, IGFs and IGF-I receptors, IGF binding proteins, TOR, swimming.

## 1. Introduction

Exercise in teleost fish has long been recognized to stimulate growth (63). The effects of sustained exercise in fish depend on the typical behavior of each species, being more important in pelagic fishes (20, 43). In salmonids and other nonsalmonid species such as Carangidae or Gadidae, exercise improves the feed conversion efficiency and thus, the specific growth rate of exercised fish compared with rested fish (20). Recently, the same effect was reported in juveniles of gilthead sea bream (28, 44, 54, 82), supporting the use of this model as a potential strategy to improve fish growth for aquaculture production.

Growth is mainly regulated by the growth hormone (GH)–insulin-like growth factors (IGFs) axis (75). Pérez-Sánchez and Le Bail (67) reported that IGF-I plasma levels increase during rapid fish growth, whereas circulating GH decreases. GH exerts its effects through activation of membrane receptors (GHR-I and GHR-II) and is mainly responsible for hepatic IGF-I secretion (50, 96). In gilthead sea bream, Saera-Vila et al. (79) suggested that GHR-I plays a key role in the tissue-specific regulation of IGFs synthesis and secretion and later showed a transcriptional correlation between GHR-II and IGFs (IGF-I and IGF-II) in response to confinement stress (80), suggesting that GHR-II rather than GHR-I is a stress-sensitive gene in gilthead sea bream (17).

It is well known that IGF-I is synthesized as a pre-pro-IGF-I composed of a signal peptide, an E-peptide, and the mature IGF-I (14). Once the signal peptide is removed to give rise to pro-IGF-I, subsequent alternative splicing in the region coding for the E-peptide leads to different IGF-I splice variants, reported to have differential physiological functions (41, 71, 86). Three splice variants named Ea, Eb, and Ec have been identified, with Ea being common to all vertebrates, whereas Eb and Ec are found exclusively in mammals (88). Ea is mostly expressed in the liver and is considered responsible for the systemic role of IGF-

I (36). In contrast, Ec is mostly expressed in muscle and is up-regulated by damage or exercise in response to the mechanical action of contraction, and for this reason, it is known as the mechano growth factor (MGF) (39, 41, 71). In the case of teleost fish, up to four IGF-I splice variants of the form named Ea in mammals, which show differential tissue-specific expression and function, including cell cycle regulation, have been described (19, 87, 95). Tiago et al. (88) reported that gilthead sea bream has three variants identified as IGF-Ia, IGF-Ib and IGF-Ic. They showed that IGF-Ic is the most highly expressed form in the liver and suggested that it could play a systemic role like that of Ea in mammals. In the case of skeletal muscle, a previous *in vitro* study in gilthead sea bream showed that the expression of the three splice variants differed during myocyte culture progression and were differentially regulated, IGF-Ic being the most abundant throughout development and IGF-Ib being the most sensitive to hormone treatments (46). Nevertheless, the existence of a MGF in fish has not yet been reported, nor have the effects of exercise on these IGF-I splice variants been studied.

In addition to the peptides IGF-I and IGF-II, the IGFs system is composed of six IGF binding proteins (IGFBPs) and the receptors (IGF-IRs, and IGF-IIR) (30, 73). In mammals, almost 75% of circulating IGF-I is bound to IGFBPs (47), thus modulating the bioavailability of the peptides (23–25). IGFBPs are produced in different cell types in a tissue-specific manner as a means of tissue-specific action (26), and their production and secretion are regulated by the same hormones and growth factors that regulate GHRs expression (75). Thus, the different IGFBPs can inhibit or potentiate IGF-I actions depending on the experimental conditions or cellular context (23, 26, 99). The anabolic effects of IGFs, such as protein synthesis and cell proliferation, are mediated through IGF-IRs. Regarding IGF-IRs, while in some fish species, only one isoform has been identified,

in many other species, two isoforms (IGF-IR1/a and IGF-IR2/b) have been reported, with differential tissue distribution, and possibly, function (27, 40, 60). In the case of gilthead sea bream, although the existence of two isoforms was suggested more than a decade ago (68, 69), their sequences have only recently been described (55, 93). Furthermore, it is known that IGF-IRs expression may be modulated by hormone levels, nutritional status and environmental factors (75). In rainbow trout, for example, both receptors are differentially regulated by the nutritional status in muscle (18, 57), and in zebrafish, IGF-IRa (but not IGF-IRb) is suppressed in muscle in response to exercise (65).

When the IGFs bind the IGF-IRs, it is mainly the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway that is activated (56, 58). Downstream of Akt the target of rapamycin (TOR) has been reported to be key in promoting protein synthesis and muscle hypertrophy (35). TOR has been described as a nutrient sensor that integrates endocrine, as well as nutritional, signals (38, 89, 97). The importance of TOR activation by amino acids and/or IGFs in relation to muscle growth has been explored both *in vivo* and *in vitro* in several fish species, including gilthead sea bream cultured myocytes (10, 83, 92). However, the effects of exercise on the gene and protein expression of these signaling molecules in fish have not been elucidated.

Recently, we reported improved growth after 5 wk of sustained and moderate exercise [five body lengths (BL)/s] in gilthead sea bream fingerlings (9). The critical swimming speed (Ucrit) diminishes as body size increases. As the Ucrit for gilthead sea bream measuring 20 cm is around 4.5 BL/s (6), we can assume that a speed of 5 BL/s for individuals of the same species 7 cm long would be below their Ucrit. The increase in growth without affecting feed intake demonstrates an improvement in metabolic efficiency in this condition (9).

The aim of the present work was to analyze the role of the GH-IGFs axis molecules that mediate the positive exercise-related effects on growth in gilthead sea bream.

## 2. Materials and methods

### 2.1. Animals and experimental trial

Five hundred and forty gilthead sea bream (*Sparus aurata* L.) fingerlings [~5 g body wt and 7 cm body length (BL)] were obtained from a hatchery located in the north of Spain and randomly distributed into eight 0.2 $m^3$ tanks. These were maintained within a temperature-controlled system (23 ± 1 ºC) and 15 h light/9 h dark photoperiod at the facilities of the School of Biology at the University of Barcelona (Spain). For the experimental trial, as described previously (9), four tanks were kept in standard rearing conditions, with a water flow of 350 l/h and vertical water inflow. In these conditions, the fish presented only spontaneous and voluntary movements (control group, CT). The other four tanks were kept in a circular and uniformly distributed flow of 700 l/h by a vertical tube with lateral holes to maintain the flux in the water column, where the fish were forced to undertake moderate and sustained swimming (5 BL/s) (exercise group, EX). The water flow was adjusted as the fish grew to maintain the speed during the whole experiment. Fish were fed with a commercial diet (Gemma Diamond, Skretting, Burgos, Spain) three times a day, representing a 5% ration (until apparent satiety). Biometric parameters (weight and length) were determined at the beginning of the experiment, and then at *week 5* to monitor fish growth. In the final sampling at *week 5*, and after an overnight fast, 12 fish of each group (3 fish per tank) were killed, and samples of plasma, liver and anterior and caudal epaxial white muscle regions were collected, frozen in liquid nitrogen, and stored at -80 ºC

until further analysis. Despite the fact that only a few fish per tank were sampled, a large number of fish was used to maintain the appropriate population in terms of fish density (kg biomass/$m^3$) similar to that used in regular fish culture conditions to avoid conflicts due to behavior (e.g., differential feed intake of individual fish, aggressive behavior among individuals).

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

### 2.2. GH and IGF-I plasma levels

Plasma GH levels were determined using a homologous gilthead sea bream radioimmunoassay (RIA), as described previously (53). Plasma IGF-I was measured using an IGF-I RIA validated for Mediterranean perciform fish, as previously described (91).

### 2.3. Gene expression analyses

#### 2.3.1. RNA extraction and cDNA synthesis

Total RNA was extracted from 40 to 500 mg of tissue (depending on tissue yield) with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain). Total RNA concentration and purity were determined using a NanoDrop2000 (Thermo Scientific, Alcobendas, Spain). The integrity of the different samples was confirmed in a 1% agarose gel (wt/vol) stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). After that, 1 µg of total RNA was treated with DNase I (Life Technologies,

Alcobendas, Spain) to remove all genomic DNA following the manufacturer's recommendations, and then the RNA was reverse transcribed with the Affinity ScriptTM QPCR cDNA Synthesis Kit (Agilent Technologies, Las Rozas, Spain).

### 2.3.2. Quantitative real-time PCR (qPCR)

According to the requirements of the MIQE guidelines (15), the mRNA transcript levels of total IGF-I, the three splice variants (IGF-Ia, IGF-Ib and IGF-Ic), IGF-II, IGFBP-1a, IGFBP-2b, IGFBP-4, IGFBP-5b, IGF-IRa, IGF-IRb, GHR-I, GHR-II, Akt, TOR, plus three reference genes [ribosomal protein S18 (RPS18), ribosomal protein L27a (RPL27a) and β-actin] were examined in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The analyses were performed in triplicate in a final volume of 5 µL including 2.5 µL of iTaq TM Universal SYBR Green Supermix (Bio-Rad), 250 nM of forward and reverse primers (Table 1), and 1 µL of cDNA for each sample. Following the conditions previously described by Salmerón et al. (81), the reactions consisted of an initial activation step of 3 min at 95 ºC, 40 cycles of 10 s at 95 ºC, 30 s at 55–68 ºC (primer dependent, see Table 1) followed by an amplicon dissociation analysis from 55 to 95 ºC with a 0.5 ºC every 30 s. Before the analyses, the appropriate cDNA dilution for each assay was determined, as well as the specificity of the reaction, and the absence of primer-dimers was confirmed by running a dilution curve with a pool of samples. Using the Pfaffl method (70), we calculated the expression level of each gene analyzed relative to the geometric mean of the reference genes RPS18 and RPL27a, the two genes that were most stable of those analyzed using the CFX Manager™ Software (Bio-Rad). Calculation of the expression of the different IGF-I splice variants was performed as described in Tiago et al.

(88). The relative expression of genes in the EX fish was represented as the fold change over the CT fish.

**Table 1.** Primers used for qPCR: Sequences, melting temperatures, and GenBank accession numbers

| Gene | Primer sequences (5'–3') | Tm, °C | Accession number | References |
|---|---|---|---|---|
| *RPS18* | **F**: GGGTGTTGGCAGACGTTAC<br>**R**: CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 | (94) |
| *RPL27a* | **F**:AAGAGGAACACAACTCACTGCCCCAC<br>**R**:GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 | (49) |
| *β-Actin* | **F**: TCCTGCGGAATCCATGAGA<br>**R**: GACGTCGCACTTCATGATGCT | 60 | X89920 | (81) |
| *IGF-Ia* | **F**:AGGACAGCACAGCAGCCAGACAAGAC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | AY996779 | (88) |
| *IGF-Iab* | **F**:AGTCATTCATCCTTCAAGGAAGTGCATCC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | EF688015 | (88) |
| *IGF-Iabc* | **F**:ACAGAATGTAGGGACGGAGCGAATGGAC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | EF688016 | (88) |
| *IGF-II* | **F**:TGGGATCGTAGAGGAGTGTTGT<br>**R**:CTGTAGAGAGGTGGCCGACA | 60 | AY996778 | (8) |
| *IGF-IRa* | **F**:AGCATCAAAGACGAACTGG<br>**R**:CTCCTCGCTGTAGAAGAAGC | 55 | KT156846 | (2) |
| *IGF-IRb* | **F**:GCTAATGCGAATGTGTTGG<br>**R**:CGTCCTTTATGCTGCTGATG | 55 | KT156847 | (2) |
| *GHR-I* | **F**:ACCTGTCAGCCACCACATGA<br>**R**:TCGTGCAGATCTGGGTCGTA | 60 | AF438176 | (16) |
| *GHR-II* | **F**:GAGTGAACCCGGCCTGACAG<br>**R**:GCGGTGGTATCTGATTCATGGT | 60 | AY573601 | (78) |
| *IGFBP-1a* | **F**:AGTGCGAGTCCTCTCTGGAT<br>**R**:TCTCTTTAAGGGCACTCGGC | 60 | KM522771 | Present work |
| *IGFBP-2b* | **F**:CGGGCTGCTGCTGACATACG<br>**R**:GTCCCGTCGCACCTCATTTG | 60 | AF377998 | (31) |
| *IGFBP-4* | **F**:TCCACAAACCAGAGAAGCAA<br>**R**:GGGTATGGGGATTGTGAAGA | 60 | F5T95CD02JMZ9K | (34) |
| *IGFBP-5b* | **F**:TTTCTCTCTCGGTGTGC<br>**R**:TCAAGTATCGGCTCCAG | 60 | AM963285 | (2) |
| *TOR* | **F**:CAGACTGACGAGGATGCTGA<br>**R**:AGTTGAGCAGCGGGTCATAG | 60 | - | García de la serrana, unpublished |
| *AKT2* | **F**:GCTCACCCCACTCTTCAGAC<br>**R**:AAATTGGGAAATGTGCTTGC | 60 | ERA047531 | (34) |

Tm, melting temperatures; F, forward; R, reverse.

### 2.4. Western blot analyses

Protein homogenates from muscle tissue were obtained, as described by García de la serrana et al. (33). The amount of protein from each sample was measured (12), and then 10–20 μg of protein were separated by electrophoresis (SDS-PAGE) on a 10%

polyacrylamide gel, as previously described (92). Then, samples were transferred to a PVDF membrane, which was blocked in 5% nonfat milk buffer and incubated with the respective primary antibodies. The dilution was 1:200 for the phosphorylated forms and 1:500 for the total forms. The rabbit polyclonal primary antibodies used were: anti-phospho Akt (Cat. No. 9271), anti-total Akt (Cat. No. 9272) and anti-phospho TOR (Cat. No. 2971), all from Cell Signaling Technology (Beverly, MA) and, anti-total TOR (Cat. No. T2949) from Sigma-Aldrich (Spain). After washing, the membranes were incubated with the peroxidase-conjugated secondary antibody (Cat. No. 31460; Thermo Scientific, Alcobendas, Spain) in 5% nonfat milk buffer. Immunoreactive bands were developed using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific). Once the phosphorylated forms were developed, primary and secondary antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific), and then, the membranes were blotted again following the same procedure with the corresponding total forms. Finally, the bands were quantified by densitometry using ImageJ software v1.47 (National Institutes of Health, Bethesda, MD).

### 2.5. Statistical analyses

Data were analyzed using IBM SPSS Statistics v.21 and were presented as mean ± SEM. The presence of a normal distribution was first analyzed using the Shapiro-Wilk test followed by Levene's test to test the homogeneity of variances. Statistical differences were analyzed using Student's T-test and were considered significant when $P < 0.05$ (*) or $P < 0.001$ (**).

## 3. Results

### 3.1. Biometric and plasma parameters

After the 5 wk of the experiment, both groups of gilthead sea bream fingerlings showed increased growth, although the fish that were subject to moderate and sustained exercise evidenced a significantly higher body weight compared to the CT fish (Table 2). Moreover, sustained swimming in the same EX fish also caused an increase in the ratio of IGF-I/GH plasma levels (Table 2).

**Table 2.** Biometric and plasma parameters. Initial and final body weights and plasma IGF-I/GH ratio in gilthead sea bream control or after 5 wk of sustained and moderate exercise

| | Initial weight, g | Final weight, g | IGF-I/GH ratio |
|---|---|---|---|
| **CT** | 4.97 ± 0.04 | 17.54 ± 0.46 | 10.60 ± 2.74 |
| **EX** | 5.14 ± 0.20 | 20.28 ± 0.38** | 26.21 ± 4.28* |

Data are shown as mean ± SEM (CT, n = 21 ; EX, n = 15). CT, control; EX, exercise; GH, growth hormone. Significant differences are indicated by * ($P < 0.05$) or ** ($P < 0.001$).

### 3.2 GH-IGFs axis gene expression in liver and muscle

The expression of total IGF-I and the splice variant IGF-Ia in the liver was significantly increased in EX fish, while IGF-Ib and IGF-Ic were significantly decreased with respect to CT fish. On the other hand, IGF-II expression remained unchanged (Fig. 1A). In the case of binding proteins, IGFBP-1a and IGFBP-2b were present in the liver, although no differences were detected between groups (Fig. 1B). Regarding the IGF-IRs, only IGF-IRb was detected in the liver and showed significantly lower expression in EX than in CT

gilthead sea bream. However, both GHRs (GHR-I and GHR-II) were expressed in this tissue, although there was significant downregulation in EX fish compared with CT fish (Fig. 1C).

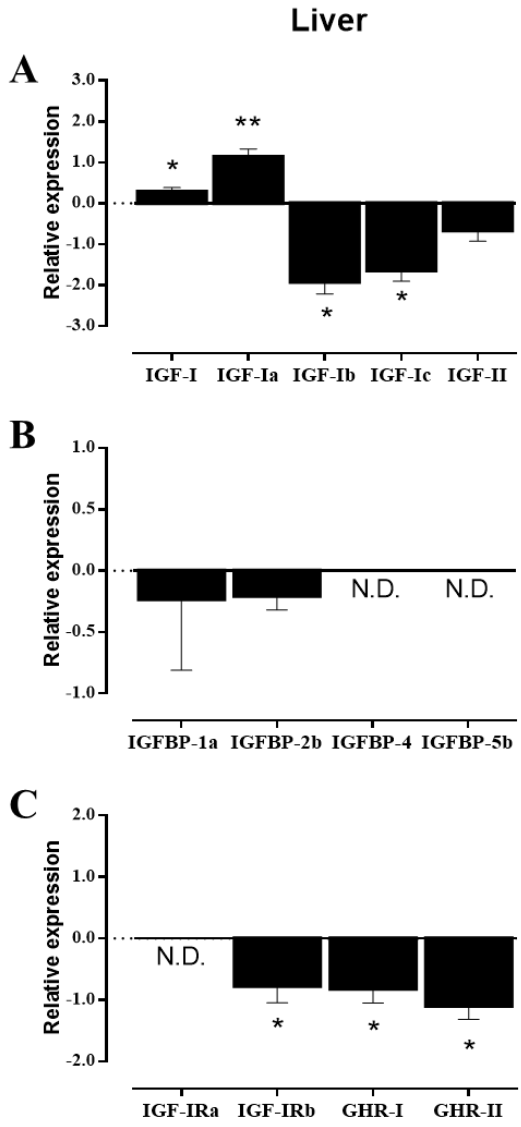


**Fig. 1.** Effects of exercise on liver GH-IGFs axis members' gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of a total IGF-I, IGF-I splice variants and IGF-II (**A**), IGF binding proteins (**B**), and IGF-I and GH receptors in liver tissue of gilthead sea bream after 5 wk of sustained and moderate exercise (EX) (**C**). Data are shown as fold change relative to control (CT) group as means ± SEM (n = 12). Significant differences are indicated by * ($P < 0.05$) or ** ($P < 0.001$). N.D., nondetected.

With regards to white skeletal muscle, the expression of total IGF-I, IGF-Ia, IGF-Ib and IGF-II in the anterior muscle was unaffected by exercise; however, IGF-Ic was significantly increased in EX fish compared to CT fish (Fig. 2A). Moreover, in contrast with the liver, IGFBP-2b was not detected in muscle, whereas IGFBP-1a, IGFBP-4 and IGFBP-5b were present, with IGFBP-5b being significantly up-regulated in the anterior muscle of EX fish (Fig. 2B). Also unlike in the liver, both IGF-IRs (IGF-IRa and IGF-IRb) were expressed in the anterior muscle, although no effects were observed in response to exercise (Fig. 2C).

On the other hand, both GHRs were expressed in the anterior muscle but showed different responses to exercise, with GHR-I being significantly increased and GHR-II significantly decreased in EX fish compared with the CT group (Fig. 2C).

The expression of the different GH-IGFs axis members studied showed similar results in the caudal muscle to those in the anterior muscle following sustained swimming, with significant increases in IGF-Ic and GHR-I expression in EX fish compared with CT fish (Fig. 2D and 2F, respectively). IGF-II expression was also significantly upregulated in the caudal muscle of EX fish (Fig. 2D). However, unlike in the anterior muscle, the mRNA levels of IGFBP-5b and GHR-II remained unchanged in the caudal region in response to exercise (Fig. 2E and 2F).

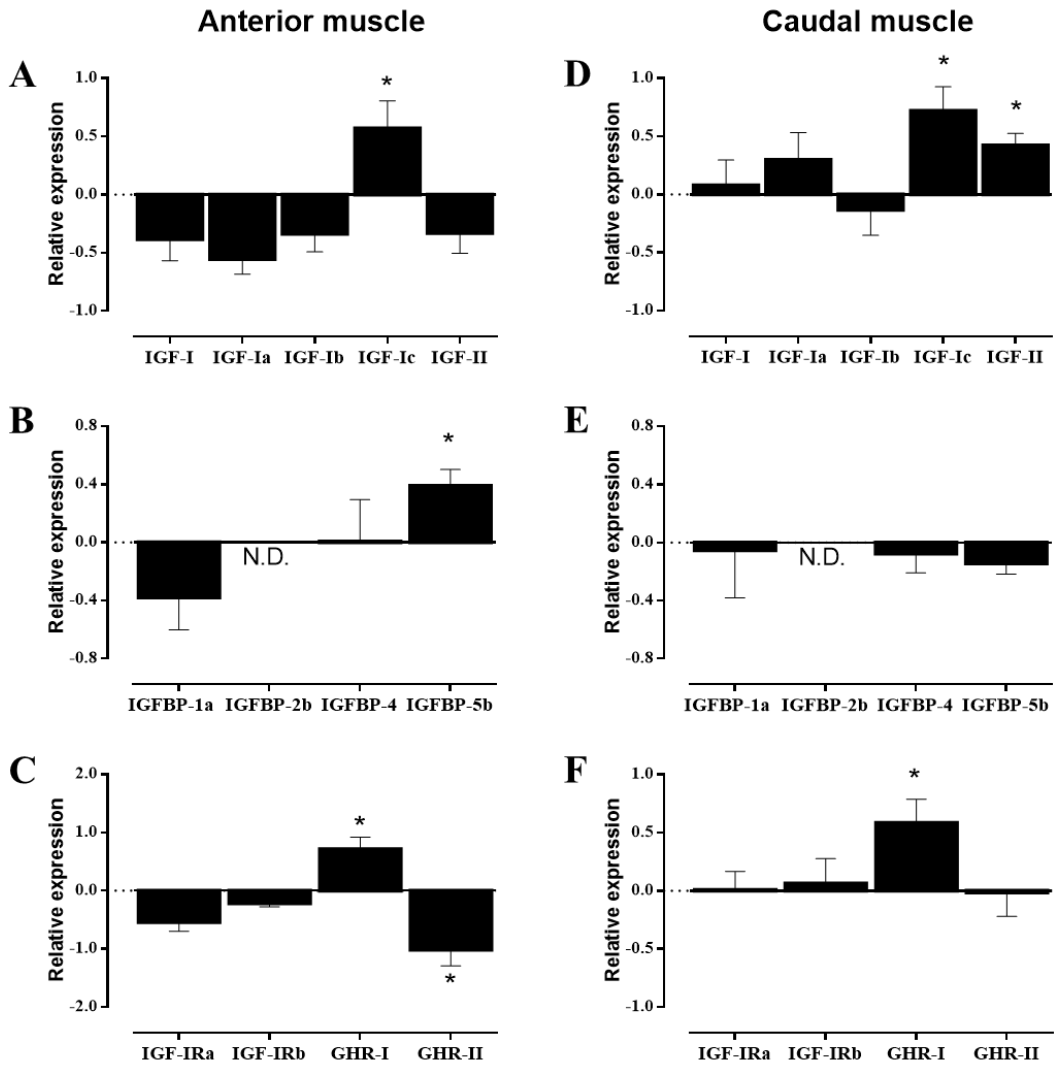


**Fig. 2.** Effects of exercise on muscle GH-IGFs axis members' gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of total IGF-I, IGF-I splice variants and IGF-II (**A**), IGF-I binding proteins (**B**), and IGF and GH receptors in anterior muscle tissue (**C**) and total IGF-I, IGF-I splice variants, and IGF-II (**D**), IGF-I binding proteins (**E**) and IGF and GH receptors in caudal muscle region of gilthead sea bream (**F**) after 5 wk of sustained and moderate EX. Data are shown as fold change relative to CT group as means ± SEM (n = 12). Significant differences are indicated by * ($P < 0.05$).

### 3.3 Akt and TOR activation

Exercise had no effect on Akt mRNA levels either in the anterior or caudal muscles; however, TOR gene expression was significantly increased in EX fish compared with CT fish in both muscle regions (Fig. 3A and 3B). With regard to protein levels, the phosphorylation of TOR was significantly increased (3.3-fold) in the anterior muscle of EX fish compared with the CT group, whereas no differences were found in Akt (Fig. 3C). In the case of the caudal muscle region, phosphorylation of both Akt and TOR was slightly increased in EX fish with respect to CT fish, but the differences were not significant (Fig. 3D).

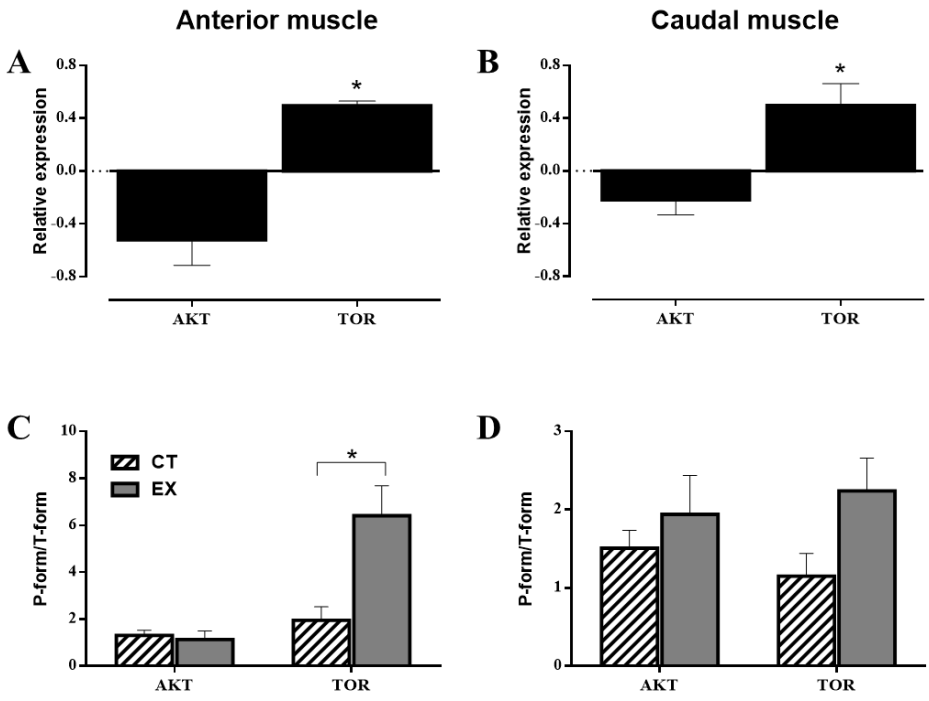


**Fig. 3.** Effects of exercise on Akt and target of rapamycin (TOR) signaling pathways. Relative mRNA expression normalized to RPS18 and RPL27a of Akt and TOR in the anterior (**A**) and caudal muscle regions (**B**) of gilthead sea bream after 5 wk of sustained and moderate EX. Data are shown as fold change relative to CT group as means ± SEM (n = 12). Significant differences were indicated with * ($P < 0.05$). Densitometric analyses of Akt and TOR phosphorylation in the anterior (**C**) and caudal (**D**) muscle regions of gilthead sea bream CT or after 5 wk of sustained and moderate EX. The intensity of each phosphorylated form was normalized to the corresponding total form for each sample. Results are presented as means ± SEM (n = 4). Significant differences are indicated by * ($P < 0.05$).

## 4. Discussion

In agreement with our previous experiments with juvenile gilthead sea bream, which demonstrated improved growth in response to exercise (28, 44, 54, 82), we recently demonstrated that 5 wk of moderate sustained swimming also improves growth in gilthead sea bream fingerlings, as well as causing a significant increase in circulating IGF-I (36.75 ± 2.63 vs. 51.10 ± 1.13 ng/ml) with a concomitant decrease in GH (7.50 ± 1.2 vs. 3.42 ± 0.55 ng/ml) plasma levels (9). Such an effect was not accompanied by changes in food intake or hepatosomatic index, although a significant decrease in mesenteric fat index was observed in EX fish compared to CT fish. In the present study, derived from the same experimental trial, we show the effects of exercise on the plasma IGF-I/GH ratio and on the tissue gene expression of components of the GH-IGFs system, aiming to provide new information on IGFs, IGFBPs, IGF-IRs, and downstream signaling molecules, as well as GHRs in liver and muscle tissues.

### Effects of sustained exercise on plasma IGF-I/GH ratio

In a review on exercise in fish, Davison (21) proposed that a reduction in catecholamines and cortisol levels together with an increase in growth factors and thyroid hormones will produce the perfect endocrine combination to enhance growth. Similarly, Pérez-Sánchez and Le Bail (67) reported that the best growth performance in fish is achieved with low plasma GH and cortisol levels, in combination with high circulating IGFs, insulin, and T3, and suggested that low GH plasma levels may be used to select fast-growing gilthead sea bream. In this context, low GH plasma levels have been reported in salmonids during high-growth periods, such as those induced by exercise (43, 85). In accordance with this,

Beckman (7) reviewed a series of experiments that demonstrated a positive correlation between IGF-I plasma levels and growth and suggested that IGF-I levels can provide a useful index of fish growth. This was also observed in exercised juvenile gilthead sea bream, in which significantly higher IGF-I plasma levels paralleled increased growth compared with control fish (82). In agreement with these findings, in our study, gilthead sea bream fingerlings exposed to moderate exercise had an increased IGF-I/GH ratio as a consequence of decreased GH, as well as increased IGF-I in the plasma (9), suggesting that moderate and sustained exercise results in a favorable endocrine condition that leads to enhanced fish growth.

**Effects of sustained exercise on hepatic GH-IGFs expression**

The expression of IGF-I in the liver reflected the results found in blood, showing an increase in the expression of total IGF-I in EX fish, which indicated that not only is hormonal secretion enhanced, but also that the corresponding gene expression is activated in these conditions. In this context, the expression of the splice variant IGF-Ia was significantly increased in EX fish, while that of IGF-Ib and IGF-Ic was decreased, suggesting differential hepatic regulation of these splice variants in this species, with IGF-Ia appearing to be the most sensitive form in response to exercise. With regard to IGFBPs, only IGFBP-1a and IGFBP-2b gene expression was detected in the gilthead sea bream liver. As reported previously in zebrafish, both IGFBP-1 and 2 have the ability to inhibit the cellular actions of IGF-I (22, 23), with IGFBP-1 being an important growth and developmental inhibitor under hypoxic conditions. Although recent data suggest that stimulation of IGFBP-2 is triggered by GH treatment in tilapia (13), in general, it is recognized that plasma levels of both IGFBPs (1 and 2) increase in catabolic conditions

and, thus, have been hypothesized as markers for negative growth in fish (48). In this context, the stable expression of these two hepatic IGFBPs with exercise, as observed in the present study, is consistent with the good growth conditions observed in EX fish.

The two IGF-IRs showed different levels of expression in the liver, with only IGF-IRb being detected by quantitative PCR, and down-regulated in EX fish. This decrease in IGF-IRb expression in the liver could be interpreted as a mechanism to avoid inhibitory effects on IGF-I secretion due to the potential negative feedback provoked by the increased levels of circulating IGF-I. Similar down-regulation in response to high circulating hormones, but at the level of binding, has been described previously in lamprey (51) and Atlantic salmon hepatocytes (74), trout cardiomyocytes (59), and brown trout and carp red muscle (5), in contrast to the upregulation observed in white muscle (66). Regarding the GHRs, levels of GHR-I and GHR-II were significantly reduced in the liver of EX fish, indicating possible GH desensitization in this tissue. GHRs transcriptional regulation has been shown to be highly variable among fish species, tissues, and physiological status. In this context, in a contrasting situation in which GH levels were increased, such as during fasting or feeding a high-plant-protein diet, a reduction in GHRs in the liver was also found in gilthead sea bream (37, 78) and hybrid striped bass (72), whereas fasted rainbow trout exhibited enhanced gene expression of GHR-I (32). Moreover, it should be noted that hepatic GH binding and signaling are also influenced by GH plasma levels, independently of GHRs expression (98).

**Effects of sustained exercise on muscle GH-IGFs expression**

In general, exercise in vertebrate muscle leads to an increase in protein synthesis and growth, a response that has been found in different fish species (52, 62), including gilthead sea bream (28, 44, 54, 82), with effects, such as hypertrophy and increased muscle vascularization. In the present study, the positive response of the GH-IGFs axis members' expression to sustained swimming was seen as a significant increase in IGF-Ic in both anterior and caudal muscle regions. IGF-Ic appears to be the most responsive splice variant to exercise in muscle tissue, suggesting that at least in gilthead sea bream, this isoform has acquired a similar role to that of the mammalian MGF, responding to mechanical stimulation (4, 71). This highlights the relative importance of IGF-Ic in muscle and will encourage further efforts to unravel its specific role in the regulation of myocyte growth.

Regarding IGFBPs, the expression of IGFBP-5b was significantly increased in the anterior white muscle of EX fish. The possible involvement of IGFBP-5 in mediating improved muscle growth by enhancing the availability or bioactivity of IGFs has been reported previously (24). IGFBP-5 is considered a promyogenic molecule and, therefore, a positive regulator of IGF-I actions (30). Furthermore, a positive correlation between IGFBP-5 mRNA levels and circulating IGF-I has been found in rainbow trout in anabolic conditions (32). In addition, Ren et al. (76) showed that IGFBP-5 induction precedes that of IGF-II, and as reviewed by Duan et al. (23), local IGFBP-5 promotes IGF-II actions during myoblast differentiation. Therefore, this IGFBP seems to play a critical role in muscle growth by stimulating the synthesis and signaling of IGF-II. According to these observations, the increase of IGFBP-5b in the anterior muscle of EX fish in our study could be linked to the increase in IGF-Ic in both anterior and caudal muscles, as well as to the increase in IGF-II expression in the caudal region during exercise, suggesting potential

cross talk between muscle regions. Moreover, although not significant, the decline in IGFBP-1a in muscle is also consistent with the increased growth rates observed in EX fish, as this IGFBP has previously been related to stressful or negative conditions (48).

Both IGF-IRs were present in muscle, although exercise had no effect on their expression, in contrast with the reduced levels of mRNA of IGF-IRa observed by Palstra et al. (65) in exercised zebrafish. Changes in the expression of IGF-IRs have been reported in relation to nutritional status, with fasting causing an increase in the expression of IGF-IRb in rainbow trout (57), or with refeeding, which increases IGF-I plasma levels, and decreases the expression of IGF-IRa in Atlantic salmon (11). In line with these findings, differences between the intensity of exercise, the physiological situation, or even the species, may explain the differences in response between our study and that on zebrafish (65). It should also be taken into account that regulation could take place at the level of IGFs binding or affinity to the receptor or activation of distinct signaling transduction pathways by different means. It has recently been shown by microarray analysis that exercise activates signaling pathways involved in the regulation of muscle mass in zebrafish, including the Akt and TOR pathways (64). In this sense, although we did not see changes in Akt phosphorylation with exercise, as in a similar trial with gilthead sea bream juveniles (82), we observed significant activation of TOR signaling at the gene and protein levels in response to exercise. In mammals, it is known that exercise activates TOR, and the most accepted hypothesis is that this involves the PI3K/Akt pathway (1, 3, 29). However, in mice, TOR can be phosphorylated by an Akt-independent mechanism following mechanical stimulation (42, 61). Moreover, after a bout of resistance exercise in the same species, signaling through Akt was a transient event, while TOR activation was sustained for a

longer period (61). On the basis of these findings, either Akt-independent activation of TOR or persistent activation of TOR with exercise may be occurring in our model.

With regards GHRs, the significant upregulation of GHR-I in anterior and caudal muscles together with body weight enhancement is consistent with the fact that GHR-I has previously been linked with anabolic pathways that are stimulated during exercise, whereas GHR-II has been linked with stress or immune responses (17, 80). In this sense, concurrent downregulation of IGF-I and GHR-I expression was observed in the head-kidney of *Enteromyxum leei* parasitized fish compared with control fish, whereas the expression of GHR-II was not altered (84). On the basis of an overwinter experiment with gilthead sea bream, the same authors reported that increased expression of GHR-I could mediate the increase in IGF-II mRNA levels (79). Similarly, Reindl and Sheridan (75) also reported upregulation of GHR-I expression in adipose tissue during fasting, pointing this out as a positive effect of growth maintenance powered by lipid metabolism. Thus, the locally increased expression of GHR-I in muscle in our study could be considered an adaptation to compensate for the reduced plasma GH levels. Furthermore, it was noticeable in our study that the two GHRs showed distinct responses to exercise, with GHR-II decreasing in the anterior muscle. These reduced levels of GHR-II were consistent with that found in exercised zebrafish (65). Moreover, muscle GHR-II was reported to be increased in fasted gilthead sea bream (78) and rainbow trout (32), supporting its role in mediating the catabolic actions of GH. This is consistent with a previous proposal that GHR-I and GHR-II have evolved as duplicated subtypes with different patterns of tissue distribution, post-receptor signaling, hormonal transcriptional regulation, and also function (45, 79).

Overall, IGF-Ic and GHR-I were up-regulated throughout the whole muscle, and in addition, the expression of IGFBP-5b and IGF-II was also increased in the anterior and

caudal muscle regions, respectively. This, together with the previously described positive relationship between GHR-I, IGFBP-5 and IGF-II availability and action, suggests that some kind of communication is taking place along the muscle, which deserves future investigation. In line with these observations, Rius-Francino et al. (77) demonstrated in gilthead sea bream that IGF-II is clearly a potent stimulator of myocyte proliferation, a condition that seems to be enhanced in this experimental model, especially in the caudal muscle area. In support of this, in a previous study, a higher number of capillaries was observed in the anterior muscle along with increased fiber size in both muscle regions in juvenile gilthead sea bream after sustained exercise (44). Besides, it is well known that under moderate and continuous exercise in Sparidae fish, the posterior zone of the muscular skeletal system is more active (90) and shows major sensitivity to swimming activity.

**Perspectives and significance**

In the present work, 5 wk of sustained and moderate exercise in gilthead sea bream fingerlings led to an increase in growth and the plasma IGF-I/GH ratio. This is consistent with the increased expression of total IGF-I in the liver, which seems to be focused on synthesizing IGF-I through upregulation of the IGF-Ia splice variant. In muscle, growth promotion seems to be hormonally mediated by the GH-GHR-I/IGFBP-5b-IGF-II axis and, mechanically, by enhancing the expression of the splice variant IGF-Ic and TOR activation. The present model provides insights into the differential regulation of GH-IGFs system members in response to sustained exercise in gilthead sea bream, and supports the use of these procedures to improve sustainable aquaculture production by modulating the endogenous endocrine system of the fish to enhance growth.

**Acknowledgments**

The authors would like to thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the School of Biology for the maintenance of the fish. E.J.V. and A.M.C are supported by a predoctoral fellowship from the "Ministerio de Ciencia e Innovación" (MICINN). This study was supported by the projects from the MICINN AGL2009-12427 and AGL2012-39768 to J.G., and the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and the SGR2009-00402 from the "Generalitat de Catalunya". The English version has been corrected by the Language Advisory Service of the University of Barcelona.

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# ARTICLE V

## Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*)

American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 312 (2017) R643-R653

Article V

# Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*)

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**Running title:** Proteolytic and myogenic markers modulation by exercise

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**Abstract**

Swimming activity primarily accelerates growth in fish by increasing protein synthesis and energy efficiency. The role of muscle in this process is remarkable and especially important in teleosts, where muscle represents a high percentage of body weight and because many fish species present continuous growth. The aim of this work was to characterize the effects of 5 wk of moderate and sustained swimming in gene and protein expression of myogenic regulatory factors, proliferation markers, and proteolytic molecules in two muscle regions (anterior and caudal) of gilthead sea bream fingerlings. Western blot results showed an increase in the proliferation marker proliferating cell nuclear antigen (PCNA), proteolytic system members calpain 1 and cathepsin D, as well as vascular-endothelial growth factor protein expression. Moreover, quantitative real-time PCR data showed that exercise increased the gene expression of proteases (calpains, cathepsins, and members of the ubiquitin-proteasome system in the anterior muscle region) and the gene expression of the proliferation marker PCNA and the myogenic factor MyoD in the caudal area compared with control fish. Overall, these data suggest a differential response of the two muscle regions during swimming adaptation, with tissue remodeling and new vessels formation occurring in the anterior muscle and enhanced cell proliferation and differentiation ocurring in the caudal area. In summary, the present study contributes to improving the knowledge of the role of proteolytic molecules and other myogenic factors in the adaptation of muscle to moderate sustained swimming in gilthead sea bream.

**Keywords:** Growth potential, myogenic regulatory factors, proteolytic molecules, swimming, VEGF, IGFs

## 1. Introduction

In fish, growth depends on the accretion of muscle, principally white skeletal muscle (48, 63), and compared with other vertebrates, differences in regulation exist among species. Whereas some fish species such as zebrafish (*Danio rerio*; 6) follow a determinate growth pattern similarly to that of mammals, most of the teleost species can grow in weight and length in an indeterminate way throughout their life (49, 92). Moreover, continuous fish muscle growth consists of hypertrophy (increase in fiber size) and hyperplasia (new muscle fibers formation; 90). These processes are the result of increased myogenesis, as well as downregulation of growth inhibitors, as is the case of some members of the transforming growth factor-β family [i.e., myostatin (MSTN); 62, 88]. During myogenesis, muscle satellite cells, which can be recognized for the expression of Pax3/7 (34), turn into myoblasts that after proliferation and differentiation, fuse together or with existing fibers to form multinucleated myofibers (14, 48). In mammals, this process is modulated by key transcription factors called myogenic regulatory factors (MRFs), which are expressed in a sequential manner, being either essential for muscle lineage determination and cell proliferation [myogenic factor 5 (Myf5) and MyoD] or responsible for the initiation of the differentiation program from myoblast to myotubes (Mrf4 and Myogenin; 18, 103). In fish, these transcription factors have also been identified, and a similar function to that in mammals has been demonstrated (9, 10, 16, 34, 36, 48, 56). In the last part of the myogenic process, and concomitant with the multinucleated myofibers formation, the necessity of structural and contractile proteins (e.g., actin or myosin) is well kown, as has been demonstrated in fish by means of both *in vivo* and *in vitro* experiments (36, 66).

Furthermore, muscle growth is the result of the positive balance between protein synthesis and degradation. The main endogenous proteolytic systems involved (ubiquitin-

proteasome, calpains, cathepsins, and caspases) are well known in mammals (5), and in the last years, different authors have identified and characterized some of these molecules in fish as well (85-87). A few studies have shown, in different species, that these molecules are regulated by nutritional status (57, 75, 81). In this line, Salmerón et al. (82) have identified a few calpain members in gilthead sea bream (*Sparus aurata*), one of the most important species in Mediterranean aquaculture (31), and demonstrated that diet composition and fasting/refeeding modulate their expression, these being changes well correlated with flesh texture. Moreover, it is commonly known that protein turnover has a greater relevance during higher growth periods (i.e., fingerlings vs. juvenile or adult fish; 72, 73, 83), and thus the proteolytic systems should be considered to completely understand development and growth in fish. In this sense, an *in vitro* study with rainbow trout (*Oncorhynchus mykiss*) myocytes has demonstrated that compared with mammals the protein degradation in fish is mainly due to the autophagy-lysosomal system (including cathepsins), at the expense of a less important role of the ubiquitin-proteasome system (85).

In addition to muscle growth, white muscle cellularity depends on various factors such as exercise (49, 50). In previous publications from this same experimental trial, Blasco et al. (8) and Vélez et al. (98) have demonstrated that 5 wk of moderate sustained swimming stimulate growth in fingerlings of gilthead sea bream without increasing food intake. Furthermore, this exercise-related growth was modulated by an increase in the aerobic capacity of the muscle and the plasma insulin-like growth factor (IGF-I)-to-growth hormone (GH) ratio, variations in the mRNA levels of GH-IGFs axis-related genes in liver and muscle, and activation of the target of rapamycin (TOR) signaling pathway (98). Overall, these changes accelerate growth by improving both muscle protein synthesis and energy efficiency (8); however, complementary information on myogenic regulators or

proteolytic systems to better picture muscle development and protein turnover was not reported, becoming the main objective of the present study.

Additionally, Egginton and Cordiner (28) have shown that swimming performance is directly related to capillary density in rainbow trout, angiogenesis being an integrated response to optimize aerobic performance. These authors also suggested that this process could be stimulated by different mechanical factors in muscle. Similarly, in zebrafish, different authors have studied the increased capillarity, mitochondrial biogenesis, and muscle remodeling induced by swimming adaptation (43, 52, 61, 70). In gilthead sea bream juveniles forced to perform sustained and moderate swimming, a differential response/adaptation to exercise between the epaxial-anterior and epaxial-caudal regions of white muscle has also been reported (44). In this sense, Vélez et al. (98) found a differential regulation between regions of several members of the GH-IGFs axis in fingerlings of this species as an effect of swimming, highlighting an increase in IGF-II gene expression in the caudal region and IGF binding protein-5 (IGFBP-5) gene expression in the anterior one. Moreover, in another study, whereas in the anterior region the number of capillaries increased, the fiber size in both anterior and caudal muscles was also enlarged with exercise (96), illustrating the different work of both muscle regions in the *Sparidae* family during swimming. Regarding this, the classical theories proposed that most of the power for fish swimming is generated by the anterior muscle (95, 99), although in later studies it seems clear that this depends on the type of muscle considered in each region. Thereby, in red muscle the power comes mainly from the posterior region (23, 40, 47, 80, 91), whereas in the case of white muscle the power is generated mostly by the anterior part (2, 29, 95). In any case, the anterior muscle has generally faster contractile properties than the posterior one for both red and white muscle types (22), and the recruitment of the different fiber

types across the axis length of the muscle depends on the swimming speed and is different among species (2, 21, 30).

The aim of this work was to characterize the gene and protein expression of MRFs, proliferation and angiogenic markers, and proteolytic molecules in the anterior and caudal skeletal muscle regions of gilthead sea bream fingerlings after 5 wk of moderate and sustained activity, completing the previous metabolic and endocrine studies reported from these fish (8, 98).

## 2. Material and methods

### 2.1. Animals and experimental trial

Five hundred and forty gilthead sea bream (*S. aurata* L.) fingerlings [~5 g body wt and 7 cm body length (BL)] were obtained from a hatchery located in the north of Spain, randomly distributed into eight 0.2 $m^3$ tanks, and maintained within a temperature-controlled seawater recirculation system at 23 ± 1 ºC at the facilities of the School of Biology at the University of Barcelona (Spain). The exchange rate of water was 35% per week, and water was exchanged through the filtering system without altering the inflow to the tanks; oxygen level was over 90% of saturation, and the photoperiod was 15 h light/9 h dark. After the acclimation period, four tanks were kept with a vertical water inflow of 350 l/h (standard rearing conditions), where the fish presented only spontaneous and voluntary movements (Control group); in the other four tanks a circular uniformly distributed flow of 700 l/h was set up, where the fish were forced to undertake moderate and sustained swimming (5 BL/s; Exercise group), as reported before (8, 98). Fish were

fed with a commercial diet (Gemma Diamond; Skretting, Burgos, Spain) three times a day, representing a 5% ration (until apparent satiety). Biometric parameters (weight and length) were determined at the beginning of the experiment and at the last time point (5 wk) to monitor fish growth. Fish were fasted for 24 h before any manipulation and sampling. As previously reported (98), in the final sampling at *week 5*, 12 fish of each group (4 fish per tank) were killed, and samples of anterior (from 25% total length behind the tip of the snout to 40% total length) and caudal (from 60% to 75% total length) epaxial white muscle regions were collected, frozen in liquid nitrogen, and stored at -80ºC until further analyses.

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

### 2.2. Gene expression

#### 2.2.1 RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of tissue with 1 ml of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) as has been described before (98). Briefly, total RNA concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain). After the samples' integrity in a 1% agarose gel (wt/vol) stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain) was verified, 1 µg of total RNA was treated with DNase I (Life Technologies) to remove all genomic DNA following the manufacturer's recommendations. Finally, RNA was reverse

transcribed with the Affinity ScriptTM QPCR cDNA Synthesis Kit (Agilent Technologies, Las Rozas, Spain).

### 2.2.2. Quantitative real-time PCR (qPCR)

The mRNA transcript levels of the following groups of interest (Table 1) were examined in a CFX384$^{TM}$ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain) according to the requirements of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (13): proliferation marker, growh inhibitors, MRFs, structural molecules, proteolytic markers, ubiquitin-proteasome system, plus three reference genes. The analyses were performed in triplicate in a final volume of 5 µL including 2.5 µL of iTaq TM Universal SYBR Green Supermix (Bio-Rad), 250 nM of forward and reverse primers (Table 1), and 1 µL of cDNA for each sample. As described before (82), the reactions consisted of the following: initial activation step of 3 min at 95 ºC, 40 cycles of 10 s at 95 ºC, 30 s at 55-68 ºC (primer dependent, Table 1) followed by an amplicon dissociation analysis from 55 to 95 ºC at 0.5 ºC increase each 30 s. Before the analyses, the appropriate cDNA dilution for each assay was determined, as well as the specificity of the reaction and the absence of primer-dimers, confirmed by running a dilution curve with a pool of samples. The expression level of each gene analyzed was calculated relative to the geometric mean of the reference genes *RPS18* and *RPL27a*, the two most stable of the genes analyzed, using the Pfaffl method (74).

**Table 1.** Primers used for quantitative PCR: sequences, melting temperatures, and GenBank accession numbers

| Gene | Primer Sequences (5'–3') | Tm, °C | Accession Number |
|---|---|---|---|
| *RPS18* | F:GGGTGTTGGCAGACGTTAC<br>R:CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 |
| *RPL27a* | F:AAGAGGAACACAACTCACTGCCCCAC<br>R:GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 |
| *β-Actin* | F: TCCTGCGGAATCCATGAGA<br>R: GACGTCGCACTTCATGATGCT | 60 | X89920 |
| *PCNA* | F:TGTTTGAGGCACGTCTGGTT<br>R:TGGCTAGGTTTCTGTCGC | 58 | AY550963.1 |
| *MSTN1* | F:GTACGACGTGCTGGGAGACG<br>R:CGTACGATTCGATTCGCTTG | 60 | AF258448.1 |
| *MSTN2* | F:ACCTGGTGAACAAAGCCAAC<br>R:TGCGGTTGAAGTAGAGCATG | 60 | AY046314 |
| *MyoD1* | F:TTTGAGGACCTGGACCC<br>R:CTTCTGCGTGGTGATGGA | 60 | AF478568.1 |
| *MyoD2* | F:CACTACAGCGGGGATTCAGAC<br>R:CGTTTGCTTCTCCTGGACTC | 60 | AF478569 |
| *Myf5* | F:CTACGAGAGCAGGTGGAGAACT<br>R:TGTCTTATCGCCCAAAGTGTC | 64 | JN034420 |
| *Myogenin* | F:CAGAGGCTGCCCAAGGTCGAG<br>R:CAGGTGCTGCCCGAACTGGGCTCG | 68 | EF462191 |
| *Mrf4* | F:CATCCCACAGCTTTAAAGGCA<br>R:GAGGACGCCGAAGATTCACT | 60 | JN034421 |
| *MHC* | F:AGCAGATCAAGAGGAACAGCC<br>R:GACTCAGAAGCCTGGCGATT | 58 | AY550963.1 |
| *MLC2a* | F:GCCCCATCAACTTCACCGTCTTT<br>R:GGTTGGTCATCTCCTCAGCGG | 60 | AF150904 |
| *MLC2b* | F:TCCCTTTGCTATTCTGCCTTC<br>R:AAATCAGCCCTATTCCCCATA | 60 | FG618631 |
| *CAPN1* | F:CCTACGAGATGAGGATGGCT<br>R:AGTTGTCAAAGTCGGCGGT | 56 | KF444899 |
| *CAPN2* | F:ACCCACGCTCAGACGGCAAA<br>R:CGTTCCCGCTGTCATCCATCA | 61 | KF444900 |
| *CAPN3* | F:AGAGGGTTTCAGCCTTGAGA<br>R:CGCTTTGATCTTTCTCCACA | 56 | ERP000874 |
| *CAPNs1a* | F:CGCAGATACAGCGATGAAAA<br>R:GTTTTGAAGGAACGGCACAT | 56 | KF444901 |
| *CAPNs1b* | F:ATGGACAGCGACAGCACA<br>R:AGAGGTATTTGAACTCGTGGAAG | 56 | ERP000874 |
| *CTSDb* | F:AAATTCCGTTCCATCAGACG<br>R:CTTCAGGGTTTCTGGAGTGG | 56 | KJ524456 |
| *MuRF1* | F:GTGACGGCGAGGATGTGC<br>R:CTTCGGCTCCTTGGTGTCTT | 60 | FM145056 |
| *MAFbx* | F:GGTCACCTGGAGTGGAAGAA<br>R:GGTGCAACTTTCTGGGTTGT | 60 | ERA047531 |
| *N3* | F:AGACACACACTGAACCCGA<br>R:TTCCTGAAGCGAACCAGA | 54 | KJ524458 |

Tm, melting temperature; F, forward; R, reverse; *RPS18*, ribosomal protein S18; *RPL27a*, ribosomal protein L27a; *PCNA*, proliferating cell nuclear antigen; *MSTN1*, myostatin 1; *MSTN2*, myostatin 2; *MyoD1*, myoblast determination protein 1; *Myod2*, myoblast determination protein 2; *Myf5*, myogenic factor 5; *Mrf4*, myogenic regulatory factor 4; *MHC*, myosin heavy chain; *MLC2a*, myosin light chain 2a; *MLC2b*, myosin light chain 2b; *CAPN1*, calpain 1; *CAPN2*, calpain 2; *CAPN3*, calpain 3; *CAPNs1a*, calpain, small subunit 1a; *CAPNs1b*, calpain, small subunit 1b; *CTSDb*, cathepsin Db; *MuRF1*, Muscle RING finger protein 1; *MAFbx*, muscle atrophy F-box or atrogin-1; *N3*, proteasome β type 4 subunit or PSMB4.

### 2.3. Western blot analysis

Protein homogenates from muscle tissue were obtained as described by García de la serrana et al. (37). The amount of protein from each sample was measured (11), and 10-20 μg of protein were separated by electrophoresis (SDS-PAGE) on 10% polyacrylamide gel (125 V for 1 h 30 min) following the procedure previously reported (97) with slight modifications. After SDS-PAGE, samples were transferred to a polyvinylidine fluoride membrane. Then, all the membranes were stained with a Ponceau S solution (Sigma-Aldrich, Tres Cantos, Spain) and scanned for posterior band quantification. Later, the membranes were washed to eliminate the Ponceau staining and then blocked in nonfat milk 5% buffer and incubated with the respective primary antibodies in a 1:200 dilution. The primary antibodies used were as follows: rabbit polyclonal anti-proliferating cell nuclear antigen (anti-PCNA; catalog no. sc-7907), rabbit polyclonal anti-muscle atrophy F-box (anti-MAFbx; catalog no. sc-33782), rabbit polyclonal anti-vascular endothelial growth factor (anti-VEGF; catalog no. sc-152), goat polyclonal anti-calpain 1 (anti-CAPN1; catalog no. sc-7530), and goat polyclonal anti-cathepsin D (anti-CTSD; catalog no. sc-6486), all from Santa Cruz Biotechnology (Santa Cruz, CA). All these antibodies have been previously demonstrated that cross-react successfully with the proteins of interest in gilthead sea bream (3, E.J. Vélez, Sh. Azizi, D. Verheyden, C. Salmerón, E. Lutfi, A. Sánchez-Moya, I. Navarro, J. Gutiérrez and E. Capilla, unpublished data); with the exception of anti-VEGF, which specificity has been tested in a pooled sample by blocking overnight with an excess (5-fold) of the immunization peptide (catalog no. sc-152 P). After washing was completed, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibody: goat anti-rabbit (catalog no. 31460, Thermo Scientific), or donkey anti-goat (catalog no. sc-2020, Santa Cruz Biotechnology). The

membranes were rewashed, and the different immunoreactive bands were developed using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific). Finally, the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Hence results from the densitometry analysis of each specific bands are presented normalized by the densitometry values of the Ponceau, since its staining showed similar amounts of loaded and transferred protein. In the case of CTSD, the densitometric analysis was performed with the sum of the bands corresponding to the immature and mature forms.

### 2.4. Statistical analyses

Data was analyzed using IBM SPSS Statistics version 21 and presented as means ± SEM. Normal distribution was first analyzed using the Shapiro-Wilk test followed by Levene's test homogeneity of variances. Statistical differences were analyzed using Student's *t*-test and considered significant when $P < 0.05$ (*) or $P < 0.001$ (**).

## 3. Results

### 3.1. Proliferation marker and growth inhibitor expression

Fig. 1A shows the effects of 5 wk of sustained swimming in fingerlings of gilthead sea bream on the gene and protein expression of the proliferation marker PCNA. PCNA gene expression was increased significantly in the caudal muscle region of exercised fish; nonetheless, the protein levels of PCNA were significantly higher in the anterior region compared with control fish.

Fig. 1B presents the mRNA levels of two MSTN paralogues in both muscle regions. Altough the expression of MSTN1 was not modified by effect of exercise, the transcript levels of MSTN2 were downregulated in the anterior muscle of exercised fish.

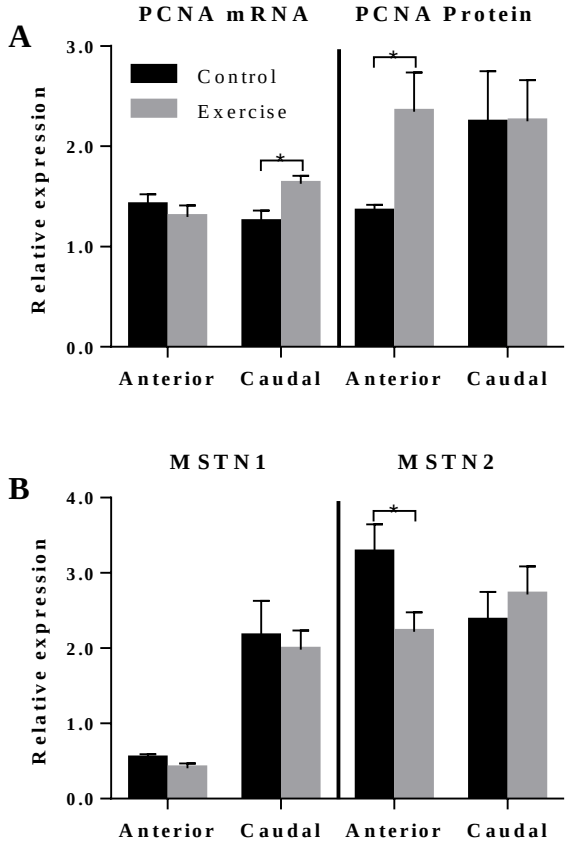


**Fig. 1.** Effects of exercise on the expression of a proliferation marker and a growth inhibitor in muscle tissue. **A**: relative mRNA expression of proliferating cell nuclear antigen (PCNA, *left*) and PCNA protein expression normalized to total protein staining with Ponceau S (*right*). **B**: relative mRNA levels of myostatin 1 (MSTN1) and MSTN2 normalized to ribosomal protein S18 (RPS18) and ribosomal protein L27a (RPL27a) in both anterior and caudal muscle regions of gilthead sea bream after 5 wk of sustained and moderate exercise (Exercise), or reared in control conditions (Control). Data are shown as means ± SEM (n = 12 for quantitative PCR; n = 4 for Western blot). *Significant difference, $P$ <0.05.

### 3.2. Myogenic regulatory factors and structural markers expression

The analysis of MRFs gene expression showed that whereas none of these factors (MyoD1, MyoD2, Myf5, Myogenin and Mrf4) were affected by swimming activity in the anterior muscle (Fig. 2A), the expression of MyoD1 was significantly increased on the caudal region (Fig. 2B).

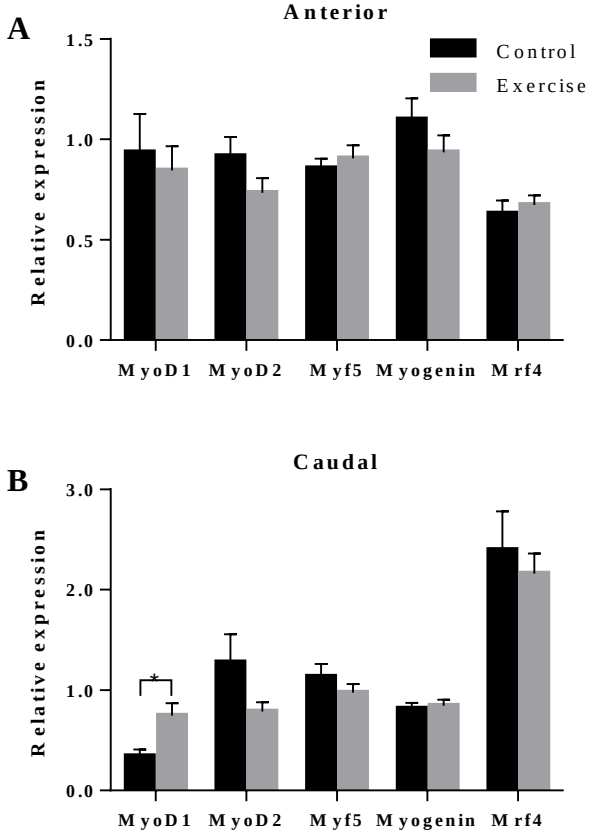


**Fig. 2.** Effects of exercise on myogenic regulatory factors gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of MyoD1, MyoD2, Myf5, myogenin, and Mrf4 in the anterior muscle (**A**) and caudal muscle (**B**) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control conditions. Data are shown as means ± SEM (n = 12). *Significant difference, $P < 0.05$.

Regarding the structural markers [myosin heavy chain (MHC), myosin light chain 2A (MLC2A), and MLC2B], in the anterior muscle of exercised fish, higher mRNA levels of

MLC2A were found (Fig. 3A). However, no differences were observed for any of these genes in the caudal muscle region (Fig. 3B).

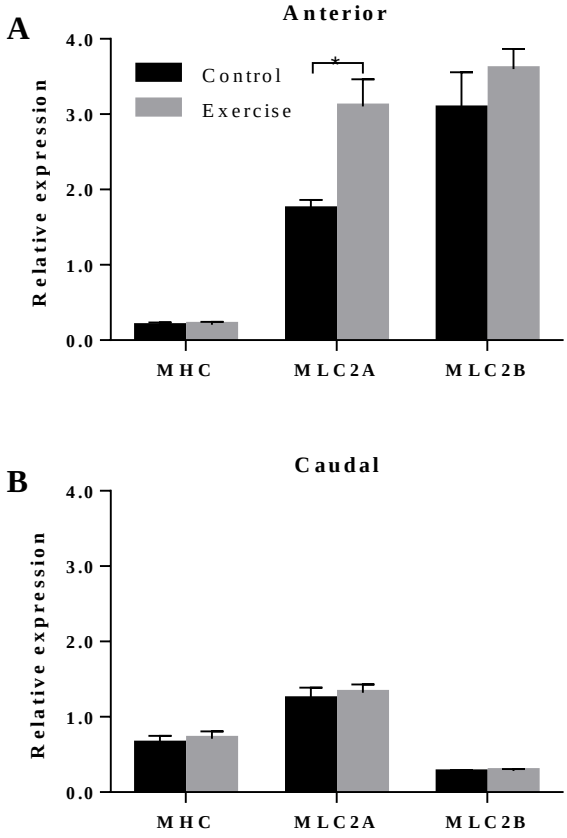


**Fig. 3.** Effects of exercise on structural markers gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of MHC, MLC2a, and MLC2b in the anterior muscle (**A**) and caudal muscle (**B**) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control conditions. Data are shown as means ± SEM (n = 12). *Significant difference, $P < 0.05$.

### 3.3. Proteolytic and angiogenesis markers

Swimming activity significantly increased the expression of several of the most important proteolytic-related genes in the anterior muscle region of the fish, such as CAPN1, CAPN2, CAPNs1a and CAPNs1b, CTSDb, muscle RING finger protein 1 (MuRF1), MAFbx, and proteasome β type 4 subunit (N3; Fig. 4A).

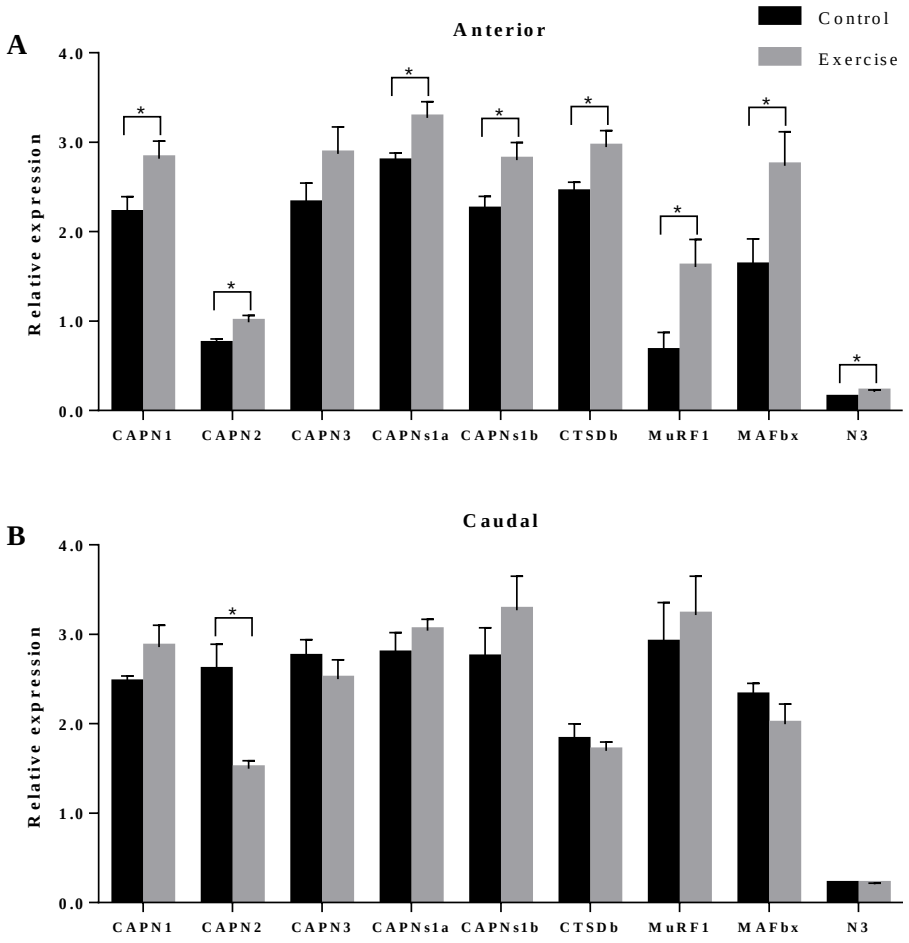


**Fig. 4.** Effects of exercise on proteolytic markers gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of CAPN1, CAPN2, CAPN3, CAPNs1a, CAPNs1b, CTSDb, MuRF1, MAFbx, and N3 in the anterior muscle (**A**) and caudal muscle (**B**) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control condition. Data are shown as means ± SEM (n = 12). *Significant difference, $P < 0.05$.

Nevertheless, in the caudal region the expression of all these molecules remained stable, except for the expression of CAPN2 being downregulated in exercised fish (Fig. 4B). These results are also supported by Western blot analysis. Thus the protein expression of CAPN1 and CTSD was significantly upregulated in the anterior region of exercised fish compared with control fish (Fig. 5A and 5B); meanwhile, no differences were found for any of these

molecules in the caudal muscle region. On the other hand, the protein levels of the ubiquitin ligase MAFbx were not modified in either muscle region (Fig. 5C).

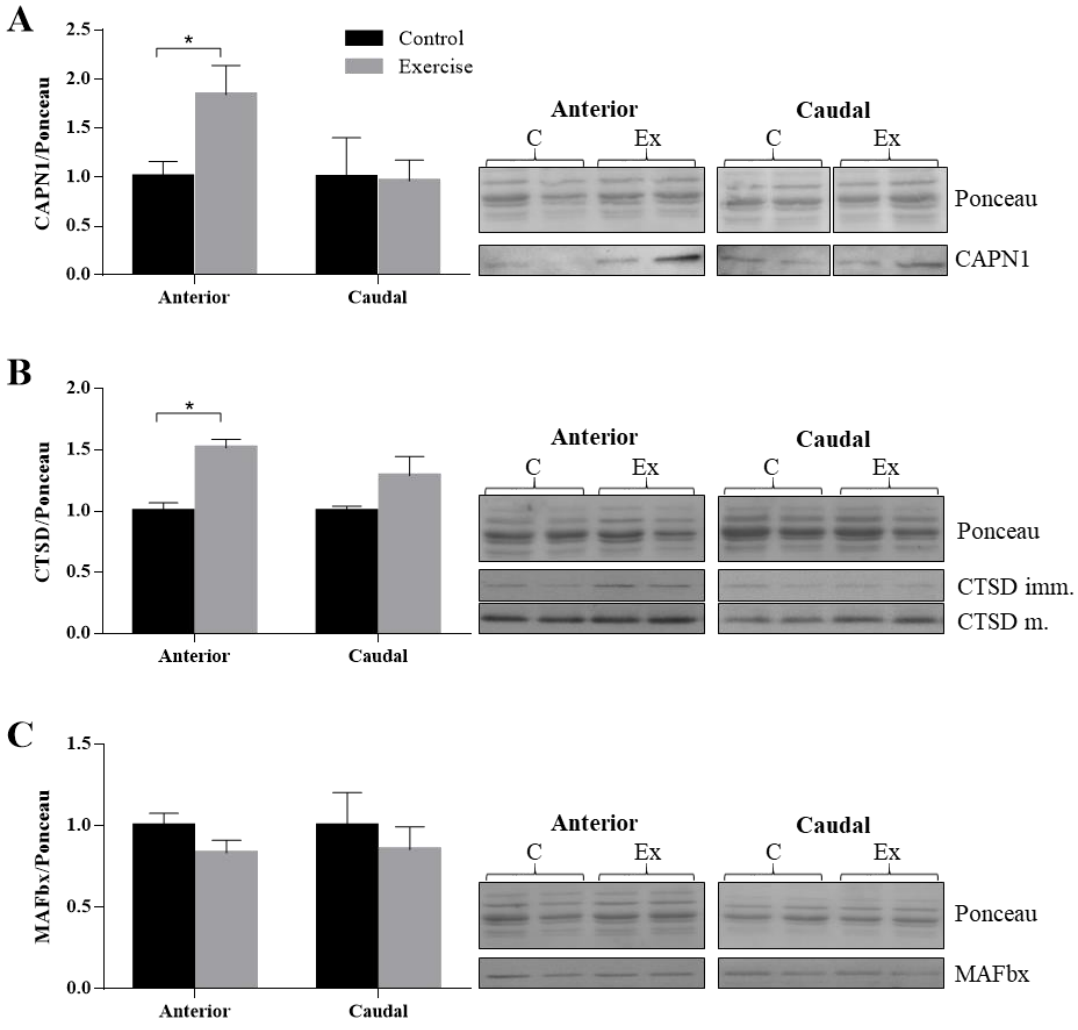


**Fig. 5.** Effects of exercise on proteolytic markers protein expression. Protein expression of CAPN1 (**A**), CTSD immature (imm.) and mature (m.) forms (**B**), and MAFbx (**C**) in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as means ± SEM (n = 4). *Significant difference, $P < 0.05$.

After validation of the specificity of the VEGF antibody using the immunization peptide to block the signal (Fig. 6A), Western blot results showed that swimming activity

significantly increased the protein expression of VEGF in the anterior muscle, whereas no differences were found in the caudal region (Fig. 6B).

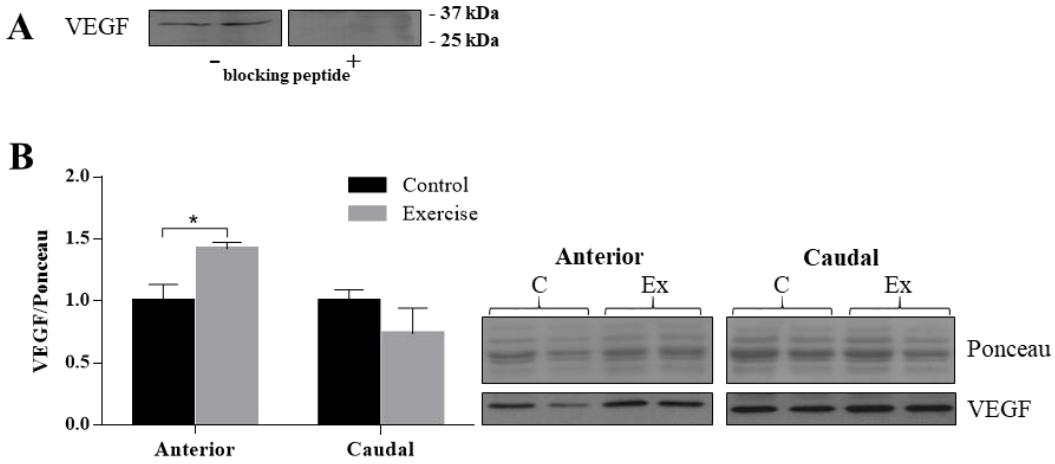


**Fig. 6.** Effects of exercise on angiogenesis marker protein expression. **A**: Western blot for VEGF specificity validation with muscle protein extract pooled samples and blocking immunization peptide. **B**: Protein expression of VEGF in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as means ± SEM (n = 4). *Significant difference, $P < 0.05$.

## 4. Discussion

The effects of exercise have been investigated in different fish species (7, 12, 25, 43, 58, 68-71, 94). Our group has demonstrated that moderate and sustained swimming activity increases body weight in rainbow trout and gilthead sea bream with clear changes in metabolism and muscle structure (8, 32, 33, 44, 59). Thus, in these studies, exercise triggered an increase in muscle aerobic capacity with rise in muscle glycogen turnover and muscle mass. Although changes in muscle protein content were not found, a significant decrease on mesenteric fat was observed in exercised gilthead sea bream fingerlings (8). Moreover, muscle structure was also adapted to exercise, with increases in hypertrophic

condition and capilarization in the anterior, but not the caudal, muscle region (44). In addition, we have also shown that swimming increases the plasma IGF-I levels (8, 84) and modulates the gene expression of several GH-IGF axis molecules, including hepatic IGF-I, and growth hormone receptor I (GHR-I) in muscle (98). Furthermore, under these conditions the TOR signaling pathway is activated at both gene and protein levels, thus stimulating protein synthesis. All these changes resulted in enhanced growth compared with control fish.

Following this line of research, in the present work we have demonstrated that the gene and protein expression of the cellular proliferation marker PCNA increases in the caudal and anterior muscle regions, respectively, with exercise. PCNA is considered a good marker of proliferation processes (1), and we have observed in gilthead sea bream cultured myocytes that the highest levels of PCNA expression coincide with the stage of maximum proliferation (36). Also, we have demonstrated that both IGF-I and IGF-II stimulate the increase of PCNA positive cells using the same cell model system (78). This concurs with the increase of IGF-II gene expression (98) parallel to that of PCNA in the caudal muscle of exercised fish. The fact that in the anterior muscle, PCNA increased in terms of protein, but not at the mRNA level, whereas in the caudal muscle the response was opposite supports the distinct response to exercise throughout the muscle already observed in our previous studies (44, 98). Moreover, this is in agreement with the longitudinal shifts in contractile properties caused by the different combination and coordination of a great variety of proteins in the white muscle of several fish species (19, 20, 26, 100), which provide faster contractile properties to the anterior muscle region (22). Nevertheless, with only these data, we cannot conclude there to be a different proliferative activity between the two muscle regions, and it becomes necessary to analyze other growth-regulating

molecules such as inhibitors, transcription factors, or structural muscle proteins. Furthermore, differences between gene and protein expression along the myogenic process have already been reported (3) and should be taken into account to understand this differential PCNA response.

In terms of growth repression, MSTN is the main inhibitor of muscle development in vertebrates (51, 79). In fish, it has been observed that fasting increases MSTN gene expression whereas refeeding decreases it (36, 64). In gilthead sea bream, as in other fish species, two main paralogs are expressed in muscle (MSTN1 and MSTN2), which can exert their function depending on physiological conditions (35, 54, 55). This is the case in this study, where only MSTN2 gene expression decreased in the anterior muscle of exercised fish but no response of MSTN gene expression was observed in the caudal region, supporting the different effect of exercise all through the muscle in fish. The significant decrease in the anterior muscle of MSTN2 would suggest the possibility that in this region the muscle is in a state of remodeling because of exercise, as we previously suggested because of the differential increase of GHR-I, IGF-I, and IGFBP-5 observed in this region (98).

In mammals, exercise determines diverse effects on myogenesis depending on its intensity and duration, which can modify the expression of MRFs, structural molecules, and IGFs (41, 101). Palstra and colleagues (70, 71) showed that zebrafish trained to perform intense exercise upregulate in muscle the expression of several MRFs, and Hasumura and Meguro (43) in the same species demonstrated very recently that short but intense exercise is able to induce the expression of MyoD and myogenin. Contrarily, in this study, 5 wk of sustained and moderate exercise in gilthead sea bream did not importantly change MRFs transcript levels. Only the increase of MyoD1 gene expression in the caudal muscle area

was significant, which could indicate the initiation of the determination stage of myogenesis in this part of the muscle that would later extend to the anterior muscle region. We should not forget that this is a dynamic process and our results only reflect a snapshot from the time of sampling. In any case, the upregulation of MyoD1 agrees with the increase in IGF-II gene expression observed in these fish (98), corroborating the stimulatory effect of this growth factor on MyoD expression previously demonstrated in gilthead sea bream myocytes (46).

With regard to the structural myofribrillar molecules, little effect was observed in the gene expression of the different myosins during exercise, with the exception of MLC2A, whose expression was increased in the anterior muscle. This coincides with the upregulation of MLC gene expression observed in the white muscle of rainbow trout (58) and zebrafish (70) under sustained swimming. Very recently, this molecule has been related to the intensive stratified formation of new fibers during muscle growth in larvae of gilthead sea bream (38). Moreover, in concordance with previous studies, it has been suggested that MLC2A is more involved in larval growth (mainly hyperplasic growth) whereas MLC2B is more related to the hypertrophic growth that takes places in the adult stages (39, 67). Similarly, Silva et al. (89) also suggested previously that MLC could be a good marker of hyperplasia in blackspot sea bream. Overall, this is in agreement with the increase in PCNA protein expression and the downregulation of MSTN2 mRNA observed in this study and with the upregulation of IGFs and GHR-I described before in anterior muscle (98), suggesting that this part of the muscle might be in a proliferative state.

The proteolytic systems play a key role in muscle, and in mammals it is known that they are necessary for normal day-to-day function. During exercise, proteolysis is increased,

proteolysis being a requirement for the remodeling and regeneration processes that occur in response to activity (24, 53, 76, 77, 102). Thus the calpain, cathepsin, and proteasome systems, including the genes MuRF or MAFbx among others, will be activated during exercise (4, 24, 27, 77, 102). In gilthead sea bream, we have recently characterized several members of the calpains, cathepsins, and ubiquitin-proteasome families and their response to nutritional changes (82, 83). However, available information on fish proteolytic systems in relation to swimming is scarce, especially in this species. Besides, as introduced before, it has been shown that in contrast to mammals, the autophagy-lysosomal system (in which cathepsins are included) has a more important contribution than the ubiquitin-proteasome on the total protein degradation in rainbow trout myotubes (85). In the present study, the anterior muscle showed a clear activation of all proteolytic systems, with the significant upregulation of most of the molecules studied. Such a uniform response also observed in the protein expression of CAPN1 and CTSD is a good indication of the proteolytic process that may be taking place in the anterior muscle, but not the caudal one, where the unique change observed was the decrease of CAPN2. In fact, muscle expression of calpains and cathepsins increased during the mobilization accompanying fasting in gilthead sea bream (82, 83). The upregulation of the calpain system observed in the present study can thus be related to two different situations known to occur in mammals. First, before myoblast fusion, it is necessary to remodel the membrane of myogenic cells at the fusion point through breaking the cytoskeletal/plasma membrane linkages (4, 42, 65), and CAPN2 is involved in this. Second, CAPN1 is more associated with the cleavage of different cytoskeletal proteins to smaller fragments (4, 102), which can be later degraded by the ubiquitin-proteasome system or cathepsins, as would be expected in fish (85) and as confirmed in this study by the increased protein levels of CTSD. Parallel to these molecules, exercise determined, in the anterior muscle, the increase of MuRF1 and MAFbx,

two muscle-specific ubiquitin ligases that participate in the last step of ubiquitination, normally induced in muscle atrophy (17). Both enzymes are frequently used as markers of muscle proteolysis in fish (9, 15, 83, 86). However, the interpretation of these results should be cautious, since at least in mammals, MAFbx is thought to exert specific control regulating MRFs such as MyoD, while MuRF1 regulates myofibrillar and sarcomeric stability (93, 102). In this sense, these results are consistent with the increased expression observed for these two molecules together with MyoD and myogenin in exercised zebrafish (43). Similarly, the β-type proteasome subunit N3 was also elevated in the anterior muscle of exercised gilthead sea bream in the present study. In this species, it has been recently demonstrated that during the greater growth stage along ontogeny (i.e., fingerlings), the fish have higher proteolysis rates than during juvenile and adult stages and, in parallel, also have higher rates of protein synthesis, producing in combination enhanced growth (83). Altogether, these data support the observation that the anterior muscle, differently from the caudal muscle, was subjected during swimming to an important remodeling.

Furthermore, the effects reported in the proteolytic systems are in agreement with the increase in PCNA protein expression and the decrease on MSTN2 gene expression in the anterior muscle. Moreover, this is also coincident with the upregulation of VEGF only in this muscle region. Although information on VEGF regulation in fish (45) is not as broad as in mammals (76, 101), VEGF is considered a good marker of muscle remodeling regulating the increase of capillary supply necessary for the new myofibers produced. Thus the observed VEGF increase in this study is another demonstration of the transformation process that is occurring in the anterior muscle region of exercised fish. Changes in capilarization were already observed in gilthead sea bream under equivalent exercise conditions. Thus, whereas in control conditions the caudal muscle showed more

capilarization than the anterior muscle, in exercised fish the capillaries in the anterior muscle increased to equal the values in caudal muscle (44). Similarly, Palstra and colleagues (70, 71) reported that in zebrafish, swimming promotes an increase in muscle hypertrophy and vascularization accompanied by upregulation of several genes including calpains and VEGF. Preliminary immunohistochemistry results have indicated that in our trial, exercised fish show a general increase also, in muscle hyperplasia and capilarization (A. Moya, J.R. Torrella, J. Fernández-Borràs, D. Rizo, A. Millán-Cubillo, E.J. Vélez, A. Ibarz, J. Gutiérrez and J. Blasco, unpublished data). Vélez et al. (98) found in anterior muscle a significant increase of both TOR gene and protein expression, which reinforces the existing equilibrium between protein degradation and synthesis; this protein balance was further confirmed by the conservation of the protein content observed in these fish (8). Overall, this is consistent with the expected muscle remodeling as effect of exercise (60).

### 4.1. Perspectives and Significance

In the present study, after 5 wk of moderate and sustained swimming in gilthead sea bream fingerlings, the significantly increased PCNA and MyoD1 gene expression in the caudal muscle region indicates the cell proliferation and differentiation condition, which correlates well with the higher gene expression levels of IGF-II and IGF-I in this region previously observed in these fish (98). Besides, in the anterior muscle region, a remodeling condition seems to exist that is confirmed by the increased gene expression of most proteolytic markers and VEGF. This overactivation of proteolytic systems corresponds with the increase in protein turnover (cathepsins and ubiquitin-proteasome) and structural changes (calpains) that are necessary for the tissue renovation and new vessels formation that take place during exercise adaptation.

Overall, the present study contributes to improving knowledge of the growth-promoting effects of swimming in fish, demonstrating for the first time the role of several proteolytic factors and the growth factor VEGF in gilthead sea bream muscle remodeling, which can help to optimize fish growth and flesh quality in this important aquaculture species and confirms the use of moderate sustained swimming as an excellent intervention for achieving this goal.

**Acknowledgments**

The authors thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the School of Biology for the maintenance of the fish. E.J.V. and E.L. are supported by a predoctoral fellowship from the "Ministerio de Ciencia e Innovación" (MICINN). This study was supported by the projects from the MICINN AGL2012-39768 and AGL2015-70679-R to J.G., and the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and the SGR2009-00402 and 2014SGR-01371 from the "Generalitat de Catalunya".

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# ARTICLE VI

## Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*)


General and Comparative Endocrinology 257 (2018) 192-202

# Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*)

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**Running title:** Growth effects of rBGH in gilthead sea bream

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**Abstract**

The growth hormone (GH)/insulin-like growth factors (IGFs) endocrine axis is the main growth-regulator system in vertebrates. Some authors have demonstrated the positive effects on growth of a sustained-release formulation of a recombinant bovine GH (rBGH) in different fish species. The aim of this work was to characterize the effects of a single injection of rBGH in fingerlings of gilthead sea bream on growth, GH-IGF axis, and both myogenic and osteogenic processes. Thus, body weight and specific growth rate were significantly increased in rBGH-treated fish respect to control fish at 6 weeks post-injection, whereas the hepatosomatic index was decreased and the condition factor and mesenteric fat index were unchanged, altogether indicating enhanced somatic growth. Moreover, rBGH injection increased the plasma IGF-I levels in parallel with a rise of hepatic mRNA from total IGF-I, IGF-Ic and IGF-II, the binding proteins IGFBP-1a and IGFBP-2b, and also the receptors IGF-IRb, GHR-I and GHR-II. In skeletal muscle, the expression of IGF-Ib and GHR-I was significantly increased but that of IGF-IRb was reduced; the mRNA levels of myogenic regulatory factors, proliferation and differentiation markers (PCNA and MHC, respectively), or that of different molecules of the signaling pathway (TOR/AKT) were unaltered. Besides, the growth inhibitor myostatin (MSTN1 and MSTN2) and the hypertrophic marker (MLC2B) expression resulted significantly enhanced, suggesting altogether that the muscle is in a non-proliferative stage of development. Contrarily in bone, although the expression of most molecules of the GH/IGF axis was decreased, the mRNA levels of several osteogenic genes were increased. The histology analysis showed a GH induced lipolytic effect with a clear decrease in the subcutaneous fat layer. Overall, these results reveal that a better growth potential can be

achieved on this species and supports the possibility to improve growth and quality through the optimization of its culture conditions.

**Keywords:** *Sparus aurata*, GH, IGF-I, growth regulation, skeletal muscle, vertebra bone

## 1. Introduction

The growth hormone (GH)/insulin-like growth factors (IGFs) axis is the most important endocrine system regulating growth in vertebrates, and this premise has also been demonstrated in fish (reviewed in: Fuentes et al., 2013; Picha et al., 2008; Reindl and Sheridan, 2012; Vélez et al., 2017). GH and IGFs stimulate somatic growth through binding their corresponding receptors (GHR and IGF-IR, respectively) widely distributed among different tissues, including muscle and bone (Le Roith et al., 2001; Reindl and Sheridan, 2012). The activation by IGFs of the PI3K-AKT-TOR (Target Of Rapamycin) signaling pathway in those tissues regulates the expression of a high number of genes involved in cell survival, proliferation and differentiation [e.g., myogenic regulatory factors (MRFs), proliferating cell nuclear antigen (PCNA), myostatin (MSTN)] among others leading to increased growth (Fuentes et al., 2013; Vélez et al., 2017).

In Mediterranean aquaculture, one of the most important cultured species is gilthead sea bream (*Sparus aurata*). In this species, the GH/IGF axis has been well studied (Benedito-Palos et al., 2007; Calduch-Giner et al., 2003; Pérez-Sánchez, 2000; Saera-Vila et al., 2005; Vélez et al., 2016b). The *in vivo* treatment with GH significantly increases the hepatic gene expression of IGF-I and IGF-II (Carnevali et al., 2005) and also has hypoosmoregulatory effects decreasing sodium levels and modifying the hepatic glucose and lipid metabolism

(Sangiao-Alvarellos et al., 2006). In *in vitro* studies, GH treatment increases myocytes proliferation and it does it at a higher level when accompanied by IGF-I (Rius-Francino et al., 2011). Moreover, this combination also stimulates the gene expression of IGF-I and two MRFs implicated in cell lineage determination and proliferation, MyoD2 and Myf5 (Azizi et al., 2016; Jiménez-Amilburu et al., 2013). Besides, direct incubation with IGF-I or IGF-II also stimulates proliferation of myocytes and osteoblasts (Capilla et al., 2011; Rius-Francino et al., 2011; Vélez et al., 2014). Overall, these studies confirm both systemic and local paracrine/autocrine actions for GH and IGFs in gilthead sea bream.

In aquaculture research, some strategies further from optimizing diet have been satisfactory in order to increase growth (reviewed in: Moomsen and Moon, 2001). This is the case of, for example, the use of anabolic steroids to stimulate protein synthesis, amino acids uptake and nitrogen retention (Higgs et al., 1982; Zohar, 1989), or the use of $\beta_2$-adrenergic agonists to heighten muscle protein while reducing muscle fat composition in rainbow trout and blue catfish (Salem et al., 2006; Webster et al., 1995). Another interesting option for growth enhancement has been the application of forced swimming (Palstra and Planas, 2013), as observed for example in gilthead sea bream, where exercise modulates the GH/IGF axis to potentiate a better physiological condition (Blasco et al., 2015; Sánchez-Gurmaches et al., 2013; Vélez et al., 2016a).

Moreover, when considering farm animals, an important background exists on beef cattle treated with a sustained-release formulation of recombinant bovine GH (rBGH), which results in increased dry matter intake, milk production, feed efficiency and protein deposition into carcass components, etc. (Dalke et al., 1992; Dohoo et al., 2003). These interesting effects made some authors to experiment with rBGH in aquaculture research

with the purpose of improving growth in some fish species. Such treatment with rBGH, which has been found to remain at detectable levels up to 140 days post-injection in coho salmon (McLean et al., 1997); and despite Leedom et al. (2002) demonstrated in tilapia that heterologous rBGH has 100-fold less affinity to the GH binding site than homologous GH, has been successfully used in rainbow trout (Biga et al., 2005, 2004a,b; Devlin et al., 2001; Gahr et al., 2008; Garber et al., 1995; Kling et al., 2012), coho and chinook salmon including transgenic coho salmon (McLean et al., 1997; Raven et al., 2012), tilapia (Kajimura et al., 2001; Leedom et al., 2002; Wille et al., 2002), different lines of catfish (Peterson et al., 2005, 2004), zebrafish and giant danio (Biga and Goetz, 2006; Biga and Meyer, 2009; Simpson et al., 2000), or more recently, sturgeon (Fenn and Small, 2015). In these studies, rBGH treatment resulted in an increase in body weight (B.W.), IGF-I plasma levels, gene expression of IGF-I in different tissues such as liver or muscle, and caused a regulation of its receptors.

However, up to date there is no information about the effects of a prolonged-release treatment with rBGH in gilthead sea bream. Therefore, the aim of this work was to analyze the effects of a single rBGH-injection in fingerlings of this species through analyzing the changes caused in somatic growth, the plasma levels of IGF-I, the expression of GH/IGF axis-, myogenic- and osteogenic-related molecules in liver, white muscle and/or bone, and the histology of muscle. All this was performed in order to expand the knowledge of GH/IGF axis and growth regulation in fish with the further intention to know how far we are from a maximum growth in this important aquaculture species.

## 2. Material and methods

### 2.1. Experimental animals and ethical statement

Two-hundred fingerlings (initial B.W. 1.01 ± 0.05 g; total body length (T.L.) 4.30 ± 0.02 cm) of gilthead sea bream (*Sparus aurata* L.) obtained from a commercial hatchery in the north of Spain were reared in the facilities of the Faculty of Biology at the University of Barcelona. Fish were distributed into 8 cages of 37 L such that two cages were put together in a larger thank (4 tanks of 200 L, each cage with 25 fish and thus, 50 fish/tank). Throughout the whole experiment, fish were maintained in a sea water recirculation system at a temperature of 23 ± 1 ºC, and were fed *ad libitum* (10% B.W./day) five times a day with a commercial diet (Gemma Diamond, Skretting, Burgos, Spain). The light cycle was 15L:9D h. After an acclimation period, the two cages of each tank were assigned randomly to a treatment group and the fish were anesthetized (MS-222 0.08 g/L) and intraperitoneally injected with a single dose (4 mg $g^{-1}$ B.W.) of rBGH (Posilac®, Elanco Animal Health, Eli Lilly and Company) diluted 1:4 with sesame oil (Sigma-Aldrich, Spain), or with the same volume of sesame oil for the controls (day 0). The rBGH dose used was chosen following the previous work of Raven et al. (2012). Biometric parameters were monitored at times 0, 2, 4 and 6 weeks. In the final sampling (6 weeks), 20 fish from each cage (80 fish per condition) were anesthetized and blood samples were taken from caudal vessels using heparinized syringes. Then fish were killed, and liver and adipose tissue were weighed to calculate both hepatosomatic (HSI) and mesenteric fat (MFI) indexes. The condition factor (C.F.) and the specific growth rate (SGR) were calculated for each tank biomass (n = 4 for each condition). Then, from three fish per tank samples of liver, anterior epaxial white muscle and vertebra (bone tissue) were collected, frozen in liquid nitrogen, and stored at -

80 °C until further analysis (n = 12 for each condition). Additionally, small samples of muscle with integument from five individuals of each group were taken and immediately fixed in 10% buffered formalin (Sigma-Aldrich, Spain) for posterior histological analysis.

Before each manipulation of the fish, the food was held for 12 h and the fish were properly anesthetized as indicated above. All procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona following the European Union, Spanish and Catalan Governments-assigned principles and legislations (permit numbers CEEA 209/14 and DAAM 7957).

**2.2. IGF-I plasma levels**

Blood was centrifuged at 1500xg for 15 min and then plasma was collected and stored at -80 ºC. Afterwards, the plasma IGFs were extracted by acid-ethanol cryoprecipitation (Shimizu et al., 2000) and the IGF-I concentration was measured using a generic IGF-I radioimmunoassay (RIA) with recombinant bream (*Pagrus auratus*) IGF-I as a tracer and standard, and polyclonal rabbit anti-barramundi (*Lates calcarifer*) IGF-I serum as a first antibody, both purchased from GroPep Bioreagents (catalogue numbers #YU020 and #PAF1, respectively). This heterologous RIA has been previously validated for Mediterranean perciform fish with a sensitivity of 0.05 ng/mL and a mid-range of 0.7-0.8 ng/mL (Mingarro et al., 2002; Vega-Rubín de Celis et al., 2004).

### 2.3. Gene expression analyses

#### 2.3.1. RNA extraction and cDNA synthesis

Total RNA extraction was performed from 30 mg of liver, or 100 mg of tissue in the case of white muscle and bone samples in 1 mL TRI reagent solution (Applied Biosystems, Alcobendas, Spain) following the manufacturer's instructions. RNA concentration and purity was determined with a Nanodrop 2000 (Thermo Scientific, Alcobendas, Spain). RNA integrity check was performed with a 1% agarose gel stained with SYBR-Safe DNA gel stain (Life Technologies, Alcobendas, Spain). In order to eliminate all genomic DNA, prior to cDNA synthesis, a DNase I (Life Technologies) treatment of 1 µg of total RNA was performed following the manufacturer's recommendations. Finally, reverse transcription was carried out using the Transcriptor First Strand cDNA synthesis kit (Roche, Sant Cugat, Spain) following the manufacturer's instructions.

#### 2.3.2. Real time quantitative-PCR (q-PCR)

Gene expression (mRNA) analyses were performed by q-PCR in a CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain) as described by Vélez et al. (2016a). All the primer sequences for the analyzed genes are showed as supplementary material (S.1 and S.2) and can be found in previous literature (Pinto et al., 2001; Rosa et al., 2010; Vélez et al., 2016a, 2014; Vieira et al., 2012). The genes can be grouped in: a) GH/IGF axis [total IGF-I and its three splice variants: IGF-Ia, IGF-Ib and IGF-Ic; IGF-II; the IGF binding proteins: IGFBP-1a, IGFBP-2b, IGFBP-4, IGFBP-5b; the receptors: IGF-IRa and IGF-IRb; GHR-I, GHR-II]; b) myogenesis [MRFs: MyoD1, MyoD2, Myf5, myogenin and MRF4;

Proliferating Cell Nuclear Antigen (PCNA); Myostatins: MSTN1, MSTN2; Myosin Heavy Chain (MHC) and Myosin Light Chain (MLC2A and MLC2B)]; c) signaling pathways [TOR; translation initiation factor 4E Binding Protein 1 (4EBP1); Ribosomal protein S6 Kinase (70S6K); AKT; Forkhead box O3 (FOXO3)]; d) osteogenesis [Runt-related transcription factor 2 (RUNX2); type 1 Collagen subunit 1-A (COL1A); Fibronectin subunit 1-A (F1B1A); Osteonectin (ON); Osteopontin (OP); Osteocalcin (OSTC); Matrix Gla Protein (MGP) and Tissue Non-specific Alkaline Phosphatase (TNAP)]; and e) reference genes [Ribosomal Protein S18 (RPS18), Ribosomal Protein L27a (RPL27a) and Elongation Factor 1α (EF1α)]. The expression levels of each gene were calculated using the Bio-Rad CFX Manager 3.1 software by the Pfaffl method (Pfaffl, 2001) relative to the geometric mean expression of the most stable genes analyzed (EF1α and RPS18). The relative expression of each gene in the rBGH condition was represented as the fold change over the control fish (log2).

**Table S1.** Primer sequences of reference and GH/IGF axis-related genes used for q-PCR.

| Gene | Primer sequences (5'–3') | Tm °C | Accession number |
|---|---|---|---|
| *RPS18* | **F**: GGGTGTTGGCAGACGTTAC<br>**R**: CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 |
| *RPL27a* | **F**:AAGAGGAACACAACTCACTGCCCCAC<br>**R**:GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 |
| *EF1a* | **F**:CTTCAACGCTCAGGTCATCAT<br>**R**:GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 |
| *IGF-Ia* | **F**:AGGACAGCACAGCAGCCAGACAAGAC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | AY996779 |
| *IGF-Iab* | **F**:AGTCATTCATCCTTCAAGGAAGTGCATCC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | EF688015 |
| *IGF-Iabc* | **F**:ACAGAATGTAGGGACGGAGCGAATGGAC<br>**R**:TTCGGACCATTGTTAGCCTCCTCTCTG | 60 | EF688016 |
| *IGF-II* | **F**:TGGGATCGTAGAGGAGTGTTGT<br>**R**:CTGTAGAGAGGTGGCCGACA | 60 | AY996778 |
| *IGFBP-1a* | **F**:AGTGCGAGTCCTCTCTGGAT<br>**R**:TCTCTTTAAGGGCACTCGGC | 60 | KM522771 |
| *IGFBP-2b* | **F**:CGGGCTGCTGCTGACATACG<br>**R**:GTCCCGTCGCACCTCATTTG | 60 | AF377998 |
| *IGFBP-4* | **F**:TCCACAAACCAGAGAAGCAA<br>**R**:GGGTATGGGGATTGTGAAGA | 60 | F5T95CD02JMZ9K |
| *IGFBP-5b* | **F**:TTTCTCTCTCGGTGTGC<br>**R**:TCAAGTATCGGCTCCAG | 60 | AM963285 |
| *IGF-IRa* | **F**:AGCATCAAAGACGAACTGG<br>**R**:CTCCTCGCTGTAGAAGAAGC | 55 | KT156846 |
| *IGF-IRb* | **F**:GCTAATGCGAATGTGTTGG<br>**R**:CGTCCTTTATGCTGCTGATG | 55 | KT156847 |
| *GHR-I* | **F**:ACCTGTCAGCCACCACATGA<br>**R**:TCGTGCAGATCTGGGTCGTA | 60 | AF438176 |
| *GHR-II* | **F**:GAGTGAACCCGGCCTGACAG<br>**R**:GCGGTGGTATCTGATTCATGGT | 60 | AY573601 |

Tm º C: Melting temperature. F: Forward. R: Reverse.

**Table S2.** Primer sequences of myogenic- and osteogenic-related genes used for q-PCR.

| Gene | Primer sequences (5'–3') | Tm ° C | Accession number |
|---|---|---|---|
| *PCNA* | **F**:TGTTTGAGGCACGTCTGGTT<br>**R**:TGGCTAGGTTTCTGTCGC | 60 | AY550963.1 |
| *MSTN1* | **F**:GTACGACGTGCTGGGAGACG<br>**R**:CGTACGATTCGATTCGCTTG | 60 | AF258448.1 |
| *MSTN2* | **F**:ACCTGGTGAACAAAGCCAAC<br>**R**:TGCGGTTGAAGTAGAGCATG | 60 | AY046314 |
| *MHC* | **F**:AGCAGATCAAGAGGAACAGCC<br>**R**:GACTCAGAAGCCTGGCGATT | 60 | AY550963.1 |
| *MLC2A* | **F**:GCCCCATCAACTTCACCGTCTTT<br>**R**:GGTTGGTCATCTCCTCAGCGG | 60 | AF150904 |
| *MLC2B* | **F**:TCCCTTTGCTATTCTGCCTTC<br>**R**:AAATCAGCCCTATTCCCCATA | 60 | FG618631 |
| *TOR* | **F**:CAGACTGACGAGGATGCTGA<br>**R**:AGTTGAGCAGCGGGTCATAG | 60 | - |
| *4EBP1* | **F**: CCAACCTGCGACTCATCTCT<br>**R**: GTTCCTCTCATCCTCCCACA | 60 | - |
| *70S6K* | **F**:GCACCAGAAAGGCATCATCT<br>**R**:AAGGTGTGGGTCACTGTTCC | 60 | - |
| *AKT2* | **F**:GCTCACCCCACTCTTCAGAC<br>**R**:AAATTGGGAAATGTGCTTGC | 60 | ERA047531 |
| *FOXO3* | **F**:CAGCAGCCTGGAGTGTGATA<br>**R**:CCAGCTCTGAGAGGTCTGCT | 60 | - |
| *RUNX2* | **F**:ACCCGTCCTACCTGAGTCC<br>**R**:AGAAGAACCTGGCAATCGTC | 60 | JX232063 |
| *COL1A* | **F**:GAGATGGCGGTGATGTGGCGGAGTC<br>**R**:GCCTGGTTTGGCTGGATGAAGAGGG | 68 | DQ324363 |
| *FIB1A* | **F**:CGGTAATAACTACAGAATCGGTGAG<br>**R**:CGCATTTGAACTCGCCCTTG | 60 | FG262933 |
| *ON* | **F**:AGGAGGAGGTCATCGTGGAAGAGCC<br>**R**:GTGGTGGTTCAGGCAGGGATTCTCA | 68 | AY239014 |
| *OP* | **F**:AAAACCCAGGAGATAAACTCAAGACAACCCA<br>**R**:AGAACCGTGGCAAAGAGCAGAACGAA | 68 | AY651247 |
| *OSTC* | **F**:TCCGCAGTGGTGAGACAGAAG<br>**R**:CGGTCCGTAGTAGGCCGTGTAG | 56 | AF048703 |
| *MGP* | **F**:TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC<br>**R**:CGGGCGGATAGTGTGAAAAATGGTTAGTG | 68 | AY065652 |
| *TNAP* | **F**:CATCGCAACCCTTTTCACAGTCACCCG<br>**R**:AACAGTGCCCAAACAGTGGTCCCATTAGC | 68 | AY266359 |

Tm º C: Melting temperature. F: Forward. R: Reverse.

### 2.4. Western Blot

To obtain protein extracts from white skeletal muscle samples, tissue homogenization was performed from 100 mg per sample in 1.5 mL RIPA Buffer supplemented with phosphatase (PMSF 1 nM and $Na_3VO_4$ 0.2 mM, Sigma-Aldrich, Tres Cantos, Spain) and protease inhibitors (Protease Inhibitor Cocktail Sc-29136, Santa Cruz Biotechnology, Inc. Santa Cruz CA., USA) using the Precellys® Evolution Homogenizer combined with Cryolys® as a cooling system (Bertin Technologies, France). Protein concentration was determined by the Bradford method (Bradford, 1976) and then, 20 µg of protein per sample were separated by SDS-PAGE electrophoresis on 10% polyacrylamide gels at 125V for 1 h 30 min. After electrophoresis, proteins were transferred to Immobilon-FL PVDF membranes (Merck Chemicals & Life Science S.A., Madrid, Spain). Prior to membrane blocking, total transferred protein was determined by REVERT™ Total Protein Stain (Li-Cor, Alcobendas, Spain) following the manufacturer's instructions and scanned with an Odyssey® FC Imaging System (Li-Cor, Alcobendas, Spain) through the 700 nm channel. After total protein quantification, the membranes were blocked 1 h 15 min in 5% non-fat milk buffer. Following blocking, the membranes were properly washed and incubated with the respective primary antibodies for the phosphorylated forms of AKT and TOR at 1:200 dilution. The selected antibodies were: rabbit polyclonal anti-phospho AKT (cat. No. 9271), and rabbit polyclonal anti-phospho mTOR (Cat. No. 2971), both obtained from Cell Signaling Technology (Beverly, MA). After washing, the membranes were incubated with peroxidase-conjugated secondary antibody: goat anti-rabbit (Cat. No. 31460) from Thermo Scientific, Alcobendas, Spain. Immunoreactive bands were developed by using an enhanced chemiluminiscence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain) and the signal detected with the Odyssey® FC Imaging System (Li-

Cor, Alcobendas, Spain). Once phosphorylated forms were analyzed, bound antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific) and then the membranes were blocked and blotted again against the total forms of the studied proteins at 1:500 dilution [rabbit polyclonal anti-total AKT (Cat. No. 9272, Cell Signaling Technology) and rabbit polyclonal anti-total mTOR (Cat. No. T2949, from Sigma-Aldrich, Spain)]. The different bands were quantified by Odissey software Image Studio ver. 5.2.5.

### 2.5. White muscle histology

For microscopic preparations, the fixed tissues were dehydrated in a graded ethanol series (70%, 80%, 90% and 100%) and embedded in paraffin blocks. The blocks were sectioned at 5 µm with a rotary microtome (pfm, ROTARY 3003, Köln, Germany). Paraffin sections of muscle tissue including subcutaneous adipose tissue and skin were stained with hematoxylin and eosin and studied by light microscopy (Olympus) at 10x magnification. The average area (FCSA), perimeter (FPER), shape factor (SF), small diameter (Feret) and the density of fibers (FD) were obtained using ImageJ software (National Institutes of Health, Bethesda, MD, USA) from three pictures of each animal (n = 5). In addition to fibers morphological parameters analysis, the subcutaneous fat content was compared between groups by visual examination.

### 2.6. Statistical analyses

Data were analyzed using the IBM SPSS Statistics v.22 software and are presented as means ± SEM. Data normality and homoscedasticity through groups was checked by the

Shapiro-Wilk test followed by Levene test. Then, a two-way ANOVA was used to confirm that a tank effect does not exists for any of the variables analyzed. Next, the differences between the two experimental groups in body weight through time were analyzed by two-way ANOVA followed by a Student's *t*-test comparison (n = 4 for each condition). The other somatic growth parameters, gene expression and western blot data, were analyzed by a Student's *t*-test [n = 12 per condition, except for HSI and MFI (n = 80), Western blot (n = 8) and histology (n = 5)]. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Biometric and plasma parameters

Fig. 1 represents the mean B.W. of control and rBGH injected fingerlings at 0, 2, 4 and 6 weeks post-injection. Although both groups of fish significantly increased their B.W. with time, the rBGH injected fish presented a significantly higher B.W. only at 6 weeks (9.5%) compared to control fish. Besides B.W., other somatic growth index results at 6 weeks post-injection are shown in Table 1.

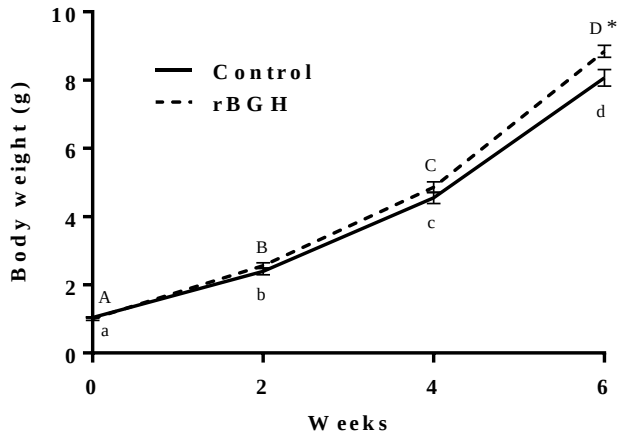


**Figure 1.** Mean body weight of control and rBGH-treated gilthead sea bream fingerlings at 0, 2, 4 and 6 weeks post-injection. Values are represented as means ± SEM (n = 4). Letters (control, lower case; rBGH, uper case) indicate time effects calculated by two way ANOVA ($p < 0.05$). Significant differences between control and rBGH groups determined by Student's *t*-test are marked with *($p < 0.05$).

The specific growth rate (SGR) at that time was significantly higher in rBGH injected fingerlings with respect to the control fish, whereas the hepatosomatic index (HSI) decreased. Nevertheless, the observed higher T.L. in rBGH injected fingerlings, and both condition factor (C.F.) and the mesenteric fat index (MFI) values, were not statistically different compared to control animals. Moreover, significantly higher IGF-I plasma levels at 6 weeks post injection were detected in the rBGH injected fish.

**Table 1.** Somatic growth parameters and plasma IGF-I levels in control and rBGH- treated gilthead sea bream fingerlings at 6 weeks post-injection.

| | SGR | T.L (cm) | C.F | HSI | MFI | IGF-I (ng/mL) |
|---|---|---|---|---|---|---|
| **Control** | 4.87 ± 0.08 | 8.06 ± 0.10 | 1.52 ± 0.01 | 1.56 ± 0.03 | 1.47 ± 0.07 | 64.37 ± 3.87 |
| **rBGH** | 5.80 ± 0.07* | 8.31 ± 0.08 | 1.50 ± 0.01 | 1.42 ± 0.03*** | 1.32 ± 0.07 | 76.75 ± 4.44* |

SGR: Specific Growth Rate % = 100 × (ln final B.W. − ln initial B.W.) $days^{-1}$; T.L: Total length; C.F: Condition Factor = 100 × B.W./$T.L^3$; HSI: Hepatosomatic Index = g liver × 100 g $B.W.^{-1}$; MFI: Mesenteric Fat Index = g fat × 100 g $B.W.^{-1}$; [IGF-I]: IGF-I plasma levels. Values are presented as means ± SEM for SGR, TL and CF (n = 4), HSI and MFI (n = 80), and IGF-I levels (n = 20). Significant differences are indicated with *($p < 0.05$) or ***($p < 0.001$).

### 3.2. GH-IGFs axis gene expression in liver, white muscle and bone tissue

The relative expression results of the main GH-IGFs axis-related genes analyzed in liver, muscle and vertebral-bone tissues at 6 weeks post-injection are shown in Figure 2. In hepatic tissue, the rBGH injection caused a significant increase in the expression of total IGF-I, IGF-Ic, IGF-II, IGFBP-1a, IGFBP-2b, IGF-IRb, GHR-I and GHR-II (Fig. 2A). In the case of white muscle, the rBGH treatment caused a significant up-regulation of IGF-Ib and GHR-I, and a significant down-regulation of IGF-IRb gene expression (Fig. 2B). In bone tissue, the rBGH injected fingerlings presented significantly lower levels of expression of IGF-Ib, IGF-Ic, and both IGF-I receptors (IGF-IRa and IGF-IRb) compared to the control fish (Fig. 2C). Regarding GH expression, the mRNA was only above

detectable levels in muscle tissue, although no differences were found between groups (data not shown).

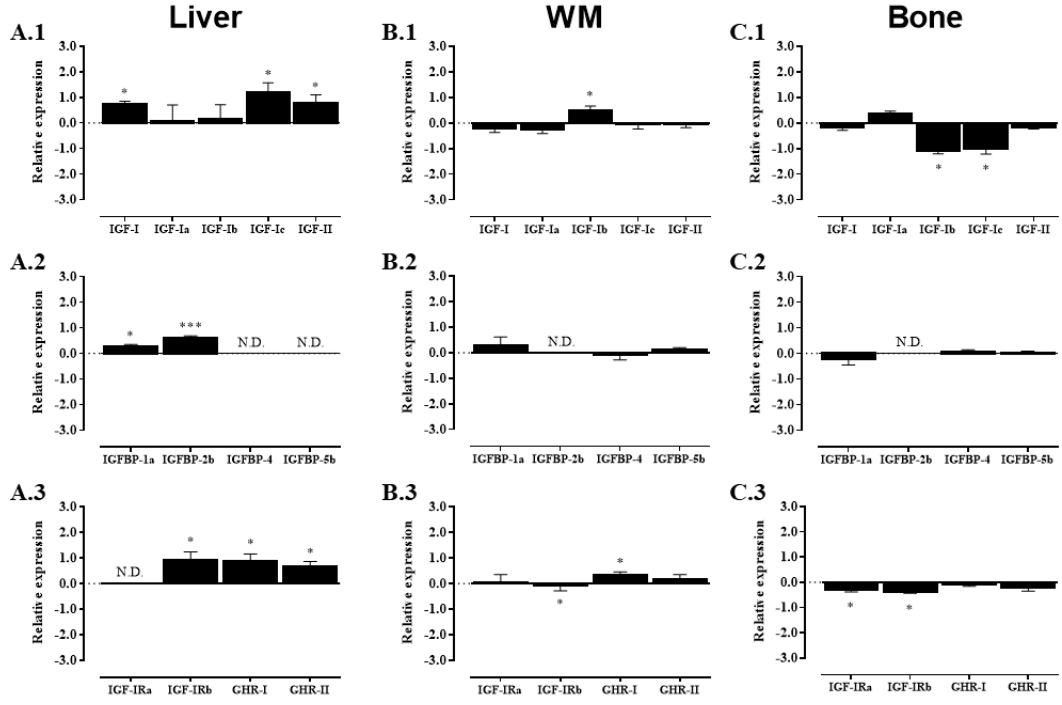


**Figure 2.** Effects of the rBGH treatment in gilthead sea bream fingerlings over the expression of the main GH-IGFs axis-related genes in liver (**A**), white muscle (**B**) and bone (**C**) at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of total IGF-I, IGF-I spliced variants and IGF-II (**A.1**, **B.1** and **C.1**); IGF-I binding proteins (**A.2**, **B.2** and **C.2**.); IGF-I and GH receptors (**A.3**, **B.3** and **C.3**). Data are shown as fold change (log2) relative to controls as means ± SEM (n = 12). Significant differences between control and rBGH groups for each gene were determined by Student's *t*-test and are marked with *($p < 0.05$) or ***($p < 0.001$). WM, white muscle; N.D., non-detected.

### 3.3. Muscle growth markers and AKT-TOR pathway gene expression and signaling

Regarding the expression of the main genes involved in the myogenic process (MyoD1, MyoD2, Myf5, myogenin and MRF4; data not shown), and the proliferation marker PCNA (Fig. 3A), significant alterations due to rBGH treatment were not observed. On the other hand, significant up-regulation of myostatin 1 and 2 (MSTN1, MSTN2) and myosin light

chain 2B (MLC2B) gene expression was detected as effect of rBGH treatment in the fingerlings of gilthead sea bream (Fig. 3A).

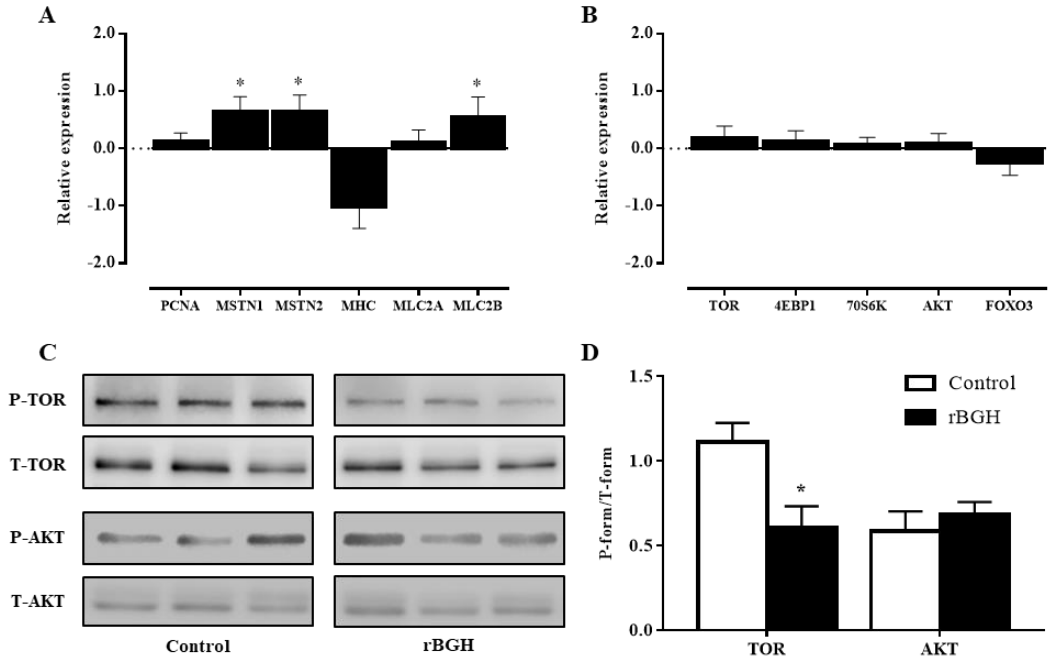


**Figure 3.** Effects of the rBGH treatment over the expression of growth markers genes and signaling pathways in white muscle of gilthead sea bream at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of (**A**) proliferation and differentiation markers (PCNA, MSTN1 (Myostatin 1), MSTN2 (Myostatin 2), MHC (Myosin Heavy Chain), MLC2A (Myosin Light Chain 2-A), MLC2B (Myosin Light Chain 2-B) and (**B**) AKT/TOR signaling molecules (TOR, 4EBP1, 70S6K, AKT, FOXO3). (**C**) Representative western blot and quantification of phosphorylated and total forms of TOR (P-TOR and T-TOR, respectively) and AKT (P-AKT and T-AKT) in control and rBGH-injected fingerlings. For **A** and **B**, data are shown as fold change (log2) relative to controls as means ± SEM (n = 12). For **C**, data are represented as means ± SEM (relative expression of the phosphorylated form of each molecule normalized to their total form, n = 8). Significant differences were determined by a Student's *t*-test and are indicated with *($p < 0.05$).

With regards to the signaling pathways studied, no differences were found in TOR, AKT and their principal downstream effectors in terms of gene expression (Fig. 3B). However, the protein expression results showed a significant decrease of TOR phosphorylation and a stable levels of AKT phosphorylation in rBGH-treated fish at 6 weeks of experiment compared to control fish (Fig. 3C, D).

### 3.4. Ostogenesis-related genes expression in bone tissue

In bone tissue, the rBGH treatment affected the expression of some of the main osteogenic genes analyzed, detecting significant up-regulation of osteopontin (OP), osteocalcin (OSTC), type 1 collagen subunit 1α (COL1A) and tissue non-specific alkaline phosphatase (TNAP) (Fig. 4).

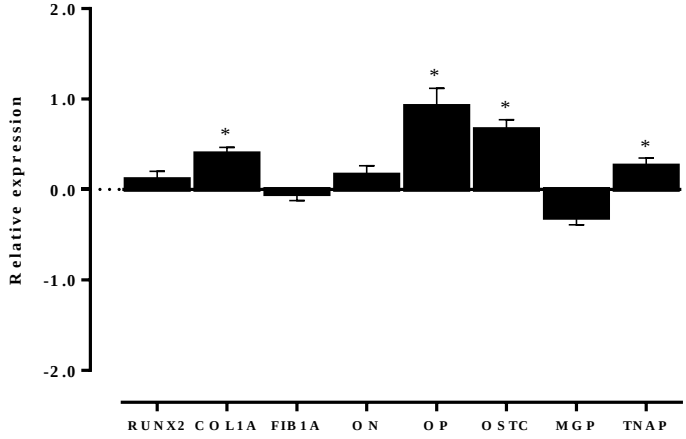


**Figure 4.** Effects of the rBGH treatment over the expression of the main osteogenesis-related genes in gilthead sea bream bone tissue at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of RUNX2 (Runt-related transcription factor 2), COL1A (type 1 Collagen subunit 1-A), FIB1A (Fibronectin subunit 1-A), ON (Osteonectin), OP (Osteopontin), OTC (Osteocalcin), MGP (Matrix Gla Protein) and TNAP (tissue non-specific alkaline phosphatase). Data are shown as fold change (log2) relative to controls as means ± SEM (n = 12). Significant differences between control and rBGH groups for each gene were determined by Student's *t*-test and are marked with * ($p < 0.05$).

### 3.5. White muscle histology

Analysis of histological white muscle tissue slides revealed that the rBGH treatment did not affect significantly muscle fiber morphometrical parameters (Table 2), but it caused a visual reduction of subcutaneous adipose tissue content in the muscle of rBGH treated fingerlings in comparison with control fish (Fig. 5).

**Table 2.** Morphometric fiber parameters in white muscle of control and rBGH-treated gilthead sea bream fingerlings at 6 weeks post-injection.

| | FCSA (µm²) | FPER (µm) | SF | Feret (µm) | FD (fibers/mm²) |
|---|---|---|---|---|---|
| **Control** | 1898.13 ± 266.02 | 164.30 ± 12.03 | 0.72 ± 0.01 | 63.09 ± 4.33 | 429.53 ± 28.03 |
| **rBGH** | 1685.51 ± 251.31 | 161.31 ± 15.75 | 0.68 ± 0.03 | 65.42 ± 7.63 | 412.75 ± 56.54 |

FCSA: fiber cross-sectional area. FPER: fiber perimeter. SF: shape factor (circularity). Feret: small diameter of the fibers. FD: fiber density. Values are represented as mean ± SEM (n=5; 3 images/animal).

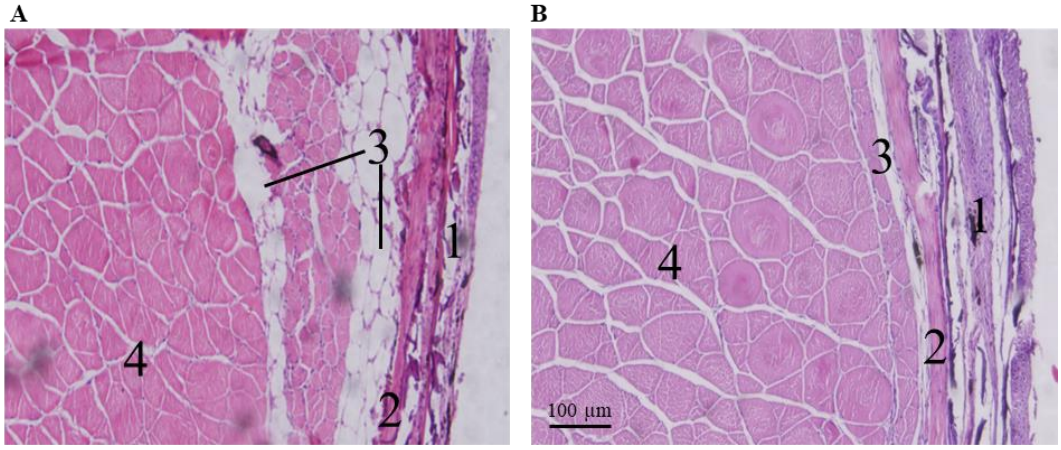


**Figure 5**. Effects of the rBGH treatment over the white muscle histology of gilthead sea bream at 6 weeks post-injection. Representative images of a hematoxylin/eosin staining of muscle histological slides of (**A**) control and (**B**) rBGH gilthead sea bream fingerlings after 6 weeks post-injection. 1. Fish skin thin section, 2. Dermis, 3. Subcutaneous fat content, 4. Skeletal muscle. Scale bar 100 µm.

## 4. Discussion

As existing literature reports, rBGH injection has been proven to be a valid approach to increase growth in various fish species (Biga and Goetz, 2006; Biga and Meyer, 2009; Biga et al., 2005, 2004a,b; Devlin et al., 2001; Fenn and Small, 2015; Gahr et al., 2008; Garber et al., 1995; Kajimura et al., 2001; Kling et al., 2012; Leedom et al., 2002; McLean et al., 1997; Peterson et al., 2005, 2004; Raven et al., 2012; Simpson et al., 2000; Wille et al., 2002). The aim of the present study was to assess for the first time in gilthead sea bream

fingerlings the effects on growth of such sustained-release formulation of rBGH. In this aspect, the significant increase obtained in B.W. and SGR, together with the reduction of HSI as effect of rBGH treatment in this species, is in agreement with those previous observations. Moreover, similarly to the present study, alteration of the C.F. was neither reported in channel catfish by Peterson et al. (2004) or coho salmon by Raven et al. (2012), thus confirming that the growth increase obtained is due mainly to somatic growth. However, a decrease on this parameter together with a reduced MFI was observed in rainbow trout (Kling et al., 2012), indicating that the effects of rBGH injection depend on multiple factors, such as species, age, type of injection (single or multiple repeated injections) or dose, among others.

### 4.1. Effects of rBGH treatment on circulating GH and IGF-I

A positive correlation between IGF-I levels and SGR in the rBGH-treated fish has been observed in different studies (Biga et al., 2005; Gahr et al., 2008; Kajimura et al., 2001; Peterson et al., 2005; Raven et al., 2012). This effect is also observed in the present work, in which a 19.23% increase of circulating IGF-I coincides with a 19.1% increase in SGR at 6 weeks post-injection. Regarding GH, although analysis of neither exogenous rBGH or endogenous gilthead sea bream GH plasma levels was possible in this study, based on previous works in trout (McLean et al., 1997; Biga et al., 2005) and mainly in tilapia (Leedom et al., 2002), as well as the IGF-I plasma levels reported here, we consider that the levels of rBGH rise in plasma just 12 h after treatment (Biga et al., 2005), and remain high at least until the final days of the trial. In fact, Leedom et al. (2002) using a dose of rBGH four times lower than that in our study (1 mg/g B.W.) found that after 70 days of

treatment, the rBGH plasma levels were very high (around 200 ng/ml), more than 10-fold higher than the normal GH levels in tilapia. Furthermore, this species is quite close phylogenetically to gilthead sea bream and is also cultured at a similar temperature, thus it makes us to assume a similar releasing rate for the injected rBGH. Moreover, with regards to the stability of rBGH in the circulation, Biga et al. (2005) demonstrated that with time, rainbow trout produce antibodies to clear the exogenous rBGH, and similarly Leedom et al. (2002) showed that treatment with rBGH induces increased IgM levels in tilapia. Nevertheless, these same authors also demonstrated that the half-life of rBGH derived from the Posilac® formulation was more than 5-fold the half-life of injected rBGH dissolved in saline, and that the homologous GH was cleared from the circulation faster than the heterologous hormone (Leedom et al., 2002). Altogether, these data suggest that after an initial peak in circulating rBGH, such levels decreased; however, even at the end of the experiment might still be much higher than the GH plasma levels present in the control conditions, knowing that circulating GH levels are on average 5-15 ng/mL in this species (Pérez-Sánchez et al., 1995; Mingarro et al., 2002; Blasco et al., 2015). Concerning endogenous GH, Raven et al. (2012) found that the rBGH treatment decreases the plasma levels of GH in coho salmon after 14 weeks post-injection (and a similar response was observed with regards to GH mRNA levels in the pituitary when normalized by B.W.). In support of this observation, in a similar experiment as the present one with gilthead sea bream juveniles, we have also found a clear down-regulation of pituitary GH gene expression. However, in rainbow trout and also in tilapia endogenous GH plasma levels were not modified by rBGH treatment in a shorter term (Biga et al., 2005; Leedom et al., 2002). These results suggest a differential species- and time-specific response to rBGH that may be also caused by differences in the affinity of GH receptors to rBGH. Overall, these

data indicates that important changes occur in response to rBGH with these circulating growth factors and points out the key role of the GH/IGF axis in somatic growth regulation.

### 4.2. Effects of rBGH treatment on GH/IGF axis gene expression

In the present work, the increased plasma IGF-I concentration agrees with the higher total IGF-I mRNA levels detected in liver, in concordance to previous studies (Biga et al., 2005, 2004b; Gahr et al., 2008; Peterson et al., 2005; Raven et al., 2012, 2008; Shamblott et al., 1995). More specifically, the rBGH treatment induced the overexpression of IGF-Ic, known as the most important splice variant in this species in terms of contributing to circulating IGF-I levels (Tiago et al., 2008). Besides IGF-I, the overexpression of IGF-II and GHR-I and GHR-II genes was in agreement with that observed in liver of rainbow trout by Gahr et al. (2008) and Shamblott et al. (1998), or gilthead sea bream by Carnevali et al. (2005), suggesting in these conditions a clear hepatic response to GH. Furthermore, these results are consistent with that found during the summer growth spurt in gilthead sea bream, where the circulating levels of GH, IGF-I, as well as the hepatic expression of GHR and IGF-I were increased (Calduch-Giner et al., 2003; Saera-Vila et al., 2007). As it is known in mammals, the GHR transcript encodes for a full length GHR that leads to the functional GHR. Then, this transcript can be post-transcriptionally truncated to generate a GHR with no intracellular signal transduction domains or GH binding proteins (GHBPs). These GHBPs can regulate bioavailability and half-life of circulating GH (Fuentes et al., 2013). Therefore, the up-regulation of GHRs expression when GH is elevated could be a transcriptional way to suppress the excess of GH signaling, as suggested by Saera-Vila et al. (2005) in this species, or in rainbow trout by Gahr et al. (2008). Regarding IGFBPs,

IGFBP-1a and IGFBP-2b are recognized in zebrafish as inhibitors of IGF-I cellular effects (Duan et al., 2010, 1999) and are considered molecules with growth-inhibitor effects in fish due to that they are usually overexpressed under catabolic conditions (Kelley et al., 2001). In the case of the present study, the overexpression found for IGFBP-2b is in agreement with previous studies in GH-treated fish species (Breves et al., 2014; Shimizu et al., 2003, 1999), which together with the increase in IGFBP-1a, it could suggest a strategy to compensate/regulate the increased levels of IGF-I.

In white muscle the expression of the GH-IGF axis-related genes was less affected. Previous studies using rBGH treatment or GH overexpressing lines, as reviewed by Fuentes et al. (2013), demonstrated stimulation of growth not only by increasing IGF-I expression in the liver, but also acting directly over muscle. In this sense, while GH-transgenesis or GH treatment in different species increases muscular expression of IGF-I (Biga and Meyer, 2009; Devlin et al., 2009; Eppler et al., 2007; Raven et al., 2008), this is not occurring or only slightly in others (Gahr et al., 2008; Kuradomi et al., 2011; Raven et al., 2012). In the present work, albeit no differences were found in the muscular expression of total IGF-I as effect of rBGH-treatment, the expression pattern of each IGF-I splice variant is in agreement with a previous study in gilthead sea bream myocytes, where a GH treatment increased the expression of IGF-Ib without detectable changes in IGF-Ia or IGF-Ic (Jiménez-Amilburu et al., 2013). Hence, these results confirm that in muscle tissue IGF-Ib is the most sensitive form to hormonal treatments in this species. Furthermore, the observed GHR-I up-regulation in muscle as effect of rBGH treatment was also observed in coho salmon (Raven et al., 2008) and it suggests that the GH anabolic effects may be induced in this tissue directly through the activation of this receptor. In the case of GHR-II, transcriptional and promoter analyses indicate that this receptor isoform is more responsive

than GHR-I to nutritional (Benedito-Palos et al., 2007) and environmental (Saera-Vila et al., 2009, 2007) stressors, which was substantiated herein by the lack of muscle response to rBGH injection. On the other hand, the decrease in IGF-IRb expression detected in muscle could be interpreted as a mechanism to regulate the effects of the increased IGF-I circulating levels. This mechanism of down-regulation has been previously observed in IGF-I binding capacity or gene expression in different fish tissues and species (Baños et al., 1997; Biga et al., 2004b; Leibush and Lappova, 1995; Moon et al., 1996; Plisetskaya et al., 1993; Vélez et al., 2016a).

Furthermore in bone, the gene expression results present a similar pattern to that observed in white muscle, especially regarding total IGF-I, IGF-II, IGF-Rs and IGFBPs. In contrast with that, the splice variant IGF-Ib and also IGF-Ic, are down-regulated, suggesting that the GH effects over the GH-IGF system in the three tissues may not completely coincide, being more pronounced in bone due to a first or more powerful action of GH over this tissue.

### 4.3. Effects of rBGH treatment on growth markers and AKT-TOR pathway in muscle

During the last decade, a large number of studies (using both *in vivo* and *in vitro* models) have focused on describing the processes that regulate muscular growth in several fish species and how different conditions may affect them. The state of the art of this research has been recently well reviewed (Fuentes et al., 2013; Vélez et al., 2016c), concluding that a direct link between the GH-IGF system and myogenesis exists. Notwithstanding, the rBGH treatment did not significantly cause any effect regarding the MRFs and other muscular proliferation and differentiation markers (e.g., PCNA and MHC, respectively) at

this time. In previous studies from our group with cultured gilthead sea bream myocytes, GH and IGF-II increased MyoD2 and Myf5 gene expression, while IGF-I increased myogenin and MRF4 gene expression (Azizi et al., 2016; Garcia de la serrana et al., 2014; Jiménez-Amilburu et al., 2013; Vélez et al., 2014). Moreover, PCNA and myogenin protein expression increased significantly after different combinations of IGFs and GH treatment at day 4 of culture (Rius-Francino et al., 2011; Vélez et al., 2014); whereas Azizi et al. (2016) using the same model did not observe an increase in PCNA gene expression but did find a stimulation of MHC gene expression after IGF-I treatment. Overall, this indicates that the effects observed can vary depending the stage of myogenesis of the muscle under study. In this sense, it is important to note that we were only able to analyze samples at a single time point after the rBGH treatment *in vivo*, and thus, the expression pattern of these regulatory factors may have varied substantially within a few days.

Furthermore, rBGH-treated fingerlings presented a significant up-regulation of both growth inhibitors MSTN1 and MSTN2, which coincides with that observed for MSTN1a in rainbow trout at three days post injection with rBGH (Biga et al., 2004a; Gahr et al., 2008), or in GH transgenic coho salmon (Overturf et al., 2010). It seems clear that after a prolonged GH exposure there is a tendency to regulate MSTN expression (especially MSTN1) to limit the GH growth promoting effects. Regarding MLC2, the two isoforms (MLC2a and MLC2b) expressed in gilthead sea bream have proven to be very important in skeletal muscle development and therefore, are recognized as good markers of muscle growth in this species. Briefly, it has been observed that MLC2a expression predominates during the hyperplastic stages (where myogenic cells proliferation predominates) and down-regulates progressively once hypertrophy appears, when MLC2b expression rises (Georgiou et al., 2016, 2014). Moreover, Georgiou et al. (2011) concluded that MLC2b has

an important role in skeletal muscle growth in gilthead sea bream juveniles, when hypertrophy predominates. Concerning endocrine regulation, Moutou et al. (2009) determined that in ovine recombinant GH-treated gilthead sea bream juveniles, MLC2a resulted up-regulated 1 day post injection, whereas no effect over MLC2b expression was detected. In this scenario, in the present work we observed that rBGH treatment caused a significant MLC2b up-regulation; while MLC2a was unaffected. Thus, it seems that rBGH may have stimulated muscle growth (a process starting with hyperplasia) during the first days post injection but at the sampling time, the muscular growth may be switching to the hypertrophic phase. This affirmation is concordant with the absence of changes observed in the expression of the proliferative marker PCNA and with the up-regulation of both MSTNs, as previously indicated (Georgiou et al., 2016).

Treatment with rBGH did not affect significantly the gene expression of the AKT-TOR pathway and its downstream molecules. GH transgenic coho salmon showed up-regulation of the gene expression of several protein-synthesis related molecules, including members of the mTORC1 signaling pathway such as 4EBP1 isoforms (Garcia de la serrana et al., 2015). However, the incubation of gilthead sea bream myocytes with IGF-I, did not determine differences in TOR or AKT gene expression (Azizi et al., 2016; Vélez et al., 2014). At a protein level, we observed that the rBGH treatment provoked a diminution of TOR phosphorylation, whereas no changes were detected in AKT. The absence of an effect in AKT phosphorylation in muscle agrees with the significantly lower expression of IGF-IRb found in this tissue and the decrease in TOR activation that would suggest a reduction in protein synthesis. In general, it is possible to link these results with the physiological state of the white skeletal muscle at the time of sampling, when a situation of negative

feedback and proliferative growth inhibition to counteract the effects induced by activation of the GH/IGF axis during the first weeks post injection could have been happened.

### 4.4. Effects of rBGH treatment on osteogenesis-related genes expression

Distinct studies have demonstrated that the osteogenic process can be stimulated by endocrine factors in mammals (reviewed in Hall, 2015). For example, IGF-I and IGF-II in cultured mammalian bone cells induce proliferation and increase type-I collagen deposition (Prisell et al., 1993; Strong et al., 1991) acting through the same signaling pathways as in muscle (Grey et al., 2003; Li et al., 2009). Although the information about the direct effects of GH treatment on bone tissue in fish is very scarce, the osteoblastogenesis process has been demonstrated to be similar to that in mammals (Ytteborg et al., 2012). Moreover, in gilthead sea bream the proliferative effects of IGF-I have been also demonstrated in cultured osteoblasts (Capilla et al., 2011). In the present study, rBGH treatment increased the expression of the extracellular matrix structural marker COL1A and the mineralization-related molecules OP and OSTC, suggesting improved osteogenesis. Thus, the gene expression results in this tissue fit with these previous findings and reinforce the idea that both white muscle and bone respond to GH treatment in order to support an enhanced harmonic musculoskeletal growth.

### 4.5. Effects of rBGH treatment on white skeletal muscle histology

There is very little information about the direct effects of GH treatment on muscle histology in gilthead sea bream, and most of data is from GH-transgenic models (Johnston et al., 2014; Kuradomi et al., 2011). Besides, in a long treatment with bovine-GH intramuscular

injection in Grass Pickerel an important increase in somatic growth, together with a significant increase in white muscle fiber diameter (hypertrophy) was achieved after 40 weeks (Weatherley and Gill, 1987). Very similar results were obtained recently by Sciara et al. (2011) in juvenile pejerrey after a weekly oral administration of recombinant pejerrey-GH during 6 weeks. However, the present histological analysis did not show significant differences in muscle fiber morphology because of rBGH treatment in gilthead sea bream fingerlings, suggesting a transition from hyperplastic to hypertrophic muscular growth at the time of sampling in agreement with the gene expression results. Thus, for the rBGH dose used, it appears that more time would have been necessary in order to achieve substantial effects over muscular histology. Finally, and as expected considering that GH has a well-known lipolytic effect (Albalat et al., 2005; Cruz-Garcia et al., 2011; Sheridan, 1986), the histological sections from rBGH-treated fingerlings presented an important reduction of the subdermal fat layer.

## 5. CONCLUSSIONS

In the present work we have deepened in the effects on growth of a 6 weeks treatment with a sustained release rBGH formulation on fingerlings of gilthead sea bream, one of the most important fish species of Mediterranean aquaculture. The experimental treatment resulted in an important increase in B.W. without changes in the C.F., thus indicating is due to somatic growth. Moreover, the liver was positively responding to rBGH treatment by up-regulating GH-IGF axis gene expression, which in turns is reflected in the increased plasma levels of IGF-I. By contrast, the most important extra hepatic tissues in terms of somatic growth, white skeletal muscle and vertebra bone seemed to have responded positively to

the high IGF-I levels (caused by rBGH) during the first weeks post injection. In this sense, the results in bone are very interesting since, on one hand reinforce the hypothesis that rBGH stimulates somatic growth, and on the other hand encourage to further work to elucidate the possible crosstalk with muscle. Altogether, the results obtained in the present study demonstrate that the maximum growth potential of such valuable species has not yet been reached via domestication, selection, breeding, etc., and that GH-IGF optimization approaches are excellent ways to obtain it without a loss in quality.

**Acknowledgments**

The authors would like to thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the School of Biology for the maintenance of the fish. Posilac was kindly provided by Miguel Abad from Elanco Animal Health, Eli Lilly and Company. E.J.V. and E.L. are supported by a predoctoral fellowship from the "Ministerio de Ciencia e Innovación" (MICINN), and A.M. by the University of Barcelona (APIF-2012). This study was supported by the project AGL2012-39768 (MICINN) to J.G., and the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and the 2014SGR-01371 from the "Generalitat de Catalunya".

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# CHAPTER 5: GENERAL DISCUSSION

During the last decades, the wild-fish captures remained relatively static although some fish species are anyway overfished. In addition, it is expected that the world population will reach overcome 9 billion people by 2050. Thus, turning the intensification of aquaculture necessary to deal with this situation, especially in some regions of southern Europe, where the production, in comparison with the northern European countries, has remained very stable (FAO, 2016). Hence, Mediterranean region faces the challenge of optimizing the production and the competitiveness of aquaculture industry. In this line, "The Strategic Plan for Spanish Aquaculture 2014-2020" approved by the Spanish administration in 2014, highlighted the special interest of increasing knowledge in nutrition and muscle development of fish aquaculture species, including gilthead sea bream, to in the last term, improve their growth and quality (OESA, 2014).

In terms of aquaculture product, skeletal muscle is the most interesting tissue (Mommsen, 2001), since it represents up to 70% of total body mass in many fish species (Bone, 1978). The muscle is a well-structured tissue that shows high plasticity and can adapt to different conditions (Relaix and Zammit, 2012; Rescan, 2001). Moreover, besides being the most valuable part for the consumer, the final quality of the product depends on the characteristics of the muscle. Thus, the enhancement of muscle growth and the improvement of the traits associated with quality, it is of great interest in gilthead sea bream.

In this sense, the *in vitro* primary culture of muscle cells has made possible the characterization of important aspects in molecular fish physiology, such as factors that control muscle development (e.g., MRFs, growth factors, myostatins, structural proteins or proteolytic systems), as well as the signaling pathways involved [Reviewed by Vélez et al. (2016)]. On other hand, through *in vivo* approaches, for many authors it has been possible

to study the role of the GH/IGF axis controlling somatic growth in different fish species, as well as to analyze the relevance of the proteolytic systems and the adaptation of muscle to exercise (Fuentes et al., 2013; Palstra et al., 2014, 2010; Vélez et al., 2017). Nonetheless, in gilthead sea bream information about endocrine and nutritional crosstalk of the main signaling pathways associated with growth, the role of the proteolytic systems in muscle development, and the effects of $\beta_2$-adrenergic agonists in myocytes is scarce. Moreover, the role of the GH/IGF axis mediating the effects of sustained swimming and the physiological adaptation of muscle to exercise, as well as the effects of a treatment with a sustained release formulation of exogenous GH, have also been poorly studied in this species.

Therefore, with the ultimate goal of contributing to improve muscle growth and to enhance the characteristics associated with quality in fish, the main objective of the present thesis is to increase knowledge on the regulation of muscle growth and flesh quality in gilthead sea bream. For this purpose, different *in vitro* and *in vivo* experiments have been performed. In this section, rather than making a detailed discussion of each chapter, it has been intended to analyze the whole set of results together trying to obtain a general overview of the outcome.

**Endocrine and nutritional regulation of somatic growth**

Different authors have postulated the GH/IGF axis as the most important endocrine system regulating muscle growth in fish [Reviewed by Fuentes et al. (2013), Mommsen and Moon (2001), and Pérez-Sánchez (2000)]. GH is involved in the regulation of protein synthesis and in both, the hypertrophic and hyperplastic muscle growth (Mommsen and Moon,

2001), but also exerts other functions through the control of IGFs secretion in the liver and muscle tissues (Pérez-Sánchez and Le Bail, 1999; Pérez-Sánchez, 2000). In what concerns to IGFs (IGF-I and IGF-II), it is commonly known that they also have an important role controling fish muscle growth [Reviewed by Fuentes et al. (2013) and Vélez et al. (2017)]. In this sense, IGFs are involved in the stimulation of cell proliferation and protein synthesis in fish myocytes, as well as in the decrease of protein degradation. In addition, IGFs also perform in fish some other functions (i.e. metabolic, such as the control of nutrients uptake) (Castillo et al., 2004; Codina et al., 2008; Fauconneau and Paboeuf, 2000; Gabillard et al., 2010; Montserrat et al., 2012, 2007b; Rius-Francino et al., 2011; Sánchez-Gurmaches et al., 2010). In the present thesis, it has been demonstrated that the incubation with IGF-I activates the AKT signaling pathway and significantly increases the proliferation of gilthead sea bream myocytes (**Article I**). Moreover, the combination of IGF-I with AA produced a synergistic effect increasing cell proliferation (Figure 38), and additionally, both IGF-I and AA, either combined or separately, enhanced the protein expression of myogenin. These results revealed the important role of these two factors, endocrine and nutritional, controlling *in vitro* myogenesis. Furthermore, in the same study AA treatment increased the phosphorylation of TOR and the gene expression of *tor* and its downstream effectors (*4ebp1* and *70s6k*), suggesting that protein synthesis is highly regulated nutritionally in gilthead sea bream (**Article I**).

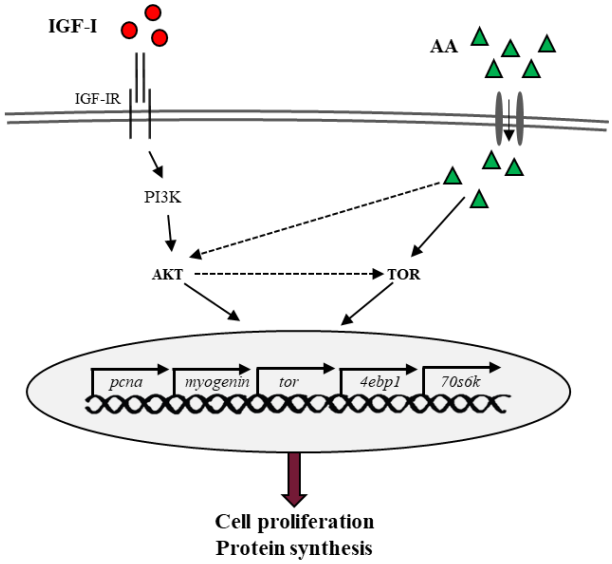


**Figure 38.** Schematic representation of the effects found for IGF-I and AA in day 4 cultured muscle cells from gilthead sea bream (**Article I**).

The positive effects of IGF-I on *in vitro* muscular development, are in agreement with the significant correlation between IGF-I levels and growth rates reported in some fish species [Reviewed by Pérez-Sánchez and Le Bail (1999) and Vélez et al. (2017)]. In fact, in this thesis, both moderate and sustained exercise and the injection of exogenous rBGH in juveniles and fingerlings of gilthead sea bream, respectively, increased the plasma levels of IGF-I in parallel with somatic growth (Figure 39) (**Article IV** and **Article VI**). Besides, this work presents the ratio IGF-I/GH as a good growth indicator in this species, since a greater proportion of IGF-I with respect to GH is associated with an enhanced growth condition (**Article IV**). This is in agreement with other authors that suggested that the best growth performance in fish is achieved with high IGF-I, insulin and T3 plasma levels, together with reduced levels of GH and cortisol (Pérez-Sánchez and Le Bail, 1999). Furthermore at the molecular level, in these two studies (**Article IV** and **Article VI**) the gene expression of *igf-1* in liver tissue was commonly increased, thus supporting the higher IGF-I circulating levels observed. On the contrary, the hepatic expression of IGF-I and GH

receptors (*igf-1rb*, *ghr-1*, and *ghr-2*) was differentially regulated between the two experiments. In the case of sustained exercise, the three receptors were down-regulated (**Article IV**), probably as a mechanism to avoid the inhibitory effects on IGF-I secretion induced by the increased circulating IGF-I; while for *ghrs* a hepatic desensitization to GH could be suggested. On the other hand, when the fingerlings of gilthead sea bream were injected with exogenous rBGH (**Article VI**), the expression of these three receptors was up-regulated. The *ghr* transcript can be post-transcriptionally truncated to generate non-functional receptors (i.e. lacking the signaling transduction domain), or GH binding proteins that regulate the bioavailability of GH [Reviewed by Fuentes et al. (2013)]. Thus, the increased expression of *ghr-1* and *ghr-2* found in the liver of rBGH-injected fish, is probably a system to suppress a GH enlarged effect, as suggested previously in the same species under a chronic GH excess induced by an undernutrition status (Saera-Vila et al., 2005). Moreover, the increased expression of *igf-1rb* in these fish can be a strategy to initiate a negative feedback in the liver to limit IGF-I secretion. This feedback, together with the inhibition mechanism by GHRs proposed above, could be putting a boundary to the endocrine functions of the GH/IGF axis (**Article VI**). In this sense, the results suggest that although it is possible to improve growth in gilthead sea bream through the enhancement of GH/IGF axis activation in the liver, there are certain limits in a way that this system presents regulatory mechanisms for the animal to reach an adequate growth.

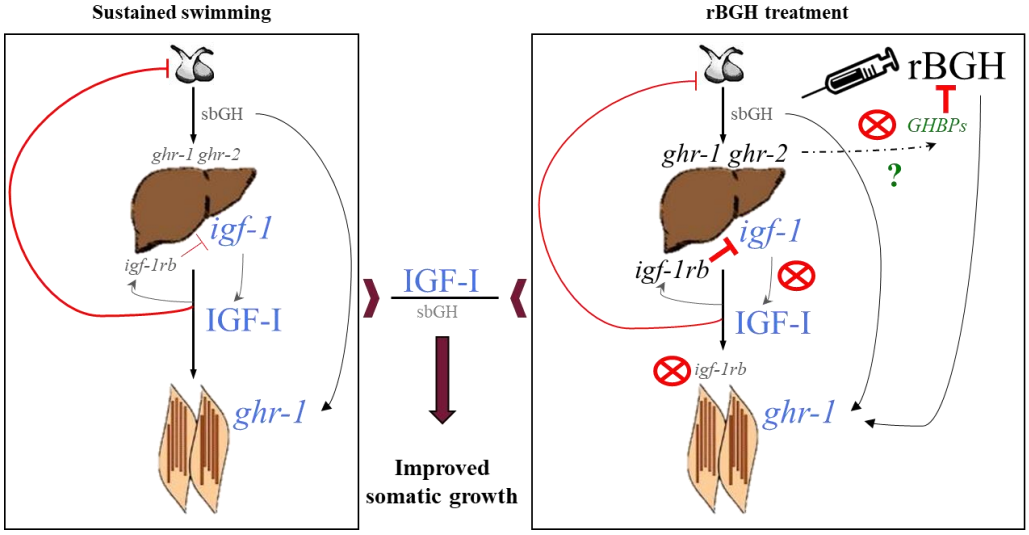


**Figure 39.** Schematic representation of GH/IGF axis actions medating the increase in growth caused by swimming (**Article IV**) and rBGH treatment (**Article VI**) in gilthead sea bream. In these experiments, it was common the increased expression of hepatic *igf-1* and that of *ghr-1* in the muscle (in blue), as well as the higher IGF-I/GH ratio in plasma, thus determining improved somatic growth. However, in the case of rBGH treatment the high levels of exogenous GH lead to the emergence of three control systems (red circles); (1) Increased GH receptors in the liver that could act as GH binding proteins (GHBPs) to limit the action of exogenous (rB) and endogenous (sb) GH. (2) Increased expression of hepatic *igf-1rb* to enhance ligand binding and to induce a negative feedback in *igf-1* expression. (3) Reduced *igf-1rb* expression in the muscle to limit IGF-I actions.

In addition to the liver, the effects of these two treatments with regards to gene expression modulation of GH/IGF axis were also analyzed in the skeletal muscle. Although differences were not found in the expression of total *igf-1* either after 5 weeks of sustained swimming nor at 6 weeks post-injection with rBGH in that tissue (**Article IV** and **Article VI**, respectively), the gene expression of the splice variants was differentially regulated. In the case of sustained swimming, the expression of *igf-1c* was increased (**Article IV**), suggesting that this form is the most responsive to exercise in the muscle tissue. Thus, this

splice variant could have acquired the capacity to respond to mechanical stimulation as it occurs with the mechano-growth factor (MGF) in mammals (Bamman et al., 2001; Philippou et al., 2007). On the other hand, in rBGH-treated fish, the expression of *igf-1b* was increased when compared with control fish in agreement with the results obtained by Jiménez-Amilburu et al. (2013) after the incubation of myocytes from the same species with GH. These results confirmed that in gilthead sea bream muscle, this splice variant is the most sensitive to hormonal treatments (**Article VI**). Concerning the muscular expression of *igf-1rs* and *ghrs* in the two experiments, the predominantly anabolic GH receptor *ghr-1* (Saera-Vila et al., 2009) was increased (**Article IV** and **Article VI**), thus confirming the growth promoting situation. Nonetheless, exercise decreased the most catabolic GH receptor isoform (*ghr-2*) (Saera-Vila et al., 2009), and enhanced the expression of the IGF binding protein *igfbp-5b*, considered a positive regulator of IGF-I actions (Duan and Xu, 2005; Fuentes et al., 2013). In addition, swimming did not change the expression of IGF-I receptors (**Article IV**). On the other hand, the injection with rBGH significantly reduced the expression of *igf-1rb* in the muscle without altering the expression of the *igfbp-5b* and *ghr-2* (**Article VI**). In a context in which IGF-I levels are increased, but the GH ones are excessive, the decreased expression of *igf-1rb* could be interpreted as a mechanism to limit in the muscle the effects of circulating IGF-I. Therefore, these results confirm the existence of also local mechanisms to regulate the GH/IGF axis actions (Figure 39). Alltogether, the obtained results revealed the importance of both nutritional and endocrine factors controlling *in vitro* myogenesis in gilthead sea bream. *In vivo*, the potentiation of the GH/IGF axis is especially relevant for the improvement of growth in this species, although as suggested there are different systemic and local regulatory mechanisms in place to guarantee proper development.

**Endogenous factors controlling muscle growth and development**

Besides the endocrine (i.e. GH/IGF axis) and nutritional (i.e. AA) signals, other factors are also important in the regulation of myogenesis. In the present thesis, the different growth condition observed with regards to the GH/IGF axis between the rBGH (**Article VI**) and the swimming (**Article IV**) experiments, suggests a possible differential modulation of other regulatory factors that deserves more investigation. In this sense, differences were found with regards to signaling pathways activation and the gene expression of the negative regulator of growth myostatin, although the expression of the MRFs was stable in both experiments (**Article V** and **Article VI**). Therefore, the treatment with rBGH did not alter the expression of *pcna* neither the phosphorylation state of AKT, but up-regulated the gene expression of the two myostatins (*mstn1* and *mstn2*) (**Article VI**), as occurred in rainbow trout (Biga et al., 2004a; Gahr et al., 2008) or in GH transgenic coho salmon (Overturf et al., 2010). This result suggests that myostatins are a complementary system to limit the GH growth-promoting effects in the muscle (Figure 40), and are in agreement with the reduced expression of *igf-1rb* hypothesized to counteract the high activation of the GH/IGF axis. In addition, the phosphorylation of TOR was reduced in rBGH-injected fish, suggesting a decrease in protein synthesis (**Article VI**). Altogether, these data confirmed a certain saturation of the processes inducing muscle growth in rBGH-treated fish. On the contrary, moderate and sustained swimming significantly activated the TOR signaling pathway at both gene and protein levels (**Article IV**). This increase in TOR occurred in parallel to the augmented PCNA protein expression and the reduced *mstn2* gene expression (**Article V**), thus supporting a situation of increased muscle growth, due to higher protein synthesis and cell proliferation (**Article I**). These results, together with the effects already commented concerning the GH/IGF axis (i.e., increased plasma IGF-I/GH ratio and higher expression

of muscular *ghr-1*), confirmed the growth-promoting effects of swimming in the muscle of fish and supported the different growth situation achieved by each one of the approaches evaluated (Figure 40).

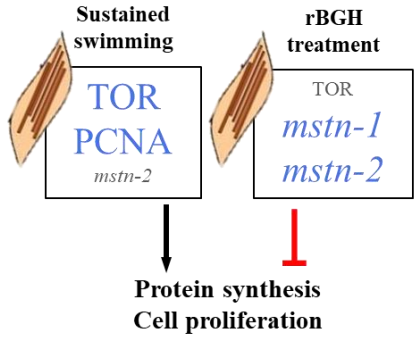


**Figure 40.** Schematic representation of the effects of both sustained swimming (**Article IV** and **Article V**) and rBGH treatment (**Article VI**) on muscle development through up-regulation (in blue) or down-regulation (in grey) of local factors in gilthead sea bream.

### Proteolytic systems and muscle adaptation to exercise

In previous studies, an interesting differential response to exercise between the anterior and caudal regions of white skeletal muscle was observed (Ibarz et al., 2011; van Leeuwen, 1995). Whereas fiber size increased in both muscle regions after exercise, the number of capillaries was only enhanced in the anterior one. To understand these differences it is important to note that in fish there is a longitudinal shift in the contractile properties of the muscle. In the anterior region, the contractions are fast, while in the caudal muscle they are slower (Coughlin, 2002; Coughlin et al., 2007). Consequently, the most active section during moderate and sustained exercise in Sparidae fish is the caudal muscle region (van Leeuwen, 1995). In the present thesis, when the effects of exercise along the white muscle were analyzed, the increased expression of *igf-2* observed in the caudal region could be

associated with increased myocyte proliferation in the area (**Article IV**). Besides, although differences in *igf-2* expression in the anterior muscle were not found (**Article IV**), the sustained swimming induced a different regulation of PCNA protein levels and *mstn2* gene expression between muscle regions that suggests the possibility that in the anterior region a situation of remodeling was occurring (**Article V**). In this sense, a process of remodeling and regeneration in response to activity or during exercise is clearly induced in mammals. This process requires protein degradation, and consequently, different members of the calpains, cathepsins and UbP systems are activated during exercise (Cunha et al., 2012; Egan and Zierath, 2013; Lira et al., 2013; Prior et al., 2004; Reid, 2005; Yang et al., 2006). In the present work, most of the molecules studied from the different endogenous proteolytic systems (e.g. *capn1*, *ctsdb*, *murf1* or mafbx) were significantly up-regulated by sustained swimming in the anterior muscle region of gilthead sea bream (**Article V**). Moreover, also the protein levels of CAPN1 and CTSD were increased in that region, whereas no significant changes were found in either gene or protein expression of proteolytic markers in the caudal muscle area. In order to understand the meaning of this up-regulation, it is necessary to first discuss the potential role of the different proteolytic systems during the myogenic process in this species. In addition, to fully understand the process of remodeling that may be taking place in response to exercise, the possible formation of new vessels in that muscle area will be further explored.

- **Calpains**

Calpains are generally involved in the cleavage of cytoskeletal proteins into smaller fragments for their posterior degradation through the UbP or the cathepsins systems

(Belcastro et al., 1998; Yang et al., 2006). However, they are also implicated in the breaking of the cytoskeletal/membrane linkage at the point that is necessary for the fusion of myoblasts (Belcastro et al., 1998; Goll et al., 2003; Moraczewski et al., 1996). In the present thesis, it has been observed in gilthead sea bream that the gene expression of calpains is in general higher at the early proliferative stages of *in vitro* myogenesis (Figure 41A) (**Article II**). Hence, the up-regulation of the calpains observed in the anterior muscle of exercised fish could be associated as hypothesized, with structural changes and tissue remodeling taking place in these fish in response or as adaptation to exercise (Figure 41B) (**Article V**). These results could reinforce the anabolic situation (i.e. increased cell proliferation and protein synthesis) of the anterior muscle region of exercised fish (**Article IV** and **Article V**).

- **Cathepsins**

The expression pattern of the different cathepsins analyzed during *in vitro* culture development suggested that these molecules are more important at the early differentiation phase of myogenesis (Figure 41A) (**Article II**). After 5 weeks of sustained swimming, the protein and gene expression of *ctsdb* was increased in the anterior muscle of gilthead sea bream (Figure 41B) (**Article V**). Interestingly, that paralog and not *ctsda* is predominatly expressed in muscle and is modulated during ontogeny, demonstrating its key role in muscle tissue development (Salmerón et al., 2015). Nevertheless, more experiments would be necessary to clarify whether these results are associated with early differentiation of myocytes in this area, increased protein turnover, or a mixture of both processes.

- **UbP system**

The UbP system is generally considered a marker of induced proteolysis in fish (Bower et al., 2010; Cleveland and Weber, 2010; Salmerón et al., 2015; Seiliez et al., 2010). In accordance with that role, in leucine or lysine deficiency in cultured myocytes up-regulated the expression of the E3 ubiquitin ligases *mafbx* and *murf1*, whereas the recovery of AA levels after a starvation period down-regulated their expression (**Article II**). However, it is also known that the proteolytic function of MAFbx plays in mammals other roles, such as for example, it is essential for myogenic stem cell function in skeletal muscle. In this process, MAFbx regulates several transcription factors such as the MRFs, MyoD or myogenin (Attaix and Baracos, 2010; Jogo et al., 2009; Tintignac et al., 2005). In the present thesis, the results obtained for *mafbx* expression during culture development (Figure 41A) (**Article II**) revealed an opposite pattern to that found by García de la serrana et al. (2014) for *myod2*. This result suggested that, probably, once the cells have determined their muscular fate by the action of MyoD, the decrease in *myod2* mRNA levels could be caused by the rise of *mafbx* (**Article II**). In exercised fish, *mafbx* expression was increased (Figure 41B) (**Article V**), together with the activation of TOR pathway in the anterior muscle (**Article IV**). Consequently, these results appear to indicate, that instead of a situation of catabolism, an advanced state of the myogenic process is taking place in that region.

Furthermore, MuRF1 regulates the sarcomeric stability through the recognition and labeling for breakdown of different myofibrillar proteins such as MHC (Murton et al., 2008; Spencer et al., 2000; Tintignac et al., 2005; Yang et al., 2006). Thus, MuRF1 is recognized as a necessary protein for the initiation and maintenance of myogenesis in

mammals. In fact, the gene expression profile observed for *murf1* in cultured myocytes (**Article II**), compared with that found previously for *mhc* in the same cellular model (García de la serrana et al., 2014), supports a functional relationship of these molecules in gilthead sea bream (Figure 41A). Hence, the higher expression of *murf1* in the anterior muscle region of exercised fish reinforces the idea that a situation of tissue remodeling induced by sustained swimming was ocurring in this area (Figure 41B) (**Article V**).

Regarding N3, this proteasome subunit is considered a good indicator of cell proliferation in mammals (Mairinger et al., 2014; Wang et al., 2013). In our *in vitro* model, the gene expression profile of *n3* during culture development (Figure 41A) (**Article II**) matched well with that of *pcna* (Azizi et al., 2016a; García de la serrana et al., 2014), and also with the percentage of PCNA-positive cells (**Article I**). Additionally, the expression of *n3* was also increased during the anabolic situation induced by the recovery of AA levels after a starvation period in myocytes (**Article II**). Overall, these results suggest the importance of *n3* during the early stages of myogenesis in gilthead sea bream, when proliferation is mostly occurring. In this sense, the increased expression of *n3* observed in the anterior muscle of exercised fish, which was parallel to the increase in PCNA protein expression (**Article V**), the up-regulation of TOR gene and protein expression (**Article IV**), and the decrease in *mstn2* gene expression (**Article V**), could be supporting the anabolic condition of this region (Figure 41B).

To put these results more into context, it was previously demonstrated that both, protein syntesis (Peragón et al., 2001), as well as the gene expression of several cathepsins and UbP system members (i.e. *ctsb*, *ctsl*, *ctsdb*, *mafbx* and *n3*) (Salmerón et al., 2015) was higher during the greatest growth stage along ontogeny (i.e., fingerlings), than in juvenile or adult fish. Therefore, these studies support the hypothesis that the anterior muscle

subjected to swimming is experiencing increased protein turnover (exemplified by cathepsins and UbP system) and structural changes (calpains) that correspond to an anabolic situation of muscle remodeling in response to such exercise (Figure 41B) (**Article IV** and **Article V**).

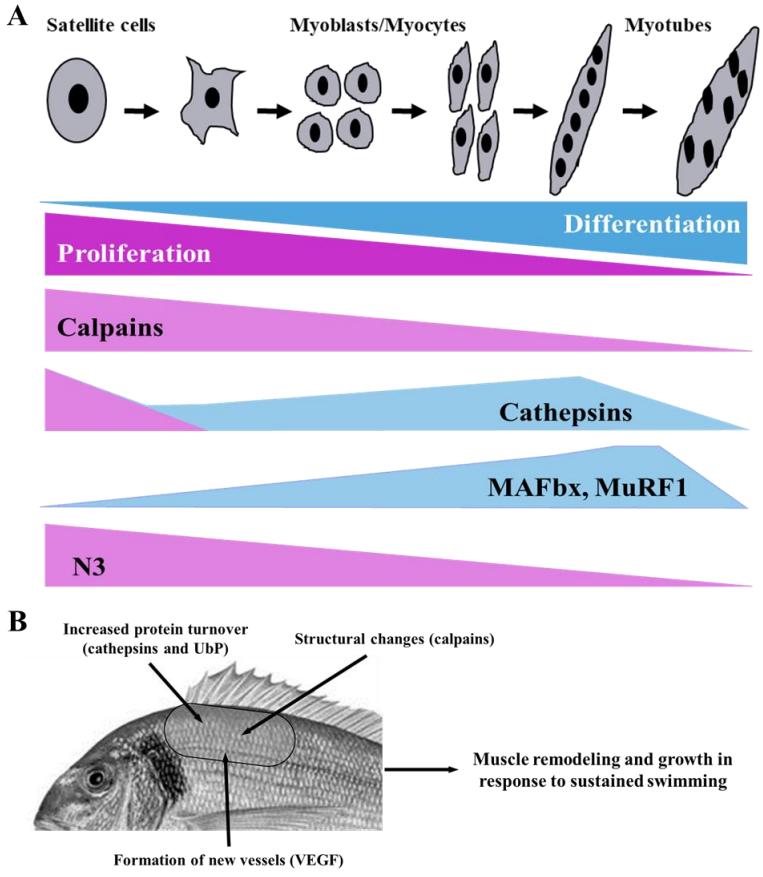


**Figure 41. A**: Schematic representation of the gene expression profiles of the different proteolytic systems members during *in vitro* myogenesis in gilthead sea bream (**Article II**). **B**: Simplification of the processes that occur in response to exercise in the anterior muscle region of this species (**Article IV** and **Article V**).

- **Angiogenesis**

In order to support the process of remodeling in the anterior muscle region of exercised fish, we could expect angiogenesis to be happening. In agreement with this hypothesis, the protein expression of the angiogenic marker VEGF was increased in response to exercise in that area (**Article V**). VEGF is considered a good marker of muscle remodeling that regulates the necessary increase of capillary supply when new myofibers are formed (Jensen et al., 2015; Prior et al., 2004; Yan et al., 2011). Therefore, angiogenesis is a necessary process when hyperplastic muscle growth occurs. In gilthead sea bream, changes in capillarization after exercise have been already reported, being the capillaries more numerous in the anterior muscle of exercised fish compared to controls (Ibarz et al., 2011). Thus, the observed VEGF increase in the present study is another demonstration of the transformation process that is occurring in the anterior muscle region of exercised fish (Figure 41B). Consequently, these results confirm the tissue renovation and formation of new vessels that take place during exercise adaptation to induce hyperplastic muscle growth in gilthead sea bream (**Article IV** and **Article V**).

**$\beta_2$-agonists strategy to increase muscle growth**

In addition to the aforementioned and discussed strategies to stimulate growth, the $\beta_2$-adrenergic agonists have been used in terrestrial farm animals to increase muscle mass (mainly by hypertrophy) with successful results (Figure 42A) [Reviewed by Lynch and Ryall (2008) and Mommsen and Moon (2001)]. In fish, some authors have demonstrated that *in vivo* administration of $\beta_2$-agonists also increases body weight (Mustin and Lovell, 1993; Oliveira et al., 2014; Salem et al., 2006; Satpathy et al., 2001; Vandenberg and

Moccia, 1998; Webster et al., 1995). In the case of rainbow trout, this effect is achieved by up-regulating protein synthesis and down-regulating proteolytic systems (Salem et al., 2006). Nevertheless, information is not available with regards to the *in vitro* effects of $\beta_2$-agonists in fish muscle cells. Thus, with the aim of deepening in that aspect, experiments have been performed in gilthead sea bream cultured myocytes to evaluate three different ligands on the regulation of myogenesis, proteolysys and lipolysis.

- **Signaling pathways activation**

In the present thesis, the effects of the incubation of myocytes with a conventional $\beta_2$-adrenoceptor ligand (i.e. NA), or with two $\beta_2$-agonists of the new generation (i.e. FOR or SALM) was evaluated. First, their capability to activate the corresponding signaling pathways through the Gα subunit or Gβγ complex was validated. All three agonists increased either the cAMP levels, or the phosphorylation of PKA or TOR, as well as the gene expression of *tor*, *70s6k* and *akt* (Figure 42B) (**Article III**). These results demonstrated that gilthead sea bream muscle cells are sensitive to $\beta_2$-adrenergic stimulation and that the signaling pathways activated are the same as in mammals (Lynch and Ryall, 2008). Interestingly, the results obtained for SALM appeared to indicate that this agonist acts with different dynamics to the other two.

- **Myogenesis**

The activation of AKT and TOR pathways at both *in vitro* and *in vivo* levels is associated with increased cell proliferation in gilthead sea bream (**Article I**, **Article IV** and **Article**

**V**); therefore, activation by $\beta_2$-agonists of these signaling pathways suggested an increase in satellite cell population (Figure 42B). This was confirmed by the higher proportion of PCNA-positive cells and *pcna* gene expression in cultured myocytes, without affecting *mstn-1* (a proliferation inhibitor) mRNA levels, after the treatment with $\beta_2$-agonists (**Article III**). Overall, these results suggest that in our model a treatment with adrenergic agonists induces the formation of new muscle cells (i.e. hyperplasia). This effect is contrary to that found in mammals, in which $\beta_2$-agonists induce hypertrophy through regulation of *myostatin* expression (Joassard et al., 2013; Lynch and Ryall, 2008). On the other hand, the data obtained concerning MRFs expression support the idea that $\beta_2$-agonists enhance the myogenic process in this species (**Article III**), being *myf5* down-regulated. This result was already previously associated with a situation of hyperplastic growth in fish (Froehlich et al., 2013b). Additionally, the $\beta_2$-agonists tested in our model, especially in the case of SALM, increased the gene expression of *igf-1* and *igf-2* (**Article III**), which could indicate an additional or multiplier effect on cell proliferation and muscle development through the regulation of these growth factors (Azizi et al., 2016b; **Article I**).

- **Proteolytic systems**

The analysis of proteolytic members after incubation of the cells with the $\beta_2$-agonists revealed that the expression of both *mafbx* and *ctsdb* was increased by SALM treatment (Figure 42B) (**Article III**). These molecules are involved in the *in vitro* differentiation of myocytes (**Article II**) and as commented above, they were up-regulated in the anterior muscle of exercised fish (**Article V**). Hence, the obtained results point out that $\beta_2$-agonists, in addition to increasing cell proliferation, also regulate myocyte differentiation in this species (**Article III**). Moreover, SALM treatment enhanced the expression of *capns1a*

(**Article III**), which is associated with the positive effects of sustained swimming in the remodelation of muscle tissue and the induced hyperplastic growth (**Article V**). Altogether, these data suggest that in fish, adrenergic agonists participate in the control of myogenesis and structural remodeling that are necessary for hyperplastic muscle growth (Figure 42B) (**Article III**) and reinforce the idea that SALM appears to act with a different tempo or intensity than the other agonists analyzed.

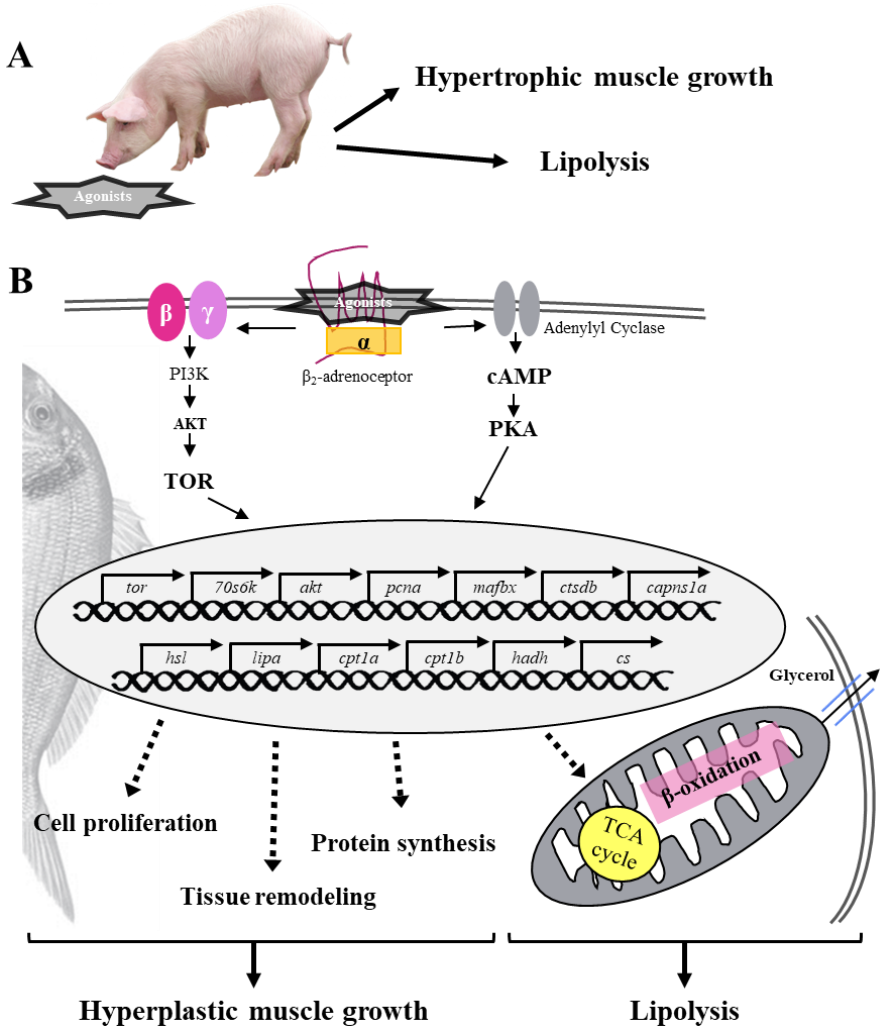


**Figure 42. A**: Schema of the typical effects of $\beta_2$-agonists in terrestrial farm animals such as pigs, beef cattle of lambs (Baker et al., 1984; Ricks et al., 1984; Sillence, 2004). **B**: Schematic representation of the effects observed in gilthead sea bream myocytes (**Article III**).

- **Lipolysis**

In addition to induce muscle growth by hyperplasia, the obtained results revealed that $\beta_2$-agonists, particularly SALM, increased the gene expression of different lipases (*hsl* and *lipa*), β-oxidation markers (*cpt1a*, *cpt1b* and *hadh*) and the mithochondrial enzyme *cs*, suggesting enhanced lipolytic and β-oxidation machinery and actively functional Krebs cycle (Figure 42B) (**Article III**). Thus, $\beta_2$-agonists improved the use of fatty acids in myocytes for ATP production. Furthermore, the increased release of glycerol into the culture media caused by the treatments (**Article III**), corroborated the ability of $\beta_2$-agonists to increase lipolysis in our *in vitro* model (Figure 42B). This effect is usually related *in vivo* to an important decrease in total body fat composition in vertebrates, including fish (Johnson et al., 2014; Mustin and Lovell, 1993; Satpathy et al., 2001; Sillence, 2004; Vandenberg et al., 1998). Thus, the effects found concerning cell proliferation and lipid metabolism in myocytes postulate that $\beta_2$-agonists, especially SALM, should be considered as an interesting strategy to test *in vivo* in order to optimize, in the last term, the growth and flesh quality in gilthead sea bream.

**Improvement of quality characteristics**

The final quality of a fish product depends on the properties of the muscle, the most abundant part at a commercial level (Bone, 1978; Mommsen, 2001); therefore, in addition to enhance muscle growth, the improvement of the characteristics associated with quality is of great importance in the aquaculture industry. In the present thesis, although the studies performed were focused to evaluate growth, they have provided valuable information in terms of the quality of the product generated as it is going to be discussed below.

- **Harmonic musculoskeletal growth**

In the first place, the treatment with rBGH revealed that in parallel to muscle growth it is necessary a coordinated development of the bone tissue in order to achieve an harmonic muscle-skeleton growth (Figure 43) (**Article VI**). Otherwise, malformations may appear in the fish and the product may present a disproportionate appearance that may be rejected by the consumer (Davidson et al., 2011). In this sense, while the effects of rBGH over the local GH/IGF axis found in the bone tissue were roughly similar to those found in muscle (i.e. down-regulation of *igf-1rb* expression and stabilization of *igfbps* mRNA levels), this treatment increased the expression of most of the osteogenesis-related genes analyzed (**Article VI**). These results then suggested improved osteogenesis in the rBGH-treated fish (Figure 43), supporting a balanced development of both, muscle and bone tissues in order to accomplish proper somatic growth.

- **Increase in texture parameters**

Regarding muscle growth, in a previous study in grass pickerel, a long treatment (40 weeks) with bovine-GH increased the muscle fiber diameter (i.e. causing hypertrophy) (Weatherley and Gill, 1987), similarly to that found in juvenile pejerrey after weekly administration of recombinant pejerrey-GH for 6 weeks (Sciara et al., 2011). However, in gilthead sea bream it was observed at 1-day post-injection with ovine recombinant GH that *mlc2a* resulted up-regulated, whereas *mlc2b* remained unaltered (Moutou et al., 2009). These two isoforms of *mlc2* are recognized as good markers of muscle growth in this species; being *mlc2a* more expressed during hyperplastic stages, and down-regulated progressively once hypertrophy appears, when *mlc2b* expression rises (Georgiou et al.,

2011, 2016, 2014). In the present study, at 6 weeks post rBGH injection, the expression of *mlc2b* was up-regulated, while *mlc2a* was unaffected (**Article VI**). This result, in agreement with the absence of changes in *pcna* expression and the up-regulation of both *myostatins*, suggests that during the first days after injection, the treatment could have induced muscle growth by hyperplasia. While after that, at sampling time, the muscular growth may be switching to hypertrophic (Figure 43). Moreover, the histological analysis did not show significant differences in muscle fiber morphology due to rBGH treatment in gilthead sea bream fingerlings (**Article VI**), reinforcing the idea that a transition from hyperplastic to hypertrophic muscular growth is taking place at the time of sampling. Overall, these results confirmed that at that time, a negative feedback and proliferative growth inhibition is occurring to counteract the effects caused by activation of the GH/IGF axis. Therefore, growth induced by rBGH is a complex condition in which hypertrophy, hyperplasia, and bone development are combined, alternated and physiologically regulated to maintain adequate somatic complexion of the fish (Figure 43). And, it should be taken into account that the consumer would consider the proportions and appearance of the fish, a quality factor.

Additionally, the size and number of fibers forming the muscle are important determinants of flesh texture (Ayala et al., 2010; Johnston, 1999; Listrat et al., 2016; Periago et al., 2005), and thus, the growth by hyperplasia is considered as means to improve muscle quality. The positive correlation between the number of fibers and the flesh textural parameters has been observed previously in gilthead sea bream (García de la serrana et al., 2013). In the present thesis, the rBGH treatment did not alter muscle cellularity at the time of sampling (**Article VI**). However, the swimming induced in the anterior white muscle region (i.e., the major muscle part in proportion), increased cell proliferation and expression of *mlc2a* and down-

regulation of *mstn2* (**Article V**), which corresponds with a situation of hyperplasia (Figure 43). Therefore, suggesting an improvement of firmness in the muscle of exercised gilthead sea bream (**Article IV** and **Article V**). In this line, the *in vitro* treatment with $\beta_2$-adrenergic agonists revealed increased PCNA protein and gene expression in myocytes, which together with the stable expression of *mstn1*, have suggested the induction of a hyperplastic growth condition in this species (**Article III**). Accordingly, the present study highlights the potential use of sustained swimming and $\beta_2$-agonists in gilthead sea bream to increase the muscle fiber density, and thus, enhance the texture and quality of the fillet (Figure 43).

- **Reduction of fat depots**

In relation to lipid stores, Blasco et al. (2015) found in a study derived from the same experimental trial of exercise (**Article IV** and **Article V**), that while the mesenteric fat index was significantly reduced by swimming activity (Figure 43), the proximate lipid composition of muscle remained unchanged. The reduction of those fat depots is a factor associated with higher production efficiency (i.e. cost reduction) and quality in farming animals [Reviewd by Sillence (2004)]. Hence, the obtained results indicate that besides increasing flesh quality by improving muscle firmness (**Article V**), exercise would reduce production costs without altering the characteristics associated with intramuscular fat (Blasco et al., 2015). In the case of the rBGH experiment, Blasco et al. (personal communication, September 2017) found in fish from the same trial a significant reduction of muscular proximate lipid composition after rBGH injection (CT: 2.2 ± 0.12 %w v *vs*. rBGH: 1.5 ± 0.08 %w v at $p < 0.001$). In this sense, as expected considering that GH has a well-known lipolytic effect (Albalat et al., 2005; Cruz-García et al., 2011; Sheridan, 1986),

the histological sections from rBGH-treated fish presented an important reduction of the subdermal fat layer (**Article VI**). These results would confirm that rBGH treatment can be used to increase growth and at the same time to limit the energy directed to fat depots (Figure 43), what in the last term optimizes the aquaculture production and quality of gilthead sea bream.

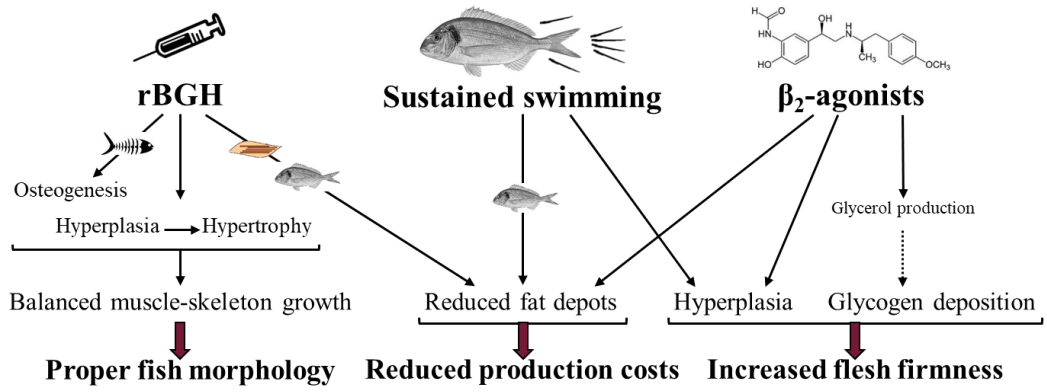


**Figure 43.** Schematic representation of rBGH, sustained swimming and $\beta_2$-agonists treatment effects in gilthead sea bream in terms of quality of the aquaculture product. (**Articles III-VI**).

In the present thesis, it has been further observed that $\beta_2$-agonists increased the lipolytic machinery and the oxidative capacity of myocytes (**Article III**). This increase in lipolysis, besides being related to a decrease in total body fat as discussed previously, can be associated with reduced lipid composition of the fish fillet, or increased glycogen deposition, therefore modifying textural parameters (Figure 43) (Mustin and Lovell, 1995, 1993; Silva et al., 2012; Webster et al., 1995). These are desirable attributes in aquaculture for its association with the quality of the final product. Altogether, the molecular mechanism induced by $\beta_2$-agonists in this work can be considered a good option to improve growth and flesh quality in gilthead sea bream. These results can serve as a starting point for the investigation in the coming years of alternative natural candidates capable to

potentiate these signaling pathways and effects, as well as those fish culture conditions that will result in the optimization of aquaculture production.

To sum up, the investigations of the present thesis using both, *in vitro* and *in vivo* approaches, have contributed to increase knowledge about the regulation of growth and flesh quality in gilthead sea bream with the ultimate purpose of providing tools to optimize in the near future the aquaculture production of this species.

# CHAPTER VI: CONCLUSIONS

1. IGF-I and AA, through activation of AKT and TOR signaling pathways, play an important role controlling myocytes proliferation, differentiation and protein synthesis; consequently, inducing muscle development in gilthead sea bream. In addition, these data demonstrate the importance of the crosstalk between endocrine and nutritional signals to regulate muscle growth.
2. Calpains appear to be more involved in the first stages of *in vitro* myogenesis in gilthead sea bream, whereas cathepsins and the UbP system seem to be more important during differentiation. Nevertheless, calpains and UbP members compared to cathepsins are differentialy regulated in response to AA availability, supporting the relevance of nutritional factors in muscle development.
3. $\beta_2$-adrenoceptor agonists, mainly SALM, activate both the PKA and PI3K/AKT/TOR signaling pathways to increase cell proliferation and lipolysis in gilthead sea bream myocytes. These effects suggest that $\beta_2$-agonist should be considered an effective strategy to stimulate muscle growth by hyperplasia while reducing fat content, thus, to improve flesh quality in fish.
4. Moderate and sustained exercise or rBGH treatment, are different useful strategies to stimulate the GH/IGF axis and enhance muscle growth in gilthead sea bream. Besides, the excess of rBGH reveals that the GH/IGF axis utilizes both systemic and local regulatory mechanisms to achieve an accurate control of somatic growth.
5. An increased ratio of IGF-I/GH in plasma is an excellent indicator of growth capacity in gilthead sea bream. In these conditions, a combination of up-regulated expression of hepatic *igf-1* with muscular *ghr-1* has appeared in our *in vivo* models as another molecular marker of fish growth potential.

6. Growth induced by rBGH is a process that combines muscle hypertrophy and hyperplasia, as well as bone development, to maintain the adequate muscle-skeleton balance that will guarantee a quality product with a proper morphology.
7. Muscle tissue remodeling in response to sustained swimming in gilthead sea bream determines the participation of endogenous proteolytic systems, which cause the increased protein turnover (cathepsins and UbP) and the structural changes (calpains) required for hyperplastic growth. Furthermore, new vessel formation also occurs, altogether resulting in the improvement of muscle quality characteristics.
8. Overall, the *in vitro* and *in vivo* experiments performed in this thesis demonstrate that it is possible to improve somatic growth and flesh quality in gilthead sea bream, being the moderate and sustained swimming and the adequate balance of nutrients (e.g., AA) in the diet, besides other potential muscle growth stimulators (such as SALM), very promising strategies to boost aquaculture production.

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# ANNEX

## ADDITIONAL PUBLICATIONS

Revista AquaTIC, nº 39 – 2013 31
Revista científica de la Sociedad Española de Acuicultura

Revista AquaTIC, nº 39, pp. 31-35. Año 2013
ISSN 1578-4541
http://www.revistaaquatic.com/aquatic/art.asp?t=p&c=280

### Control hormonal y nutricional del crecimiento y desarrollo muscular en dorada (*Sparus aurata*)

E.J. Vélez*, E. Lutfi*, E. Capilla, M. Riera, I. Navarro y J. Gutiérrez

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Data in Brief 6 (2016) 507–513

Contents lists available at ScienceDirect

Data in Brief

journal homepage: www.elsevier.com/locate/dib

Data Article

### Characterization data of gilthead sea bream (*Sparus aurata*) IGF-I receptors (IGF-IRa/Rb)

Emilio J. Vélez [a,1], Sheida Azizi [c,d,1], Cristina Salmerón [a], Shu Jin Chan [b], Mohammad Ali Nematollahi [c], Bagher Mojazi Amiri [c], Isabel Navarro [a], Encarnación Capilla [a], Joaquim Gutiérrez [a,*]

Comparative Biochemistry and Physiology, Part B 199 (2016) 67–73

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

### Contribution of *in vitro* myocytes studies to understanding fish muscle physiology☆

Emilio J. Vélez, Esmail Lutfi, Sheida Azizi, Núria Montserrat, Miquel Riera-Codina, Encarnación Capilla, Isabel Navarro, Joaquim Gutiérrez *

Aquaculture 467 (2017) 28–40

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

## Understanding fish muscle growth regulation to optimize aquaculture production

E.J. Vélez, E. Lutfi, Sh. Azizi, M. Perelló, C. Salmerón, M. Riera-Codina, A. Ibarz, J. Fernández-Borràs, J. Blasco, E. Capilla, I. Navarro, J. Gutiérrez *

J Comp Physiol B (2015) 185:859–868
DOI 10.1007/s00360-015-0933-5

ORIGINAL PAPER

## Growth-promoting effects of sustained swimming in fingerlings of gilthead sea bream (*Sparus aurata* L.)

**Josefina Blasco[1] · A. Moya[1] · A. Millán-Cubillo[1] · E. J. Vélez[1] · E. Capilla[1] · J. Pérez-Sánchez[2] · J. Gutiérrez[1] · J. Fernández- Borrás[1]**

General and Comparative Endocrinology 232 (2016) 7–16

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

## IGF-I and IGF-II effects on local IGF system and signaling pathways in gilthead sea bream (*Sparus aurata*) cultured myocytes

Sheida Azizi [a,b], Mohammad Ali Nematollahi [a,*], Bagher Mojazi Amiri [a], Emilio J. Vélez [b], Cristina Salmerón [b], Shu Jin Chan [c], Isabel Navarro [b], Encarnación Capilla [b], Joaquim Gutiérrez [b,*]

PLOS | ONE

RESEARCH ARTICLE

# Lysine and Leucine Deficiencies Affect Myocytes Development and IGF Signaling in Gilthead Sea Bream (*Sparus aurata*)

**Sheida Azizi[1,2], Mohammad Ali Nematollahi[1]*, Bagher Mojazi Amiri[1], Emilio J. Vélez[2], Esmail Lutfi[2], Isabel Navarro[2], Encarnación Capilla[2], Joaquim Gutiérrez[2]***

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OPEN ACCESS

**Citation:** Azizi S, Nematollahi MA, Mojazi Amiri B, Vélez EJ, Lutfi E, Navarro I, et al. (2016) Lysine and Leucine Deficiencies Affect Myocytes Development and IGF Signaling in Gilthead Sea Bream (*Sparus aurata*). PLoS ONE 11(1): e0147618. doi:10.1371/journal.pone.0147618

Aquaculture 471 (2017) 80–91

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# Eating for two: Consequences of parental methionine nutrition on offspring metabolism in rainbow trout (*Oncorhynchus mykiss*)

Iban Seiliez [a,*], Emilio J. Vélez [b], Esmail Lutfi [b], Karine Dias [a], Elisabeth Plagnes-Juan [a], Lucie Marandel [a], Stéphane Panserat [a], Inge Geurden [a], Sandrine Skiba-Cassy [a]

**ARTICLES INCLUDED IN THIS THESIS**

General and Comparative Endocrinology 205 (2014) 296–304

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

# IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes

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ARTICLE INFO

*Article history:*
Available online 2 June 2014

*Keywords:*
*Sparus aurata*
Growth and myogenic factors
4EBP1
70S6K
AKT
Myocytes

ABSTRACT

Skeletal muscle growth and development is controlled by nutritional (amino acids, AA) as well as hormonal factors (insulin-like growth factor, IGF-I); however, how its interaction modulates muscle mass in fish is not clearly elucidated. The purpose of this study was to analyze the development of gilthead sea bream cultured myocytes to describe the effects of AA and IGF-I on proliferating cell nuclear antigen (PCNA) and myogenic regulatory factors (MRFs) expression, as well as on the transduction pathways involved in its signaling (TOR/AKT). Our results showed that AA and IGF-I separately increased the number of PCNA-positive cells and, together produced a synergistic effect. Furthermore, AA and IGF-I, combined or separately, increased significantly Myogenin protein expression, whereas MyoD was not affected. These results indicate a role for these factors in myocyte proliferation and differentiation. At the mRNA level, AA significantly enhanced PCNA expression, but no effects were observed on the expression of the MRFs or AKT2 and FOXO3 upon treatment. Nonetheless, we demonstrated for the first time in gilthead sea bream that AA significantly increased the gene expression of TOR and its downstream effectors 4EBP1 and 70S6K, with IGF-I having a supporting role on 4EBP1 up-regulation. Moreover, AA and IGF-I also activated TOR and AKT by phosphorylation, respectively, being this activation decreased by specific inhibitors. In summary, the present study demonstrates the importance of TOR signaling on the stimulatory role of AA and IGF-I in gilthead sea bream myogenesis and contributes to better understand the potential regulation of muscle growth and development in fish.



## 1. Introduction

The gilthead sea bream (*Sparus aurata* L.) has become one of the most important species for Mediterranean aquaculture over the last 30 years, overcoming the capture fisheries production (FAO, 2012). However, despite its commercial interest, more research is needed to better understand its growth and development.

The growth pattern in fish differs from other vertebrates, since most fish can grow in length and weight until they die (Johnston et al., 2011; Talbot, 1993). The majority of this growth is due to accretion of muscle tissue, mostly the white skeletal muscle (Johnston, 2006; Mommsen, 2001). Contrary to most vertebrates, this muscle growth implies not only muscle hypertrophy (increase in fiber size), but also hyperplasia (new muscle fibers formation), which is mediated by muscle satellite cells (Stoiber and Sänger, 1996). During skeletal muscle development, precursor cells become myoblasts, which undergo proliferation, cell cycle exit, differentiation and then, fusion to form multinucleated myofibers (Chargé and Rudnicki, 2004; Johnston, 2006).

Myogenesis in fish is regulated by several growth and transcription factors expressed in a sequential manner (García de la serrana et al., 2014; Johnston, 2006). Some of these myogenic regulatory factors (MRFs) are essential for muscle lineage determination and cell proliferation (Myf5 and MyoD) while others contribute to the initiation and maintenance of the differentiation program, which turns myoblasts into myotubes (MRF4 and Myogenin). In addition to MRFs, growth is hormonally regulated mainly by the hypothalamic-pituitary axis through the growth hormone (GH) and the insulin-like growth factors (IGFs) system (Fuentes et al., 2013; Le Roith et al., 2001; Montserrat et al., 2007a; Reindl and Sheridan, 2012; Reinecke et al., 2005; Wood et al., 2005). Interestingly, it has been demonstrated that IGFs (IGF-I and IGF-II) stimulate *in vitro* nutrients uptake and protein synthesis (Castillo et al., 2004; Codina et al., 2008; Montserrat et al., 2012), as well as

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http://dx.doi.org/10.1016/j.ygcen.2014.05.024
0016-6480/

myoblast proliferation, which has been observed in gilthead sea bream myocytes using a proliferating cell nuclear antigen (PCNA) immunocytochemical technique (Rius-Francino et al., 2011) and in rainbow trout myocytes by BrdU labeling (Gabillard et al., 2010).

In addition to the endocrine factors, fish growth is a multifactorial process influenced by nutritional, genetic and environmental factors (García de la serrana et al., 2012a). Amino acids (AA) are important precursors that stimulate protein synthesis mainly activating the target of rapamycin (TOR) nutrient-sensitive signaling pathway (Kim, 2009; Meijer, 2003). Insulin and growth factors (e.g. IGFs) also activate the TOR signaling pathway through its ability to induce the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (Glass, 2010), resulting in an increase in protein synthesis via indirect activation of TOR (Vander Haar et al., 2007). In vertebrates, it is commonly known that TOR integrates signals from nutrients, energy status and growth factors, and that is an essential regulator of cell growth by controlling cell cycle, gene transcription, cytoskeleton organization and protein synthesis among other functions (Destefano and Jacinto, 2013; Dowling et al., 2010; Edinger and Thompson, 2002; Gough, 2012; van Dam et al., 2011; Yang et al., 2008). TOR is present in two distinct multiprotein complexes: TORC1 (Raptor) that is rapamycin and nutrient-sensitive, and TORC2 (Rictor), which is rapamycin and nutrient-insensitive. TORC1 promotes cellular growth, proliferation and metabolism by stimulating protein synthesis through 4EBP1 and 70S6K either in fish as in mammals (Sarbassov et al., 2005; Seiliez et al., 2008). Moreover, TORC2 controls various metabolic processes and promotes cell proliferation and survival by facilitating the phosphorylation of the active loop of AKT by the phosphoinositide-dependent kinase-1 (PDK1), which is necessary for AKT activation (Destefano and Jacinto, 2013; Foster and Fingar, 2010; Liao et al., 2008; Manning and Cantley, 2007; Sarbassov et al., 2005). Hence, AKT also regulates cell metabolism, growth and survival by inhibiting the forkhead family of transcription factors, FOXO (Calnan and Brunet, 2008; Héron-Milhavet et al., 2006; Manning and Cantley, 2007; Vadlakonda et al., 2013). Nevertheless, studies exploring the role of AA on fish myogenesis as well as the signaling pathways involved are very scarce. Seiliez et al. (2008) showed in rainbow trout *in vivo* and *in vitro* the importance of nutritional factors on the activation of TOR and its downstream effectors. Moreover, a study in Atlantic salmon using cultured myocytes has demonstrated the effects of AA alone or combined with IGF-I up-regulating the expression of different members of the IGF system (Bower and Johnston, 2010); thus supporting a nutritional stimulatory role on muscle growth.

In this framework, the main aim of the present study was to investigate the role of AA and growth factors in muscle growth and development in gilthead sea bream *in vitro* using a primary cell culture system. Thus, we analyzed the effects of IGF-I and AA on protein expression of PCNA and the myogenic factors, MyoD and Myogenin, as well as the gene expression of PCNA and MRFs (MyoD1, MyoD2, Myf5, Myogenin). Furthermore, we investigated the action of these molecules on the mRNA expression of TOR, AKT2 and its downstream effectors (4EBP1, 70S6K and FOXO3, respectively), as well as the activation by phosphorylation of these signaling pathways.

## 2. Materials and methods

### 2.1. Animals

Fish were obtained from a commercial fishery located in the north of Spain and maintained at the facilities of the School of Biology at the University of Barcelona (Spain) in 0.4 $m^3$ tanks with a temperature-controlled seawater recirculation system (21 ± 1 °C) and 12L:12D photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (Skretting, Burgos, Spain) and fasted for 24 h previously to the isolation of muscle cells. All animal handling procedures were conducted with the Ethics and Animal Care Committee of the University of Barcelona approval, following the EU, Spanish and Catalan Government-established norms and procedures.

### 2.2. Myocyte cell culture

For the different studies a total of twenty independent primary cultures of muscle satellite cells were performed as described by Montserrat et al. (2007b). We used approximately 40 gilthead sea bream (*S. aurata* L.) juveniles from 4 to 24 g body mass per culture. Although the size of the fish influenced the recovery efficiency, since a lower number of cells is obtained per gram of tissue as the fish increases in body size, all the cells extracted were muscle satellite cells and the cultures were very homogeneous. Cells were cultured in 6 well-plates (9.6 $cm^2$/well) for Western blot and quantitative real-time PCR (qPCR) studies and in 12 well-plates containing glass cover slips (2.55 $cm^2$) for immunofluorescence and immunocytochemistry analyses. Cells were counted, diluted and plated to a final density of 0.2–0.25 $10^6$ cells/$cm^2$ for both types of plates. The cultures were maintained at 23 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.11% NaCl, 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (A/A). The development of the cells was first analyzed throughout the culture (days 2, 4, 8 and 12) in order to describe the proliferation and differentiation patterns using immunocytochemical and immunofluorescence assays as described below.

### 2.3. Experimental treatments

For the different studies, myocytes at day 4 were used. This day of culture development was chosen because at this point, the cells retain the ability to proliferate and have also the capacity to start fusing and differentiating (Montserrat et al., 2007b). The cells were held for 12 h with DMEM containing 0.02% FBS and 1% A/A, then starved for 5 h with medium B (without amino acids, AA) containing 10% Earle's Balanced Salt Solution (EBSS, E7510) + 1% MEM vitamins (M6895) + 0.9% NaCl + 0.13% BSA. Subsequently, the cells were maintained in medium B alone (Control) or were treated with human IGF-I at 100 nM and/or an AA cocktail, which contained the concentration of AA of a standard cell culture medium, previously reported by Lansard et al. (2010) (1% MEM Amino Acids Solution (M5550) + 1% MEM Non-essentials Amino Acids Solution (M7145)). The incubations lasted for 3 h in the case of Western blot experiments and for 6 h for the rest of analyses. When using for Western blot studies the specific inhibitors of TOR, rapamycin (R8781) at 100 nM, and AKT, wortmannin (W3144) at 1 μM, these were added for the last 30 min of starvation and during subsequent incubation with the different treatments. Once the incubation time passed, wells were washed twice with cold phosphate buffered saline (PBS) and the samples were recovered accordingly depending on the assay type to be performed. All reagents were obtained from Sigma–Aldrich (Tres Cantos, Spain) except human IGF-I that was from Bachem (Weil am Rhein, Germany) and all plastic ware and glass cover slips were from Nunc (LabClinics, Barcelona, Spain).

### 2.4. Immunocytochemistry

Cell proliferation was analyzed by immunostaining using a commercial PCNA staining kit (Cat. No. 93-1143. Life Technologies, Alcobendas, Spain). After washing, cells were fixed at room temperature in 4% paraformaldehyde (PFA, Sigma–Aldrich, Spain) for 15 min, washed, and postfixed for 5 min in 50% and 70% ethanol. Briefly, coverslips were incubated in PCNA staining reagents following the suggested manufacturer's protocol. Coverslips were

incubated in a blocking solution to prevent non-specific binding before incubation with anti-PCNA primary antibody for 1 h and a biotinylated secondary antibody for 5 min, both at room temperature. Finally, cells were dehydrated in a graded alcohol series and mounted with histomount. The amount of PCNA-positive cells was calculated by dividing the PCNA-positive stained cells by the total number of nuclei in 6–8 images per coverslip using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Digital images were acquired with a CC2 camera coupled to a microscope at 40× using analySIS (Soft Imaging System) software. All images were analyzed by the same researcher.

### 2.5. Immunofluorescence

Immunofluorescence was performed based on the protocol described by Gabillard et al. (2010) with minor modifications. After washing with PBS, cells were fixed in 4% PFA for 30 min, and permeabilized in 0.2% Triton X-100/PBS for 3 min. Then, cells were rinsed three times in PBS and blocked for 1 h with 3% BSA, 0.1% Tween20 in PBS (PBST) at room temperature. Cells were incubated with primary antibodies diluted in blocking solution (3% BSA in PBST) for 24 h at 4 °C. Polyclonal rabbit anti-Myogenin (M-225; 1:100 dilution) and polyclonal rabbit anti-MyoD (M-318; 1:100 dilution) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody incubation (Goat anti-rabbit 568, A21069, Life Technologies, Alcobendas, Spain) was carried out in PBST for 1 h at room temperature. Nuclei were counterstained with Hoechst (H1399, Life Technologies, Alcobendas, Spain). Images were obtained at 36× magnification on a Leica TCS SP2 confocal microscope. Protein expression was evaluated by nuclei fluorescence intensity quantification normalized to the total number of nuclei (Hoechst stained) in each field by using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and 5–10 images per coverslip were analyzed.

### 2.6. Gene expression

#### 2.6.1. RNA extraction and cDNA synthesis

RNA samples were collected and processed with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using the procedure previously described (Jiménez-Amilburu et al., 2013). Concentration and RNA purity was determined using a NanoDrop ND2000 (Thermo Scientific, Alcobendas, Spain) and integrity of the samples was confirmed in a 1% agarose gel and staining with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). Afterwards, 500 ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain) following the manufacturer's recommendation to remove all genomic DNA, and further the RNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain).

#### 2.6.2. Quantitative real-time PCR (qPCR)

The mRNA transcript levels of PCNA, MyoD1, MyoD2, Myf5, Myogenin, TOR, 4EBP1, 70S6K, AKT2 and FOXO3, plus three reference genes (ribosomal protein S18 (RPS18), elongation factor 1 alpha (EF1α) and β-actin) were examined according to the requirements of the MIQE guidelines (Bustin et al., 2009) in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The analyses were performed using 2.5 μL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1) and 1 μL of cDNA for each sample in a final volume of 5 μL. Reactions were performed in triplicate in 384-well plates in the same conditions previously described by Salmerón et al. (2013). Shortly: initial activation for 3 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at 58–68 °C (primer dependent, Table 1) followed by an amplicon dissociation analysis from 55 to 95 °C at 0.5 °C increase each 30 s. Prior to analyses, a dilution curve with a pool of samples was run to confirm specificity of the reaction, absence of primer–dimers and to determine the appropriate cDNA dilution. The expression level of each gene analyzed was calculated relative to the reference genes RPS18 and β-actin, the two most stable of the genes analyzed, using the Pfaffl method (Pfaffl, 2001).

### 2.7. Western blot analysis

Protein homogenates from cells were obtained as described by Codina et al. (2008). The amount of protein from each sample was measured (Bradford, 1976) and 10–20 μg of protein were separated by electrophoresis (SDS–PAGE) on 10% polyacrylamide gel

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

| Gene | Primer sequences (5′–3′) | Tm °C | Accession number | References |
|---|---|---|---|---|
| *EF1α* | **F**: CTTCAACGCTCAGGTCATCAT<br>**R**: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | Salmerón et al. (2013) |
| *RPS18* | **F**: GGGTGTTGGCAGACGTTAC<br>**R**: CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 | Vieira et al. (2012) |
| β-actin | **F**: TCCTGCGGAATCCATGAGA<br>**R**: GACGTCGCACTTCATGATGCT | 60 | X89920 | Salmerón et al. (2013) |
| PCNA | **F**: TGTTTGAGGCACGTCTGGTT<br>**R**: TGGCTAGGTTTCTGTCGC | 58 | AY550963.1 | García de la serrana et al. (2014) |
| *MyoD1* | **F**: TTTGAGGACCTGGACCC<br>**R**: CTTCTGCGTGGTGATGGA | 60 | AF478568.1 | Present study |
| MyoD2 | **F**: CACTACAGCGGGGATTCAGAC<br>**R**: CGTTTGCTTCTCCTGGACTC | 60 | AF478569 | Jiménez-Amilburu et al. (2013) |
| *Myf5* | **F**: CTACGAGAGCAGGTGGAGAACT<br>**R**: TGTCTTATCGCCCAAAGTGTC | 64 | JN034420 | Jiménez-Amilburu et al. (2013) |
| *Myogenin* | **F**: CAGAGGCTGCCCAAGGTCGAG<br>**R**: CAGGTGCTGCCCGAACTGGGCTCG | 68 | EF462191 | Jiménez-Amilburu et al. (2013) |
| *TOR* | **F**: CAGACTGACGAGGATGCTGA<br>**R**: AGTTGAGCAGCGGGTCATAG | 60 | – | García de la serrana (unpublished) |
| 70S6K | **F**: GCACCAGAAAGGCATCATCT<br>**R**: AAGGTGTGGGTCACTGTTCC | 60 | – | García de la serrana (unpublished) |
| E4EBP1 | **F**: CCAACCTGCGACTCATCTCT<br>**R**: GTTCCTCTCATCCTCCCACA | 60 | – | García de la serrana (unpublished) |
| *AKT2* | **F**: GCTCACCCCACTCTTCAGAC<br>**R**: AAATTGGGAAATGTGCTTGC | 60 | ERA047531 | García de la serrana et al. (2012b) |
| *FOXO3* | **F**: CAGCAGCCTGGAGTGTGATA<br>**R**: CCAGCTCTGAGAGGTCTGCT | 60 | – | García de la serrana (unpublished) |

(125 V for 1 h 30 min) in electrophoresis buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3). Then, samples were transferred to a PVDF membrane overnight at 100 mA in transfer buffer (25 mM Tris–HCl, 192 mM Glycine, 20% Methanol, pH 8.3). After transfer, the membrane was blocked in non-fat milk 5% buffer at room temperature for 2 h and then incubated overnight at 4 °C with the respective primary antibodies diluted in washing buffer (20 mM Tris·HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6). The primary antibodies used were as follows: rabbit polyclonal anti-phospho AKT (Cat. No. 9271); rabbit polyclonal anti-total AKT (Cat. No. 9272); rabbit polyclonal anti-phospho TOR (Cat. No. 2971), all from Cell Signaling Technology (Beverly, MA); and rabbit polyclonal anti-total TOR (Cat. No. T2949. Sigma–Aldrich, Spain). For the phosphorylated forms 1:200 dilution was used and a 1:500 for the total forms. After washing (3 times for 15 min), the membranes were incubated with the peroxidase-conjugated secondary antibody (Cat. No. 31460. Thermo Scientific, Alcobendas, Spain) for 1 h at room temperature. The membranes were rewashed and the different immunoreactive bands were developed by using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain). Once the phosphorylated forms were developed, primary and secondary antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Alcobendas, Spain) incubating 10 min at room temperature and then, the membranes were blotted again following the same procedure with the total forms for both AKT and TOR. Finally, the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### 2.8. Statistical analyses

Data was analyzed using IBM SPSS Statistics v.20 and presented as means ± SEM. Normal distribution was first analyzed using Shapiro–Wilk test followed by Levene's to test homogeneity of variances. Treatments effects between groups were tested by T-test or one-way analysis of variance (ANOVA), followed by the Tukey test. When data did not follow a normal distribution non-parametric Kruskal–Wallis ANOVA and Mann–Whitney test were

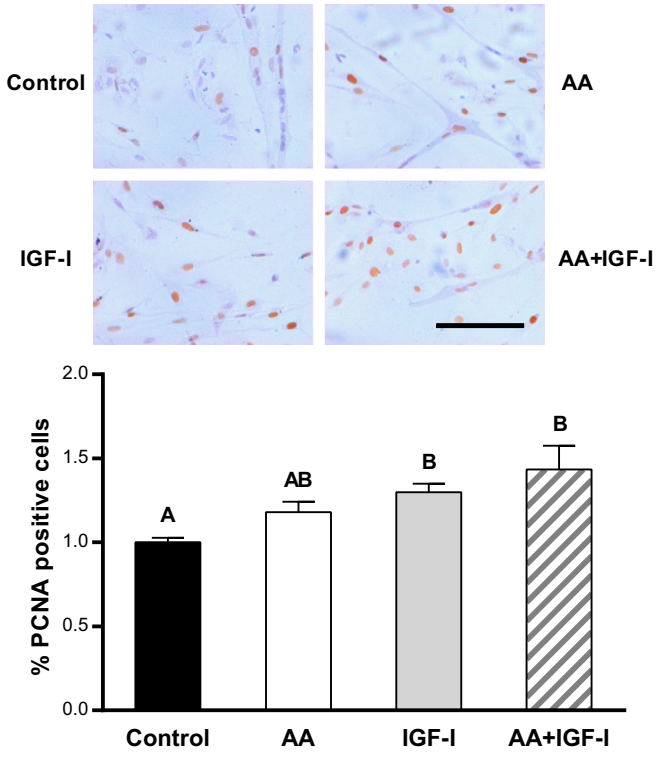


**Fig. 2.** Effects of amino acids (AA) and IGF-I on PCNA protein expression in gilthead sea bream cultured muscle cells. Representative images and quantification of PCNA-positive cells in gilthead sea bream muscle cells at day 4 incubated with AA, IGF-I and AA + IGF-I. PCNA was detected by immunostaining as described in Section 2 (40×). Results are presented as fold change over Control. Mean ± SEM ($n = 3$). Different letters indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

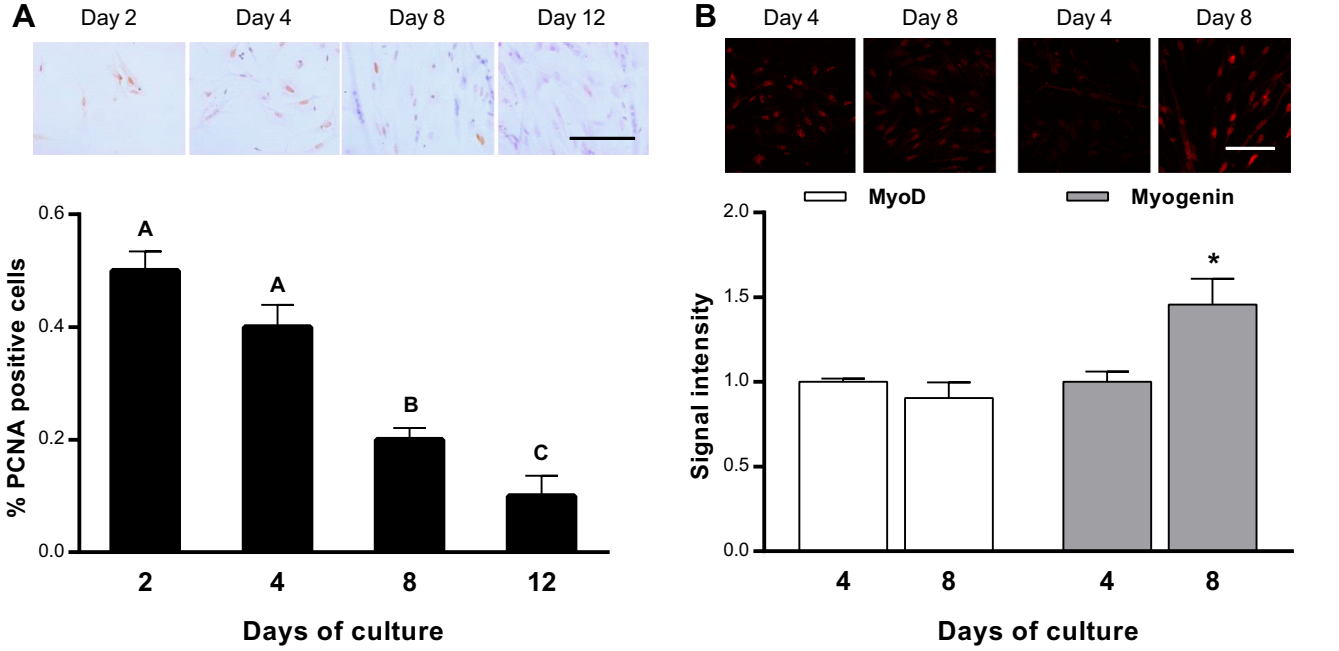


**Fig. 1.** Characterization of gilthead sea bream myocyte culture development. (A) Representative images and quantification of PCNA-positive cells from gilthead sea bream muscle cells at different days in culture (2, 4, 8 and 12). Slides were immunostained for PCNA as described in material and methods showing the different stages of development from myoblasts to myotubes (40×). (B) Representative images and quantification of MyoD and Myogenin protein expression at days 4 and 8 of culture. MyoD and Myogenin were detected by immunofluorescence and the expression intensity in the nuclei quantified as described in Section 2. Data are shown as mean values ± SEM ($n = 3$). Significant differences are shown by different letters or an asterisk ($P < 0.05$). Scale bar = 100 μm.

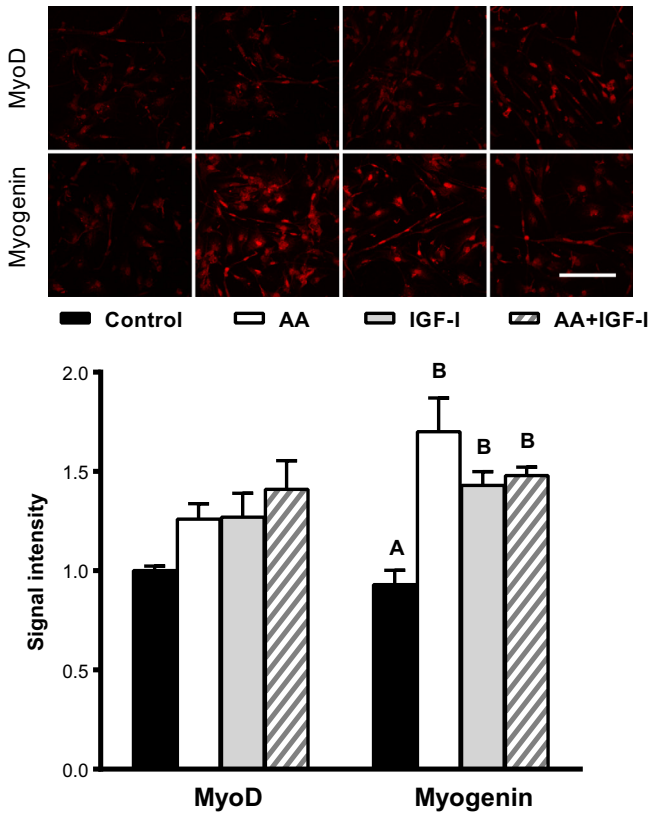


**Fig. 3.** Effects of amino acids (AA) and IGF-I on MyoD and Myogenin protein expression in gilthead sea bream cultured muscle cells. Representative images and quantification of nuclei MyoD and Myogenin protein expression in gilthead sea bream cultured muscle cells at day 4 incubated with AA, IGF-I and AA + IGF-I. MyoD and Myogenin were detected by immunofluorescence as described in Section 2 (40×). Results are presented as fold change over Control. Mean ± SEM ($n = 3$). Different letters indicate significant differences ($P < 0.05$). Scale bar = 100 μm.

applied. Statistical differences were considered significant when *P*-value <0.05 or <0.01.

## 3. Results

### 3.1. Cell culture development characterization

In order to determine the developmental patterns of myocyte culture in gilthead sea bream we examined cell proliferation throughout the culture at days 2, 4, 8 and 12 and, the protein expression of two MRFs, MyoD and Myogenin, at days 4 and 8. The amount of PCNA-positive cells was significantly higher during the first days of culture (days 2 and 4) and progressively decreased over time (Fig. 1A). On the other hand, the measurement of MRFs protein expression by immunofluorescence showed that MyoD was similar at both days (4 and 8), whereas Myogenin abundance was significantly higher during the differentiation phase of the culture (day 8) than at day 4 (Fig. 1B).

### 3.2. Effects of AA and IGF-I incubation on cultured myocytes

#### 3.2.1. Myogenic developmental markers protein and gene expression

The percentage of PCNA-positive cells significantly increased after 6 h of treatment with IGF-I alone or in combination with AA, whereas it was not altered by only AA (Fig. 2). Furthermore, the immunofluorescence analyses showed that MyoD showed a tendency to increase upon treatment, while both AA and IGF-I treatments, in conjunction or separately, significantly increased Myogenin protein expression over the Control (Fig. 3).

Regarding the gene expression of proliferation and progression markers of culture development, AA and the combined treatment of AA + IGF-I significantly increased PCNA mRNA levels over the Control, whereas the expression levels remained unaltered in the case of IGF-I treatment (Fig. 4A). Moreover, the mRNA levels of the different MRFs, MyoD1, MyoD2, Myf5 and Myogenin appeared unaltered by any treatment (Fig. 4B).

#### 3.2.2. AKT and TOR signaling pathways

Next, we examined the gene expression of two key signal transduction molecules; TOR and AKT2, and its principal substrates. The results showed that AA significantly increased TOR gene expression while IGF-I or AA combined with IGF-I caused no effects (Fig. 5A). Then, both AA and AA + IGF-I treatments significantly increased the expression of 4EBP1 (Fig. 5B). In the case of 70S6K, AA treatment significantly increased its gene expression, whereas AA + IGF-I only caused a slight increase (Fig. 5C). On the other hand, neither AA nor IGF-I, in combination or separately, altered the expression of AKT2 and its downstream effector FOXO3 (Fig. 6A and B).

Additionally, Western blot results showed that AA, alone or combined with IGF-I, significantly increased the phosphorylation of TOR over the Control (Fig. 7A) whereas IGF-I had no effects. Moreover, the treatment with the TOR specific inhibitor, rapamycin (R), decreased significantly TOR phosphorylation *versus* the samples without inhibitor (N.I.) in the Control group, although

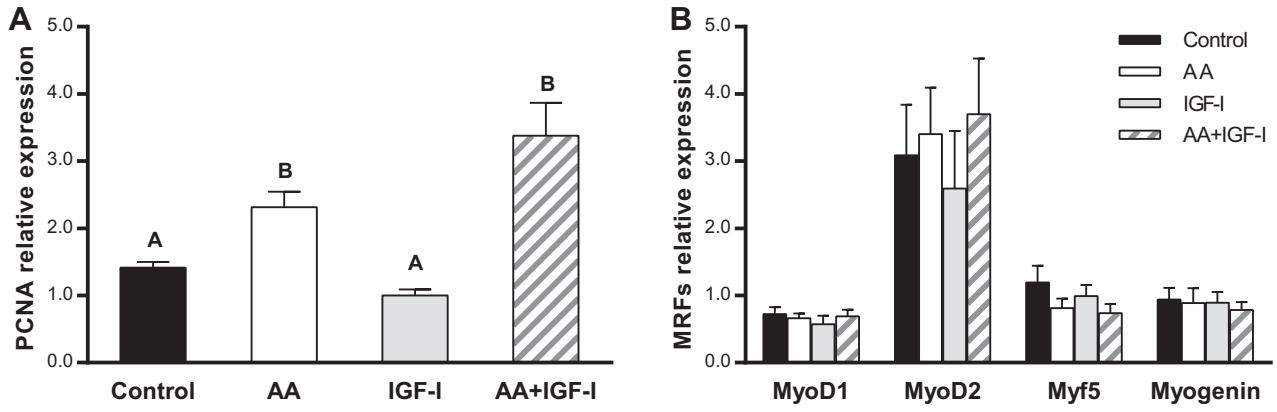


**Fig. 4.** Effects of amino acids (AA) and IGF-I on the gene expression of PCNA and MRFs in gilthead sea bream cultured muscle cells. (A) PCNA and (B) MRFs (MyoD1, MyoD2, Myf5 and Myogenin) expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM ($n = 4–7$). Different letters indicate significant differences ($P < 0.05$).

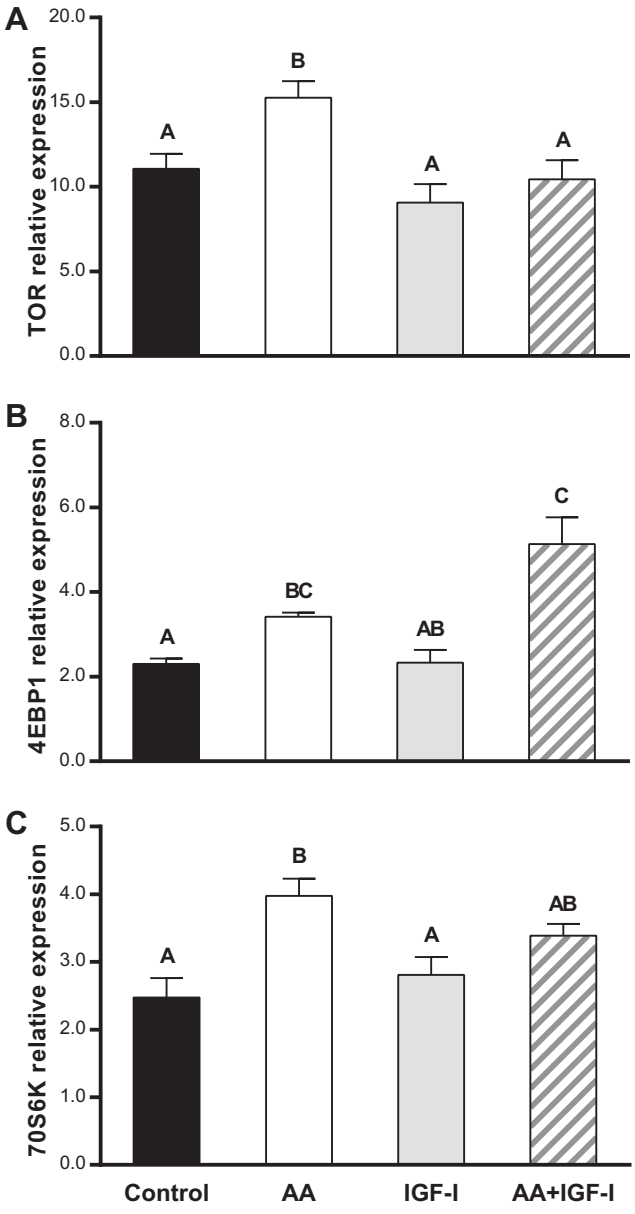


**Fig. 5.** Effects of amino acids (AA) and IGF-I on the gene expression of key molecules in the TOR signaling pathway in gilthead sea bream cultured muscle cells. (A) TOR, (B) 4EBP1 and (C) 70S6K expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM ($n$ = 4–7). Different letters indicate significant differences ($P$ < 0.05).

not in the other groups; whereas TOR was not altered by wortmannin (W) treatment in any case (Fig. 7A).

Regarding AKT, incubation with IGF-I alone or in combination with AA significantly increased AKT activation, whereas AA although induced a minor stimulation did not change significantly the level of phosphorylation (Fig. 7B). These effects disappeared upon incubation with the AKT's inhibitor, wortmannin (W), whereas rapamycin (R) did not produce any changes in AKT activation except in the case of the Control group, where it caused also decrease in AKT phosphorylation (Fig. 7B).

## 4. Discussion

Skeletal muscle biology is studied in many fields, from physiology to immunology through genetic diseases, tissue regeneration and even meat production, which is the most interesting from the view point of the industry (Muñoz-Cánoves and Michele, 2013). Skeletal muscle development has a well-ordered structure with high plasticity, being able to adapt to different conditions (Pisconti et al., 2012; Relaix and Zammit, 2012; Rescan, 2001). In this sense, the nutritional status has been shown to affect protein synthesis, and thus, muscle mass, both in mammals and teleost fish (Martin-Perez et al., 2013; Tirapegui et al., 2012).

In this study, we used as a model system a primary culture of muscle cells from gilthead sea bream to investigate the effects of AA and IGF-I on the gene and protein expression of proliferative and myogenic regulatory factors. Moreover, we analyzed the activation of transduction pathways involved in its signaling to better understand the process of myogenesis and its regulation by these nutritional and hormonal factors in this species.

PCNA is an auxiliary protein of DNA polymerase that helps to increase the processivity of the leading strand synthesis during DNA replication (Strzalka and Ziemienowicz, 2011). Specific PCNA-staining is observed in proliferating cells nuclei during normal muscle growth (Veggetti et al., 1999). Our results showed a progressive decrease in the percentage of PCNA-positive cells in culture, indicating that proliferation occurs mainly at early stages of development, in agreement with that observed in gene expression (García de la serrana et al., 2014). Nevertheless, the protein expression of the MRF involved in satellite cell specification, MyoD showed no differences. The fact that the polyclonal antibody used does not distinguish between the two MyoD isoforms and that they present different gene expression patterns throughout the culture (Vélez et al. unpublished data), could explain the little changes in MyoD signal intensity as suggested by Froehlich et al. (2013) in zebrafish. On the other hand, Myogenin protein expression was higher at day 8 than at day 4, confirming that myoblast differentiation takes place when proliferation decreases, supporting a role for this MRF in the late phases of culture development when formation of myotubes occurs. It is important to note that these results are consistent with the common knowledge about the process of myogenesis in teleosts (Johnston et al., 2008; Rescan, 2001) and specifically, in agreement with the results reported recently in gilthead sea bream (García de la serrana et al., 2014; Montserrat et al., 2007b) and rainbow trout (Gabillard et al., 2010).

With regards to the hormonal and nutritional regulation of myocyte development, our data indicated that a treatment with IGF-I alone or in combination with AA increased the amount of PCNA-positive cells as previously demonstrated for IGFs in gilthead sea bream and rainbow trout (Gabillard et al., 2010; Rius-Francino et al., 2011), supporting a role for IGF-I stimulating proliferation. Furthermore, the immunofluorescence results revealed that although MyoD did not change, both AA and IGF-I, in conjunction or separately, enhanced Myogenin protein expression, which is related to cell differentiation (Gabillard et al., 2010; García de la serrana et al., 2014). At the level of gene expression, only AA and the AA + IGF-I combination increased PCNA gene expression, meanwhile IGF-I alone had no effects; suggesting that the regulatory role of IGF-I on proliferation seems to occurs primarily at a post-transcriptional level. Moreover, none of the treatments altered the expression of MyoD1, MyoD2 or Myf5 as it was also observed previously (Jiménez-Amilburu et al., 2013). However, in that study it was shown that IGF-I, and mainly IGF-I plus GH increased Myogenin expression, effect not observed here. Nevertheless, different responses in MRFs expression after IGF-I treatment were also observed in rainbow trout myocytes depending on the incubation time (Garikipati and Rodgers, 2012a, 2012b).

Next, we analyzed the gene expression of TOR and AKT and its downstream effectors, as well as the activation of TOR and AKT by phosphorylation in response to AA and IGF-I. Our results indicated

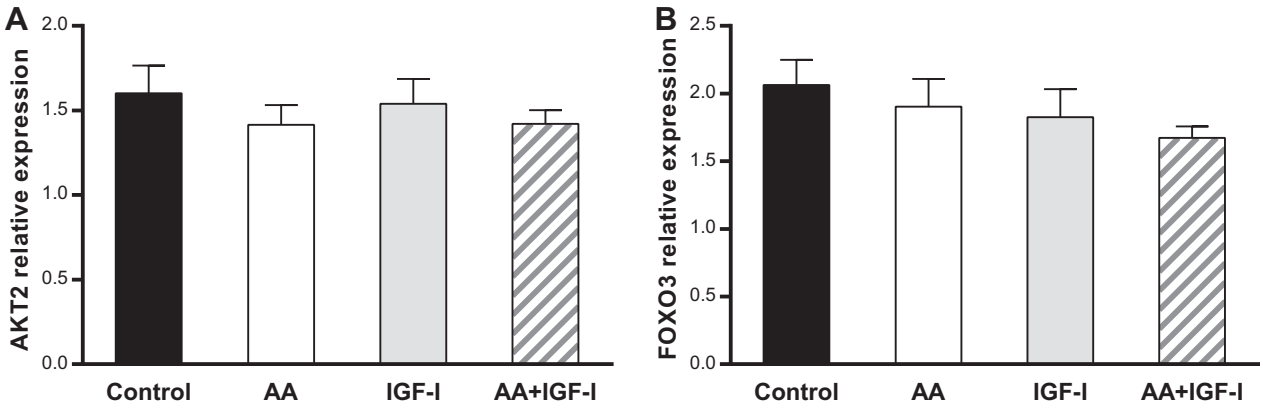


**Fig. 6.** Effects of amino acids (AA) and IGF-I on the gene expression of key molecules in the AKT signaling pathway in gilthead sea bream cultured muscle cells. (A) AKT2 and (B) FOXO3 expression in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I. Data are shown as mean values ± SEM ($n$ = 4–7). Different letters indicate significant differences ($P$ < 0.05).

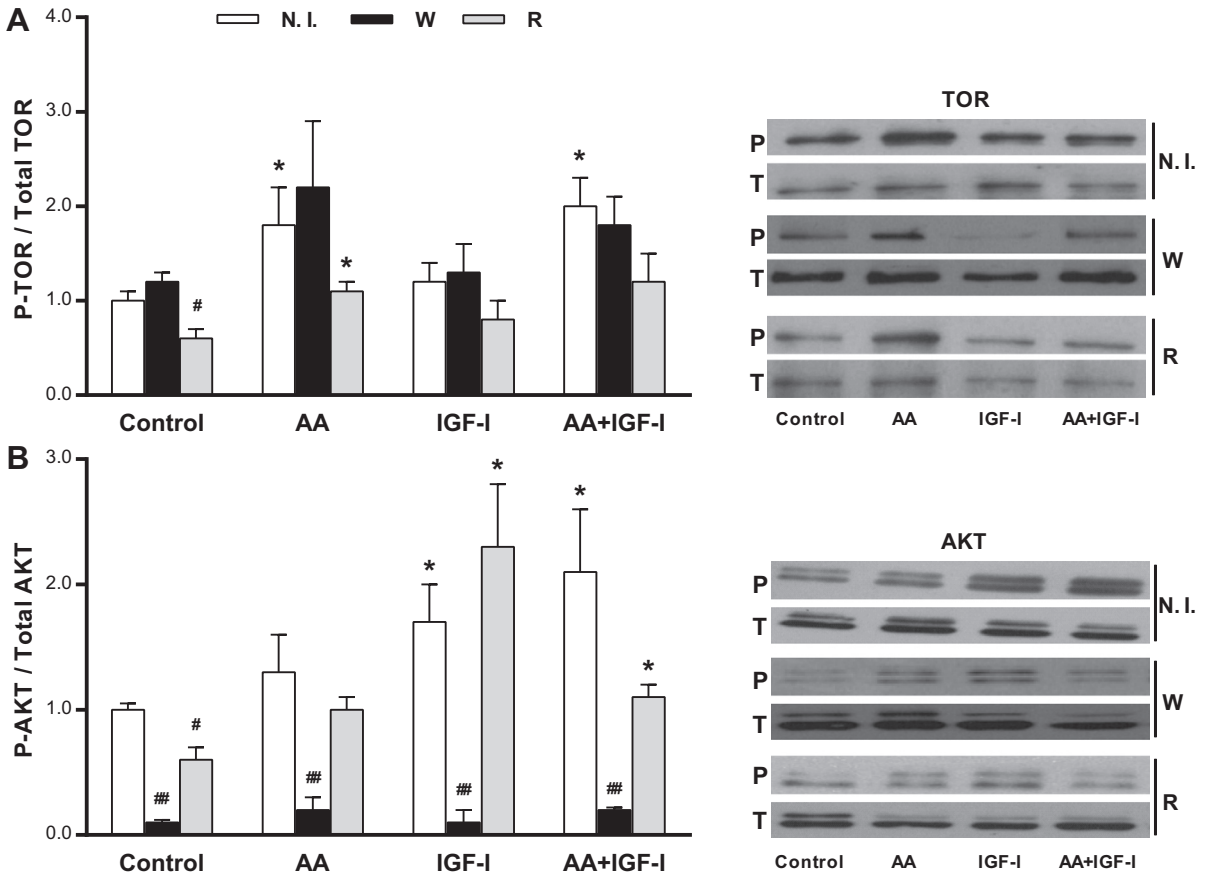


**Fig. 7.** Effects of amino acids (AA) and IGF-I on TOR and AKT signaling pathways activation in gilthead sea bream cultured muscle cells. Representative Western blots and quantification of TOR (A) and AKT (B) signaling pathways evaluated in myoblast cells at day 4 incubated with AA, IGF-I and AA + IGF-I, in the absence (N.I.) or presence of rapamycin (R) or wortmannin (W). Phosphorylated forms (P) band intensity was normalized to total forms (T). Results are presented as a fold change over Control. Mean ± SEM ($n$ = 3–15). Asterisks indicate significant differences with the Control for each treatment and # indicates significant inhibition over N.I. within each treatment. Significant differences were considered at $P$ < 0.05 (*/#) or $P$ < 0.01 (##).

that AA have the ability to regulate the mRNA expression of TOR and its effectors while IGF-I did not cause any changes in the expression of these molecules. Nevertheless, the combination of AA with IGF-I also increased 4EBP1 expression, suggesting that IGF-I may have a supporting regulatory role in TOR pathway. Interestingly AA also enhanced TOR phosphorylation as previously reported for TOR and its downstream effectors in different fish species (García de la serrana and Johnston, 2013; Seiliez et al., 2008), therefore showing a double effect on TOR simultaneously increasing its expression and activity. Although IGF-I alone had no effects activating TOR, the combination of AA and IGF-I stimulated also TOR phosphorylation as in recent reports in mammals (Destefano

and Jacinto, 2013; Kim and Guan, 2011). Similarly, in previous studies in Atlantic salmon myocytes and rainbow trout hepatocytes, IGF-I or insulin alone, respectively, only slightly increased TOR signaling but when combined with AA caused a greater stimulation (García de la serrana and Johnston, 2013; Lansard et al., 2010). The lack of effects on TOR phosphorylation by IGF-I alone could be due to species or cell type differences or to the incubation time, 3 h in our study *versus* 24 h or 15 min in those studies. Nevertheless, overall our results are in agreement with the effects of AA and growth factors on TOR activation, although little information exists at the level of gene expression, therefore the present study permits to demonstrate the important effect of AA not only on protein activation but also on gene expression of this signaling pathway.

This study was completed with a rapamycin treatment, the TORC1 specific inhibitor (Nobukuni et al., 2005). Rapamycin decreased TOR phosphorylation in all conditions although only significantly in the Control group, in agreement with previous results (Lansard et al., 2010), confirming the specificity of the pathway, but also indicating that perhaps the sensitivity of the TORC1 complex in gilthead sea bream seems to be lower than that of mammals or other fish species. In this sense, the understanding of the complete regulation of TOR complexes in this species deserves more investigation.

Concerning AKT results, we have proved that IGF-I stimulates the PI3K/AKT pathway in myocytes by increasing AKT phosphorylation, in agreement with the results related by our group in gilthead sea bream (Montserrat et al., 2012) and rainbow trout (Codina et al., 2008), or in mammals by others (Laviola et al., 2007; Philippou et al., 2007). Although there was no significant effect of AA, the combined treatment with AA + IGF-I maintained the activation of this signaling pathway. However, since effects were not found on AKT2 or FOXO3 gene expression in response to any treatment, we hypothesize that the regulation of AKT takes place at a post-traductional level.

Moreover, wortmannin the specific inhibitor of PI3K, did not affect TOR activation, but dramatically decreased AKT phosphorylation in all conditions, as it has been previously shown (Montserrat et al., 2012). Contrariwise, rapamycin treatment did not affect AKT activity, except in the case of the Control group, indicating that AKT phosphorylation is mostly independent of AA action through TORC1, as in mammals (Kim and Guan, 2011). Although more studies are needed to confirm it, the significant suppressive effects of rapamycin in Control myocytes in our study could suggest mechanistic regulatory differences in the activation of this signaling pathway between fish and mammals.

In summary, this work shows the important role of IGF-I and AA in muscle growth regulation including the stimulation of the processes of proliferation and differentiation and the conservation of the main signaling pathways involved through vertebrates. However, it should be taken into consideration that some particularities among fish species could be related to the physiological and metabolic differences, as well as to the importance of proteins in the diet. Altogether, it contributes to improve the knowledge on the regulation of myogenesis in gilthead sea bream and provides potential nutritional treatments to optimize muscle growth in this cultured species.

## Acknowledgments

The authors would like to thank Carlos Mazorra from Tinamenor S.L and the personnel from the facilities at the School of Biology for the maintenance of the fish. E.C. is a "Ramón y Cajal" researcher fellow from the "Ministerio de Ciencia e Innovación" (MICINN). E.J.V. and E.L. are supported by a predoctoral fellowship from the MICINN. This study was supported by the projects from the MICINN AGL2009-12427 and AGL2012-39768 to J.G. and AGL2010-17324 to E.C., the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and SGR2009-00402 from the "Generalitat de Catalunya", and by funds from the European Union through the project LIFECYCLE (EU-FP7 222719).

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PLOS ONE

RESEARCH ARTICLE

# Proteolytic systems' expression during myogenesis and transcriptional regulation by amino acids in gilthead sea bream cultured muscle cells

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OPEN ACCESS

**Citation:** Vélez EJ, Azizi S, Verheyden D, Salmerón C, Lutfi E, Sánchez-Moya A, et al. (2017) Proteolytic systems' expression during myogenesis and transcriptional regulation by amino acids in gilthead sea bream cultured muscle cells. PLoS ONE 12(12): e0187339. https://doi.org/10.1371/journal.pone.0187339

**Editor:** Silvia Martínez-Llorens, Universitat Politècnica de València, SPAIN

**Received:** May 19, 2017

**Accepted:** October 18, 2017

**Published:** December 20, 2017



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** E.J.V. and E.L. are supported by predoctoral fellowships (BES-2013-062949 and BES-2012-061867) from the "Ministerio de Economía y Competitividad" (MINECO). This study was supported by the projects from the MINECO AGL2014-57974-R to I.N. and E.C., AGL2012-39768 and AGL2015-70679-R to J.G., the "Xarxa

## Abstract

Proteolytic systems exert an important role in vertebrate muscle controlling protein turnover, recycling of amino acids (AA) or its use for energy production, as well as other functions like myogenesis. In fish, proteolytic systems are crucial for the relatively high muscle somatic index they possess, and because protein is the most important dietary component. Thus in this study, the molecular profile of proteolytic markers (calpains, cathepsins and ubiquitin-proteasome system (UbP) members) were analyzed during gilthead sea bream (*Sparus aurata*) myogenesis *in vitro* and under different AA treatments. The gene expression of calpains (*capn1*, *capn3* and *capns1b*) decreased progressively during myogenesis together with the proteasome member *n3*; whereas *capn2*, *capns1a*, *capns1b* and ubiquitin (*ub*) remained stable. Contrarily, the cathepsin D (*ctsd*) paralogs and E3 ubiquitin ligases *mafbx* and *murf1*, showed a significant peak in gene expression at day 8 of culture that slightly decreased afterwards. Moreover, the protein expression analyzed for selected molecules presented in general the same profile of the mRNA levels, which was confirmed by correlation analysis. These data suggest that calpains seem to be more important during proliferation, while cathepsins and the UbP system appear to be required for myogenic differentiation. Concerning the transcriptional regulation by AA, the recovery of their levels after a short starvation period did not show effects on cathepsins expression, whereas it down-regulated the expression of *capn3*, *capns1b*, *mafbx*, *murf1* and up-regulated *n3*. With regards to AA deficiencies, the major changes occurred at day 2, when leucine limitation suppressed *ctsb* and *ctsl* expression. Besides at the same time, both leucine and lysine deficiencies increased the expression of *mafbx* and *murf1* and decreased that of *n3*. Overall, the opposite nutritional regulation observed, especially for the UbP members, points out an efficient and complementary role of these factors that could be useful in gilthead sea bream diets optimization.

de Refèrencia d'R+D+I en Aqüicultura" and 2014SGR-01371 from the "Generalitat de Catalunya", and by funds from the European Union through the project LIFECYCLE (EU-FP7 222719). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Muscle growth regulation in vertebrates requires an equilibrium among protein synthesis and degradation (proteolysis). This balance acquires special interest in fish, since they mostly present indeterminate growth, and thus increase their muscle mass throughout their life, as well as they are naturally exposed to periods of low food availability, when metabolic mobilization becomes important to survive fasting.

Fish muscle growth differs in many species from other vertebrates in so most mammals can develop muscle mass only by hypertrophy after sexual maturation, whereas fish still continue to increase their muscle mass with important rates of muscle hyperplasia (myogenesis). Understanding on the regulation of fish myogenesis has increased significantly in the last years (reviewed by Fuentes et al. [1]; Johnston et al. [2]; Vélez et al. [3]), as well as the importance of proteolysis on muscle growth *in vivo*; however, the investigation involving the proteolytic systems *in vitro* has been only limited [4–8]. Furthermore, muscle proteolysis research is important in aquaculture because the proteolytic systems play a key role in determining the fish flesh quality. During the *post mortem* period, the muscle tissue is subjected to changes caused by many factors such as temperature, pH or microbial activity, which in conjunction with the action of endogenous proteases can modify muscle properties [5]. Therefore, due to its significance in regulating both, muscle growth and value, to fully elucidate the role of the different proteolytic systems in fish is of utmost importance.

The four chief endogenous proteolytic systems in vertebrates include: cathepsins, calpains, the ubiquitin-proteasome (UbP) and caspases [9, 10]; although the caspases will not be considered in this study as they are mostly linked to cellular apoptosis [11].

The cathepsin family contains several classes of proteases comprising: 1) cysteine proteases (CTSB, L, H, K, S and O), 2) aspartyl proteases (CTSD and E) and, 3) serine proteases (CTSG). Most cathepsins are lysosomal enzymes and part of the autophagic-lysosome system (ALS) involved in cellular degradation. In fact, they are characterized as regulators of an enormous number of biological processes like bone remodeling or angiogenesis, and have been implicated in the development of different pathological conditions (e.g. inflammation and cancer) [12]. In fish, Seiliez and co-workers have recently demonstrated in rainbow trout (*Oncorhynchus mykiss*) myotubes that the ALS is responsible for up to 50% of total protein degradation in contrast to mammals, in which this system appears to be proportionally less important [8].

The calpain system is composed by intracellular proteases that are $Ca^{2+}$-dependent and belong to the papain superfamily of cysteine proteases. The catalytic CAPN1 and CAPN2 subunits bind a common regulatory member, CAPN4 or calpain small subunit (CAPNS) to form an active heterodimer, that has different biological functions during myogenesis depending on the catalytic member [13]. While CAPN1 may be involved in the myogenic regulation via its action on myogenin, ezrin, vimentin and caveolin 3 [14], CAPN2 participates in the fusion of mononuclear myoblasts to multinucleated myotubes in muscle cell cultures [15]. To date, members of this proteolytic system have been characterized in several teleost fish including gilthead sea bream (*Sparus aurata*). In addition in this species, the expression of *capn1* and *capns1a*, was shown to be inversely correlated with muscle texture, indicating they may serve as potential genetic markers of flesh quality [16].

In the UbP system, a large proportion of the proteins intended for degradation in the cell (representing up to 50% in mammals) are tagged by ubiquitination, and then recognized by the 26S proteasome complex, where they are degraded to oligopeptides [9]. Nonetheless in fish, the UbP system is only responsible for 17% of the protein degradation as demonstrated in rainbow trout myotubes [8]. Among the members that conform this system, the muscle specific F-box protein (MAFbx, a.k.a. Atrogin1/Fbox-32) and the muscle RING-finger protein 1

(MuRF1) are key E3 ubiquitin ligases specifically expressed in skeletal, cardiac and smooth muscle that perform multiple functions [17], and have been found up-regulated in situations of muscular atrophy [18]. Similarly, although ubiquitin has multiple functions either proteolytic or non-proteolytic, its expression has been found to increase with age in mammals, which has been related with the poorer healing capacity of the muscle in the elderly [19]. Furthermore, N3 (a.k.a. PSMB4) is a β type proteasome subunit that has been previously used as a proteolysis marker of this degradation system in fish [16, 20–22].

To study skeletal muscle *in vitro* development in mammals, several cell lines have been characterized (e.g. C2C12, L6 or HSkM), but equivalent models are not available in farmed fish, turning primary cultures essential. Therefore, during the last decade primary cultures of myocytes derived from isolated white muscle satellite cells have been established for some economically important fish species, like rainbow trout [23], gilthead sea bream [24], Atlantic salmon (*Salmo salar*) [25], giant danio (*Devario cf. aequipinnatus*) [26] and even, zebrafish (*Danio rerio*) [27]. These fish models represent a useful tool to study not only the conserved mechanisms taking place during myogenesis, but also can facilitate the identification of specific-critical factors involved in this process. In this sense, for example the regulation of myogenic development by nutritional factors such as amino acids (AA) has been investigated in several fish species including gilthead sea bream [28, 29]. These studies demonstrated the stimulatory effect of AA on myocytes proliferation and differentiation, as well as the critical negative effect on such processes of lysine limitation. Notwithstanding, information regarding the function of cathepsins, calpains and UbP members on fish myogenesis and how these catabolic systems respond to either AA supplementation or limitation is scarce, and most of the studies reported to date have been performed in salmonids [8, 30, 31].

Thus, the aim of this study was to characterize these 3 main proteolytic systems in gilthead sea bream during *in vitro* myogenesis and the transcriptional modulation of its members by AA to better understand the overall regulation of muscle development and growth in this important farmed species.

## Material and methods

### Experimental animals and ethical statement

The gilthead sea bream were provided by a commercial hatchery in northern Spain (Tinamenor S.L., Pesués, Cantabria). The fish were kept in tanks of 0.4 $m^3$ with a closed-water flow circuit at the facilities of the Faculty of Biology at the University of Barcelona. Conditions in the tanks, such as temperature of the sea water (21 ± 1˚C), photoperiod (12 h light: 12 h dark) and pH (7.5–8), were kept stable at all times. Twice a day fish were fed *ad libitum* with a commercial diet (Skretting, Burgos, Spain). The animal handling procedures were carried out with the specific approval of the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 168/14 and DAAM 7749), following the EU, Spanish and Catalan Government-assigned principles and legislations.

### Myocyte cell culture

A total of fifteen independent white muscle satellite cell cultures were performed following the method described previously by Montserrat et al. [24]. Around 40 juvenile fish weighing 5 to 15 g were used for each culture. The fish were sacrificed by a blow to the head, weighed and immediately, their external surfaces were sterilized by immersion in 70% ethanol during 0.5 to 1 min. Then, fish were dissected and the epaxial white muscle tissue was collected in cold Dulbecco's Modified Eagle's Medium (DMEM), containing 9 mM $NaHCO_3$, 20 mM HEPES, 0.11% NaCl, and 1% (v/v) antibiotic/antimycotic solution, and in this case supplemented with

15% (v/v) horse serum (HS) at a rate of 5 mL/g of tissue. Subsequently, muscle was minced to small fragments and centrifuged (3000 xg, 5 min), washed twice in DMEM and afterwards, the muscle shreds were enzymatically digested with 0.2% collagenase type IA dissolved in DMEM with gentle agitation during 80 min at 21˚C. The obtained suspension was centrifuged and the pellet washed with DMEM medium (300 xg, 5 min), resuspended again and triturated by repeated pipetting. After centrifuged once more (300 xg, 5 min), the tissue fragments were digested twice during 20 min at 21˚C, with 0.1% trypsin solution prepared in DMEM and gentle agitation. After each digestion the remained fragments were pelleted (300 xg, 1 min) to collect the supernatants, which were pooled and diluted in complete medium (DMEM supplemented with 15% of HS) to block trypsin activity. Then, the supernatant was centrifuged (300 xg, 20 min) and the obtained pellet resuspended, forced to trituration by pipetting and then, the suspension was filtered first on a 100 μm, and subsequently on a 40 μm nylon cell strainer, and finally centrifuged one last time (300 xg, 20 min). Later, the obtained cells were diluted in growth media (DMEM supplemented with 10% fetal bovine serum (FBS) and seeded in six well-plates (9.6 $cm^2$/well) at a final density of 1–2 x $10^6$ cells per well. Cultures were kept at 23˚C in growth medium with medium change every 2–4 days. To characterize the role of the different proteolytic systems during myogenesis, cell samples for gene and protein expression were taken at days 2, 4, 8 and 12 of culture. These days were chosen because they represent well the different stages of myogenesis, which can be followed according to cell morphology, and are supported by data reported in previous publications [24, 28, 32, 33].

## Experimental treatments

To study the effects of AA recovery, as described previously by Vélez et al. [28], cells at day 4 were first maintained for 12 h with DMEM with 0.02% FBS, and then, starved during 5 h with a medium deficient in AA (medium B: 10% Earle's Balanced Salt Solution (EBSS, E7510) with 1% MEM vitamins (M6895), 0.9% NaCl and 0.13% bovine serum albumin (BSA)). Next, cells were held 6 h in medium B alone (Control) or supplemented with an AA cocktail (1% MEM Amino Acids Solution (M5550) and 1% MEM Non-essentials Amino Acids Solution (M7145)) before samples were collected. In the case of the leucine or lysine deficiency experiments, as described before by Azizi et al. [29] other 3 different media were prepared (control, without leucine or without lysine) using DMEM/F12HAM (D9785) devoid of leucine and lysine as a base media, and adding 10% FBS, and the missing AA. The concentration of either leucine (24.2 μM) or lysine (24.7 μM) provided by the FBS in each corresponding deficient medium was reduced in a 93.8% respect to the control condition (where total concentration was 389.6 μM and 398.0 μM for leucine and lysine, respectively). In this experiment, the growth medium was replaced with the corresponding media at day 1 of culture for samplings at days 2 and 4, and at day 7 for the sampling at day 8.

All plastic ware were obtained from Nunc (LabClinics, Barcelona, Spain) and all reagents were from Sigma-Aldrich (Tres Cantos, Spain) unless stated otherwise.

## Gene expression

**RNA extraction and cDNA synthesis.** Cell samples for RNA extraction from each independent culture were collected from 3 replicate wells pooled together per sampling point during myogenesis characterization and from 2 replicate wells pooled together per condition in both AA experiments using 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) and processed following the manufacturer's instructions. A NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain) was used to determine total RNA concentration and purity. Confirmation of RNA integrity was performed in a 1% (m/v) agarose gel stained with SYBR-Safe DNA Gel Stain (Life

Technologies, Alcobendas, Spain). In order to obtain cDNA, 500 ng of the total RNA was first exposed to a DNase I enzyme (Life Technologies, Alcobendas, Spain) to remove all genomic DNA and after reversely transcribed by using a Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendation.

**Quantitative real-time PCR (qPCR).** Levels of mRNA transcripts of different cathepsins (*ctsda*, *ctsdb*, *ctsb* and *ctsl*), calpains (*capn1*, *capn2*, *capn3*, *capns1a* and *capns1b*) and UbP members (*mafbx*, *murf*, *n3* and *ub*), as well as the reference genes ribosomal protein S18 (*rps18*), elongation factor 1 alpha (*ef1α*) and beta-actin (*β-actin*) were analyzed according to the MIQE guidelines requirements [34] in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). The qPCR reactions were performed using 2.5 μL of iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1) and 1 μL cDNA of each sample at the corresponding dilution for an efficient measurement in a final volume of 5 μL. Each run was performed in triplicate using 384-well plates and conditions were the same as those described previously [16]. Briefly, a short initial activation of 3 min at 95˚C was followed by 40 cycles of 10 sec at 95˚C, 30 sec at 54–61˚C (primer dependent, Table 1) and ended with an amplification dissociation analysis from 55 up to 95˚C with a 0.5˚C increase

**Table 1. Primer sequences used for qPCR.**

| Gene | Primer sequences (5'-3') | Accession No. | Ta (˚C) | Amplicon (bp) | E (%) |
|---|---|---|---|---|---|
| *ef1a* | **F:** CTTCAACGCTCAGGTCATCAT<br>**R:** GCACAGCGAAACGACCAAGGGGA | AF184170 | 60 | 263 | 96.6 |
| *β-actin* | **F:** TCCTGCGGAATCCATGAGA<br>**R:** GACGTCGCACTTCATGATGCT | X89920 | 68 | 50 | 98.3 |
| *rps18* | **F:** GGGTGTTGGCAGACGTTAC<br>**R:** CTTCTGCCTGTTGAGGAACCA | AM490061.1 | 60 | 160 | 97.7 |
| *ctsda* | **F:** CCTCCATTCACTGCTCCTTC<br>**R:** ACCGGATGGAAAACTCTGTG | AF036319 | 56 | 107 | 102.1 |
| *ctsdb* | **F:** AAATTCCGTTCCATCAGACG<br>**R:** CTTCAGGGTTTCTGGAGTGG | KJ524456 | 56 | 131 | 95.6 |
| *ctsb* | **F:** GCAGCCTTCCTGTTATTGG<br>**R:** AGGTCCCTTCAGCATCGTA | KJ524457 | 57 | 185 | 95.0 |
| *ctsl* | **F:** ACTCCTTGGGCAAACACA<br>**R:** CCTTGAACTTCCTCTCCGT | DQ875329 | 54 | 116 | 94.5 |
| *capn1* | **F:** CCTACGAGATGAGGATGGCT<br>**R:** AGTTGTCAAAGTCGGCGGT | KF444899 | 56 | 114 | 103.2 |
| *capn2* | **F:** ACCCACGCTCAGACGGCAAA<br>**R:** CGTTCCCGCTGTCATCCATCA | KF444900 | 61 | 405 | 91.3 |
| *capn3* | **F:** AGAGGGTTTCAGCCTTGAGA<br>**R:** CGCTTTGATCTTTCTCCACA | ERP000874 | 56 | 113 | 97.2 |
| *capns1a* | **F:** CGCAGATACAGCGATGAAAA<br>**R:** GTTTTGAAGGAACGGCACAT | KF444901 | 56 | 92 | 100.2 |
| *capns1b* | **F:** ATGGACAGCGACAGCACA<br>**R:** AGAGGTATTTGAACTCGTGGAAG | ERP000874 | 56 | 51 | 99.7 |
| *mafbx* | **F:** GGTCACCTGGAGTGGAAGAA<br>**R:** GGTGCAACTTTCTGGGTTGT | ERA047531 | 60 | 158 | 94.3 |
| *murf1* | **F:** GTGACGGCGAGGATGTGC<br>**R:** CTTCGGCTCCTTGGTGTCTT | FM145056 | 60 | 50 | 98.5 |
| *n3* | **F:** AGACACACACTGAACCCGA<br>**R:** TTCCTGAAGCGAACCAGA | KJ524458 | 54 | 118 | 99.1 |
| *ub* | **F:** ACTGGCAAGACCATTACCTT<br>**R:** TGGATGTTGTAGTCGGAAAG | KJ524459 | 54 | 160 | 97.2 |

F: forward; R: reverse; Accession No.: GenBank accession numbers; Ta: annealing temperature; Amplicon: product size (base pairs); E: qPCR efficiency.

https://doi.org/10.1371/journal.pone.0187339.t001

every 30 sec. Although all the primers have been previously validated [16, 22], a dilution curve with a pooled sample was made before the analyses to confirm reaction specificity, absence of primer-dimers, efficiency of the primers pairs (Table 1) and to determine the appropriate cDNA dilution to work with. Transcript abundance of each studied gene was calculated relative to the geometric mean of the three reference genes (*rps18*, *β-actin* and *ef1α*) since they were all stable (confirmed by the geNorm algorithm) using the method described by Pfaffl [35] with the Bio-Rad CFX Manager 3.1 software.

## Protein expression

**Protein extraction.** Protein isolation was carried out from the same samples taken at different time points during myogenesis and obtained from triplicate wells pooled together from each independent culture using the interphase and organic phase produced during the RNA isolation and following the TRI Reagent Solution manufacturer protocol. The protein pellets were resuspended with 60 μL of RIPA buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 2 mM, NP-40 1%, SDS 0.1% and Na-deoxycholate 0.5% plus protease inhibitor cocktail P8340) and homogenated with a Pellet pestle (Sigma-Aldrich). Then, the samples were kept for 1 h in an orbital at 4˚C and centrifuged at 15,000 xg for 30 min at 4˚C. Finally, the supernatant was transferred to a new tube and stored at -80˚C until further analysis.

**Western blot analysis.** Protein concentration of each sample was measured using the Bradford assay, with BSA as reference protein. Four to ten μg of soluble fraction protein were subjected to a SDS-PAGE gel electrophoresis on a 12% acrylamide gel (1 h 30 min at 125 V) following the procedure previously described [28]. After blocking with 5% non-fat milk in washing buffer (20 mM Tris HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6), the membranes were incubated with the primary antibodies diluted in washing buffer overnight at 4˚C. The primary polyclonal antibodies used were CAPN1 (sc-7530), CTSD (sc-6486), CTSL (sc-6501) and MAFbx (sc-33782), all from Santa Cruz Biotechnology (Santa Cruz, CA, US). CAPN1, CTSD and CTSL were used at 1:200 and MAFbx at 1:400 final concentration. Afterwards, the membranes were washed and incubated with the respective secondary antibodies (sc-2020 and sc-2004, also provided by Santa Cruz Biotechnology) in a 5% non-fat milk washing buffer solution at a final concentration of 1:10000. After washing, an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain) was used to develop the bands. When required, the membranes were stripped for 15 min at 65˚C and 30 min at 37˚C on a roller with a commercial stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific). The software ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the obtained bands by densitometry. Since the band corresponding to the immature form of cathepsin D (CTSD imm) was stable during myogenesis, the expression of this protein was used as a loading control to normalize the expression of all the other proteins analyzed.

## Statistical analyses

IBM SPSS Statistics v.20 was used to analyze the data. The results are presented as means ± SEM. All the raw data underlying the obtained results can be found in the S1 File. 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. When normality existed, data was subjected to a one-way ANOVA followed by a Tukey or Dunnet T3 *post-hoc* test depending if respectively there was homogeneity of variances or not. Nevertheless, when normality was not assumed, the non-parametric Kruskal-Wallis test was used followed by a Mann-Whitney U test. Similarly, correlations between mRNA and protein levels were established either with a Spearman's rank correlation coefficient (ρ) or a Pearson correlation (PC). Differences were considered significant at $p<0.05$.

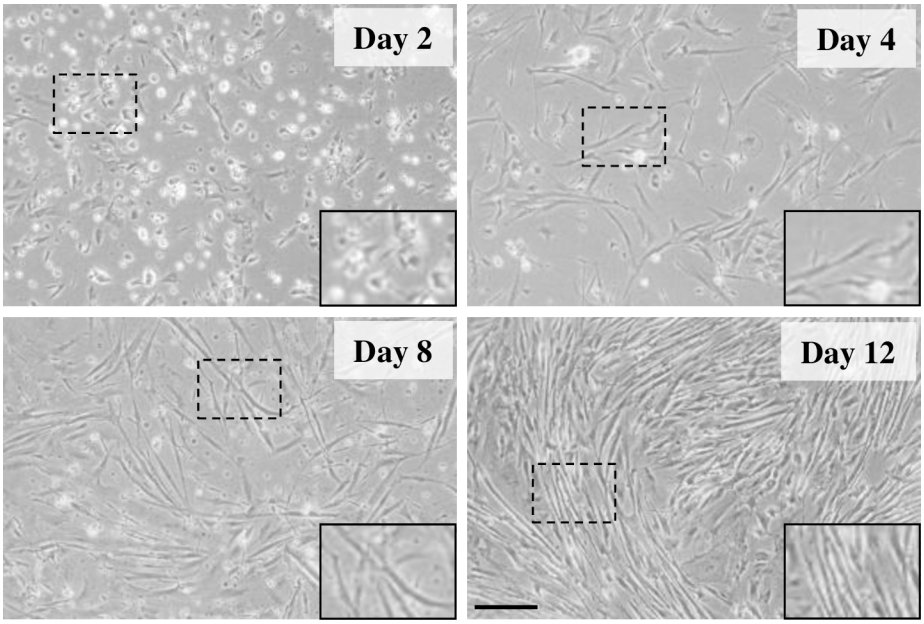


**Fig 1. Representative images of gilthead sea bream cultured myocytes at days 2, 4, 8 and 12 of development.** Images were taken with an EOS 1000D Canon digital camera coupled to an Axiovert 40C inverted microscope (Carl Zeiss, Germany). Objective: 10x. Scale bar: 50 μm. Insets in each image are enlarged views of cells from each panel.

https://doi.org/10.1371/journal.pone.0187339.g001

## Results

### Gene and protein expression profiles of proteolytic markers during myogenesis

The transcriptional profile of several members of the three endogenous proteolytic systems was studied in gilthead sea bream myocytes at days 2, 4, 8 and 12 of culture. On day 2, activated mononucleated myoblasts cells are undergoing active proliferation to become myocytes (day 4). Then, cells subsequently differentiate and fuse to form small myotubes (day 8), and later on (day 12) some large myotubes can be observed (Fig 1).

Concerning the cathepsins gene expression, although the profile of the two *ctsd* paralogs throughout the culture was quite similar, *ctsda* showed increased mRNA expression at day 8 compared to the other days, while the changes observed in the expression of *ctsdb* were not significantly different (Fig 2A). *ctsb* and *ctsl* showed as well a similar profile to that of *ctsdb*, with *ctsl* being significantly down-regulated in the last stage of myocyte differentiation (Fig 2B). Regarding calpains gene expression, a significant decrease during myogenesis was observed for *capn1*, *capn3* and *capns1b*, while *capn2* and *capns1a* remained stable (Fig 2C and 2D). In the case of the UbP members, the gene expression data showed that while ubiquitin E3 ligases (*mafbx* and *murf*) significantly increased up to day 8 to decrease afterwards (Fig 2E), *ub* remained stable and the proteasome beta-type subunit *n3* was significantly decreased along with myogenesis (Fig 2F).

In contrast to the gene expression results, the immunoblotting data did not show any significant differences. In this sense, the protein levels of the immature form of CTSD remained very stable throughout myocyte differentiation, although both the mature and intermediate enzymes showed a tendency to increase at day 8 (Fig 3). MAFbx presented as well a peak on protein expression at day 8 of culture (Fig 4C), whereas both CTSL and CAPN1 were gradually increased reaching a maximum of expression at day 12 (Fig 4A and 4B).

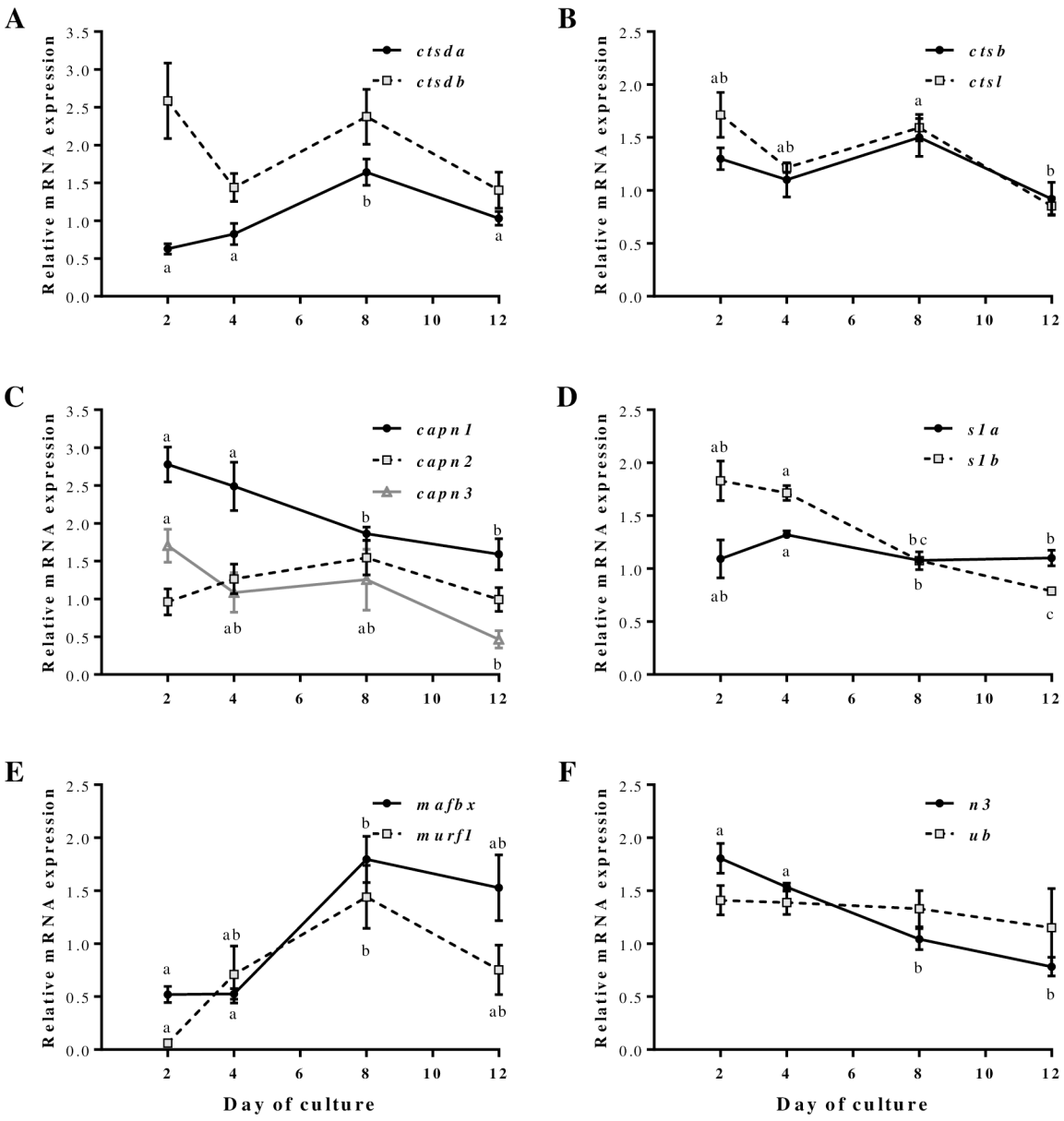


**Fig 2. Cathepsins, calpains and ubiquitin-proteasome members mRNA levels during *in vitro* myogenesis in gilthead sea bream.** Quantitative gene expression relative to the geometric mean of *β-actin*, *rps18* and *ef1α* of (**A**) *ctsda* and *ctsdb*, (**B**) *ctsb* and *ctsl*, (**C**) *capn1*, *capn2* and *capn3*, (**D**) *capns1a* (*s1a*) and *capns1b* (*s1b*), (**E**) *mafbx* and *murf1*, and (**F**) *n3* and *ub*. Results are shown as mean ± SEM (n = 4 independent cultures). Different letters indicate significant differences at p<0.05.

https://doi.org/10.1371/journal.pone.0187339.g002

## Correlation between gene and protein expression of proteolytic markers during myogenesis

Despite the absence of significant changes on protein expression during myogenesis, when the data were plotted against the corresponding gene expression levels, a significant positive correlation was found between *ctsda* and mature CTSD (Fig 5A), *ctsda* and intermediate

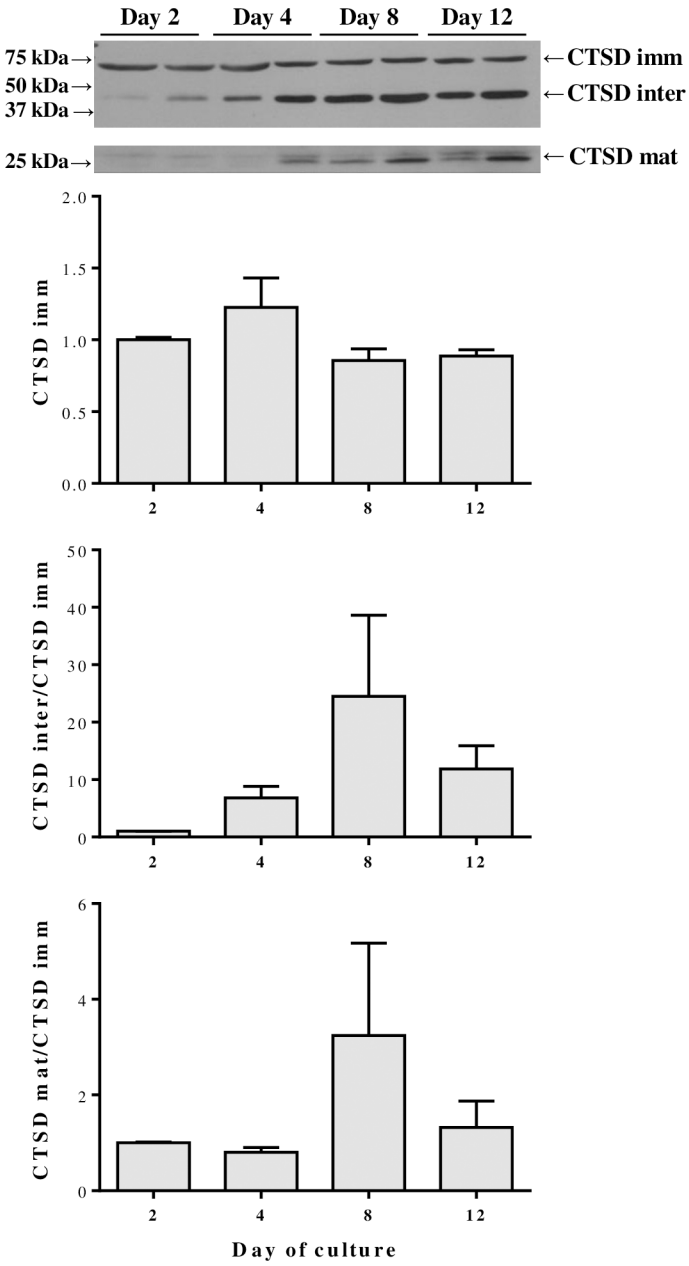


**Fig 3. Cathepsin D protein abundance in gilthead sea bream during *in vitro* myogenesis.** Representative Western blot showing the immature (top, CTSD imm), intermediate (middle, CTSD inter) and mature (bottom, CTSD mat) forms at days 2, 4, 8 and 12 of myocytes culture. The densitometric data for CTSD inter and CTSD mat was normalized to the corresponding CTSD imm band. Results are shown as mean ± SEM (n = 3 independent cultures). Note: Although all three bands are from the same Western blot, the mature form is shown separated because the image comes from a longer exposed film for better visualization.

https://doi.org/10.1371/journal.pone.0187339.g003

CTSD ($R^2$ = 0.4399, PC = 0.662, p = 0.019; S1A Fig) and *mafbx* with MAFbx (Fig 5C). On the other hand, a significant negative correlation was found between *ctsl* mRNA and CTSL protein levels (Fig 5B), whereas also a negative but non-statistically different correlation was found between *capn1* and CAPN1 ($R^2$ = 0.1116, ρ = -0.500, p = 0.098; S1B Fig).

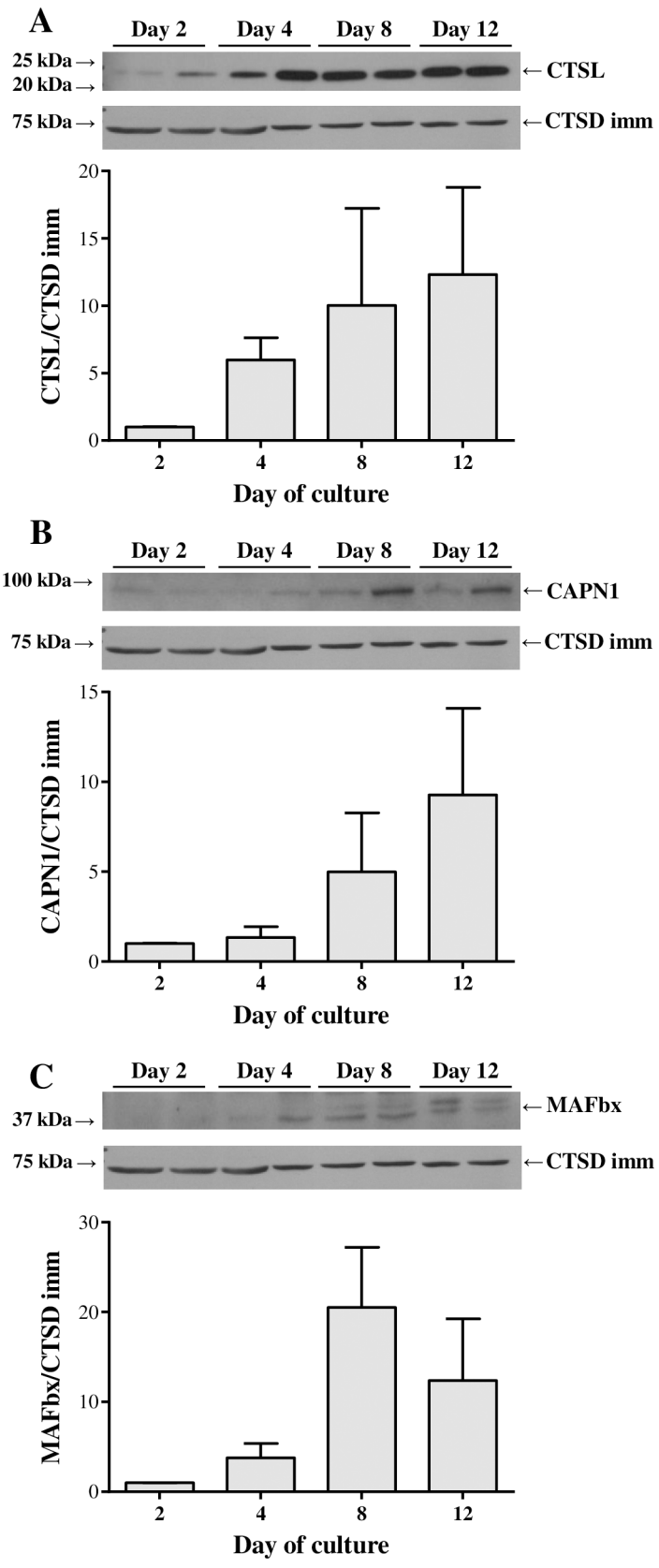
A
Day 2
Day 4
Day 8
Day 12
25 kDa →
20 kDa →
← CTSL
75 kDa →
← CTSD imm
CTSL/CTSD imm
20
15
10
5
0
2
4
8
12
Day of culture
B
Day 2
Day 4
Day 8
Day 12
100 kDa →
← CAPN1
75 kDa →
← CTSD imm
CAPN1/CTSD imm
15
10
5
0
2
4
8
12
Day of culture
C
Day 2
Day 4
Day 8
Day 12
37 kDa →
← MAFbx
75 kDa →
← CTSD imm
MAFbx/CTSD imm
30
20
10
0
2
4
8
12
Day of culture

**Fig 4. Cathepsin L, calpain 1 and MAFbx protein abundance in gilthead sea bream cultured myocytes.** Representative Western blots from (**A**) CTSL, (**B**) CAPN1 and (**C**) MAFbx at days 2, 4, 8 and 12 of myocytes culture development. The densitometric data was normalized relative to the corresponding cathepsin D immature form (CTSD imm). Results are shown as mean ± SEM (n = 3 independent cultures).

https://doi.org/10.1371/journal.pone.0187339.g004

## Proteolytic genes expression regulation by recovery or deficiency in selected amino acids

The expression of all cathepsin genes studied remained unchanged when the culture medium was supplemented with a cocktail to recover the AA levels at day 4 (Fig 6A). Similarly, differences were not observed for the calpains *capn1*, *capn2* and *capns1a* (Fig 6B). Contrarily, AA recovery caused a significant decrease on *capn3* and *capns1b* gene expression (Fig 6B) and the same effect was found for *mafbx* and *murf1* (Fig 6C). Nevertheless, this response to recovered AA was not general to all the UbP genes because *ub* was not affected and *n3* was significantly increased.

Next, the deficiency of leucine or lysine on the proteolytic gene markers expression through *in vitro* myocytes development was examined (Fig 7). Deficiency in leucine significantly decreased *ctsb* and *ctsl* gene expression in day 2 myocytes (Fig 7A). Otherwise, lysine deficiency did not provoke such an inhibitory effect and contrarily at day 8 *ctsb* expression resulted significantly increased. Furthermore, AA limitation provoked little effects in the gene expression of calpains and only *capn3* was significantly decreased at day 4 in lysine deficient medium (Fig 7B). Among the UbP genes, *mafbx* and *murf1* were the most affected (Fig 7C) with significant up-regulation at day 2 in response to both deficiencies, and at day 8 for *mafbx* when incubated in a medium deficient in leucine. Moreover, *ub* gene expression was not affected at any time upon any condition, while *n3* was significantly decreased after two days in both AA deficiencies.

# Discussion

The first objective of this study was to analyze the mRNA and protein levels of various cathepsins, calpains and UbP members throughout *in vitro* myogenesis in gilthead sea bream in order to unravel the phase in which those systems are required for the adequate progression of the process. Second, we assessed the involvement of AA regulating the gene expression of these three catabolic systems' members to define how crucial they are and to consider that for fish feeds formulation.

## Characterization of proteolytic markers gene and protein expression during gilthead sea bream myogenesis

The comparison of protein and gene expression of selected proteolytic members revealed the intricate control of these factors. In this sense, correlation analysis for cathepsin D and MAFbx confirmed a parallelism between gene and protein levels, while in the case of cathepsin L and calpain 1 the results indicated an opposite pattern. Such negative correlation could indicate a complex network of mRNA regulation at different levels: transcription, translation and degradation. The same opposite pattern for calpain 1 was observed during L8 rat myoblast fusion [36, 37] with increase of CAPN1 protein abundance in the maturation phase as in this study. Besides, it is interesting to note that even without showing significant differences, most of the molecules studied presented their highest protein levels at the end of myogenesis, suggesting an active role for these enzymes in muscle stabilization/consolidation. This means that these proteolytic molecules might be essential for the remodeling that occurs during muscle

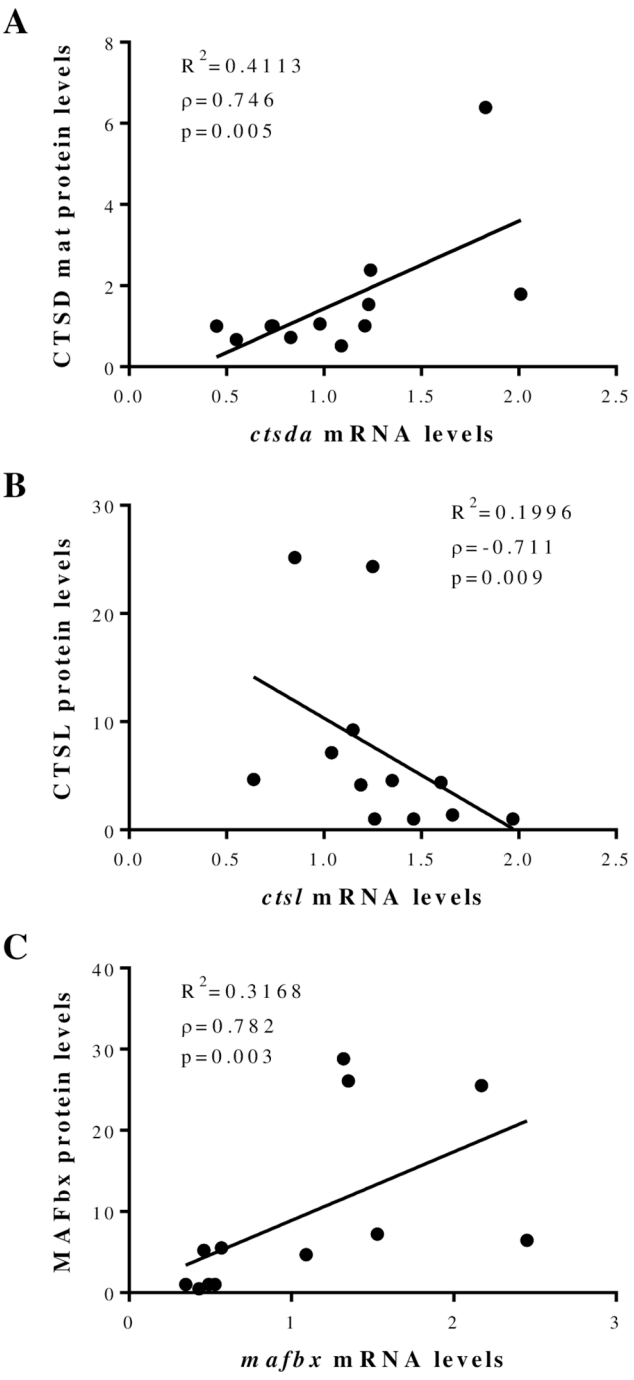


**Fig 5. Correlations between mRNA and protein levels of some cathepsins and ubiquitin-proteasome members in gilthead sea bream during *in vitro* myogenesis.** Significant correlations between mRNA relative expression and protein abundance in cultured myocytes between (**A**) cathepsin Da (*ctsda*) with cathepsin D mature form (CTSD mat), (**B**) cathepsin L (*ctsl*) with CTSL and (**C**) muscle atrophy F-box (*mafbx*) with MAFbx. Data are from n = 3 independent cultures. The $R^2$ of the linear regression, the Spearman's rank correlation coefficient (ρ) and the p-value are shown.

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formation (i.e. breaking of the cytoskeletal/plasma membranes linkages necessary to create points for myoblast fusion [13, 38]).

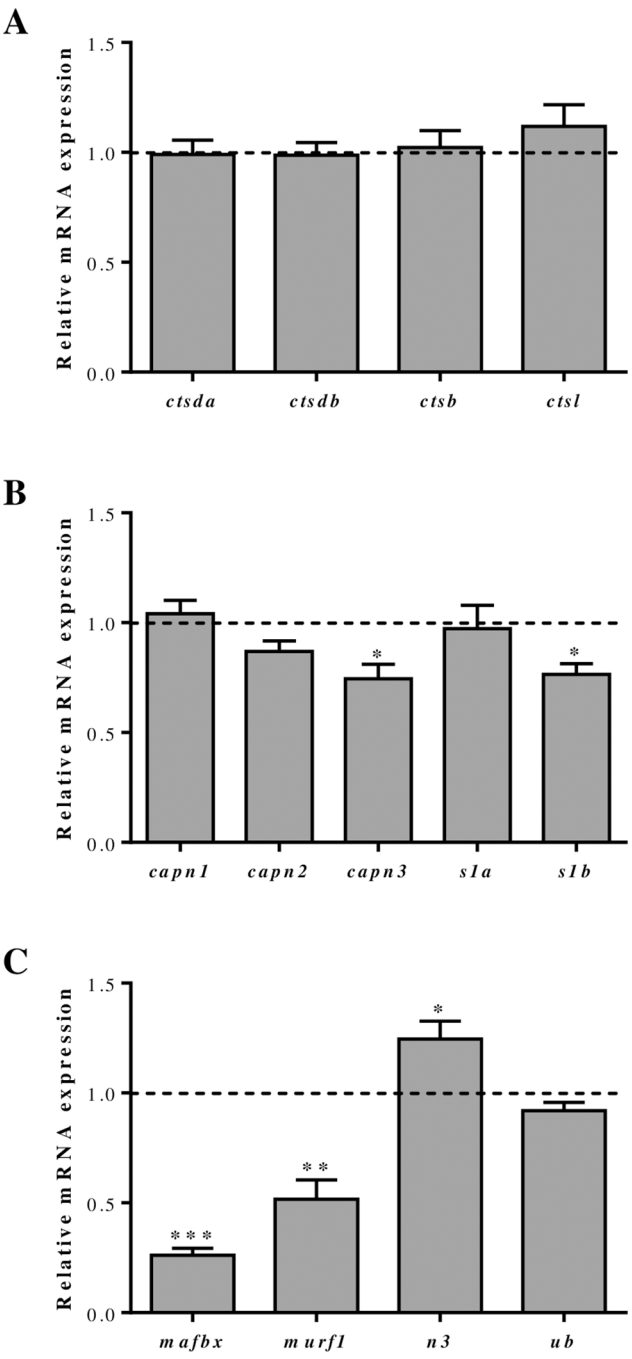


**Fig 6. Effects of amino acids (AA) recovery on proteolytic molecules gene expression in gilthead sea bream cultured myocytes.** Quantitative gene expression of (**A**) cathepsins (*ctsda*, *ctsdb*, *ctsb*, *ctsl*), (**B**) calpains (*capn1*, *capn2*, *capn3*, *capns1a* (*s1a*), *capns1b* (*s1b*)) and, (**C**) UbP members (*mafbx*, *murf1*, *n3*, *ub*) relative to the geometric mean of *ef1α* and *rps18* in day 4 cultured myocytes supplemented with a cocktail of AA for 6 h after a 12 h starvation period. Results are shown as fold change relative to the control condition (cells maintained without AA for the 18 h period including starvation and treatment), represented by the dotted line. Mean ± SEM (n = 4–7 independent cultures). Asterisks indicate significant differences compared to the control (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

https://doi.org/10.1371/journal.pone.0187339.g006

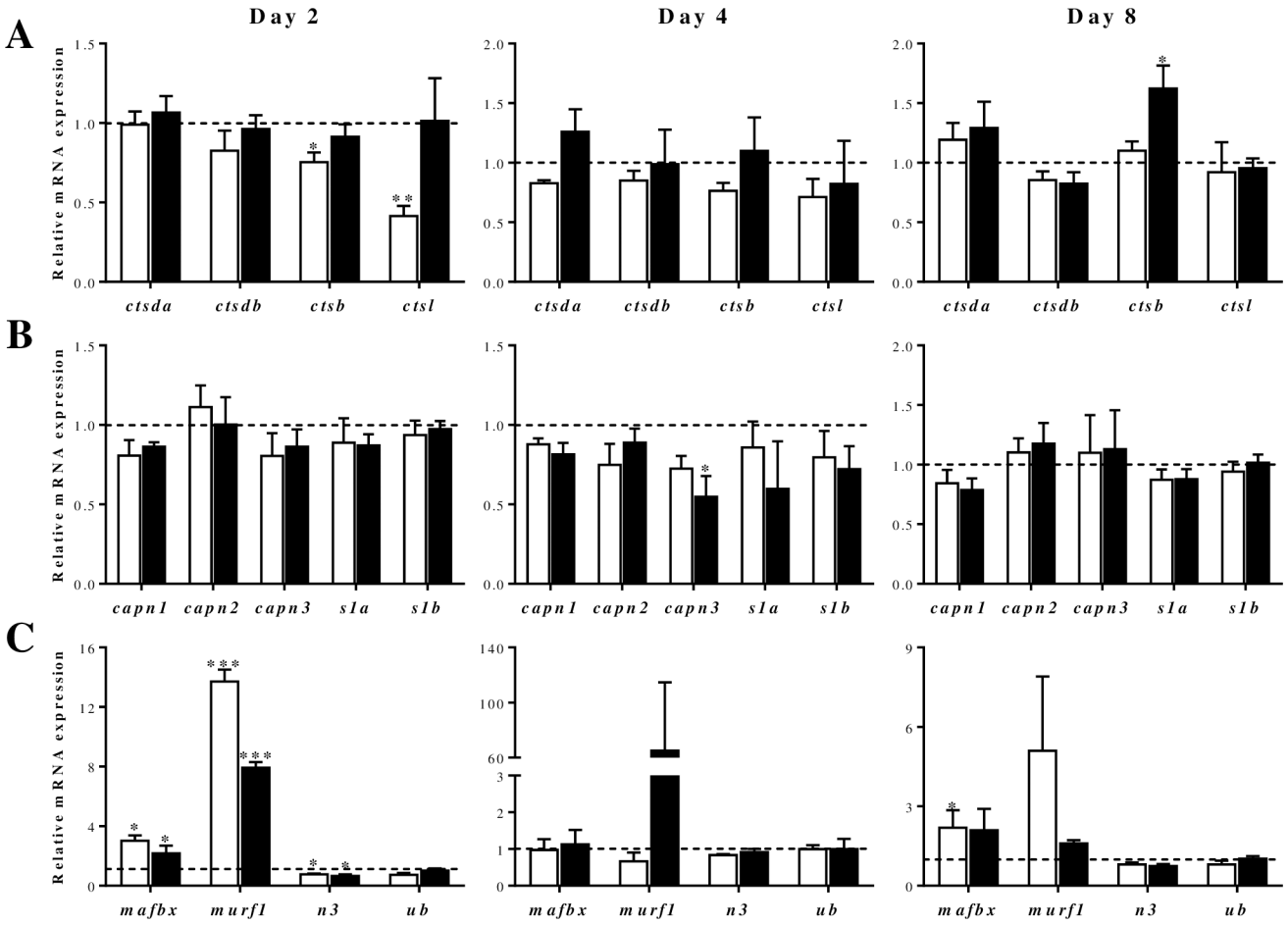


**Fig 7. Effects of leucine or lysine deficient media on proteolytic molecules gene expression in gilthead sea bream cultured myocytes.** Quantitative gene expression of (**A**) cathepsins (*ctsda*, *ctsdb*, *ctsb*, *ctsl*), (**B**) calpains (*capn1*, *capn2*, *capn3*, *capns1a* (*s1a*), *capns1b* (*s1b*)) and (**C**), UbP members (*mafbx*, *murf1*, *n3*, *ub*) relative to the geometric mean of *ef1α* and *rps18* in myocytes at days 2, 4 or 8 of culture after incubation from day 1 (for samples at days 2 and 4) or day 7 (for samples at day 8) with a growth medium deficient in leucine (open bars) or lysine (filled bars). Data are shown as fold change relative to control condition (growth medium without AA deficiencies), represented by the dotted line. Mean ± SEM (n = 3–4 independent cultures). Asterisks indicate significant differences compared to the control at each time (*: p<0.05; **: p<0.01; ***: p<0.001).

https://doi.org/10.1371/journal.pone.0187339.g007

Concerning gene expression, the data suggest that cathepsins would have greater importance during the early differentiation phase of myogenesis. In agreement with that, Colella et al. [37] found that *ctsb* gene expression decreased after fusion of myotubes in the L6 rat myogenic line, and Ebisui et al. [39] that the differentiation of C2C12 myoblasts involved up-regulation of lysosomal cathepsins. Contrarily, in chick myoblasts, *ctsb* showed the highest mRNA levels at the proliferative phase [40]. In fish, little information exists, but during salmon myocytes development, Bower and Johnston [41] described the increase of *ctsl1* expression with a peak at day 8, followed by a decrease at day 11 as in our study, and a new increase later at days 17 or 20, suggesting overall a relevant role for CTSL1 in differentiation and formation of myotubes.

Regarding calpains, in the present study expression of *capn1*, *capn3* and *capns1b* decreased progressively during myogenesis but *capn2* and *capns1a* remained stable. Similarly, Nakashima et al. [40] found in chick myoblasts a progressive decrease of *capn1* gene expression through *in vitro* development; whereas in rat muscle primary culture, Stockholm et al. [42]

found that *capn1* and *capn2* increased while *capn3* decreased, indicating that the various calpains can be regulated in an opposite way as it occurs in our model. Moreover, Van Ba and Inho [43] also demonstrated that CAPN1 is involved in proliferation and survival during myogenesis in bovine muscle cells. Overall these data indicate that although differences exist among the different vertebrate groups, the main trend is to observe higher gene expression of calpains at the early myogenic stages.

The E3 ubiquitin ligases, MAFbx and MuRF1, are important members of the UbP system. There is evidence that MuRF1 is necessary for the initiation and stabilization of myogenesis [44], being its actions located mainly in the cytoplasm of muscle cells, where it recognizes myofibrillar proteins, such as myosin heavy chain (MHC), and targets them for breakdown [10, 45]. In gilthead sea bream, *mhc* gene expression increased up to day 9 in cultured myocytes and became stable afterwards [32], which is parallel to *murf1* expression and supports also in this species the functional relationship of these molecules. On the other hand, MAFbx is essential for myogenic stem cell function in adult skeletal muscle, as it identifies and targets for ubiquitination several transcription factors with key roles in the control of skeletal muscle development (i.e. myogenic differentiation 1 (MyoD1) or myogenin) [45–47]. García de la serrana et al. [32] found for *myod2* expression in gilthead sea bream myocytes a profile opposite to *mafbx*. This opposed relationship can be explained by the fact that at the start of development the stem cells have to determine their fate and so *myod* levels have to be high, while contrarily, when muscle cells become differentiated, MyoD is no longer needed, and its expression decreases probably due to the up-regulation of *mafbx*. Overall, the profiles of both E3 ligases are similar, which agrees with the findings of Spencer et al. [44] and Perera et al. [48] in mouse skeletal muscle and C2C12 cells, respectively demonstrating that *murf1* is required for myoblast differentiation and myotube fusion, pointing out very well the conserved role of this UbP molecule as well in muscle development.

Concerning the other members of the UbP system, it is well accepted that *n3* is a good marker of proliferation [49, 50], which is in agreement with it showing the same pattern of gene expression as that reported for the proliferation marker *pcna* in gilthead sea bream [29, 32]. Finally, Nakashima et al. [40] observed a significant reduction on *ub* gene expression during chicken myoblast differentiation, although we found it unaltered in gilthead sea bream myocytes. In support of this absence of changes in gene expression, Kimura and Tanaka [51] suggested that ubiquitin plays multiple roles controlled by complex regulatory mechanisms to actually maintain its levels stable.

In summary, as far as we know, the present study shows for the first time in cultured fish myocytes the expression of several proteolytic members that seems to be in agreement with a more relevant role of calpains during the proliferative phase of myogenesis and of cathepsins and the UbP system in muscle cells differentiation. This in concordance with the more anabolic aspect of calpains in comparison to cathepsins and the UbP system, since they do not degrade proteins up to small peptides or AA, but only disassemble the sarcomeric structure of the muscle [10, 13, 15, 44]. Moreover, the expression of *ctsb*, *ctsl*, *ctsdb*, *mafbx* and *n3* was reported greater in the muscle of fingerlings than in juvenile or adult gilthead sea bream, pointing out a major role for these two endogenous systems (cathepsins and UbP) when the myogenic process is more active [22].

## Regulatory effects of recovery or deficiency of selected AA in proteolytic markers gene expression in gilthead sea bream myocytes

Previous studies have demonstrated in gilthead sea bream that almost all cathepsins and UbP system-related genes are up- and down-regulated during fasting and refeeding, respectively

[22], and similar results were observed, although to a lesser extent, with regards to calpains [16]. Besides, it has been shown that forced swimming provokes in gilthead sea bream up-regulation of cathepsins and UbP members [38], supporting that muscle remodeling is taking place under both catabolic and anabolic conditions. In this sense, we have found now in myocytes of the same species that specifically the AA seem to have an important role controlling proteolytic systems, although mostly the expression of UbP members.

In agreement with that, Cleveland and Weber [4] found that *ctsd* and *ctsl* expression was not affected by a leucine treatment in rainbow trout myocytes; while contrarily, lysine supplementation had an inhibitory effect on ALS activity in C2C12 myotubes [52]. With regards to calpains, response to AA in this study was observed only for *capn3* and *capns1b*, the same genes modified in response to fasting and refeeding in the same species [16]. In the case of halibut (*Hippoglossus hippoglossus*) and channel catfish (*Ictalurus punctatus*), skeletal muscle *capn3* mRNA was at its lowest level during fasting, and highest in refeeding [53, 54], providing overall these data an evidence for species-specific differences concerning the activity of this gene. Notwithstanding, considering that calpain 3 is a muscle specific regulator of other calpains' expression and activity, as well as its levels have been correlated with bovine and ovine muscle tenderness [55, 56], these variable responses in fish deserve to be further investigated.

Moving to the expression of UbP genes affected by AA, it is interesting to emphasize that in our study, AA levels recovery decreased the expression of *mafbx* and *murf1* but increased *n3* whereas contrarily, leucine and lysine deficiencies stimulated, mainly at day 2, *mafbx* and *murf1* expression while inhibiting *n3*. These results suggest that both MAFbx and MuRF1 could be increasing the amount of proteins sent to the proteasome when AA are lacking; however, the opposite response of *n3* and the stable *ub* expression might be indicating that the flux of ubiquitinated proteins through the proteasome is constrained (or slowed down). Then, these proteins would be probably degraded by autophagy, as it has been observed in mammals, demonstrating that there is an important cross-talk regulation within the proteolytic systems [56]. This hypothesis makes even more sense in fish, in which in contrast to mammals, the ALS is responsible for around two to three times more protein degradation than the UbP system [8].

Furthermore in *in vitro* models, AA limitation increases proteolysis in an UbP-dependent manner in C2C12 myotubes, although an increase in AA or leucine alone down-regulates protein degradation and the expression of components of the UbP pathway [57]. However, also in C2C12 cells, the expression of *murf1* was not affected after incubation with lysine [52]. Similarly, in rainbow trout myocytes, leucine supplementation did not affect *murf1* while serum deprivation increased the expression of the ubiquitin ligases *mafbx*, *fbx25* and *murf1* [4]. In the case of salmon muscle cells, an starving of AA caused down-regulation of *mafbx* [31], whereas in a previous study analyzing two different splice variants in the same cell model, it was demonstrated that serum and AA starvation resulted in a 6-fold increase in the expression of *mafbx-α*. This isoform expression declined subsequently in response to an AA treatment [58], but *mafbx-β* appeared to be less sensitive to AA since its expression remained similar to the control, and only was altered when insulin or insulin-like growth factors (IGFs) were present in the culture media. Probably, this differential response between isoforms is due to their specific roles during salmon *in vitro* myogenesis, where *mafbx-α* gene expression is highest in differentiated myotubes (similarly to our data), and *mafbx-β* mRNA is more abundant at myoblast stage [58]. Moreover, serum depletion and specifically AA withdrawal in rainbow trout myocytes induced the expression of the autophagy-proteasome genes (*lc3b*, *gabarapl1*, and *atg4b*) [7, 30], suggesting an important role for the AA released by muscle mobilization during fasting, to regulate proteolytic genes.

In this sense, considering our experimental model, Vélez et al. [28] after AA recovery found increases on proliferation, differentiation and protein synthesis markers such as *pcna*, *myogenin*, *tor*, *4ebp1* and *70s6k*, while the expression of *foxo*, a factor involved in the activation of the proteolytic pathway, remained unaffected. After 2 days of leucine limitation, Azizi et al. [29] found that the expression of the AA deficiency indicator *chop* was increased, whereas *4ebp1* and *foxo* diminished. Furthermore, after 8 days of lysine deficiency, an increased expression of other two AA-limitation markers (i.e. *atf4* and *as*) was observed; and also, a decrease in important proteogenic/anabolic pathways' molecules including members of the IGF system (i.e. *pcna*, *igf-1*, *igf-2*, *igf-1rb*, *akt*, *erk* and *70s6k*) [29]. These data confirm an overall negative effect of the reduced AA levels, especially lysine, on protein turnover and thus, muscle growth in gilthead sea bream, which is supported by our results.

The present study provides new information about the potential role of key members of the endogenous proteolytic systems (cathepsins, calpains and UbP) in gilthead sea bream cultured muscle cells. We can suggest that there is a functional distribution between the different proteolytic system molecules throughout the *in vitro* development of muscle cells at least until the phases of myocyte differentiation and small myotube formation (day 8). Besides, it is interesting to note the up-regulation of *mafbx* and *murf1* in response to AA deficiencies and their down-regulation with AA recovery and the reverse response of *n3*, pointing out to an efficient and complementary role of these UbP system members to AA supply.

In summary, the research on the function of proteolytic systems in fish offers interesting information on the evolution of myogenesis regulation and the effects of AA on such process that can have valuable application in aquaculture in order to optimize diet composition for this species.

## Supporting information

**S1 File. Raw data.**
(XLSX)

**S1 Fig. Correlations between mRNA and protein levels of some cathepsins and calpains in gilthead sea bream during *in vitro* myogenesis.** (A) cathepsin Da (*ctsda*) with cathepsin D intermediate form (CTSD inter), and (B) calpain 1 (*capn1*) with CAPN1. Data are from n = 3 independent cultures. The $R^2$ of the linear regression, the Spearman's rank correlation coefficient (ρ) and the p-value are shown.
(TIF)

## Acknowledgments

The authors would like to thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the Faculty of Biology for the supply and maintenance of the fish, as well as José Rives for his help with the cell cultures and laboratory analyses.

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*Am J Physiol Regul Integr Comp Physiol* 310: R313–R322, 2016.
First published December 9, 2015; doi:10.1152/ajpregu.00230.2015.

# Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*)

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Submitted 26 May 2015; accepted in final form 8 December 2015

**Vélez EJ, Azizi Sh, Millán-Cubillo A, Fernández-Borràs J, Blasco J, Chan SJ, Calduch-Giner JA, Pérez-Sánchez J, Navarro I, Capilla E, Gutiérrez J.** Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*). *Am J Physiol Regul Integr Comp Physiol* 310: R313–R322, 2016. First published December 9, 2015; doi:10.1152/ajpregu.00230.2015.—The endocrine system regulates growth mainly through the growth hormone (GH)/insulin-like growth factors (IGFs) axis and, although exercise promotes growth, little is known about its modulation of these factors. The aim of this work was to characterize the effects of 5 wk of moderate sustained swimming on the GH-IGFs axis in gilthead sea bream fingerlings. Plasma IGF-I/GH ratio and tissue gene expression of total IGF-I and three splice variants, IGF-II, three IGF binding proteins, two GH receptors, two IGF-I receptors, and the downstream molecules were analyzed. Fish under exercise (EX) grew more than control fish (CT), had a higher plasma IGF-I/GH ratio, and showed increased hepatic IGF-I expression (mainly IGF-Ia). Total IGF-I expression levels were similar in the anterior and caudal muscles; however, IGF-Ic expression increased with exercise, suggesting that this splice variant may be the most sensitive to mechanical action. Moreover, IGFBP-5b and IGF-II increased in the anterior and caudal muscles, respectively, supporting enhanced muscle growth. Furthermore, in EX fish, hepatic IGF-IRb was reduced together with both GHRs; GHR-II was also reduced in anterior muscle, while GHR-I showed higher expression in the two muscle regions, indicating tissue-dependent differences and responses to exercise. Exercise also increased gene and protein expression of target of rapamycin (TOR), suggesting enhanced muscle protein synthesis. Altogether, these data demonstrate that moderate sustained activity may be used to increase the plasma IGF-I/GH ratio and to potentiate growth in farmed gilthead sea bream, modulating the gene expression of different members of the GH-IGFs axis (i.e., IGF-Ic, IGF-II, IGFBP-5b, GHR-I, and TOR).

growth hormone; GH receptors; IGFs; IGF-I receptors; IGF binding proteins; TOR; swimming

EXERCISE IN TELEOST FISH HAS long been recognized to stimulate growth (63). The effects of sustained exercise in fish depend on the typical behavior of each species, being more important in pelagic fishes (20, 43). In salmonids and other nonsalmonid species, such as Carangidae or Gadidae, exercise improves the feed conversion efficiency and thus, the specific growth rate of exercised fish compared with rested fish (20). Recently, the same effect was reported in juveniles of gilthead sea bream (28, 44, 54, 82), supporting the use of this model as a potential strategy to improve fish growth for aquaculture production.

Growth is mainly regulated by the growth hormone (GH)-insulin-like growth factors (IGFs) axis (75). Pérez-Sánchez and Le Bail (67) reported that IGF-I plasma levels increase during rapid fish growth, whereas circulating GH decreases. GH exerts its effects through activation of membrane receptors (GHR-I and GHR-II) and is mainly responsible for hepatic IGF-I secretion (50, 96). In gilthead sea bream, Saera-Vila et al. (79) suggested that GHR-I plays a key role in the tissue-specific regulation of IGFs synthesis and secretion and later showed a transcriptional correlation between GHR-II and IGFs (IGF-I and IGF-II) in response to confinement stress (80), suggesting that GHR-II rather than GHR-I is a stress-sensitive gene in gilthead sea bream (17).

It is well known that IGF-I is synthesized as a pre-pro-IGF-I composed of a signal peptide, an E-peptide, and the mature IGF-I (14). Once the signal peptide is removed to give rise to pro-IGF-I, subsequent alternative splicing in the region coding for the E-peptide leads to different IGF-I splice variants, reported to have differential physiological functions (41, 71, 86). Three splice variants named Ea, Eb, and Ec have been identified, with Ea being common to all vertebrates, whereas Eb and Ec are found exclusively in mammals (88). Ea is mostly expressed in the liver and is considered responsible for the systemic role of IGF-I (36). In contrast, Ec is mostly expressed in muscle and is upregulated by damage or exercise in response to the mechanical action of contraction, and for this reason, it is known as the mechano growth factor (MGF) (39, 41, 71). In the case of teleost fish, up to four IGF-I splice variants of the form named Ea in mammals, which show differential tissue-specific expression and function, including cell cycle regulation, have been described (19, 87, 95). Tiago et al. (88) reported that gilthead sea bream has three variants identified as IGF-Ia, IGF-Ib, and IGF-Ic. They showed that IGF-Ic is the most highly expressed form in the liver and suggested that it could play a systemic role like that of Ea in mammals. In the case of skeletal muscle, a previous in vitro study in gilthead sea bream showed that the expression of the three splice variants differed during myocyte culture progression and were differentially regulated, IGF-Ic being the most abundant throughout development and IGF-Ib being the most sensitive to hormone treatments (46). Nevertheless, the existence of a MGF in fish

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has not yet been reported, nor have the effects of exercise on these IGF-I splice variants been studied.

In addition to the peptides IGF-I and IGF-II, the IGFs system is composed of six IGF binding proteins (IGFBPs) and the receptors (IGF-IRs and IGF-IIR) (30, 73). In mammals, almost 75% of circulating IGF-I is bound to IGFBPs (47), thus modulating the bioavailability of the peptides (23–25). IGFBPs are produced in different cell types in a tissue-specific manner as a means of tissue-specific action (26), and their production and secretion are regulated by the same hormones and growth factors that regulate GHR expression (75). Thus, the different IGFBPs can inhibit or potentiate IGF-I actions depending on the experimental conditions or cellular context (23, 26, 99). The anabolic effects of IGFs, such as protein synthesis and cell proliferation, are mediated through IGF-IRs. Regarding IGF-IRs, while in some fish species, only one isoform has been identified, in many other species, two isoforms (IGF-IR1/a and IGF-IR2/b) have been reported, with differential tissue distribution, and possibly, function (27, 40, 60). In the case of gilthead sea bream, although the existence of two isoforms was suggested more than a decade ago (68, 69), their sequences have only recently been described (55, 93). Furthermore, it is known that IGF-IRs expression may be modulated by hormone levels, nutritional status, and environmental factors (75). In rainbow trout, for example, both receptors are differentially regulated by the nutritional status in muscle (18, 57), and in zebrafish, IGF-IRa (but not IGF-IRb) is suppressed in muscle in response to exercise (65).

When the IGFs bind the IGF-IRs, it is mainly the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway that is activated (56, 58). Downstream of Akt, the target of rapamycin (TOR) has been reported to be key in promoting protein synthesis and muscle hypertrophy (35). TOR has been described as a nutrient sensor that integrates endocrine, as well as nutritional, signals (38, 89, 97). The importance of TOR activation by amino acids and/or IGFs in relation to muscle growth has been explored both in vivo and in vitro in several fish species, including gilthead sea bream cultured myocytes (10, 83, 92). However, the effects of exercise on the gene and protein expression of these signaling molecules in fish have not been elucidated.

Recently, we reported improved growth after 5 wk of sustained and moderate exercise [five body lengths (BL)/s] in gilthead sea bream fingerlings (9). The critical swimming speed (Ucrit) diminishes as body size increases. As the Ucrit for gilthead sea bream measuring 20 cm is around 4.5 BL/s (6), we can assume that a speed of 5 BL/s for individuals of the same species 7 cm long would be below their Ucrit. The increase in growth without affecting feed intake demonstrates an improvement in metabolic efficiency in this condition (9).

The aim of the present work was to analyze the role of the GH-IGF axis molecules that mediate the positive exercise-related effects on growth in gilthead sea bream.

## MATERIALS AND METHODS

### *Animals and Experimental Trial*

Five hundred and forty gilthead sea bream (*Sparus aurata* L.) fingerlings [~5 g body wt and 7 cm body length (BL)] were obtained from a hatchery located in the north of Spain and randomly distributed into eight 0.2 $m^3$ tanks. These were maintained within a temperature-controlled system (23 ± 1°C) and 15 h light/9 h dark photoperiod at the facilities of the School of Biology at the University of Barcelona (Spain). For the experimental trial, as described previously (9), four tanks were kept in standard rearing conditions, with a water flow of 350 l/h and vertical water inflow. In these conditions, the fish presented only spontaneous and voluntary movements (control group, CT). The other four tanks were kept in a circular and uniformly distributed flow of 700 l/h by a vertical tube with lateral holes to maintain the flux in the water column, where the fish were forced to undertake moderate and sustained swimming (5 BL/s) (exercise group, EX). The water flow was adjusted as the fish grew to maintain the speed during the whole experiment. Fish were fed with a commercial diet (Gemma Diamond, Skretting, Burgos, Spain) three times a day, representing a 5% ration (until apparent satiety). Biometric parameters (weight and length) were determined at the beginning of the experiment, and then at *week 5* to monitor fish growth. In the final sampling at *week 5*, and after an overnight fast, 12 fish from each group (three fish per tank) were killed, and samples of plasma, liver, and anterior and caudal epaxial white muscle regions were collected, frozen in liquid nitrogen, and stored at −80°C until further analysis. Despite the fact that only a few fish per tank were sampled, a large number of fish was used to maintain the appropriate population in terms of fish density (kg biomass/$m^3$) similar to that used in regular fish culture conditions to avoid conflicts due to behavior (e.g., differential feed intake of individual fish, aggressive behavior among individuals).

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

### *GH and IGF-I Plasma Levels*

Plasma GH levels were determined using a homologous gilthead sea bream radioimmunoassay (RIA), as described previously (53). Plasma IGF-I was measured using an IGF-I RIA validated for Mediterranean perciform fish, as previously described (91).

### *Gene Expression Analyses*

*RNA extraction and cDNA synthesis.* Total RNA was extracted from 40 to 500 mg of tissue (depending on tissue yield) with 1 ml of TRI reagent solution (Applied Biosystems, Alcobendas, Spain). Total RNA concentration and purity were determined using a NanoDrop2000 (Thermo Scientific, Alcobendas, Spain). The integrity of the different samples was confirmed in a 1% agarose gel (wt/vol) stained with SYBR-Safe DNA gel stain (Life Technologies, Alcobendas, Spain). After that, 1 μg of total RNA was treated with DNase I (Life Technologies) to remove all genomic DNA following the manufacturer's recommendations, and then the RNA was reverse transcribed with the Affinity Script QPCR cDNA synthesis kit (Agilent Technologies, Las Rozas, Spain).

*Quantitative real-time PCR.* According to the requirements of the MIQE guidelines (15), the mRNA transcript levels of total IGF-I, the three splice variants (IGF-Ia, IGF-Ib, and IGF-Ic), IGF-II, IGFBP-1a, IGFBP-2b, IGFBP-4, IGFBP-5b, IGF-IRa, IGF-IRb, GHR-I, GHR-II, Akt, TOR, plus three reference genes [ribosomal protein S18 (RPS18), ribosomal protein L27a (RPL27a), and β-actin] were examined in a CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain). The analyses were performed in triplicate in a final volume of 5 μl, including 2.5 μl of iTaq Universal SYBR Green Supermix (Bio-Rad), 250 nM of forward and reverse primers (Table 1), and 1 μl of cDNA for each sample. Following the conditions previously described by Salmerón et al. (81), the reactions consisted of an initial activation step of 3 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 55–68°C (primer dependent, see Table 1) followed by an amplicon dissociation analysis from 55 to 95°C with a 0.5°C every 30 s. Before

Table 1. *Primers used for qPCR: Sequences, melting temperatures, and GenBank accession numbers*

| Gene | Primer Sequences (5′–3′) | Tm, °C | Accession Number | References |
|---|---|---|---|---|
| *RPS18* | F: GGGTGTTGGCAGACGTTAC | 60 | AM490061.1 | (94) |
| | R: CTTCTGCCTGTTGAGGAACCA | | | |
| *RPL27a* | F: AAGAGGAACACAACTCACTGCCCCAC | 68 | AY188520 | (49) |
| | R: GCTTGCCTTTGCCCAGAACTTTGTAG | | | |
| *β-Actin* | F: TCCTGCGGAATCCATGAGA | 60 | X89920 | (81) |
| | R: GACGTCGCACTTCATGATGCT | | | |
| *IGF-Ia* | F: AGGACAGCACAGCAGCCAGACAAGAC | 60 | AY996779 | (88) |
| | R: TTCGGACCATTGTTAGCCTCCTCTCTG | | | |
| *IGF-Iab* | F: AGTCATTCATCCTTCAAGGAAGTGCATCC | 60 | EF688015 | (88) |
| | R: TTCGGACCATTGTTAGCCTCCTCTCTG | | | |
| *IGF-Iabc* | F: ACAGAATGTAGGGACGGAGCGAATGGAC | 60 | EF688016 | (88) |
| | R: TTCGGACCATTGTTAGCCTCCTCTCTG | | | |
| *IGF-II* | F: TGGGATCGTAGAGGAGTGTTGT | 60 | AY996778 | (8) |
| | R: CTGTAGAGAGGTGGCCGACA | | | |
| *IGF-IRa* | F: AGCATCAAAGACGAACTGG | 55 | KT156846 | (2) |
| | R: CTCCTCGCTGTAGAAGAAGC | | | |
| *IGF-IRb* | F: GCTAATGCGAATGTGTTGG | 55 | KT156847 | (2) |
| | R: CGTCCTTTATGCTGCTGATG | | | |
| *GHR-I* | F: ACCTGTCAGCCACCACATGA | 60 | AF438176 | (16) |
| | R: TCGTGCAGATCTGGGTCGTA | | | |
| *GHR-II* | F: GAGTGAACCCGGCCTGACAG | 60 | AY573601 | (78) |
| | R: GCGGTGGTATCTGATTCATGGT | | | |
| *IGFBP-1a* | F: AGTGCGAGTCCTCTCTGGAT | 60 | KM522771 | Present work |
| | R: TCTCTTTAAGGGCACTCGGC | | | |
| *IGFBP-2b* | F: CGGGCTGCTGCTGACATACG | 60 | AF377998 | (31) |
| | R: GTCCCGTCGCACCTCATTTG | | | |
| *IGFBP-4* | F: TCCACAAACCAGAGAAGCAA | 60 | F5T95CD02JMZ9K | (34) |
| | R: GGGTATGGGGATTGTGAAGA | | | |
| *IGFBP-5b* | F: TTTCTCTCTCGGTGTGC | 60 | AM963285 | (2) |
| | R: TCAAGTATCGGCTCCAG | | | |
| *TOR* | F: CAGACTGACGAGGATGCTGA | 60 | — | García de la serrana, unpublished |
| | R: AGTTGAGCAGCGGGTCATAG | | | |
| *AKT2* | F: GCTCACCCCACTCTTCAGAC | 60 | ERA047531 | (34) |
| | R: AAATTGGGAAATGTGCTTGC | | | |

Tm, melting temperatures; F, forward; R, reverse.

the analyses, the appropriate cDNA dilution for each assay was determined, as well as the specificity of the reaction, and the absence of primer-dimers was confirmed by running a dilution curve with a pool of samples. Using the Pfaffl method (70), we calculated the expression level of each gene analyzed relative to the geometric mean of the reference genes RPS18 and RPL27a, the two genes that were most stable of those analyzed using the CFX Manager Software (Bio-Rad). Calculation of the expression of the different IGF-I splice variants was performed as described by Tiago et al. (88). The relative expression of genes in the EX fish was represented as the fold change over the CT fish.

### *Western Blot Analyses*

Protein homogenates from muscle tissue were obtained, as described by García de la serrana et al. (33). The amount of protein from each sample was measured (12), and then 10–20 μg of protein were separated by electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, as previously described (92). Then, samples were transferred to a PVDF membrane, which was blocked in 5% nonfat milk buffer and incubated with the respective primary antibodies. The dilution was 1:200 for the phosphorylated forms and 1:500 for the total forms. The rabbit polyclonal primary antibodies used were anti-phospho Akt (cat. no. 9271), anti-total Akt (cat. no. 9272), and anti-phospho TOR (cat. no. 2971), all from Cell Signaling Technology (Beverly, MA) and, anti-total TOR (cat. no. T2949) from Sigma-Aldrich (Spain). After washing, the membranes were incubated with the peroxidase-conjugated secondary antibody (cat. no. 31460; Thermo Scientific, Alcobendas, Spain) in 5% nonfat milk buffer. Immunoreactive bands were developed using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific). Once the phosphorylated forms were developed, primary and secondary antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific), and then, the membranes were blotted again following the same procedure with the corresponding total forms. Finally, the bands were quantified by densitometry using ImageJ software v1.47 (National Institutes of Health, Bethesda, MD).

### *Statistical Analyses*

Data were analyzed using IBM SPSS Statistics v.21 and were presented as means ± SE. The presence of a normal distribution was first analyzed using the Shapiro-Wilk test followed by Levene's test to test the homogeneity of variances. Statistical differences were analyzed using Student's *t*-test and were considered significant when $P < 0.05$ (*) or $P < 0.001$ (**).

## RESULTS

### *Biometric and Plasma Parameters*

After the 5 wk of the experiment, both groups of gilthead sea bream fingerlings showed increased growth, although the fish that were subject to moderate and sustained exercise evidenced a significantly higher body weight compared with the CT fish (Table 2). Moreover, sustained swimming in the same EX fish also caused an increase in the ratio of IGF-I/GH plasma levels (Table 2).

Table 2. *Biometric and plasma parameters. Initial and final body weights and plasma IGF-I/GH ratio in gilthead sea bream control or after 5 wk of sustained and moderate exercise*

| | Initial Weight, g | Final Weight, g | IGF-I/GH ratio |
|---|---|---|---|
| CT | 4.97 ± 0.04 | 17.54 ± 0.46 | 10.60 ± 2.74 |
| EX | 5.14 ± 0.20 | 20.28 ± 0.38** | 26.21 ± 4.28* |

Data are shown as mean ± SE (CT, $n = 21$; EX, $n = 15$). CT, control; EX, exercise; GH, growth hormone. Significant differences are indicated by *($P < 0.05$) or **($P < 0.001$).

### *GH-IGFs Axis Gene Expression in Liver and Muscle*

The expression of total IGF-I and the splice variant IGF-Ia in the liver was significantly increased in EX fish, while IGF-Ib and IGF-Ic were significantly decreased with respect to CT fish. On the other hand, IGF-II expression remained unchanged (Fig. 1*A*). In the case of binding proteins, IGFBP-1a and IGFBP-2b were present in the liver, although no differences were detected between groups (Fig. 1*B*). Regarding the IGF-IRs, only IGF-IRb was detected in the liver and showed significantly lower expression in EX than in CT gilthead sea bream. However, both GHRs (GHR-I and GHR-II) were expressed in this tissue, although there was significant downregulation in EX fish compared with CT fish (Fig. 1*C*).

With regard to white skeletal muscle, the expression of total IGF-I, IGF-Ia, IGF-Ib, and IGF-II in the anterior muscle was unaffected by exercise; however, IGF-Ic was significantly increased in EX fish compared with CT fish (Fig. 2*A*). Moreover, in contrast with the liver, IGFBP-2b was not detected in muscle, whereas IGFBP-1a, IGFBP-4, and IGFBP-5b were present, with IGFBP-5b being significantly upregulated in the anterior muscle of EX fish (Fig. 2*B*). Also unlike in the liver, both IGF-IRs (IGF-IRa and IGF-IRb) were expressed in the anterior muscle, although no effects were observed in response to exercise (Fig. 2*C*). On the other hand, both GHRs were expressed in the anterior muscle but showed different responses to exercise, with GHR-I being significantly increased and GHR-II significantly decreased in EX fish compared with the CT group (Fig. 2*C*).

The expression of the different GH-IGFs axis members studied showed similar results in the caudal muscle to those in the anterior muscle following sustained swimming, with significant increases in IGF-Ic and GHR-I expression in EX fish compared with CT fish (Fig. 2, *D* and *F*, respectively). IGF-II expression was also significantly upregulated in the caudal muscle of EX fish (Fig. 2*D*). However, unlike in the anterior muscle, the mRNA levels of IGFBP-5b and GHR-II remained unchanged in the caudal region in response to exercise (Fig. 2, *E* and *F*).

### *Akt and TOR Activation*

Exercise had no effect on Akt mRNA levels either in the anterior or caudal muscles; however, TOR gene expression was significantly increased in EX fish compared with CT fish in both muscle regions (Fig. 3, *A* and *B*). With regard to protein levels, the phosphorylation of TOR was significantly increased (3.3-fold) in the anterior muscle of EX fish compared with the CT group, whereas no differences were found in Akt (Fig. 3*C*). In the case of the caudal muscle region, phosphorylation of both Akt and TOR was slightly increased in EX fish with respect to CT fish, but the differences were not significant (Fig. 3*D*).

## DISCUSSION

In agreement with our previous experiments with juvenile gilthead sea bream, which demonstrated improved growth in response to exercise (28, 44, 54, 82), we recently demonstrated that 5 wk of moderate sustained swimming also improves

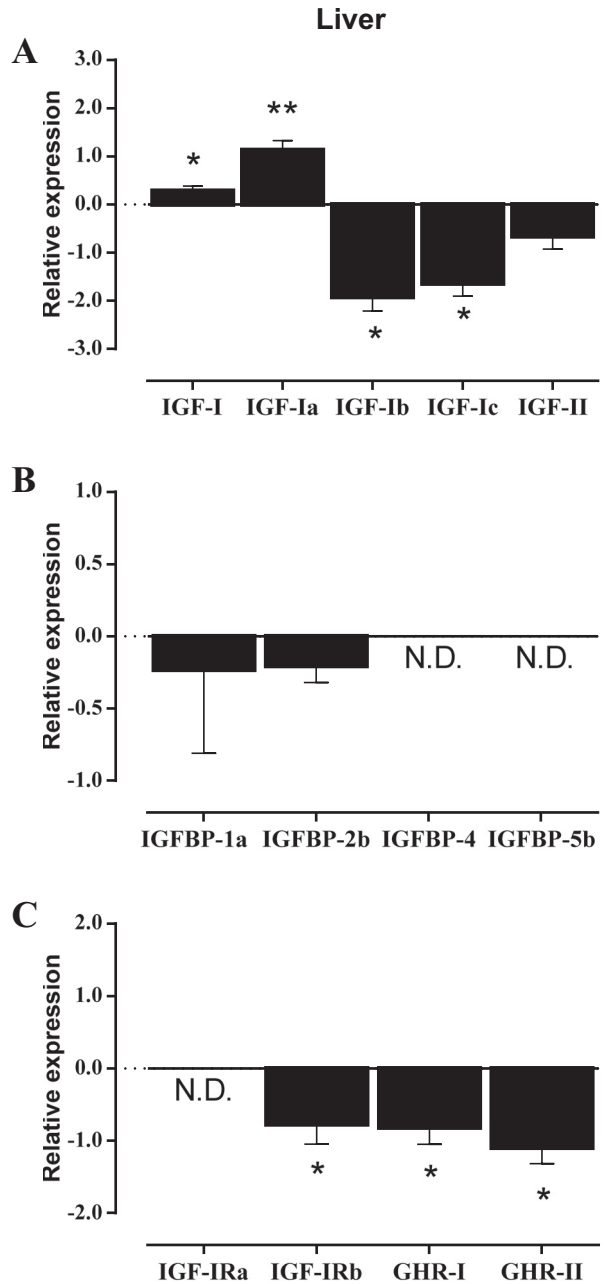


Fig. 1. Effects of exercise on liver GH-IGFs axis members' gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of a total of IGF-I, IGF-I splice variants, and IGF-II (*A*), IGF binding proteins (*B*), and IGF-I and GH receptors in liver tissue of gilthead sea bream after 5 wk of sustained and moderate exercise (EX) (*C*). Data are shown as fold change relative to control (CT) group as means ± SE ($n = 12$). Significant differences are indicated by * ($P < 0.05$) or ** ($P < 0.001$). N.D., nondetected.

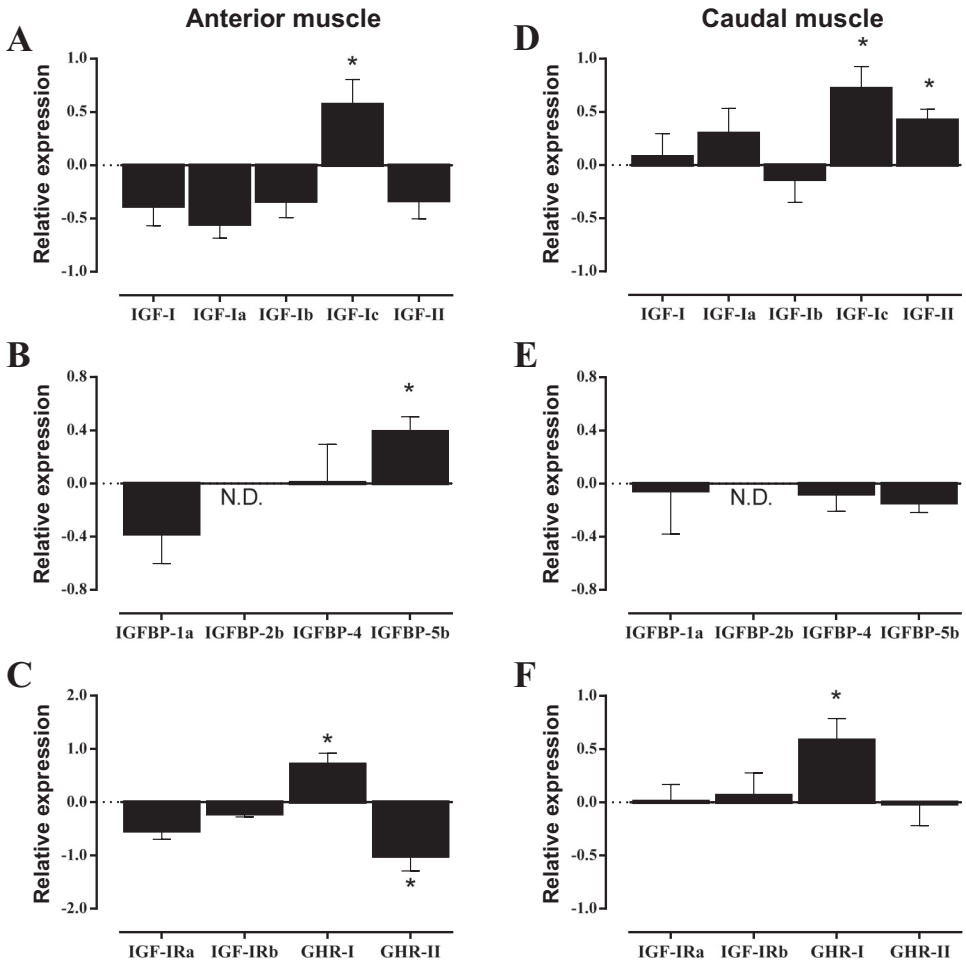


Fig. 2. Effects of exercise on muscle GH-IGFs axis members' gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of total IGF-I, IGF-I splice variants and IGF-II (*A*), IGF-I binding proteins (*B*), and IGF and GH receptors in anterior muscle tissue (*C*) and total IGF-I, IGF-I splice variants, and IGF-II (*D*), IGF-I binding proteins (*E*) and IGF and GH receptors in caudal muscle region of gilthead sea bream (*F*) after 5 wk of sustained and moderate EX. Data are shown as fold change relative to CT group as means ± SE ($n = 12$). Significant differences are indicated by * ($P < 0.05$).

growth in gilthead sea bream fingerlings, as well as causing a significant increase in circulating IGF-I (36.75 ± 2.63 vs. 51.10 ± 1.13 ng/ml) with a concomitant decrease in GH (7.50 ± 1.2 vs. 3.42 ± 0.55 ng/ml) plasma levels (9). Such an effect was not accompanied by changes in food intake or hepatosomatic index, although a significant decrease in mesenteric fat index was observed in EX fish compared with CT fish. In the present study, derived from the same experimental trial, we show the effects of exercise on the plasma IGF-I/GH ratio and on the tissue gene expression of components of the GH-IGFs system, aiming to provide new information on IGFs, IGFBPs, IGF-IRs, and downstream signaling molecules, as well as GHRs in liver and muscle tissues.

### *Effects of Sustained Exercise on Plasma IGF-I/GH Ratio*

In a review on exercise in fish, Davison (21) proposed that a reduction in catecholamines and cortisol levels together with an increase in growth factors and thyroid hormones will produce the perfect endocrine combination to enhance growth. Similarly, Pérez-Sánchez and Le Bail (67) reported that the best growth performance in fish is achieved with low plasma GH and cortisol levels, in combination with high circulating IGFs, insulin, and T3, and suggested that low GH plasma levels may be used to select fast-growing gilthead sea bream. In this context, low GH plasma levels have been reported in salmonids during high-growth periods, such as those induced by exercise (43, 85). In accordance with this, Beckman (7) reviewed a series of experiments that demonstrated a positive correlation between IGF-I plasma levels and growth and suggested that IGF-I levels can provide a useful index of fish growth. This was also observed in exercised juvenile gilthead sea bream, in which significantly higher IGF-I plasma levels paralleled increased growth compared with control fish (82). In agreement with these findings, in our study, gilthead sea bream fingerlings exposed to moderate exercise had an increased IGF-I/GH ratio as a consequence of decreased GH, as well as increased IGF-I in the plasma (9), suggesting that moderate and sustained exercise results in a favorable endocrine condition that leads to enhanced fish growth.

### *Effects of Sustained Exercise on Hepatic GH-IGFs Expression*

The expression of IGF-I in the liver reflected the results found in blood, showing an increase in the expression of total IGF-I in EX fish, which indicated that not only is hormonal secretion enhanced, but also that the corresponding gene expression is activated in these conditions. In this context, the expression of the splice variant IGF-Ia was significantly increased in EX fish, while that of IGF-Ib and IGF-Ic was decreased, suggesting differential hepatic regulation of these splice variants in this species, with IGF-Ia appearing to be the most sensitive form in response to exercise. With regard to

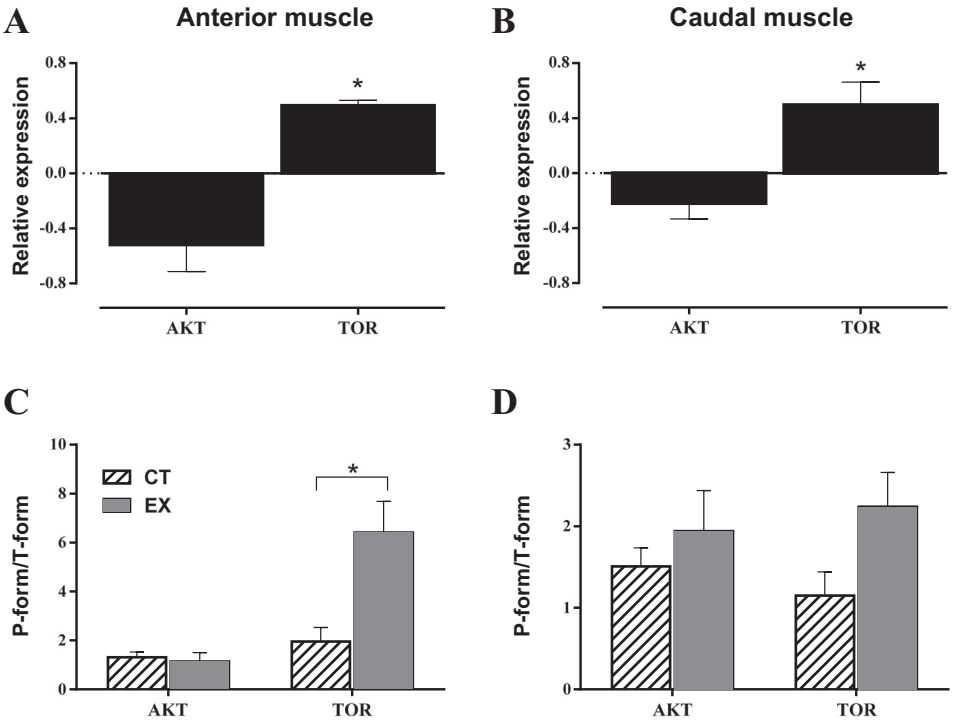


Fig. 3. Effects of exercise on Akt and target of rapamycin (TOR) signaling pathways. Relative mRNA expression normalized to RPS18 and RPL27a of Akt and TOR in the anterior (*A*) and caudal muscle regions (*B*) of gilthead sea bream after 5 wk of sustained and moderate EX. Data are shown as fold change relative to CT group as means ± SE ($n = 12$). Significant differences were indicated by * ($P < 0.05$). Densitometric analyses of Akt and TOR phosphorylation in the anterior (*C*) and caudal (*D*) muscle regions of gilthead sea bream CT or after 5 wk of sustained and moderate EX. The intensity of each phosphorylated form was normalized to the corresponding total form for each sample. Results are presented as means ± SE ($n = 4$). Significant differences are indicated by * ($P < 0.05$).

IGFBPs, only IGFBP-1a and IGFBP-2b gene expression was detected in the gilthead sea bream liver. As reported previously in zebrafish, both IGFBP-1 and IGFBP-2 have the ability to inhibit the cellular actions of IGF-I (22, 23), with IGFBP-1 being an important growth and developmental inhibitor under hypoxic conditions. Although recent data suggest that stimulation of IGFBP-2 is triggered by GH treatment in tilapia (13), in general, it is recognized that plasma levels of both IGFBPs (1 and 2) increase in catabolic conditions and, thus, have been hypothesized as markers for negative growth in fish (48). In this context, the stable expression of these two hepatic IGFBPs with exercise, as observed in the present study, is consistent with the good growth conditions observed in EX fish.

The two IGF-IRs showed different levels of expression in the liver, with only IGF-IRb being detected by quantitative PCR, and downregulated in EX fish. This decrease in IGF-IRb expression in the liver could be interpreted as a mechanism to avoid inhibitory effects on IGF-I secretion due to the potential negative feedback provoked by the increased levels of circulating IGF-I. Similar downregulation in response to high circulating hormones, but at the level of binding, has been described previously in lamprey (51) and Atlantic salmon hepatocytes (74), trout cardiomyocytes (59), and brown trout and carp red muscle (5), in contrast to the upregulation observed in white muscle (66). Regarding the GHRs, levels of GHR-I and GHR-II were significantly reduced in the liver of EX fish, indicating possible GH desensitization in this tissue. GHRs transcriptional regulation has been shown to be highly variable among fish species, tissues, and physiological status. In this context, in a contrasting situation in which GH levels were increased, such as during fasting or feeding a high-plant-protein diet, a reduction in GHRs in the liver was also found in gilthead sea bream (37, 78) and hybrid striped bass (72), whereas fasted rainbow trout exhibited enhanced gene expression of GHR-I (32). Moreover, it should be noted that hepatic GH binding and signaling are also influenced by GH plasma levels, independently of GHR expression (98).

### *Effects of Sustained Exercise on Muscle GH-IGF Expression*

In general, exercise in vertebrate muscle leads to an increase in protein synthesis and growth, a response that has been found in different fish species (52, 62), including gilthead sea bream (28, 44, 54, 82), with effects, such as hypertrophy and increased muscle vascularization. In the present study, the positive response of the GH-IGFs axis members' expression to sustained swimming was seen as a significant increase in IGF-Ic in both anterior and caudal muscle regions. IGF-Ic appears to be the most responsive splice variant to exercise in muscle tissue, suggesting that at least in gilthead sea bream, this isoform has acquired a similar role to that of the mammalian MGF, responding to mechanical stimulation (4, 71). This highlights the relative importance of IGF-Ic in muscle and will encourage further efforts to unravel its specific role in the regulation of myocyte growth.

Regarding IGFBPs, the expression of IGFBP-5b was significantly increased in the anterior white muscle of EX fish. The possible involvement of IGFBP-5 in mediating improved muscle growth by enhancing the availability or bioactivity of IGFs has been reported previously (24). IGFBP-5 is considered a promyogenic molecule and, therefore, a positive regulator of IGF-I actions (30). Furthermore, a positive correlation between IGFBP-5 mRNA levels and circulating IGF-I has been found in rainbow trout in anabolic conditions (32). In addition, Ren et al. (76) showed that IGFBP-5 induction precedes that of IGF-II, and as reviewed by Duan et al. (23), local IGFBP-5

promotes IGF-II actions during myoblast differentiation. Therefore, this IGFBP seems to play a critical role in muscle growth by stimulating the synthesis and signaling of IGF-II. According to these observations, the increase of IGFBP-5b in the anterior muscle of EX fish in our study could be linked to the increase in IGF-Ic in both anterior and caudal muscles, as well as to the increase in IGF-II expression in the caudal region during exercise, suggesting potential cross talk between muscle regions. Moreover, although not significant, the decline in IGFBP-1a in muscle is also consistent with the increased growth rates observed in EX fish, as this IGFBP has previously been related to stressful or negative conditions (48).

Both IGF-IRs were present in muscle, although exercise had no effect on their expression, in contrast with the reduced levels of mRNA of IGF-IRa observed by Palstra et al. (65) in exercised zebrafish. Changes in the expression of IGF-IRs have been reported in relation to nutritional status, with fasting causing an increase in the expression of IGF-IRb in rainbow trout (57), or with refeeding, which increases IGF-I plasma levels, and decreases the expression of IGF-IRa in Atlantic salmon (11). In line with these findings, differences between the intensity of exercise, the physiological situation, or even the species, may explain the differences in response between our study and that on zebrafish (65). It should also be taken into account that regulation could take place at the level of IGFs binding or affinity to the receptor or activation of distinct signaling transduction pathways by different means. It has recently been shown by microarray analysis that exercise activates signaling pathways involved in the regulation of muscle mass in zebrafish, including the Akt and TOR pathways (64). In this sense, although we did not see changes in Akt phosphorylation with exercise, as in a similar trial with gilthead sea bream juveniles (82), we observed significant activation of TOR signaling at the gene and protein levels in response to exercise. In mammals, it is known that exercise activates TOR, and the most accepted hypothesis is that this involves the PI3K/Akt pathway (1, 3, 29). However, in mice, TOR can be phosphorylated by an Akt-independent mechanism following mechanical stimulation (42, 61). Moreover, after a bout of resistance exercise in the same species, signaling through Akt was a transient event, while TOR activation was sustained for a longer period (61). On the basis of these findings, either Akt-independent activation of TOR or persistent activation of TOR with exercise may be occurring in our model.

With regard to GHRs, the significant upregulation of GHR-I in anterior and caudal muscles together with body weight enhancement is consistent with the fact that GHR-I has previously been linked with anabolic pathways that are stimulated during exercise, whereas GHR-II has been linked with stress or immune responses (17, 80). In this sense, concurrent downregulation of IGF-I and GHR-I expression was observed in the head-kidney of *Enteromyxum leei* parasitized fish compared with control fish, whereas the expression of GHR-II was not altered (84). On the basis of an overwinter experiment with gilthead sea bream, the same authors reported that increased expression of GHR-I could mediate the increase in IGF-II mRNA levels (79). Similarly, Reindl and Sheridan (75) also reported upregulation of GHR-I expression in adipose tissue during fasting, pointing this out as a positive effect of growth maintenance powered by lipid metabolism. Thus, the locally increased expression of GHR-I in muscle in our study could be considered an adaptation to compensate for the reduced plasma GH levels. Furthermore, it was noticeable in our study that the two GHRs showed distinct responses to exercise, with GHR-II decreasing in the anterior muscle. These reduced levels of GHR-II were consistent with that found in exercised zebrafish (65). Moreover, muscle GHR-II was reported to be increased in fasted gilthead sea bream (78) and rainbow trout (32), supporting its role in mediating the catabolic actions of GH. This is consistent with a previous proposal that GHR-I and GHR-II have evolved as duplicated subtypes with different patterns of tissue distribution, postreceptor signaling, hormonal transcriptional regulation, and also function (45, 79).

Overall, IGF-Ic and GHR-I were upregulated throughout the whole muscle, and in addition, the expression of IGFBP-5b and IGF-II was also increased in the anterior and caudal muscle regions, respectively. This, together with the previously described positive relationship between GHR-I, IGFBP-5, and IGF-II availability and action, suggests that some kind of communication is taking place along the muscle, which deserves future investigation. In line with these observations, Rius-Francino et al. (77) demonstrated in gilthead sea bream that IGF-II is clearly a potent stimulator of myocyte proliferation, a condition that seems to be enhanced in this experimental model, especially in the caudal muscle area. In support of this, in a previous study, a higher number of capillaries was observed in the anterior muscle along with increased fiber size in both muscle regions in juvenile gilthead sea bream after sustained exercise (44). Besides, it is well known that under moderate and continuous exercise in Sparidae fish, the posterior zone of the muscular skeletal system is more active (90) and shows major sensitivity to swimming activity.

### *Perspectives and Significance*

In the present work, 5 wk of sustained and moderate exercise in gilthead sea bream fingerlings led to an increase in growth and the plasma IGF-I/GH ratio. This is consistent with the increased expression of total IGF-I in the liver, which seems to be focused on synthesizing IGF-I through upregulation of the IGF-Ia splice variant. In muscle, growth promotion seems to be hormonally mediated by the GH-GHR-I/IGFBP-5b-IGF-II axis and, mechanically, by enhancing the expression of the splice variant IGF-Ic and TOR activation. The present model provides insights into the differential regulation of GH-IGFs system members in response to sustained exercise in gilthead sea bream, and supports the use of these procedures to improve sustainable aquaculture production by modulating the endogenous endocrine system of the fish to enhance growth.

**ACKNOWLEDGMENTS**

The authors thank Carlos Mazorra from Tinamenor Sociedad Limitada and the personnel from the facilities at the School of Biology for the maintenance of the fish. The English version has been corrected by the Language Advisory Service of the University of Barcelona.

**GRANTS**

E. J. Vélez and A. Millán-Cubillo are supported by a predoctoral fellowship from the Ministerio de Ciencia e Innovación (MICINN). This study was supported by the projects from the MICINN AGL2009-12427 and AGL2012-39768 to J. Gutiérrez, and the Xarxa de Refèrencia d'R+D+I en Aqüicultura and the SGR2009-00402 from the Generalitat de Catalunya.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Author contributions: E.J.V., Sh.A., A.M.-C., J.A.C.-G., J.P.-S., and E.C. performed experiments; E.J.V., Sh.A., and E.C. analyzed data; E.J.V., J.F.-B., J.B., J.A.C.-G., J.P.-S., E.C., and J.G. interpreted results of experiments; E.J.V. prepared figures; E.J.V., E.C., and J.G. drafted manuscript; E.J.V., Sh.A., A.M.-C., J.F.-B., J.B., S.J.C., J.A.C.-G., J.P.-S., I.N., E.C., and J.G. edited and revised manuscript; E.J.V., Sh.A., A.M.-C., J.F.-B., J.B., S.J.C., J.A.C.-G., J.P.-S., I.N., E.C., and J.G. approved final version of manuscript; J.F.-B., J.B., and J.G. conception and design of research.

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*Am J Physiol Regul Integr Comp Physiol* 312: R643–R653, 2017.
First published February 22, 2017; doi:10.1152/ajpregu.00308.2016.

**RESEARCH ARTICLE** | *Hormones, Reproduction and Development*

# Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*)

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Submitted 8 July 2016; accepted in final form 15 February 2017

**Vélez EJ, Azizi Sh, Lutfi E, Capilla E, Moya A, Navarro I, Fernández-Borràs J, Blasco J, Gutiérrez J.** Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*). *Am J Physiol Regul Integr Comp Physiol* 312: R643–R653, 2017. First published February 22, 2017; doi:10.1152/ajpregu.00308.2016.—Swimming activity primarily accelerates growth in fish by increasing protein synthesis and energy efficiency. The role of muscle in this process is remarkable and especially important in teleosts, where muscle represents a high percentage of body weight and because many fish species present continuous growth. The aim of this work was to characterize the effects of 5 wk of moderate and sustained swimming in gene and protein expression of myogenic regulatory factors, proliferation markers, and proteolytic molecules in two muscle regions (anterior and caudal) of gilthead sea bream fingerlings. Western blot results showed an increase in the proliferation marker proliferating cell nuclear antigen (PCNA), proteolytic system members calpain 1 and cathepsin D, as well as vascular endothelial growth factor protein expression. Moreover, quantitative real-time PCR data showed that exercise increased the gene expression of proteases (calpains, cathepsins, and members of the ubiquitin-proteasome system in the anterior muscle region) and the gene expression of the proliferation marker PCNA and the myogenic factor MyoD in the caudal area compared with control fish. Overall, these data suggest a differential response of the two muscle regions during swimming adaptation, with tissue remodeling and new vessel formation occurring in the anterior muscle and enhanced cell proliferation and differentiation occurring in the caudal area. In summary, the present study contributes to improving the knowledge of the role of proteolytic molecules and other myogenic factors in the adaptation of muscle to moderate sustained swimming in gilthead sea bream.

growth potential; myogenic regulatory factors; proteolytic molecules; swimming; VEGF; IGFs

IN FISH, GROWTH DEPENDS on the accretion of muscle, principally white skeletal muscle (48, 63), and compared with other vertebrates, differences in regulation exist among species. Whereas some fish species such as zebrafish (*Danio rerio*; 6) follow a determinate growth pattern similar to that of mammals, most of the teleost species can grow in weight and length in an indeterminate way throughout their life (49, 92). Moreover, continuous fish muscle growth consists of hypertrophy (increase in fiber size) and hyperplasia (new muscle fiber formation; 90). These processes are the result of increased myogenesis, as well as downregulation of growth inhibitors, as is the case of some members of the transforming growth factor-β family [i.e., myostatin (MSTN); 62, 88]. During myogenesis, muscle satellite cells, which can be recognized for the expression of Pax3/7 (34), turn into myoblasts that after proliferation and differentiation, fuse together or with existing fibers to form multinucleated myofibers (14, 48). In mammals, this process is modulated by key transcription factors called myogenic regulatory factors (MRFs), which are expressed in a sequential manner, being either essential for muscle lineage determination and cell proliferation [myogenic factor 5 (Myf5) and MyoD] or responsible for the initiation of the differentiation program from myoblast to myotubes (Mrf4 and myogenin; 18, 103). In fish, these transcription factors have also been identified, and a similar function to that in mammals has been demonstrated (9, 10, 16, 34, 36, 48, 56). In the last part of the myogenic process, and concomitant with multinucleated myofiber formation, the necessity of structural and contractile proteins (e.g., actin or myosin) is well known, as has been demonstrated in fish by means of both in vivo and in vitro experiments (36, 66).

Furthermore, muscle growth is the result of the positive balance between protein synthesis and degradation. The main endogenous proteolytic systems involved (ubiquitin-proteasome, calpains, cathepsins, and caspases) are well known in mammals (5), and in the last years, different authors have identified and characterized some of these molecules in fish as well (85–87). A few studies have shown, in different species, that these molecules are regulated by nutritional status (57, 75, 81). In this line, Salmerón et al. (82) have identified a few calpain members in gilthead sea bream (*Sparus aurata*), one of the most important species in Mediterranean aquaculture (31), and demonstrated that diet composition and fasting/refeeding modulate their expression, these being changes well correlated with flesh texture. Moreover, it is commonly known that protein turnover has a greater relevance during higher growth periods (i.e., fingerlings vs. juvenile or adult fish; 72, 73, 83), and thus the proteolytic systems should be considered to completely understand development and growth in fish. In this sense, an in vitro study with rainbow trout (*Oncorhynchus mykiss*) myocytes has demonstrated that compared with mammals the protein degradation in fish is mainly due to the autophagy-lysosomal system (including cathepsins), at the ex-

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pense of a less important role of the ubiquitin-proteasome system (85).

In addition to muscle growth, white muscle cellularity depends on various factors such as exercise (49, 50). In previous publications from this same experimental trial, Blasco et al. (8) and Vélez et al. (98) have demonstrated that 5 wk of moderate sustained swimming stimulate growth in fingerlings of gilthead sea bream without increasing food intake. Furthermore, this exercise-related growth was modulated by an increase in the aerobic capacity of the muscle and the plasma insulin-like growth factor I (IGF-I)-to-growth hormone (GH) ratio, variations in the mRNA levels of GH-IGF axis-related genes in liver and muscle, and activation of the target of rapamycin (TOR) signaling pathway (98). Overall, these changes accelerate growth by improving both muscle protein synthesis and energy efficiency (8); however, complementary information on myogenic regulators or proteolytic systems to better picture muscle development and protein turnover was not reported, becoming the main objective of the present study.

Additionally, Egginton and Cordiner (28) have shown that swimming performance is directly related to capillary density in rainbow trout, angiogenesis being an integrated response to optimize aerobic performance. These authors also suggested that this process could be stimulated by different mechanical factors in muscle. Similarly, in zebrafish, different authors have studied the increased capillarity, mitochondrial biogenesis, and muscle remodeling induced by swimming adaptation (43, 52, 61, 70). In gilthead sea bream juveniles forced to perform sustained and moderate swimming, a differential response/adaptation to exercise between the epaxial-anterior and epaxial-caudal regions of white muscle has also been reported (44). In this sense, Vélez et al. (98) found a differential regulation between regions of several members of the GH-IGF axis in fingerlings of this species as an effect of swimming, highlighting an increase in IGF-II gene expression in the caudal region and IGF binding protein-5 (IGFBP-5) gene expression in the anterior one. Moreover, in another study, whereas in the anterior region the number of capillaries increased, the fiber size in both anterior and caudal muscles was also enlarged with exercise (96), illustrating the different work of both muscle regions in the *Sparidae* family during swimming. Regarding this, the classical theories proposed that most of the power for fish swimming is generated by the anterior muscle (95, 99), although in later studies it seems clear that this depends on the type of muscle considered in each region. Thereby, in red muscle the power comes mainly from the posterior region (23, 40, 47, 80, 91), whereas in the case of white muscle the power is generated mostly by the anterior part (2, 29, 95). In any case, the anterior muscle has generally faster contractile properties than the posterior one for both red and white muscle types (22), and the recruitment of the different fiber types across the axis length of the muscle depends on the swimming speed and is different among species (2, 21, 30).

The aim of this work was to characterize the gene and protein expression of MRFs, proliferation and angiogenic markers, and proteolytic molecules in the anterior and caudal skeletal muscle regions of gilthead sea bream fingerlings after 5 wk of moderate and sustained activity, completing the previous metabolic and endocrine studies reported from these fish (8, 98).

## MATERIALS AND METHODS

### *Animals and Experimental Trial*

Five hundred forty gilthead sea bream (*S. aurata* L.) fingerlings [~5 g body wt and 7 cm body length (BL)] were obtained from a hatchery located in the north of Spain, randomly distributed into eight 0.2-$m^3$ tanks, and maintained within a temperature-controlled seawater recirculation system at 23 ± 1°C at the facilities of the School of Biology at the University of Barcelona (Spain). The exchange rate of water was 35% per week, and water was exchanged through the filtering system without altering the inflow to the tanks; oxygen level was over 90% of saturation, and the photoperiod was 15 h light/9 h dark. After the acclimation period, four tanks were kept with a vertical water inflow of 350 l/h (standard rearing conditions), where the fish presented only spontaneous and voluntary movements (Control group); in the other four tanks a circular uniformly distributed flow of 700 l/h was set up, where the fish were forced to undertake moderate and sustained swimming (5 BL/s; Exercise group), as reported before (8, 98). Fish were fed with a commercial diet (Gemma Diamond; Skretting, Burgos, Spain) three times a day, representing a 5% ration (until apparent satiety). Biometric parameters (weight and length) were determined at the beginning of the experiment and at the last time point (5 wk) to monitor fish growth. Fish were fasted for 24 h before any manipulation and sampling. As previously reported (98), in the final sampling at *week 5*, 12 fish of each group (4 fish per tank) were killed, and samples of anterior (from 25% total length behind the tip of the snout to 40% total length) and caudal (from 60 to 75% total length) epaxial white muscle regions were collected, frozen in liquid nitrogen, and stored at −80°C until further analyses.

All animal-handling procedures were conducted following the European Union Council (86/609/EU) and Spanish and Catalan government-established norms and procedures and with Ethics and Animal Care Committee of the University of Barcelona approval (permit no. DAAM 7644).

### *Gene Expression*

*RNA extraction and cDNA synthesis.* Total RNA was extracted from 100 mg of tissue with 1 ml of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) as has been described before (98). Briefly, total RNA concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain). After the samples' integrity in a 1% agarose gel (wt/vol) stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain) was verified, 1 μg of total RNA was treated with DNase I (Life Technologies) to remove all genomic DNA following the manufacturer's recommendations. Finally, RNA was reverse transcribed with the Affinity Script qPCR cDNA Synthesis Kit (Agilent Technologies, Las Rozas, Spain).

*Quantitative real-time PCR.* The mRNA transcript levels of the following groups of interest (Table 1) were examined in a CFX384 Real-Time System (Bio-Rad, El Prat de Llobregat, Spain) according to the requirements of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (13): proliferation marker, growth inhibitors, MRFs, structural molecules, proteolytic markers, ubiquitin-proteasome system, plus three reference genes. The analyses were performed in triplicate in a final volume of 5 μl including 2.5 μl of iTaq Universal SYBR Green Supermix (Bio-Rad), 250 nM of forward and reverse primers (Table 1), and 1 μl of cDNA for each sample. As described before (82), the reactions consisted of the following: initial activation step of 3 min at 95°C, 40 cycles of 10 s at 95°C, and 30 s at 55–68°C (primer dependent, Table 1) followed by an amplicon dissociation analysis from 55 to 95°C at 0.5°C increase each 30 s. Before the analyses, the appropriate cDNA dilution for each assay was determined, as well as the specificity of the reaction and the absence of primer dimers, confirmed by running a dilution curve with a pool of samples. The expression level of each

Table 1. *Primers used for quantitative PCR: sequences, melting temperatures, and GenBank accession numbers*

| Gene | Primer Sequences (5′–3′) | Tm, °C | Accession Number |
|---|---|---|---|
| *RPS18* | F: GGGTGTTGGCAGACGTTAC | 60 | AM490061.1 |
| | R: CTTCTGCCTGTTGAGGAACCA | | |
| *RPL27a* | F: AAGAGGAACACAACTCACTGCCCCAC | 68 | AY188520 |
| | R: GCTTGCCTTTGCCCAGAACTTTGTAG | | |
| *β-Actin* | F: TCCTGCGGAATCCATGAGA | 60 | X89920 |
| | R: GACGTCGCACTTCATGATGCT | | |
| *PCNA* | F: TGTTTGAGGCACGTCTGGTT | 58 | AY550963.1 |
| | R: TGGCTAGGTTTCTGTCGC | | |
| *MSTN1* | F: GTACGACGTGCTGGGAGACG | 60 | AF258448.1 |
| | R: CGTACGATTCGATTCGCTTG | | |
| *MSTN2* | F: ACCTGGTGAACAAAGCCAAC | 60 | AY046314 |
| | R: TGCGGTTGAAGTAGAGCATG | | |
| *MyoD1* | F: TTTGAGGACCTGGACCC | 60 | AF478568.1 |
| | R: CTTCTGCGTGGTGATGGA | | |
| *MyoD2* | F: CACTACAGCGGGGATTCAGAC | 60 | AF478569 |
| | R: CGTTTGCTTCTCCTGGACTC | | |
| *Myf5* | F: CTACGAGAGCAGGTGGAGAACT | 64 | JN034420 |
| | R: TGTCTTATCGCCCAAAGTGTC | | |
| *Myogenin* | F: CAGAGGCTGCCCAAGGTCGAG | 68 | EF462191 |
| | R: CAGGTGCTGCCCGAACTGGGCTCG | | |
| *Mrf4* | F: CATCCCACAGCTTTAAAGGCA | 60 | JN034421 |
| | R: GAGGACGCCGAAGATTCACT | | |
| *MHC* | F: AGCAGATCAAGAGGAACAGCC | 58 | AY550963.1 |
| | R: GACTCAGAAGCCTGGCGATT | | |
| *MLC2a* | F: GCCCCATCAACTTCACCGTCTTT | 60 | AF150904 |
| | R: GGTTGGTCATCTCCTCAGCGG | | |
| *MLC2b* | F: TCCCTTTGCTATTCTGCCTTC | 60 | FG618631 |
| | R: AAATCAGCCCTATTCCCCATA | | |
| *CAPN1* | F: CCTACGAGATGAGGATGGCT | 56 | KF444899 |
| | R: AGTTGTCAAAGTCGGCGGT | | |
| *CAPN2* | F: ACCCACGCTCAGACGGCAAA | 61 | KF444900 |
| | R: CGTTCCCGCTGTCATCCATCA | | |
| *CAPN3* | F: AGAGGGTTTCAGCCTTGAGA | 56 | ERP000874 |
| | R: CGCTTTGATCTTTCTCCACA | | |
| *CAPNs1a* | F: CGCAGATACAGCGATGAAAA | 56 | KF444901 |
| | R: GTTTTGAAGGAACGGCACAT | | |
| *CAPNs1b* | F: ATGGACAGCGACAGCACA | 56 | ERP000874 |
| | R: AGAGGTATTTGAACTCGTGGAAG | | |
| *CTSDb* | F: AAATTCCGTTCCATCAGACG | 56 | KJ524456 |
| | R: CTTCAGGGTTTCTGGAGTGG | | |
| *MuRF1* | F: GTGACGGCGAGGATGTGC | 60 | FM145056 |
| | R: CTTCGGCTCCTTGGTGTCTT | | |
| *MAFbx* | F: GGTCACCTGGAGTGGAAGAA | 60 | ERA047531 |
| | R: GGTGCAACTTTCTGGGTTGT | | |
| *N3* | F: AGACACACACTGAACCCGA | 54 | KJ524458 |
| | R: TTCCTGAAGCGAACCAGA | | |

$T_m$, melting temperature; F, forward; R, reverse; *RPS18*, ribosomal protein S18; *RPL27a*, ribosomal protein L27a; *PCNA*, proliferating cell nuclear antigen; *MSTN1*, myostatin 1; *MSTN2*, myostatin 2; *MyoD1*, myoblast determination protein 1; *MyoD2*, myoblast determination protein 2; *Myf5*, myogenic factor 5; *Mrf4*, myogenic regulatory factor 4; *MHC*, myosin heavy chain; *MLC2a*, myosin light chain 2a; *MLC2b*, myosin light chain 2b; *CAPN1*, calpain 1; *CAPN2*, calpain 2; *CAPN3*, calpain 3; *CAPNs1a*, calpain, small subunit 1a; *CAPNs1b*, calpain, small subunit 1b; *CTSDb*, cathepsin Db; *MuRF1*, muscle RING finger protein 1; *MAFbx*, muscle atrophy F-box or atrogin-1; *N3*, proteasome β type 4 subunit or PSMB4.

gene analyzed was calculated relative to the geometric mean of the reference genes *RPS18* and *RPL27a*, the two most stable of the genes analyzed, using the Pfaffl method (74).

### *Western Blot Analysis*

Protein homogenates from muscle tissue were obtained as described by García de la serrana et al. (37). The amount of protein from each sample was measured (11), and 10–20 μg of protein were separated by electrophoresis (SDS-PAGE) on 10% polyacrylamide gel (125 V for 1 h 30 min) following the procedure previously reported (97) with slight modifications. After SDS-PAGE, samples were transferred to a polyvinylidene fluoride membrane. Then, all the membranes were stained with a Ponceau S solution (Sigma-Aldrich, Tres Cantos, Spain) and scanned for posterior band quantification. Later, the membranes were washed to eliminate the Ponceau staining and then blocked in nonfat milk 5% buffer and incubated with the respective primary antibodies in a 1:200 dilution. The primary antibodies used were as follows: rabbit polyclonal anti-proliferating cell nuclear antigen (anti-PCNA; catalog no. sc-7907), rabbit polyclonal anti-muscle atrophy F-box (anti-MAFbx; catalog no. sc-33782), rabbit polyclonal anti-VEGF (catalog no. sc-152), goat polyclonal anti-calpain 1 (anti-CAPN1; catalog no. sc-7530), and goat polyclonal anti-cathepsin D (anti-CTSD; catalog no. sc-6486), all from Santa Cruz Biotechnology (Santa Cruz, CA). All these antibodies have been previously demonstrated to cross-react successfully with the proteins of interest in gilthead sea bream (3; E.J. Vélez, Sh. Azizi, D. Verheyden, C. Salmerón, E. Lutfi, A. Sánchez-Moya, I. Navarro, J. Gutiérrez and E. Capilla, unpublished data), with the exception of anti-VEGF, whose specificity has been tested in a pooled sample by blocking overnight with an excess (5-fold) of the immunization peptide (catalog no. sc-152 P). After washing was completed, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibody: goat anti-rabbit (catalog no. 31460; Thermo Scientific), or donkey anti-goat (catalog no. sc-2020, Santa Cruz Biotechnology). The membranes were rewashed, and the different immunoreactive bands were developed using an enhanced chemi-

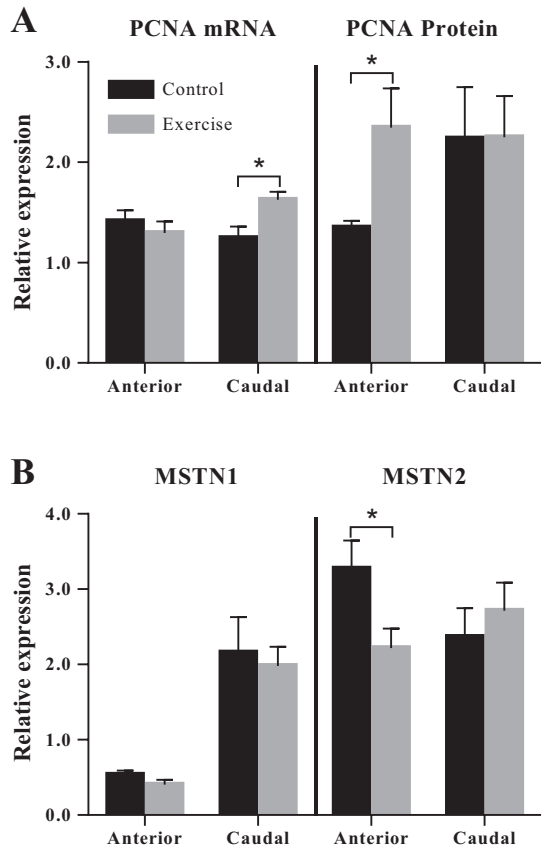


Fig. 1. Effects of exercise on the expression of a proliferation marker and a growth inhibitor in muscle tissue. *A*: relative mRNA expression of proliferating cell nuclear antigen (PCNA, *left*) and PCNA protein expression normalized to total protein staining with Ponceau S (*right*). *B*: relative mRNA levels of myostatin (MSTN)1 and MSTN2 normalized to ribosomal protein S18 (RPS18) and ribosomal protein L27a (RPL27a) in both anterior and caudal muscle regions of gilthead sea bream after 5 wk of sustained and moderate exercise (Exercise), or reared in control conditions (Control). Data are shown as means ± SE ($n = 12$ for quantitative PCR; $n = 4$ for Western blot). *Significant difference, $P < 0.05$.

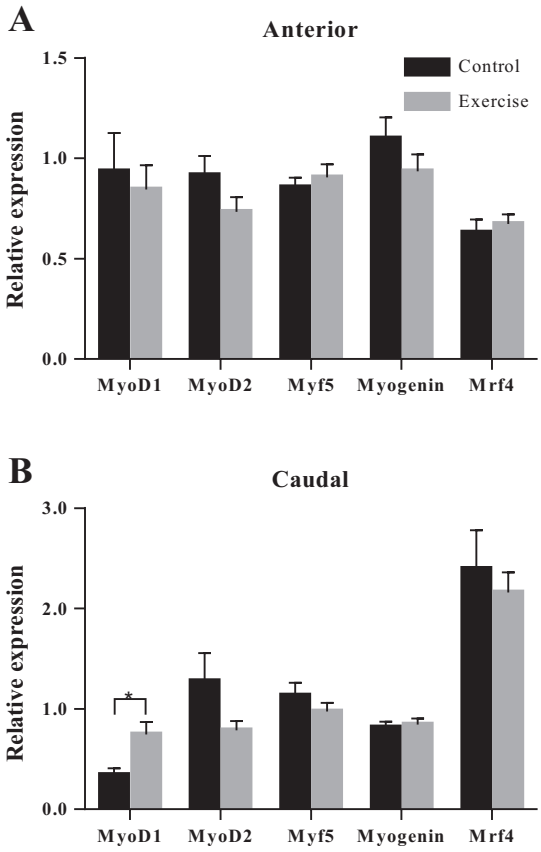


Fig. 2. Effects of exercise on myogenic regulatory factor gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of MyoD1, MyoD2, Myf5, myogenin, and Mrf4 in the anterior muscle (*A*) and caudal muscle (*B*) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control conditions. Data are shown as means ± SE ($n = 12$). *Significant difference, $P < 0.05$.

luminescence kit (Pierce ECL WB Substrate; Thermo Scientific). Finally, the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Hence results from the densitometry analysis of each specific band are presented normalized by the densitometry values of the Ponceau, since its staining showed similar amounts of loaded and transferred protein. In the case of CTSD, the densitometric analysis was performed with the sum of the bands corresponding to the immature and mature forms.

### *Statistical Analyses*

Data were analyzed using IBM SPSS Statistics version 21 and are presented as means ± SE. Normal distribution was first analyzed using the Shapiro-Wilk test followed by Levene's test to test homogeneity of variances. Statistical differences were analyzed using Student's *t*-test and considered significant when $P < 0.05$ (*) or $P < 0.001$ (**).

## RESULTS

### *Proliferation Marker and Growth Inhibitor Expression*

Figure 1*A* shows the effects of 5 wk of sustained swimming in fingerlings of gilthead sea bream on the gene and protein expression of the proliferation marker PCNA. PCNA gene expression was increased significantly in the caudal muscle region of exercised fish; nonetheless, the protein levels of PCNA were significantly higher in the anterior region compared with control fish.

Figure 1*B* presents the mRNA levels of two MSTN paralogs in both muscle regions. Although the expression of MSTN1 was not modified by the effect of exercise, the transcript levels of MSTN2 were downregulated in the anterior muscle of exercised fish.

### *Myogenic Regulatory Factor and Structural Marker Expression*

The analysis of MRFs gene expression showed that whereas none of these factors (MyoD1, MyoD2, Myf5, myogenin, and Mrf4) were affected by swimming activity in the anterior muscle (Fig. 2*A*), the expression of MyoD1 was significantly increased in the caudal region (Fig. 2*B*).

Regarding the structural markers [myosin heavy chain (MHC), myosin light chain 2A (MLC2A), and MLC2B], in the anterior muscle of exercised fish, higher mRNA levels of MLC2A were found (Fig. 3*A*). However, no differences were observed for any of these genes in the caudal muscle region (Fig. 3*B*).

### *Proteolytic and Angiogenesis Markers*

Swimming activity significantly increased the expression of several of the most important proteolytic-related genes in the

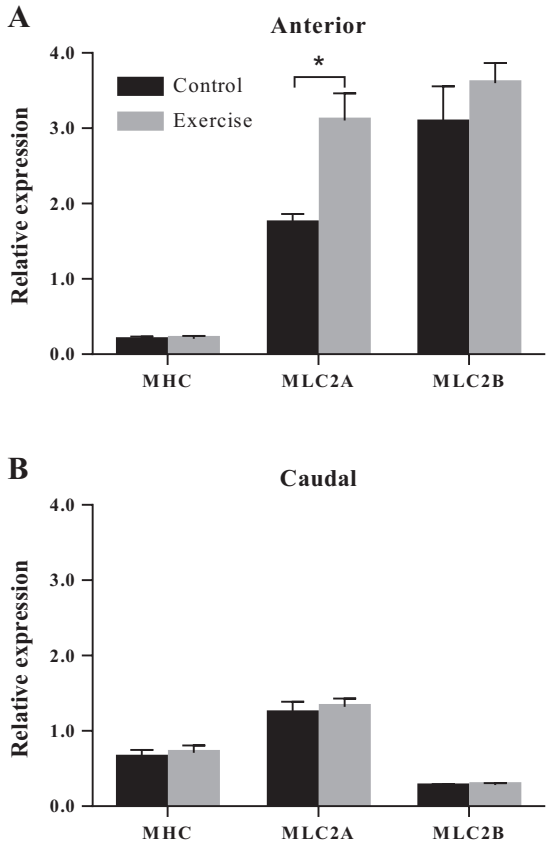


Fig. 3. Effects of exercise on structural marker gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of MHC, MLC2A, and MLC2B in the anterior muscle (*A*) and caudal muscle (*B*) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control conditions. Data are shown as means ± SE ($n = 12$). *Significant difference, $P < 0.05$.

anterior muscle region of the fish, such as CAPN1, CAPN2, CAPNs1a and CAPNs1b, CTSDb, muscle RING finger protein 1 (MuRF1), MAFbx, and proteasome β type 4 subunit (N3; Fig. 4*A*). Nevertheless, in the caudal region the expression of all these molecules remained stable, except for the expression of CAPN2 being downregulated in exercised fish (Fig. 4*B*). These results are also supported by Western blot analysis. Thus the protein expression of CAPN1 and CTSD was significantly upregulated in the anterior region of exercised fish compared with control fish (Fig. 5, *A* and *B*); meanwhile, no differences were found for any of these molecules in the caudal muscle region. On the other hand, the protein levels of the ubiquitin ligase MAFbx were not modified in either muscle region (Fig. 5*C*).

After validation of the specificity of the VEGF antibody using the immunization peptide to block the signal (Fig. 6*A*), Western blot results showed that swimming activity significantly increased the protein expression of VEGF in the anterior muscle, whereas no differences were found in the caudal region (Fig. 6*B*).

## DISCUSSION

The effects of exercise have been investigated in different fish species (7, 12, 25, 43, 58, 68–71, 94). Our group has demonstrated that moderate and sustained swimming activity increases body weight in rainbow trout and gilthead sea bream with clear changes in metabolism and muscle structure (8, 32, 33, 44, 59). Thus, in these studies, exercise triggered an increase in muscle aerobic capacity with rise in muscle glycogen turnover and muscle mass. Although changes in muscle protein content were not found, a significant decrease in mesenteric fat was observed in exercised gilthead sea bream fingerlings (8). Moreover, muscle structure was also adapted to exercise, with increases in hypertrophic condition and capillarization in the anterior, but not the caudal, muscle region (44). In addition, we have also shown that swimming increases the plasma IGF-I levels (8, 84) and modulates the gene expression of several GH-IGF axis molecules, including hepatic IGF-I, and growth hormone receptor I (GHR-I) in muscle (98). Furthermore, under these conditions the TOR signaling pathway is activated at both gene and protein levels, thus stimulating protein synthesis. All these changes resulted in enhanced growth compared with control fish.

Following this line of research, in the present work we have demonstrated that the gene and protein expression of the cellular proliferation marker PCNA increases in the caudal and anterior muscle regions, respectively, with exercise. PCNA is considered a good marker of proliferation processes (1), and

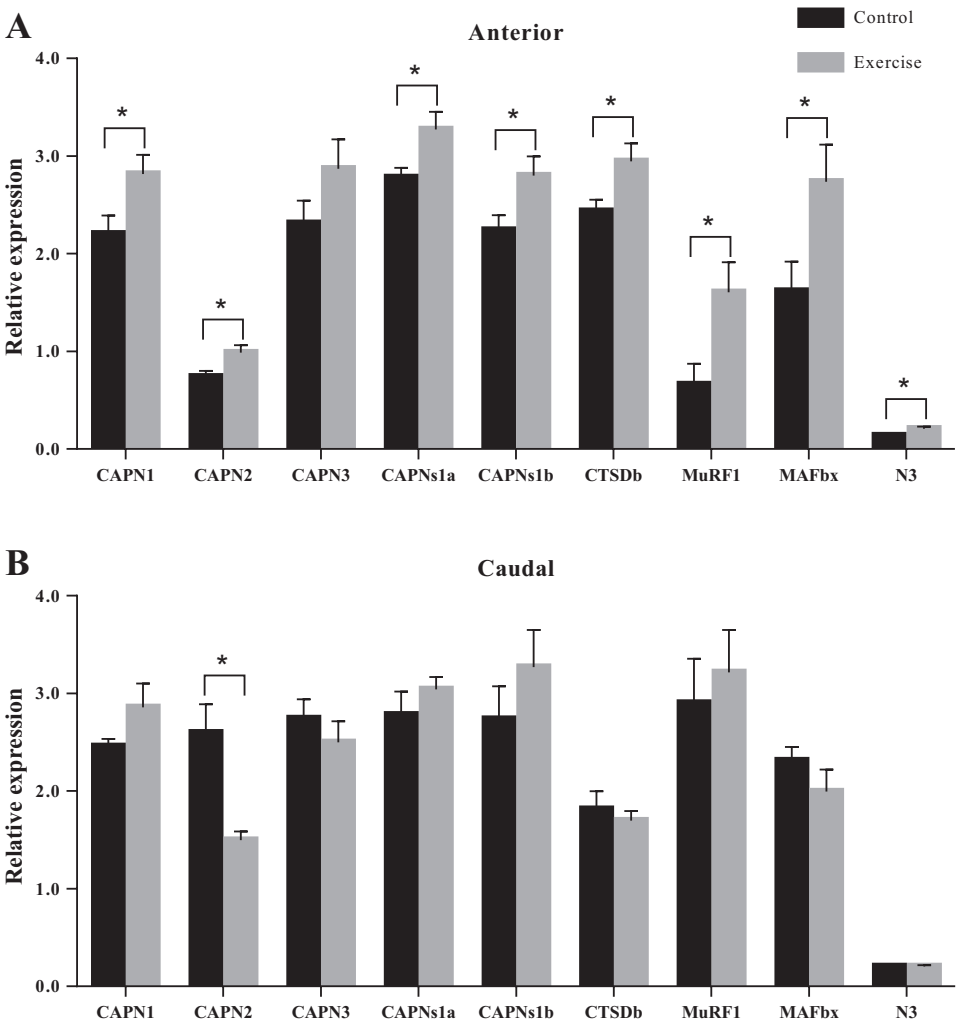


Fig. 4. Effects of exercise on proteolytic marker gene expression. Relative mRNA expression normalized to RPS18 and RPL27a of CAPN1, CAPN2, CAPN3, CAPNs1a, CAPNs1b, CTSDb, MuRF1, MAFbx, and N3 in the anterior muscle (*A*) and caudal muscle (*B*) regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise, or in control conditions. Data are shown as means ± SE ($n = 12$). *Significant difference, $P < 0.05$.

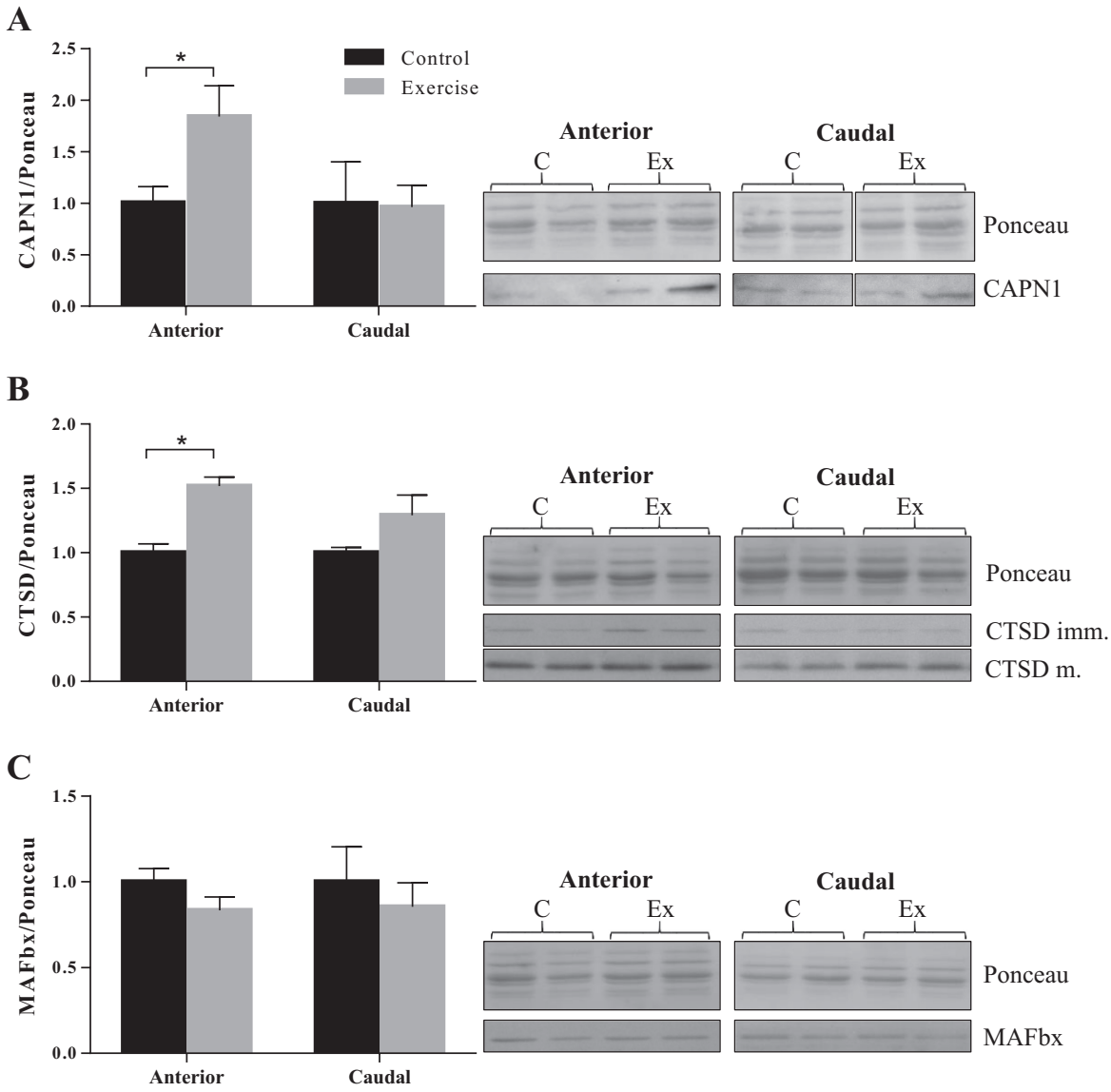


Fig. 5. Effects of exercise on proteolytic marker protein expression. Protein expression of CAPN1 (*A*), CTSD immature (imm.) and mature (m.) forms (*B*), and MAFbx (*C*) in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as means ± SE ($n = 4$). *Significant difference, $P < 0.05$.

we have observed in gilthead sea bream cultured myocytes that the highest levels of PCNA expression coincide with the stage of maximum proliferation (36). Also, we have demonstrated that both IGF-I and IGF-II stimulate the increase of PCNA-positive cells using the same cell model system (78). This concurs with the increase of IGF-II gene expression (98) parallel to that of PCNA in the caudal muscle of exercised fish. The fact that in the anterior muscle, PCNA increased in terms of protein, but not at the mRNA level, whereas in the caudal muscle the response was opposite supports the distinct response to exercise throughout the muscle already observed in our previous studies (44, 98). Moreover, this is in agreement with the longitudinal shifts in contractile properties caused by the different combination and coordination of a great variety of proteins in the white muscle of several fish species (19, 20, 26, 100), which provide faster contractile properties to the anterior muscle region (22). Nevertheless, with only these data, we cannot conclude there to be a different proliferative activity between the two muscle regions, and it becomes necessary to analyze other growth-regulating molecules such as inhibitors, transcription factors, or structural muscle proteins. Furthermore, differences between gene and protein expression along the myogenic process have already been reported (3) and should be taken into account to understand this differential PCNA response.

In terms of growth repression, MSTN is the main inhibitor of muscle development in vertebrates (51, 79). In fish, it has been observed that fasting increases MSTN gene expression whereas refeeding decreases it (36, 64). In gilthead sea bream, as in other fish species, two main paralogs are expressed in muscle (MSTN1 and MSTN2), which can exert their function depending on physiological conditions (35, 54, 55). This is the case in this study, where only MSTN2 gene expression decreased in the anterior muscle of exercised fish but no response

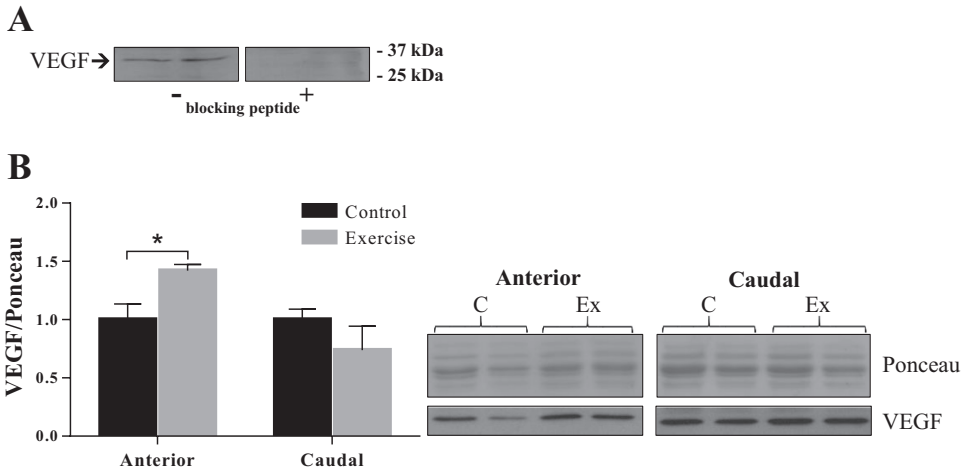


Fig. 6. Effects of exercise on angiogenesis marker protein expression. *A*: Western blot for VEGF specificity validation with muscle protein extract pooled samples and blocking immunization peptide. *B*: protein expression of VEGF in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 wk of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as means ± SE ($n$ = 4). *Significant difference, $P < 0.05$.

of MSTN gene expression was observed in the caudal region, supporting the different effect of exercise all through the muscle in fish. The significant decrease in the anterior muscle of MSTN2 would suggest the possibility that in this region the muscle is in a state of remodeling because of exercise, as we previously suggested because of the differential increase of GHR-I, IGF-I, and IGFBP-5 observed in this region (98).

In mammals, exercise determines diverse effects on myogenesis depending on its intensity and duration, which can modify the expression of MRFs, structural molecules, and IGFs (41, 101). Palstra and colleagues (70, 71) showed that zebrafish trained to perform intense exercise upregulate in muscle the expression of several MRFs, and Hasumura and Meguro (43) in the same species demonstrated very recently that short but intense exercise is able to induce the expression of MyoD and myogenin. Contrarily, in this study, 5 wk of sustained and moderate exercise in gilthead sea bream did not importantly change MRFs transcript levels. Only the increase of MyoD1 gene expression in the caudal muscle area was significant, which could indicate the initiation of the determination stage of myogenesis in this part of the muscle that would later extend to the anterior muscle region. We should not forget that this is a dynamic process and our results only reflect a snapshot from the time of sampling. In any case, the upregulation of MyoD1 agrees with the increase in IGF-II gene expression observed in these fish (98), corroborating the stimulatory effect of this growth factor on MyoD expression previously demonstrated in gilthead sea bream myocytes (46).

With regard to the structural myofibrillar molecules, little effect was observed in the gene expression of the different myosins during exercise, with the exception of MLC2A, whose expression was increased in the anterior muscle. This coincides with the upregulation of MLC gene expression observed in the white muscle of rainbow trout (58) and zebrafish (70) under sustained swimming. Very recently, this molecule has been related to the intensive stratified formation of new fibers during muscle growth in larvae of gilthead sea bream (38). Moreover, in concordance with previous studies, it has been suggested that MLC2A is more involved in larval growth (mainly hyperplasic growth) whereas MLC2B is more related to the hypertrophic growth that takes places in the adult stages (39, 67). Similarly, Silva et al. (89) also suggested previously that MLC could be a good marker of hyperplasia in blackspot sea bream. Overall, this is in agreement with the increase in PCNA protein expression and the downregulation of MSTN2 mRNA observed in this study and with the upregulation of IGFs and GHR-I described before in anterior muscle (98), suggesting that this part of the muscle might be in a proliferative state.

The proteolytic systems play a key role in muscle, and in mammals it is known that they are necessary for normal day-to-day function. During exercise, proteolysis is increased, proteolysis being a requirement for the remodeling and regeneration processes that occur in response to activity (24, 53, 76, 77, 102). Thus the calpain, cathepsin, and proteasome systems, including the genes *MuRF* or *MAFbx* among others, will be activated during exercise (4, 24, 27, 77, 102). In gilthead sea bream, we have recently characterized several members of the calpains, cathepsins, and ubiquitin-proteasome families and their response to nutritional changes (82, 83). However, available information on fish proteolytic systems in relation to swimming is scarce, especially in this species. Besides, as introduced before, it has been shown that in contrast to mammals, the autophagy-lysosomal system (in which cathepsins are included) has a more important contribution than the ubiquitin-proteasome system on the total protein degradation in rainbow trout myotubes (85). In the present study, the anterior muscle showed a clear activation of all proteolytic systems, with the significant upregulation of most of the molecules studied. Such a uniform response also observed in the protein expression of CAPN1 and CTSD is a good indication of the proteolytic process that may be taking place in the anterior muscle, but not the caudal one, where the unique change observed was the decrease of CAPN2. In fact, muscle expression of calpains and cathepsins increased during the mobilization accompanying fasting in gilthead sea bream (82, 83). The upregulation of the calpain system observed in the present study can thus be related to two different situations known to occur in mammals. First, before myoblast fusion, it is necessary to remodel the membrane of myogenic cells at the fusion point through breaking the cytoskeletal/plasma membrane linkages (4, 42, 65), and CAPN2 is involved in this. Second, CAPN1 is more associated with the cleavage of different cytoskeletal proteins to smaller fragments (4, 102), which can be later degraded by the ubiquitin-proteasome system or cathepsins, as would be expected in fish (85) and as confirmed in this study by the increased protein levels of CTSD. Parallel to these molecules,

exercise determined, in the anterior muscle, the increase of MuRF1 and MAFbx, two muscle-specific ubiquitin ligases that participate in the last step of ubiquitination, normally induced in muscle atrophy (17). Both enzymes are frequently used as markers of muscle proteolysis in fish (9, 15, 83, 86). However, the interpretation of these results should be cautious, since at least in mammals, MAFbx is thought to exert specific control regulating MRFs such as MyoD, whereas MuRF regulates myofibrillar and sarcomeric stability (93, 102). In this sense, these results are consistent with the increased expression observed for these two molecules together with MyoD and myogenin in exercised zebrafish (43). Similarly, the β-type proteasome subunit N3 was also elevated in the anterior muscle of exercised gilthead sea bream in the present study. In this species, it has been recently demonstrated that during the greater growth stage along ontogeny (i.e., fingerlings), the fish have higher proteolysis rates than during juvenile and adult stages and, in parallel, also have higher rates of protein synthesis, producing in combination enhanced growth (83). Altogether, these data support the observation that the anterior muscle, differently from the caudal muscle, was subjected during swimming to an important remodeling.

Furthermore, the effects reported in the proteolytic systems are in agreement with the increase in PCNA protein expression and the decrease in MSTN2 gene expression in the anterior muscle. Moreover, this is also coincident with the upregulation of VEGF only in this muscle region. Although information on VEGF regulation in fish (45) is not as broad as in mammals (76, 101), VEGF is considered a good marker of muscle remodeling regulating the increase of capillary supply necessary for the new myofibers produced. Thus the observed VEGF increase in this study is another demonstration of the transformation process that is occurring in the anterior muscle region of exercised fish. Changes in capillarization were already observed in gilthead sea bream under equivalent exercise conditions. Thus, whereas in control conditions the caudal muscle showed more capillarization than the anterior muscle, in exercised fish the capillaries in the anterior muscle increased to equal the values in caudal muscle (44). Similarly, Palstra and colleagues (70, 71) reported that in zebrafish, swimming promotes an increase in muscle hypertrophy and vascularization accompanied by upregulation of several genes including calpains and VEGF. Preliminary immunohistochemistry results have indicated that in our trial, exercised fish show a general increase also, in muscle hyperplasia and capillarization (A. Moya, J.R. Torrella, J. Fernández-Borràs, D. Rizo, A. Millán-Cubillo, E.J. Vélez, A. Ibarz, J. Gutiérrez and J. Blasco, unpublished data). Vélez et al. (98) found in anterior muscle a significant increase of both TOR gene and protein expression, which reinforces the existing equilibrium between protein degradation and synthesis; this protein balance was further confirmed by the conservation of the protein content observed in these fish (8). Overall, this is consistent with the expected muscle remodeling as effect of exercise (60).

### *Perspectives and Significance*

In the present study, after 5 wk of moderate and sustained swimming in gilthead sea bream fingerlings, the significantly increased PCNA and MyoD1 gene expression in the caudal muscle region indicates the cell proliferation and differentiation condition, which correlates well with the higher gene expression levels of IGF-II and IGF-I in this region previously observed in these fish (98). Besides, in the anterior muscle region, a remodeling condition seems to exist that is confirmed by the increased gene expression of most proteolytic markers and VEGF. This overactivation of proteolytic systems corresponds with the increase in protein turnover (cathepsins and ubiquitin-proteasome) and structural changes (calpains) that are necessary for the tissue renovation and new vessel formation that take place during exercise adaptation.

Overall, the present study contributes to improving knowledge of the growth-promoting effects of swimming in fish, demonstrating for the first time the role of several proteolytic factors and the growth factor VEGF in gilthead sea bream muscle remodeling, which can help to optimize fish growth and flesh quality in this important aquaculture species and confirms the use of moderate sustained swimming as an excellent intervention for achieving this goal.

## ACKNOWLEDGMENTS

The authors thank Carlos Mazorra from Tinamenor and the personnel from the facilities at the School of Biology for the maintenance of the fish.

## GRANTS

E. J. Vélez and E. Lutfi are supported by a predoctoral fellowship from the Ministerio de Ciencia e Innovación (MICINN). This study was supported by MICINN Projects AGL2012-39768 and AGL2015-70679-R to J. Gutiérrez and Xarxa de Refèrencia d'R+D+I en Aqüicultura, SGR2009-00402, and 2014SGR-01371 from the Generalitat de Catalunya.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

J.F.-B., J.B., and J.G. conceived and designed research; E.J.V., Sh.A., E.L., and E.C. performed experiments and laboratory analyses; E.J.V., Sh.A., E.L., E.C., A.M., I.N., J.F.-B., J.B., and J.G. analyzed data; E.J.V., Sh.A., E.L., E.C., A.M., I.N., J.F.-B., J.B., and J.G. edited and revised manuscript; E.J.V., Sh.A., E.L., E.C., A.M., I.N., J.F.-B., J.B., and J.G. approved final version of manuscript.

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General and Comparative Endocrinology 257 (2018) 192–202

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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Research paper

# Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*)

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ARTICLE INFO

*Article history:*
Received 20 January 2017
Revised 16 June 2017
Accepted 22 June 2017
Available online 27 June 2017

*Keywords:*
*Sparus aurata*
GH
IGF-I
Growth regulation
Skeletal muscle
Vertebra bone

ABSTRACT

The growth hormone (GH)/insulin-like growth factors (IGFs) endocrine axis is the main growth-regulator system in vertebrates. Some authors have demonstrated the positive effects on growth of a sustained-release formulation of a recombinant bovine GH (rBGH) in different fish species. The aim of this work was to characterize the effects of a single injection of rBGH in fingerlings of gilthead sea bream on growth, GH-IGF axis, and both myogenic and osteogenic processes. Thus, body weight and specific growth rate were significantly increased in rBGH-treated fish respect to control fish at 6 weeks post-injection, whereas the hepatosomatic index was decreased and the condition factor and mesenteric fat index were unchanged, altogether indicating enhanced somatic growth. Moreover, rBGH injection increased the plasma IGF-I levels in parallel with a rise of hepatic mRNA from total IGF-I, IGF-Ic and IGF-II, the binding proteins IGFBP-1a and IGFBP-2b, and also the receptors IGF-IRb, GHR-I and GHR-II. In skeletal muscle, the expression of IGF-Ib and GHR-I was significantly increased but that of IGF-IRb was reduced; the mRNA levels of myogenic regulatory factors, proliferation and differentiation markers (PCNA and MHC, respectively), or that of different molecules of the signaling pathway (TOR/AKT) were unaltered. Besides, the growth inhibitor myostatin (MSTN1 and MSTN2) and the hypertrophic marker (MLC2B) expression resulted significantly enhanced, suggesting altogether that the muscle is in a non-proliferative stage of development. Contrarily in bone, although the expression of most molecules of the GH/IGF axis was decreased, the mRNA levels of several osteogenic genes were increased. The histology analysis showed a GH induced lipolytic effect with a clear decrease in the subcutaneous fat layer. Overall, these results reveal that a better growth potential can be achieved on this species and supports the possibility to improve growth and quality through the optimization of its culture conditions.



## 1. Introduction

The growth hormone (GH)/insulin-like growth factors (IGFs) axis is the most important endocrine system regulating growth in vertebrates, and this premise has also been demonstrated in fish (reviewed in: Fuentes et al., 2013; Picha et al., 2008; Reindl and Sheridan, 2012; Vélez et al., 2017). GH and IGFs stimulate somatic growth through binding their corresponding receptors (GHR and IGF-IR, respectively) widely distributed among different tissues, including muscle and bone (Le Roith et al., 2001; Reindl and Sheridan, 2012). The activation by IGFs of the PI3K-AKT-TOR (Target Of Rapamycin) signaling pathway in those tissues regulates the expression of a high number of genes involved in cell survival, proliferation and differentiation (e.g. myogenic regulatory factors (MRFs), proliferating cell nuclear antigen (PCNA), myostatin (MSTN)) among others leading to increased growth (Fuentes et al., 2013; Vélez et al., 2017).

In Mediterranean aquaculture, one of the most important cultured species is gilthead sea bream (*Sparus aurata*). In this species,

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http://dx.doi.org/10.1016/j.ygcen.2017.06.019
0016-6480/

the GH/IGF axis has been well studied (Benedito-Palos et al., 2007; Calduch-Giner et al., 2003; Pérez-Sánchez, 2000; Saera-Vila et al., 2005; Vélez et al., 2016b). The *in vivo* treatment with GH significantly increases the hepatic gene expression of IGF-I and IGF-II (Carnevali et al., 2005) and also has hypoosmoregulatory effects decreasing sodium levels and modifying the hepatic glucose and lipid metabolism (Sangiao-Alvarellos et al., 2006). In *in vitro* studies, GH treatment increases myocytes proliferation and it does it at a higher level when accompanied by IGF-I (Rius-Francino et al., 2011). Moreover, this combination also stimulates the gene expression of IGF-I and two MRFs implicated in cell lineage determination and proliferation, MyoD2 and Myf5 (Azizi et al., 2016; Jiménez-Amilburu et al., 2013). Besides, direct incubation with IGF-I or IGF-II also stimulates proliferation of myocytes and osteoblasts (Capilla et al., 2011; Rius-Francino et al., 2011; Vélez et al., 2014). Overall, these studies confirm both systemic and local paracrine/autocrine actions for GH and IGFs in gilthead sea bream.

In aquaculture research, some strategies further from optimizing diet have been satisfactory in order to increase growth (reviewed in: Moomsen and Moon, 2001). This is the case of, for example, the use of anabolic steroids to stimulate protein synthesis, amino acids uptake and nitrogen retention (Higgs et al., 1982; Zohar, 1989), or the use of $\beta_2$-adrenergic agonists to heighten muscle protein while reducing muscle fat composition in rainbow trout and blue catfish (Salem et al., 2006; Webster et al., 1995). Another interesting option for growth enhancement has been the application of forced swimming (Palstra and Planas, 2013), as observed for example in gilthead sea bream, where exercise modulates the GH/IGF axis to potentiate a better physiological condition (Blasco et al., 2015; Sánchez-Gurmaches et al., 2013; Vélez et al., 2016a).

Moreover, when considering farm animals, an important background exists on beef cattle treated with a sustained-release formulation of recombinant bovine GH (rBGH), which results in increased dry matter intake, milk production, feed efficiency and protein deposition into carcass components, etc. (Dalke et al., 1992; Dohoo et al., 2003). These interesting effects made some authors to experiment with rBGH in aquaculture research with the purpose of improving growth in some fish species. Such treatment with rBGH, which has been found to remain at detectable levels up to 140 days post-injection in coho salmon (McLean et al., 1997); and despite Leedom et al. (2002) demonstrated in tilapia that heterologous rBGH has 100-fold less affinity to the GH binding site than homologous GH, has been successfully used in rainbow trout (Biga et al., 2005, 2004a,b; Devlin et al., 2001; Gahr et al., 2008; Garber et al., 1995; Kling et al., 2012), coho and chinook salmon including transgenic coho salmon (McLean et al., 1997; Raven et al., 2012), tilapia (Kajimura et al., 2001; Leedom et al., 2002; Wille et al., 2002), different lines of catfish (Peterson et al., 2005, 2004), zebrafish and giant danio (Biga and Goetz, 2006; Biga and Meyer, 2009; Simpson et al., 2000), or more recently, sturgeon (Fenn and Small, 2015). In these studies, rBGH treatment resulted in an increase in body weight (B.W.), IGF-I plasma levels, gene expression of IGF-I in different tissues such as liver or muscle, and caused a regulation of its receptors.

However, up to date there is no information about the effects of a prolonged-release treatment with rBGH in gilthead sea bream. Therefore, the aim of this work was to analyze the effects of a single rBGH-injection in fingerlings of this species through analyzing the changes caused in somatic growth, the plasma levels of IGF-I, the expression of GH/IGF axis-, myogenic- and osteogenic-related molecules in liver, white muscle and/or bone, and the histology of muscle. All this was performed in order to expand the knowledge of GH/IGF axis and growth regulation in fish with the further intention to know how far we are from a maximum growth in this important aquaculture species.

## 2. Material and methods

### 2.1. Experimental animals and ethical statement

Two-hundred fingerlings (initial B.W. 1.01 ± 0.05 g; total body length (T.L.) 4.30 ± 0.02 cm) of gilthead sea bream (*Sparus aurata* L.) obtained from a commercial hatchery in the north of Spain were reared in the facilities of the Faculty of Biology at the University of Barcelona. Fish were distributed into 8 cages of 37 L such that two cages were put together in a larger thank (4 tanks of 200 L, each cage with 25 fish and thus, 50 fish/tank). Throughout the whole experiment, fish were maintained in a sea water recirculation system at a temperature of 23 ± 1 °C, and were fed *ad libitum* (10% B.W./day) five times a day with a commercial diet (Gemma Diamond, Skretting, Burgos, Spain). The light cycle was 15 L:9D h. After an acclimation period, the two cages of each tank were assigned randomly to a treatment group and the fish were anesthetized (MS-222 0.08 g/L) and intraperitoneally injected with a single dose (4 mg $g^{-1}$ B.W.) of rBGH (Posilac®, Elanco Animal Health, Eli Lilly and Company) diluted 1:4 with sesame oil (Sigma-Aldrich, Spain), or with the same volume of sesame oil for the controls (day 0). The rBGH dose used was chosen following the previous work of Raven et al. (2012). Biometric parameters were monitored at times 0, 2, 4 and 6 weeks. In the final sampling (6 weeks), 20 fish from each cage (80 fish per condition) were anesthetized and blood samples were taken from caudal vessels using heparinized syringes. Then fish were killed, and liver and adipose tissue were weighed to calculate both hepatosomatic (HSI) and mesenteric fat (MFI) indexes. The condition factor (C.F.) and the specific growth rate (SGR) were calculated for each tank biomass (n = 4 for each condition). Then, from three fish per tank samples of liver, anterior epaxial white muscle and vertebra (bone tissue) were collected, frozen in liquid nitrogen, and stored at −80 °C until further analysis (n = 12 for each condition). Additionally, small samples of muscle with integument from five individuals of each group were taken and immediately fixed in 10% buffered formalin (Sigma-Aldrich, Spain) for posterior histological analysis.

Before each manipulation of the fish, the food was held for 12 h and the fish were properly anesthetized as indicated above. All procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona following the European Union, Spanish and Catalan Governments-assigned principles and legislations (permit numbers CEEA 209/14 and DAAM 7957).

### 2.2. IGF-I plasma levels

Blood was centrifuged at 1500×*g* for 15 min and then plasma was collected and stored at −80 °C. Afterwards, the plasma IGFs were extracted by acid-ethanol cryoprecipitation (Shimizu et al., 2000) and the IGF-I concentration was measured using a generic IGF-I radioimmunoassay (RIA) with recombinant bream (*Pagrus auratus*) IGF-I as a tracer and standard, and polyclonal rabbit anti-barramundi (*Lates calcarifer*) IGF-I serum as a first antibody, both purchased from GroPep Bioreagents (catalogue numbers #YU020 and #PAF1, respectively). This heterologous RIA has been previously validated for Mediterranean perciform fish with a sensitivity of 0.05 ng/mL and a mid-range of 0.7–0.8 ng/mL (Mingarro et al., 2002; Vega-Rubín de Celis et al., 2004).

### 2.3. Gene expression analyses

#### 2.3.1. RNA extraction and cDNA synthesis

Total RNA extraction was performed from 30 mg of liver, or 100 mg of tissue in the case of white muscle and bone samples in 1 mL TRI reagent solution (Applied Biosystems, Alcobendas,

Spain) following the manufacturer's instructions. RNA concentration and purity was determined with a Nanodrop 2000 (Thermo Scientific, Alcobendas, Spain). RNA integrity check was performed with a 1% agarose gel stained with SYBR-Safe DNA gel stain (Life Technologies, Alcobendas, Spain). In order to eliminate all genomic DNA, prior to cDNA synthesis, a DNase I (Life Technologies, Alcobendas, Spain) treatment of 1 μg of total RNA was performed following the manufacturer's recommendations. Finally, reverse transcription was carried out using the Transcriptor First Strand cDNA synthesis kit (Roche, Sant Cugat, Spain) following the manufacturer's instructions.

*2.3.2. Real time quantitative-PCR (q-PCR)*

Gene expression (mRNA) analyses were performed by q-PCR in a CFX384 real-time system (Bio-Rad, El Prat de Llobregat, Spain) as described by Vélez et al. (2016a). All the primer sequences for the analyzed genes are showed as Supplementary material (S.1 and S.2) and can be found in previous literature (Pinto et al., 2001; Rosa et al., 2010; Vélez et al., 2016a, 2014, 2012). The genes can be grouped in: a) GH/IGF axis [total IGF-I and its three splice variants: IGF-Ia, IGF-Ib and IGF-Ic; IGF-II; the IGF binding proteins: IGFBP-1a, IGFBP-2b, IGFBP-4, IGFBP-5b; the receptors: IGF-IRa and IGF-IRb; GHR-I, GHR-II]; b) myogenesis [MRFs: MyoD1, MyoD2, Myf5, myogenin and MRF4; Proliferating Cell Nuclear Antigen (PCNA); Myostatins: MSTN1, MSTN2; Myosin Heavy Chain (MHC) and Myosin Light Chain (MLC2A and MLC2B)]; c) signaling pathways [TOR; translation initiation factor 4E Binding Protein 1 (4EBP1); Ribosomal protein S6 Kinase (70S6K); AKT; Forkhead box O3 (FOXO3)]; d) osteogenesis [Runt-related transcription factor 2 (RUNX2); type 1 Collagen subunit 1-A (COL1A); Fibronectin subunit 1-A (F1B1A); Osteonectin (ON); Osteopontin (OP); Osteocalcin (OSTC); Matrix Gla Protein (MGP) and Tissue Non-specific Alkaline Phosphatase (TNAP)]; and e) reference genes [Ribosomal Protein S18 (RPS18), Ribosomal Protein L27a (RPL27a) and Elongation Factor 1α (EF1α)]. The expression levels of each gene were calculated using the Bio-Rad CFX Manager 3.1 software by the Pfaffl method (Pfaffl, 2001) relative to the geometric mean expression of the most stable genes analyzed (EF1α and RPS18). The relative expression of each gene in the rBGH condition was represented as the fold change over the control fish (log2).

*2.4. Western blot*

To obtain protein extracts from white skeletal muscle samples, tissue homogenization was performed from 100 mg per sample in 1.5 mL RIPA Buffer supplemented with phosphatase (PMSF 1 nM and $Na_3VO_4$ 0.2 mM, Sigma-Aldrich, Tres Cantos, Spain) and protease inhibitors (Protease Inhibitor Cocktail Sc-29136, Santa Cruz Biotechnology, Inc. Santa Cruz CA., USA) using the Precellys® Evolution Homogenizer combined with Cryolys® as a cooling system (Bertin Technologies, France). Protein concentration was determined by the Bradford method (Bradford, 1976) and then, 20 μg of protein per sample were separated by SDS-PAGE electrophoresis on 10% polyacrylamide gels at 125 V for 1 h 30 min. After electrophoresis, proteins were transferred to Immobilon-FL PVDF membranes (Merck Chemicals & Life Science S.A., Madrid, Spain). Prior to membrane blocking, total transferred protein was determined by REVERT™ Total Protein Stain (Li-Cor, Alcobendas, Spain) following the manufacturer's instructions and scanned with an Odyssey® FC Imaging System (Li-Cor, Alcobendas, Spain) through the 700 nm channel. After total protein quantification, the membranes were blocked 1 h 15 min in 5% non-fat milk buffer. Following blocking, the membranes were properly washed and incubated with the respective primary antibodies for the phosphorylated forms of AKT and TOR at 1:200 dilution. The selected antibodies were: rabbit polyclonal anti-phospho AKT (cat. No. 9271), and rabbit polyclonal anti-phospho mTOR (Cat. No. 2971), both obtained from Cell Signaling Technology (Beverly, MA). After washing, the membranes were incubated with peroxidase-conjugated secondary antibody: goat anti-rabbit (Cat. No. 31460) from Thermo Scientific, Alcobendas, Spain. Immunoreactive bands were developed by using an enhanced chemiluminiscence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain) and the signal detected with the Odyssey® FC Imaging System (Li-Cor, Alcobendas, Spain). Once phosphorylated forms were analyzed, bound antibodies were removed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Alcobendas, Spain) and then the membranes were blocked and blotted again against the total forms of the studied proteins at 1:500 dilution (rabbit polyclonal anti-total AKT (Cat. No. 9272, Cell Signaling Technology) and rabbit polyclonal anti-total mTOR (Cat. No. T2949, from Sigma-Aldrich, Spain)). The different bands were quantified by Odissey software Image Studio ver. 5.2.5.

*2.5. White muscle histology*

For microscopic preparations, the fixed tissues were dehydrated in a graded ethanol series (70%, 80%, 90% and 100%) and embedded in paraffin blocks. The blocks were sectioned at 5 μm with a rotary microtome (pfm, ROTARY 3003, Köln, Germany). Paraffin sections of muscle tissue including subcutaneous adipose tissue and skin were stained with hematoxylin and eosin and studied by light microscopy (Olympus) at 10× magnification. The average area (FCSA), perimeter (FPER), shape factor (SF), small diameter (Feret) and the density of fibers (FD) were obtained using ImageJ software (National Institutes of Health, Bethesda, MD, USA) from three pictures of each animal (n = 5). In addition to fibers morphological parameters analysis, the subcutaneous fat content was compared between groups by visual examination.

*2.6. Statistical analyses*

Data were analyzed using the IBM SPSS Statistics v.22 software and are presented as means ± SEM. Data normality and homoscedasticity through groups was checked by the Shapiro-Wilk test followed by Levene test. Then, a two-way ANOVA was used to confirm that a tank effect does not exists for any of the variables analyzed. Next, the differences between the two experimental groups in body weight through time were analyzed by two-way ANOVA followed by a Student's *t*-test comparison (n = 4 for each condition). The other somatic growth parameters, gene expression and western blot data, were analyzed by a Student's *t*-test (n = 12 per condition, except for HSI and MFI (n = 80), western blot (n = 8) and histology (n = 5)). Differences were considered significant at $p < 0.05$.

## 3. Results

*3.1. Biometric and plasma parameters*

Fig. 1 represents the mean B.W. of control and rBGH injected fingerlings at 0, 2, 4 and 6 weeks post-injection. Although both groups of fish significantly increased their B.W. with time, the rBGH injected fish presented a significantly higher B.W. only at 6 weeks (9.5%) compared to control fish. Besides B.W., other somatic growth index results at 6 weeks post-injection are shown in Table 1. The specific growth rate (SGR) at that time was significantly higher in rBGH injected fingerlings with respect to the control fish, whereas the hepatosomatic index (HSI) decreased. Nevertheless, the observed higher T.L. in rBGH injected fingerlings, and both condition factor (C.F.) and the mesenteric fat index (MFI)

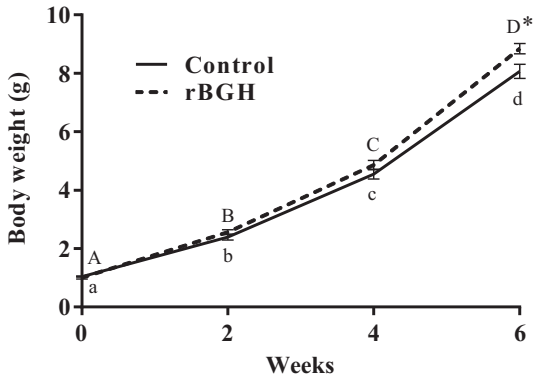


**Fig. 1.** Mean body weight of control and rBGH-treated gilthead sea bream fingerlings at 0, 2, 4 and 6 weeks post-injection. Values are represented as mean ± SEM (n = 4). Letters (control, lower case; rBGH, uper case) indicate time effects calculated by two way ANOVA ($p < 0.05$). Significant differences between control and rBGH groups determined by Student's *t*-test are marked with $^{*}(p < 0.05)$.

values, were not statistically different compared to control animals. Moreover, significantly higher IGF-I plasma levels at 6 weeks post injection were detected in the rBGH injected fish.

### 3.2. GH-IGFs axis gene expression in liver, white muscle and bone tissue

The relative expression results of the main GH-IGFs axis-related genes analyzed in liver, muscle and vertebral-bone tissues at 6 weeks post-injection are shown in Fig. 2. In hepatic tissue, the rBGH injection caused a significant increase in the expression of total IGF-I, IGF-Ic, IGF-II, IGFBP-1a, IGFBP-2b, IGF-IRb, GHR-I and GHR-II (Fig. 2A). In the case of white muscle, the rBGH treatment caused a significant up-regulation of IGF-Ib and GHR-I, and a significant down-regulation of IGF-IRb gene expression (Fig. 2B). In bone tissue, the rBGH injected fingerlings presented significantly lower levels of expression of IGF-Ib, IGF-Ic, and both IGF-I receptors (IGF-IRa and IGF-IRb) compared to the control fish (Fig. 2C). Regarding GH expression, the mRNA was only above detectable

**Table 1**
Somatic growth parameters and plasma IGF-I levels in control and rBGH- treated gilthead sea bream fingerlings at 6 weeks post-injection. SGR: Specific Growth Rate% = 100 × (ln final B.W. − ln initial B.W.) days$^{-1}$; T.L: Total length; C.F: Condition Factor = 100 × B.W./T.L$^{3}$; HSI: Hepatosomatic Index = g liver × 100 g B.W.$^{-1}$; MFI: Mesenteric Fat Index = g fat × 100 g B.W.$^{-1}$; [IGF-I]: IGF-I plasma levels. Values are presented as mean ± SEM for SGR, TL and CF (n = 4), HSI and MFI (n = 80), and IGF-I levels (n = 20). Significant differences are indicated with $^{*}(p < 0.05)$ or $^{***}(p < 0.001)$.

| | SGR | T.L (cm) | C.F | HSI | MFI | IGF-I (ng/mL) |
|---|---|---|---|---|---|---|
| Control | 4.87 ± 0.08 | 8.06 ± 0.10 | 1.52 ± 0.01 | 1.56 ± 0.03 | 1.47 ± 0.07 | 64.37 ± 3.87 |
| rBGH | 5.80 ± 0.07$^{*}$ | 8.31 ± 0.08 | 1.50 ± 0.01 | 1.42 ± 0.03$^{***}$ | 1.32 ± 0.07 | 76.75 ± 4.44$^{*}$ |

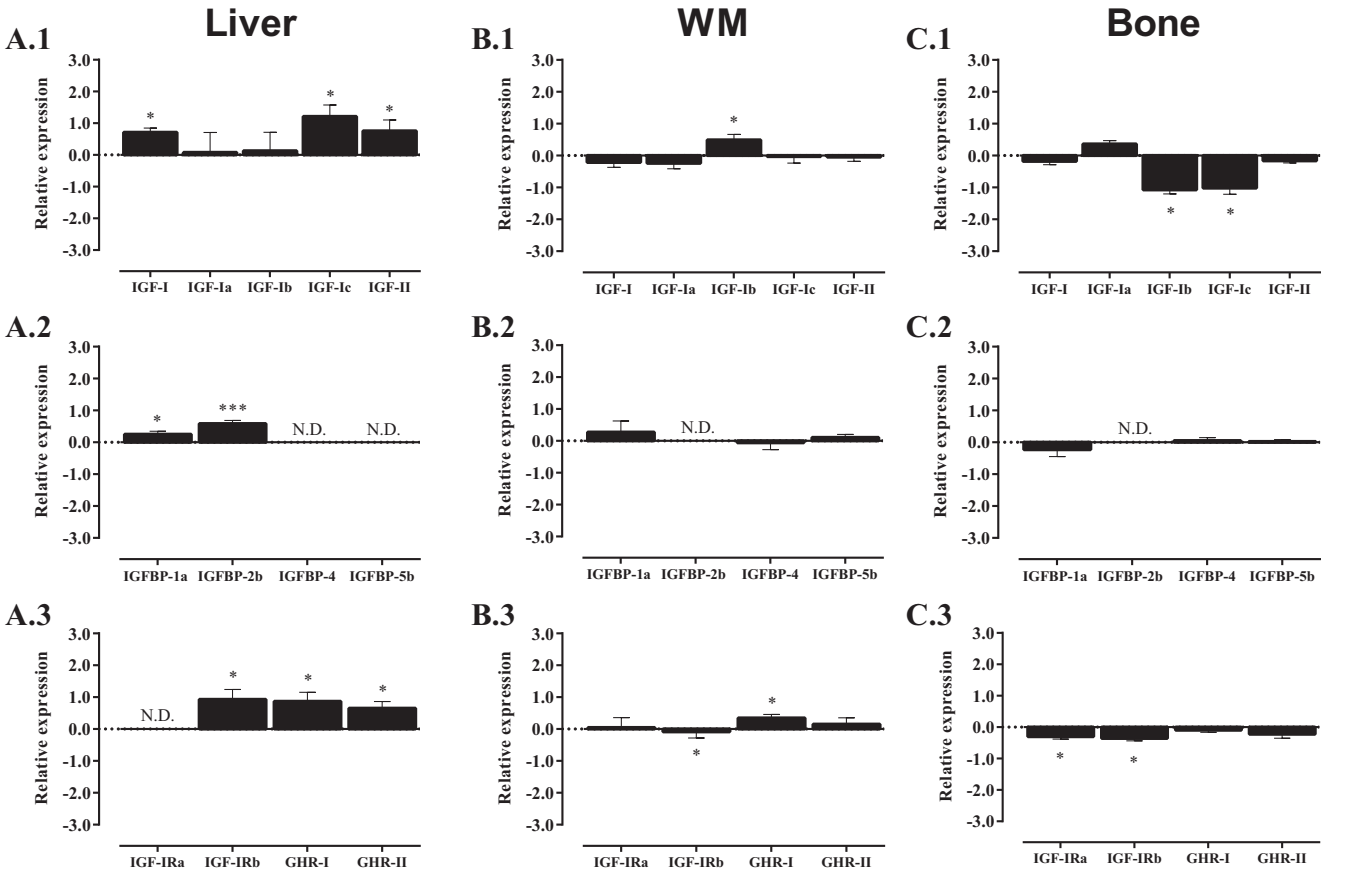


**Fig. 2.** Effects of the rBGH treatment in gilthead sea bream fingerlings over the expression of the main GH-IGFs axis-related genes in liver (A), white muscle (B) and bone (C) at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of total IGF-I, IGF-I spliced variants and IGF-II (A.1, B.1 and C.1); IGF-I binding proteins (A.2, B.2 and C.2.); IGF-I and GH receptors (A.3, B.3 and C.3). Data are shown as fold change (log2) relative to controls as mean ± SEM (n = 12). Significant differences between control and rBGH groups for each gene were determined by Student's *t*-test and are marked with $^{*}(p < 0.05)$ or $^{***}(p < 0.001)$. WM, white muscle; N.D., non-detected.

levels in muscle tissue, although no differences were found between groups (data not shown).

### 3.3. Muscle growth markers and AKT-TOR pathway gene expression and signaling

Regarding the expression of the main genes involved in the myogenic process (MyoD1, MyoD2, Myf5, myogenin and MRF4; data not shown), and the proliferation marker PCNA (Fig. 3A), significant alterations due to rBGH treatment were not observed. On the other hand, significant up-regulation of myostatin 1 and 2 (MSTN1, MSTN2) and myosin light chain 2B (MLC2B) gene expression was detected as effect of rBGH treatment in the fingerlings of gilthead sea bream (Fig. 3A). With regards to the signaling pathways studied, no differences were found in TOR, AKT and their principal downstream effectors in terms of gene expression (Fig. 3B). However, the protein expression results showed a significant decrease of TOR phosphorylation and a stable levels of AKT phosphorylation in rBGH-treated fish at 6 weeks of experiment compared to control fish (Fig. 3C, D).

### 3.4. Ostogenesis-related genes expression in bone tissue

In bone tissue, the rBGH treatment affected the expression of some of the main osteogenic genes analyzed, detecting significant up-regulation of osteopontin (OP), osteocalcin (OSTC), type 1 collagen subunit 1α (COL1A) and tissue non-specific alkaline phosphatase (TNAP) (Fig. 4).

### 3.5. White muscle histology

Analysis of histological white muscle tissue slides revealed that the rBGH treatment did not affect significantly muscle fiber

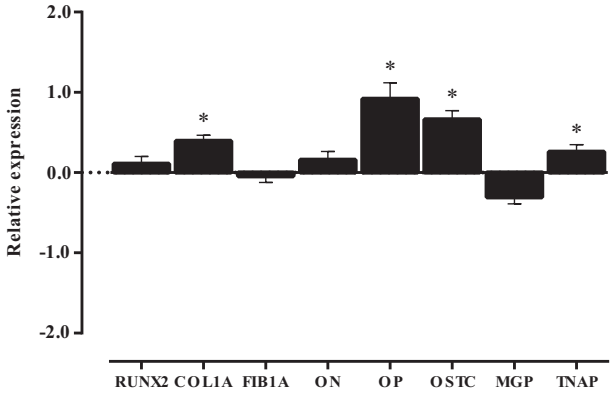


**Fig. 4.** Effects of the rBGH treatment over the expression of the main osteogenesis-related genes in gilthead sea bream bone tissue at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of RUNX2 (Runt-related transcription factor 2), COL1A (type 1 Collagen subunit 1-A), FIB1A (Fibronectin subunit 1-A), ON (Osteonectin), OP (Osteopontin), OTC (Osteocalcin), MGP (Matrix Gla Protein) and TNAP (tissue non-specific alkaline phosphatase). Data are shown as fold change (log2) relative to controls as mean ± SEM (n = 12). Significant differences between control and rBGH groups for each gene were determined by Student's *t*-test and are marked with *($p < 0.05$).

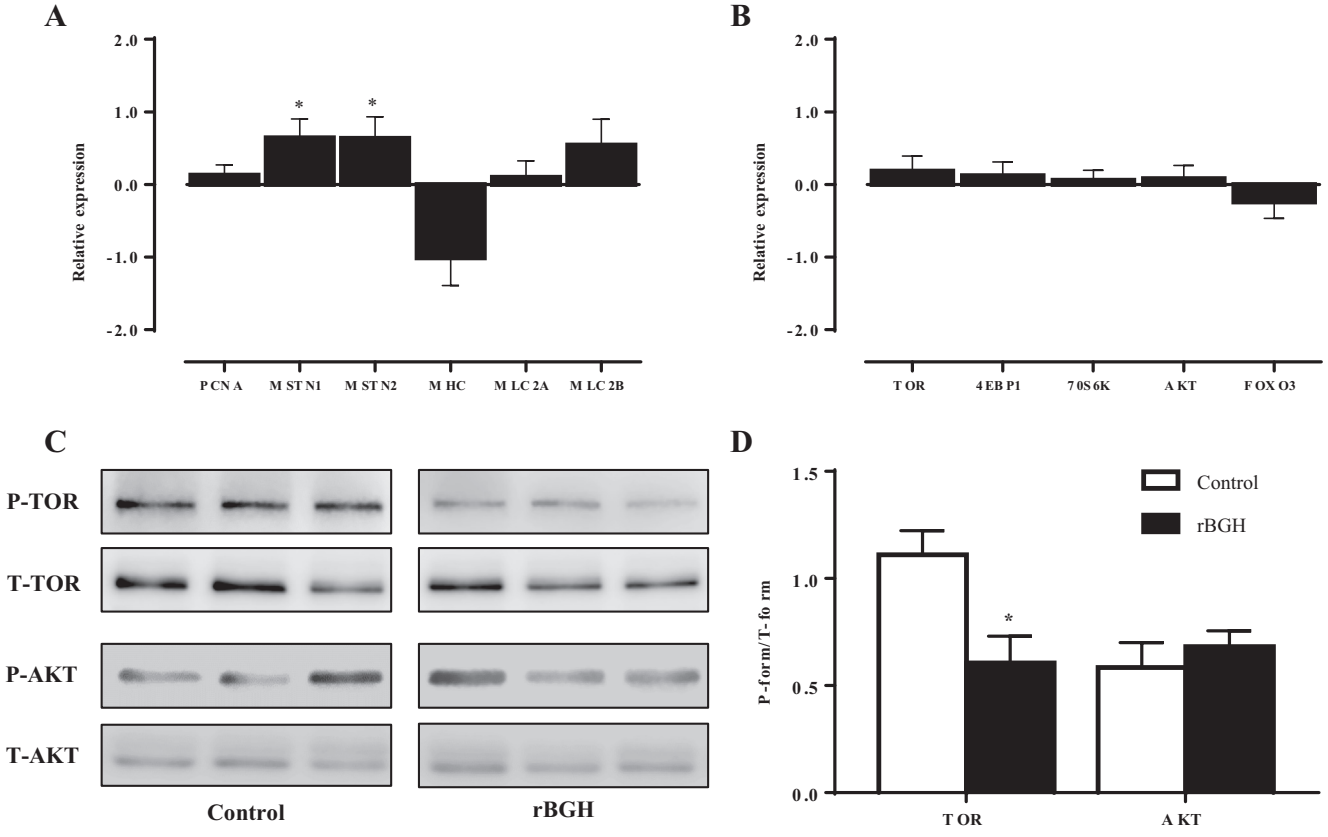


**Fig. 3.** Effects of the rBGH treatment over the expression of growth markers genes and signaling pathways in white muscle of gilthead sea bream at 6 weeks post-injection. Relative mRNA expression normalized to EF1α and RPS18 of (A) proliferation and differentiation markers (PCNA, MSTN1 (Myostatin 1), MSTN2 (Myostatin 2), MHC (Myosin Heavy Chain), MLC2A (Myosin Light Chain 2-A), MLC2B (Myosin Light Chain 2-B) and (B) AKT/TOR signaling molecules (TOR, 4EBP1, 70S6K, AKT, FOXO3). (C) Representative western blot and quantification of phosphorylated and total forms of TOR (P-TOR and T-TOR, respectively) and AKT (P-AKT and T-AKT) in control and rBGH-injected fingerlings. For A and B, data are shown as fold change (log2) relative to controls as mean ± SEM (n = 12). For C, data are represented as mean ± SEM (relative expression of the phosphorylated form of each molecule normalized to their total form, n = 8). Significant differences were determined by a Student's *t*-test and are indicated with *($p < 0.05$).

**Table 2**
Morphometric fiber parameters in white muscle of control and rBGH-treated gilthead sea bream fingerlings at 6 weeks post-injection. FCSA: fiber cross-sectional area. FPER: fiber perimeter. SF: shape factor (circularity). Feret: small diameter of the fibers. FD: fiber density. Values are represented as mean ± SEM (n = 5; 3 images/animal).

| | FCSA (μm2) | FPER (μm) | SF | Feret (μm) | FD (fibers/mm2) |
|---|---|---|---|---|---|
| Control | 1898.13 ± 266.02 | 164.30 ± 12.03 | 0.72 ± 0.01 | 63.09 ± 4.33 | 429.53 ± 28.03 |
| rBGH | 1685.51 ± 251.31 | 161.31 ± 15.75 | 0.68 ± 0.03 | 65.42 ± 7.63 | 412.75 ± 56.54 |

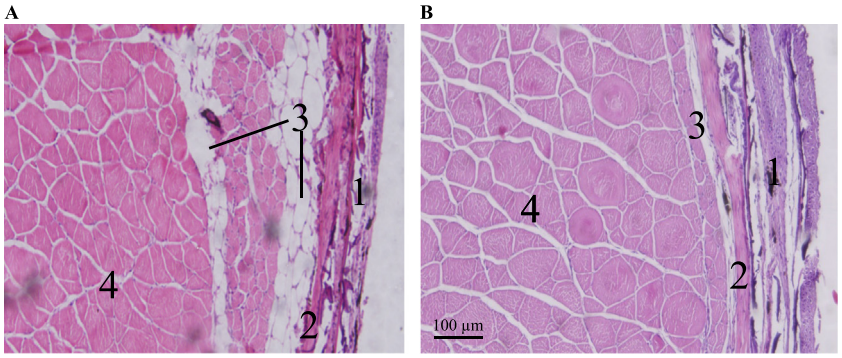


**Fig. 5.** Effects of the rBGH treatment over the white muscle histology of gilthead sea bream at 6 weeks post-injection. Representative images of a hematoxylin/eosin staining of muscle histological slides of (A) control and (B) rBGH gilthead sea bream fingerlings after 6 weeks post-injection. 1. Fish skin thin section, 2. Dermis, 3. Subcutaneous fat content, 4. Skeletal muscle. Scale bar 100 μm.

morphometrical parameters (Table 2), but it caused a visual reduction of subcutaneous adipose tissue content in the muscle of rBGH treated fingerlings in comparison with control fish (Fig. 5).

## 4. Discussion

As existing literature reports, rBGH injection has been proven to be a valid approach to increase growth in various fish species (Biga and Goetz, 2006; Biga and Meyer, 2009; Biga et al., 2005, 2004a,b; Devlin et al., 2001; Fenn and Small, 2015; Gahr et al., 2008; Garber et al., 1995; Kajimura et al., 2001; Kling et al., 2012; Leedom et al., 2002; McLean et al., 1997; Peterson et al., 2005, 2004; Raven et al., 2012; Simpson et al., 2000; Wille et al., 2002). The aim of the present study was to assess for the first time in gilthead sea bream fingerlings the effects on growth of such sustained-release formulation of rBGH. In this aspect, the significant increase obtained in B.W. and SGR, together with the reduction of HSI as effect of rBGH treatment in this species, is in agreement with those previous observations. Moreover, similarly to the present study, alteration of the C.F. was neither reported in channel catfish by Peterson et al. (2004) or coho salmon by Raven et al. (2012), thus confirming that the growth increase obtained is due mainly to somatic growth. However, a decrease on this parameter together with a reduced MFI was observed in rainbow trout (Kling et al., 2012), indicating that the effects of rBGH injection depend on multiple factors, such as species, age, type of injection (single or multiple repeated injections) or dose, among others.

### 4.1. Effects of rBGH treatment on circulating GH and IGF-I

A positive correlation between IGF-I levels and SGR in the rBGH-treated fish has been observed in different studies (Biga et al., 2005; Gahr et al., 2008; Kajimura et al., 2001; Peterson et al., 2005; Raven et al., 2012). This effect is also observed in the present work, in which a 19.23% increase of circulating IGF-I coincides with a 19.1% increase in SGR at 6 weeks post-injection. Regarding GH, although analysis of neither exogenous rBGH or endogenous gilthead sea bream GH plasma levels was possible in this study, based on previous works in trout (McLean et al., 1997; Biga et al., 2005) and mainly in tilapia (Leedom et al., 2002), as well as the IGF-I plasma levels reported here, we consider that the levels of rBGH rise in plasma just 12 h after treatment (Biga et al., 2005), and remain high at least until the final days of the trial. In fact, Leedom et al. (2002) using a dose of rBGH four times lower than that in our study (1 mg/g B.W.) found that after 70 days of treatment, the rBGH plasma levels were very high (around 200 ng/ml), more than 10-fold higher than the normal GH levels in tilapia. Furthermore, this species is quite close phylogenetically to gilthead sea bream and is also cultured at a similar temperature, thus it makes us to assume a similar releasing rate for the injected rBGH. Moreover, with regards to the stability of rBGH in the circulation, Biga et al. (2005) demonstrated that with time, rainbow trout produce antibodies to clear the exogenous rBGH, and similarly Leedom et al. (2002) showed that treatment with rBGH induces increased IgM levels in tilapia. Nevertheless, these same authors also demonstrated that the half-life of rBGH derived from the Posilac® formulation was more than 5-fold the half-life of injected rBGH dissolved in saline, and that the homologous GH was cleared from the circulation faster than the heterologous hormone (Leedom et al., 2002). Altogether, these data suggest that after an initial peak in circulating rBGH, such levels decreased; however, even at the end of the experiment might still be much higher than the GH plasma levels present in the control conditions, knowing that circulating GH levels are on average 5–15 ng/mL in this species (Pérez-Sánchez et al., 1995; Mingarro et al., 2002; Blasco et al., 2015). Concerning endogenous GH, Raven et al. (2012) found that the rBGH treatment decreases the plasma levels of GH in coho salmon after 14 weeks post-injection (and a similar response was observed with regards to GH mRNA levels in the pituitary when normalized by B.W.). In support of this observation, in a similar experiment as the present one with gilthead sea bream juveniles, we have also found a clear down-regulation of pituitary GH gene expression. However, in rainbow trout and also in tilapia endogenous GH plasma levels were not modified by rBGH treatment in a shorter term (Biga et al., 2005; Leedom et al., 2002). These results suggest a differential species- and time-specific response to rBGH that may be also caused by differences in the affinity of GH receptors to rBGH. Overall, these data indicates that important changes occur in response to rBGH with these

circulating growth factors and points out the key role of the GH/IGF axis in somatic growth regulation.

### 4.2. Effects of rBGH treatment on GH/IGF axis gene expression

In the present work, the increased plasma IGF-I concentration agrees with the higher total IGF-I mRNA levels detected in liver, in concordance to previous studies (Biga et al., 2005, 2004b; Gahr et al., 2008; Peterson et al., 2005; Raven et al., 2012, 2008; Shamblott et al., 1995). More specifically, the rBGH treatment induced the overexpression of IGF-Ic, known as the most important splice variant in this species in terms of contributing to circulating IGF-I levels (Tiago et al., 2008). Besides IGF-I, the overexpression of IGF-II and GHR-I and GHR-II genes was in agreement with that observed in liver of rainbow trout by Gahr et al. (2008) and Shamblott et al. (1998), or gilthead sea bream by Carnevali et al. (2005), suggesting in these conditions a clear hepatic response to GH. Furthermore, these results are consistent with that found during the summer growth spurt in gilthead sea bream, where the circulating levels of GH, IGF-I, as well as the hepatic expression of GHR and IGF-I were increased (Calduch-Giner et al., 2003; Saera-Vila et al., 2007). As it is known in mammals, the GHR transcript encodes for a full length GHR that leads to the functional GHR. Then, this transcript can be post-transcriptionally truncated to generate a GHR with no intracellular signal transduction domains or GH binding proteins (GHBPs). These GHBPs can regulate bioavailability and half-life of circulating GH (Fuentes et al., 2013). Therefore, the up-regulation of GHRs expression when GH is elevated could be a transcriptional way to suppress the excess of GH signaling, as suggested by Saera-Vila et al. (2005) in this species, or in rainbow trout by Gahr et al. (2008). Regarding IGFBPs, IGFBP-1a and IGFBP-2b are recognized in zebrafish as inhibitors of IGF-I cellular effects (Duan et al., 2010, 1999) and are considered molecules with growth-inhibitor effects in fish due to that they are usually overexpressed under catabolic conditions (Kelley et al., 2001). In the case of the present study, the overexpression found for IGFBP-2b is in agreement with previous studies in GH-treated fish species (Breves et al., 2014; Shimizu et al., 2003, 1999), which together with the increase in IGFBP-1a, it could suggest a strategy to compensate/regulate the increased levels of IGF-I.

In white muscle the expression of the GH-IGF axis-related genes was less affected. Previous studies using rBGH treatment or GH overexpressing lines, as reviewed by Fuentes et al. (2013), demonstrated stimulation of growth not only by increasing IGF-I expression in the liver, but also acting directly over muscle. In this sense, while GH-transgenesis or GH treatment in different species increases muscular expression of IGF-I (Biga and Meyer, 2009; Devlin et al., 2009; Eppler et al., 2007; Raven et al., 2008), this is not occurring or only slightly in others (Gahr et al., 2008; Kuradomi et al., 2011; Raven et al., 2012). In the present work, albeit no differences were found in the muscular expression of total IGF-I as effect of rBGH-treatment, the expression pattern of each IGF-I splice variant is in agreement with a previous study in gilthead sea bream myocytes, where a GH treatment increased the expression of IGF-Ib without detectable changes in IGF-Ia or IGF-Ic (Jiménez-Amilburu et al., 2013). Hence, these results confirm that in muscle tissue IGF-Ib is the most sensitive form to hormonal treatments in this species. Furthermore, the observed GHR-I up-regulation in muscle as effect of rBGH treatment was also observed in coho salmon (Raven et al., 2008) and it suggests that the GH anabolic effects may be induced in this tissue directly through the activation of this receptor. In the case of GHR-II, transcriptional and promoter analyses indicate that this receptor isoform is more responsive than GHR-I to nutritional (Benedito-Palos et al., 2007) and environmental (Saera-Vila et al., 2009, 2007) stressors, which was substantiated herein by the lack of muscle response to rBGH injection. On the other hand, the decrease in IGF-IRb expression detected in muscle could be interpreted as a mechanism to regulate the effects of the increased IGF-I circulating levels. This mechanism of down-regulation has been previously observed in IGF-I binding capacity or gene expression in different fish tissues and species (Baños et al., 1997; Biga et al., 2004b; Leibush and Lappova, 1995; Moon et al., 1996; Plisetskaya et al., 1993; Vélez et al., 2016a).

Furthermore in bone, the gene expression results present a similar pattern to that observed in white muscle, especially regarding total IGF-I, IGF-II, IGF-Rs and IGFBPs. In contrast with that, the splice variant IGF-Ib and also IGF-Ic, are down-regulated, suggesting that the GH effects over the GH-IGF system in the three tissues may not completely coincide, being more pronounced in bone due to a first or more powerful action of GH over this tissue.

### 4.3. Effects of rBGH treatment on growth markers and AKT-TOR pathway in muscle

During the last decade, a large number of studies (using both *in vivo* and *in vitro* models) have focused on describing the processes that regulate muscular growth in several fish species and how different conditions may affect them. The state of the art of this research has been recently well reviewed (Fuentes et al., 2013; Vélez et al., 2016c), concluding that a direct link between the GH-IGF system and myogenesis exists. Notwithstanding, the rBGH treatment did not significantly cause any effect regarding the MRFs and other muscular proliferation and differentiation markers (e.g. PCNA and MHC, respectively) at this time. In previous studies from our group with cultured gilthead sea bream myocytes, GH and IGF-II increased MyoD2 and Myf5 gene expression, while IGF-I increased myogenin and MRF4 gene expression (Azizi et al., 2016; García de la serrana et al., 2014; Jiménez-Amilburu et al., 2013; Vélez et al., 2014). Moreover, PCNA and myogenin protein expression increased significantly after different combinations of IGFs and GH treatment at day 4 of culture (Rius-Francino et al., 2011; Vélez et al., 2014); whereas Azizi et al. (2016) using the same model did not observe an increase in PCNA gene expression but did find a stimulation of MHC gene expression after IGF-I treatment. Overall, this indicates that the effects observed can vary depending the stage of myogenesis of the muscle under study. In this sense, it is important to note that we were only able to analyze samples at a single time point after the rBGH treatment *in vivo*, and thus, the expression pattern of these regulatory factors may have varied substantially within a few days.

Furthermore, rBGH-treated fingerlings presented a significant up-regulation of both growth inhibitors MSTN1 and MSTN2, which coincides with that observed for MSTN1a in rainbow trout at three days post injection with rBGH (Biga et al., 2004a; Gahr et al., 2008), or in GH transgenic coho salmon (Overturf et al., 2010). It seems clear that after a prolonged GH exposure there is a tendency to regulate MSTN expression (especially MSTN1) to limit the GH growth promoting effects. Regarding MLC2, the two isoforms (MLC2a and MLC2b) expressed in gilthead sea bream have proven to be very important in skeletal muscle development and therefore, are recognized as good markers of muscle growth in this species. Briefly, it has been observed that MLC2a expression predominates during the hyperplastic stages (where myogenic cells proliferation predominates) and down-regulates progressively once hypertrophy appears, when MLC2b expression rises (Georgiou et al., 2016, 2014). Moreover, Georgiou et al. (2011) concluded that MLC2b has an important role in skeletal muscle growth in gilthead sea bream juveniles, when hypertrophy predominates. Concerning endocrine regulation, Moutou et al. (2009) determined that in ovine recombinant GH-treated gilthead sea bream juveniles, MLC2a resulted up-regulated 1 day post injection, whereas no

effect over MLC2b expression was detected. In this scenario, in the present work we observed that rBGH treatment caused a significant MLC2b up-regulation; while MLC2a was unaffected. Thus, it seems that rBGH may have stimulated muscle growth (a process starting with hyperplasia) during the first days post injection but at the sampling time, the muscular growth may be switching to the hypertrophic phase. This affirmation is concordant with the absence of changes observed in the expression of the proliferative marker PCNA and with the up-regulation of both MSTNs, as previously indicated (Georgiou et al., 2016).

Treatment with rBGH did not affect significantly the gene expression of the AKT-TOR pathway and its downstream molecules. GH transgenic coho salmon showed up-regulation of the gene expression of several protein-synthesis related molecules, including members of the mTORC1 signaling pathway such as 4EBP1 isoforms (García de la serrana et al., 2015). However, the incubation of gilthead sea bream myocytes with IGF-I, did not determine differences in TOR or AKT gene expression (Azizi et al., 2016; Vélez et al., 2014). At a protein level, we observed that the rBGH treatment provoked a diminution of TOR phosphorylation, whereas no changes were detected in AKT. The absence of an effect in AKT phosphorylation in muscle agrees with the significantly lower expression of IGF-IRb found in this tissue and the decrease in TOR activation that would suggest a reduction in protein synthesis. In general, it is possible to link these results with the physiological state of the white skeletal muscle at the time of sampling, when a situation of negative feedback and proliferative growth inhibition to counteract the effects induced by activation of the GH/IGF axis during the first weeks post injection could have been happened.

### *4.4. Effects of rBGH treatment on osteogenesis-related genes expression*

Distinct studies have demonstrated that the osteogenic process can be stimulated by endocrine factors in mammals (reviewed in Hall, 2015). For example, IGF-I and IGF-II in cultured mammalian bone cells induce proliferation and increase type-I collagen deposition (Prisell et al., 1993; Strong et al., 1991) acting through the same signaling pathways as in muscle (Grey et al., 2003; Li et al., 2009). Although the information about the direct effects of GH treatment on bone tissue in fish is very scarce, the osteoblastogenesis process has been demonstrated to be similar to that in mammals (Ytteborg et al., 2012). Moreover, in gilthead sea bream the proliferative effects of IGF-I have been also demonstrated in cultured osteoblasts (Capilla et al., 2011). In the present study, rBGH treatment increased the expression of the extracellular matrix structural marker COL1A and the mineralization-related molecules OP and OSTC, suggesting improved osteogenesis. Thus, the gene expression results in this tissue fit with these previous findings and reinforce the idea that both white muscle and bone respond to GH treatment in order to support an enhanced harmonic musculoskeletal growth.

### *4.5. Effects of rBGH treatment on white skeletal muscle histology*

There is very little information about the direct effects of GH treatment on muscle histology in gilthead sea bream, and most of data is from GH-transgenic models (Johnston et al., 2014; Kuradomi et al., 2011). Besides, in a long treatment with bovine-GH intramuscular injection in Grass Pickerel an important increase in somatic growth, together with a significant increase in white muscle fiber diameter (hypertrophy) was achieved after 40 weeks (Weatherley and Gill, 1987). Very similar results were obtained recently by Sciara et al. (2011) in juvenile pejerrey after a weekly oral administration of recombinant pejerrey-GH during 6 weeks. However, the present histological analysis did not show significant differences in muscle fiber morphology because of rBGH treatment in gilthead sea bream fingerlings, suggesting a transition from hyperplastic to hypertrophic muscular growth at the time of sampling in agreement with the gene expression results. Thus, for the rBGH dose used, it appears that more time would have been necessary in order to achieve substantial effects over muscular histology. Finally, and as expected considering that GH has a well-known lipolytic effect (Albalat et al., 2005; Cruz-Garcia et al., 2011; Sheridan, 1986), the histological sections from rBGH-treated fingerlings presented an important reduction of the subdermal fat layer.

## 5. Conclussions

In the present work we have deepened in the effects on growth of a 6 weeks treatment with a sustained release rBGH formulation on fingerlings of gilthead sea bream, one of the most important fish species of Mediterranean aquaculture. The experimental treatment resulted in an important increase in B.W. without changes in the C. F., thus indicating is due to somatic growth. Moreover, the liver was positively responding to rBGH treatment by up-regulating GH-IGF axis gene expression, which in turns is reflected in the increased plasma levels of IGF-I. By contrast, the most important extra hepatic tissues in terms of somatic growth, white skeletal muscle and vertebra bone seemed to have responded positively to the high IGF-I levels (caused by rBGH) during the first weeks post injection. In this sense, the results in bone are very interesting since, on one hand reinforce the hypothesis that rBGH stimulates somatic growth, and on the other hand encourage to further work to elucidate the possible crosstalk with muscle. Altogether, the results obtained in the present study demonstrate that the maximum growth potential of such valuable species has not yet been reached via domestication, selection, breeding, etc., and that GH-IGF optimization approaches are excellent ways to obtain it without a loss in quality.

## Acknowledgments

The authors would like to thank Carlos Mazorra from Tinamenor S.L. and the personnel from the facilities at the School of Biology for the maintenance of the fish. Posilac was kindly provided by Miguel Abad from Elanco Animal Health, Eli Lilly and Company. E.J.V. and E.L. are supported by a predoctoral fellowship from the "Ministerio de Ciencia e Innovación" (MICINN), and A.M. by the University of Barcelona (APIF-2012). This study was supported by the project AGL2012-39768 (MICINN) to J.G., and the "Xarxa de Refèrencia d'R+D+I en Aqüicultura" and the 2014SGR-01371 from the "Generalitat de Catalunya".

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.06.019.

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