UNIVERSITAT DE BARCELONA

Control of the expression of key genes to spare protein and increase the use of dietary carbohydrates in gilthead sea bream (*Sparus aurata*)

Ania Rashidpour

Aquesta tesi doctoral està subjecta a la llicència ***Reconeixement- NoComercial – SenseObraDerivada 4.0. Espanya de Creative Commons***.

Esta tesis doctoral está sujeta a la licencia ***Reconocimiento - NoComercial – SinObraDerivada 4.0. España de Creative* Commons**.

This doctoral thesis is licensed under the ***Creative Commons Attribution-NonCommercial-NoDerivs 4.0. Spain License***.

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Control of the expression of key genes to spare protein and increase the use of dietary carbohydrates in gilthead sea bream (*Sparus aurata*)

Ania Rashidpour

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

DEPARTAMENT DE BIOQUÍMICA I FISIOLOGIA

SECCIÓ DE BIOQUÍMICA I BIOLOGIA MOLECULAR

PROGRAMA DE DOCTORAT EN BIOTECNOLOGIA

Control of the expression of key genes to spare protein and increase the use of dietary carbohydrates in gilthead sea bream (*Sparus aurata*)

Memòria presentada per Ania Rashidpour per a optar al títol de doctor per la Universitat de Barcelona

Isidoro Metón Teijeiro
Director

María Pilar Almajano Pablos
Director

Ania Rashidpour
Doctorand

Barcelona, 2024

Dedicated to

My Dear Family

I am writing to express my gratitude to you, who are the reason my little heart beats.

My dear mother and father, you have always been my companions and supports throughout my life. When I decided to immigrate to Spain and asked for your help, you accepted my decision without the slightest hesitation. During the seven years away from my homeland, you never left me alone and supported me completely, both financially and spiritually.

My dear sister, you are no less than our parents. During my seven years of studying in Spain, from North America to Europe, I received the love and support of you and your young and lovely family.

My dearest Professor Dr. Isidoro Metón! When I came to your country, I didn't know anyone here, but from the first meeting with you, I realized that I would never be alone here. Your influence in my life was not only through my education, but I also learned many lessons from you. No one knows what will happen in the future, but for the moment that my life continues on this earth, I am indebted to you and your immeasurable kindness.

My dear and gentle Professor Dr. María Pilar Almajano. For me, you are not only a good professor but also a friend who can be counted on for the rest of my life. You are a star!

I would like to thank all the respected professors and kind friends who were by my side with kindness and honesty during this time and who supported me from the moment I entered the university and welcomed me back with open arms, Specially my colleague Yuanbing Wu, Lab technicians Christopher Domínguez Vela and Ignasi Mata Saumell and Agustina Baladrón Pino.

Abstract

Aquaculture raises several concerns about environmental welfare and sustainability. To increase the current knowledge of the control of glucose homeostasis in carnivorous fish, we studied the effect of metformin, an anti-diabetic drug for humans, on serum metabolites, and rate-limiting enzymes in key pathways and lipogenic factors in the liver of gilthead sea bream (*Sparus aurata*). Our findings showed that metformin improved glucose homeostasis in *S. aurata* by counteracting glucose-dependent activation on key enzymes in glycolysis and the citric acid cycle, and the expression of lipogenic factors. In addition to advance in the characterization of the intermediary metabolism of a carnivorous and glucose intolerant fish, the results of this study suggest that metformin reduced gluconeogenesis by decreasing hepatic transdeamination and amino acid entrance into the citric acid cycle and their subsequent use as gluconeogenic substrates. To induce a protein-sparing effect in *S. aurata* by boosting conversion of carbohydrates into lipids, an additional aim of this thesis was to study the effect of chitosan-tripolyphosphate (TPP) nanoparticles complexed with a plasmid expressing the N-terminus of hamster SREBP1a (pSG5-SREBP1a) by periodical intraperitoneal injection (every 4 weeks; 3 doses in total) to *S. aurata* fed diets differing in macronutrient composition. Following 70 days of treatment, chitosan-TPP-pSG5-SREBP1a nanoparticles hugely up-regulated SREBP1a mRNA levels in the liver of *S. aurata*. Consistent with improved conversion of dietary carbohydrates into lipids, overexpression of SREBP1a in the liver increased serum triglycerides and cholesterol as well as hepatic glucose oxidation via glycolysis and the pentose phosphate pathway, while not affecting gluconeogenesis and transamination. Furthermore, up-regulation of SREBP1a significantly increased weight gain, specific growth rate and protein efficiency ratio, while decreased feed conversion ratio even in fish fed a low protein-high carbohydrate diet. In addition to show that chitosan-TPP-DNA nanoparticles constitute an efficient method to express exogenous genes in fish avoiding the use of genetically modified organisms, the results of this study support that periodical administration of chitosan-TPP-DNA nanoparticles to overexpress SREBP1a in the liver enhanced growth performance of *S. aurata* through a mechanism that enabled protein sparing by enhancing metabolization of dietary carbohydrates in a carnivorous glucose intolerant fish.

Resumen

La acuicultura plantea importantes retos ambientales y de sostenibilidad. Para profundizar en el conocimiento del control de la homeostasis de glucosa en peces carnívoros, hemos estudiado el efecto de metformina, fármaco antidiabético para humanos, sobre metabolitos, enzimas clave y factores lipogénicos en hígado de dorada (*Sparus aurata*). El tratamiento con metformina mejoró la homeostasis de glucosa en *S. aurata*, contrarrestando la activación dependiente de glucosa de enzimas clave en glucólisis y ciclo del ácido cítrico, y la expresión de factores lipogénicos. Además de avanzar en la caracterización del metabolismo intermediario de un pez carnívoro intolerante a glucosa, nuestros resultados sugieren que la metformina redujo la gluconeogénesis al disminuir transdesaminación hepática y la entrada de aminoácidos al ciclo del ácido cítrico para su uso como sustratos gluconeogénicos. Para ahorrar proteína en *S. aurata* promoviendo la conversión de carbohidratos en lípidos, un objetivo adicional de esta tesis fue estudiar el efecto de nanopartículas de quitosano-tripolifosfato (TPP) encapsulando un plásmido de expresión del extremo N-terminal de SREBP1a de hámster (pSG5-SREBP1a), mediante inyecciones intraperitoneales periódicas (cada 4 semanas; 3 dosis en total) a doradas alimentadas con diferentes dietas. Tras 70 días, la administración de nanopartículas de quitosano-TPP-pSG5-SREBP1a aumentó considerablemente la expresión de SREBP1a en hígado. Consistentemente con una mayor conversión de carbohidratos de la dieta en lípidos, la sobreexpresión hepática de SREBP1a aumentó triglicéridos y colesterol séricos, así como la oxidación hepática de glucosa mediante glucólisis y la vía de las pentosas fosfato, sin afectar la gluconeogénesis ni la transaminación. Además, la expresíon de SREBP1a aumentó significativamente el peso, la tasa de crecimiento específico y el índice de eficiencia proteica, mientras que disminuyó el índice de conversión alimenticia, incluso en peces alimentados con una dieta baja en proteínas y alta en carbohidratos. Además de demostrar que las nanopartículas de quitosano constituyen un método eficiente para expresar genes exógenos en peces evitando el uso de organismos genéticamente modificados, nuestros resultados indican que la administración periódica de quitosano-TPP-DNA para sobreexpresar SREBP1a en hígado mejoró el crecimiento de *S. aurata*, ahorrando proteína mediante la inducción de la metabolización de los carbohidratos de la dieta en un pez carnívoro intolerante a la glucosa.

Table of contents

List of abbreviations

4E-BP1	4E-Binding protein 1
ACC	Acetyl-CoA carboxylase
ACC1	Acetyl-CoA carboxylase 1
ACC2	Acetyl-CoA carboxylase 2
AGPAT	1-Acyl-sn-glycerol-3-phosphate acyltransferase
ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate-activated protein kinase
AST	Aspartate aminotransferase
ATF4	Activating transcription factor 4
bHLH-Zip	Basic-helix-loop-helix leucine zippers
C/EBPβ	CCAAT/Enhancer-binding protein beta
cALT	Cytosolic alanine aminotransferase
CAT	Catalase
ChREBP	Carbohydrate-responsive element-binding protein
COS	Chitooligosaccharide
CPT1	Carnitine palmitoyltransferase I
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DNL	*De novo* lipogenesis
EF1α	Elongation factor 1α
EPA	Eicosapentaenoic acid
FASN	Fatty acid synthase
FBP	Fructose-1,6-bisphosphatase
FCR	Feed conversion ratio
Fru-2,6-P_2	Fructose-2,6-bisphosphate
Fru-6-P	Fructose-6-phosphate
G6PC	Glucose-6-phosphatase
G6PD	Glucose-6-phosphate dehydrogenase
GCK	Glucokinase
GDH	Glutamate dehydrogenase
GH	Growth hormone
GHR	Growth hormone receptor
Glu-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
GLUT4	Glucose facilitative transporter type 4
GLUT	Glucose transporter
GMO	Genetically modified organism
GPAT	Glycerol-3-phosphate acyltransferase
GPx	Glutathione peroxidase

GT	Glycol chitosan-taurocholic acid
HDL	High-density lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme-A
HSI	Hepatosomatic index
i.p.	Intraperitoneal
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IGFBP	IGF-Binding protein
IMTA	Integrated multitrophic aquaculture
iNOS	Inducible nitric oxide synthase
Insig	Insulin-induced gene protein
LC-PUFA	Long chain omega-3 polyunsaturated fatty acids
LDL	Low-density lipoprotein
lncRNA	Long non-coding RNA
LPL	Lipoprotein lipase
LR	Lipid retention
MDH	Malate dehydrogenase
miRNA	MicroRNA
mTOR	Mechanistic target of rapamycin
OGDH	Oxoglutarate dehydrogenase
PAT	Proton-coupled amino acid transporter
PCK1	Phosphoenolpyruvate carboxykinase
PEI	Polyethylenimine
PEP	Phosphoenolpyruvate
PER	Protein efficiency ratio
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	6-Phosphofructo-1-kinase
PFKFB1	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase liver isozyme
PGC1β	Peroxisome proliferator-activated receptor-gamma coactivator 1β
PGD	Phosphogluconate dehydrogenase
pIgR	Polymeric Ig receptor
PK	Pyruvate kinase
PPAR	Peroxisome proliferator activated receptor
PPP	Pentose phosphate pathway
PR	Protein retention
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
S6K	Ribosomal protein S6 kinase
Scap	SREBP-Cleavage activating protein
SCD	Stearoyl Δ9 fatty acid desaturase

SGLT	Sodium-dependent glucose transporter
SGR	Specific growth rate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNAT	Sodium-dependent neutral amino acid transporter
SOD	Superoxide dismutase
SREBP	Sterol regulatory element-binding protein
SREBP1a	Sterol regulatory element binding protein 1a
TCA	Tricarboxylic acid cycle
TNF-α	Tumor necrosis factor-alpha
TPP	Chitosan-tripolyphosphate
VLDL	Very-low-density lipoprotein

1. Introduction

1.1. Aquaculture

1.1.1. History and Development of Aquaculture

Aquaculture, the controlled cultivation of aquatic organisms, has a rich and diverse history that spans thousands of years. From its humble beginnings in ancient civilizations to the sophisticated operations of the modern era, the evolution of aquaculture mirrors humanity's relentless pursuit of sustainable food sources and economic development. Ancient civilizations, such as the Egyptians, Chinese, and Romans, were among the first to practice rudimentary forms of aquaculture. Evidence suggests that the ancient Egyptians reared fish in managed ponds along the Nile River, while the Chinese cultivated common carp in artificial ponds as early as 2000 BC. These early practices laid the foundation for the development of aquaculture techniques that would later revolutionize food production. Throughout the Middle Ages, monasteries in Europe maintained fish ponds to supplement their diets during periods of fasting. The monks' meticulous management of these ponds contributed to the sustainable production of fish, highlighting the integration of aquaculture with agricultural practices (Smith & Peterson, 2019).

The advent of the Industrial Revolution in the 19th century brought significant advancements to aquaculture. Steam-powered pumps were introduced for water circulation, improving oxygen levels and enhancing fish growth rates. Furthermore, the use of artificial fertilizers revolutionized pond management, boosting productivity and paving the way for more intensive aquaculture practices. The 20th century marked a turning point in the history of aquaculture, with rapid technological advancements and scientific innovations driving its expansion. Researchers in Japan played a pivotal role in the development of intensive aquaculture techniques, particularly in the farming of salmon and trout. These breakthroughs revolutionized the global aquaculture industry, leading to the widespread adoption of modern production methods. Today, aquaculture is a thriving and dynamic industry that encompasses a wide range of species and production systems. From freshwater ponds to offshore fish farms, aquaculture plays a vital role in meeting the world's growing demand for seafood. However, the industry faces numerous challenges, including disease management, environmental sustainability, and genetic improvement of cultured species (Engle et al., 2022).

1.1.2. Culture of *Sparus aurata*

Aquaculture encompasses the cultivation of a wide variety of aquatic species, ranging

from finfish to shellfish and aquatic plants. The selection of species for aquaculture depends on factors such as market demand, environmental suitability, and technological feasibility.

Gilthead sea bream, also known simply as sea bream, is a highly valued marine fish species in aquaculture. Native to the Mediterranean Sea and the Eastern Atlantic Ocean, gilthead sea bream has been cultured for centuries due to its delicious taste, fast growth rate, and adaptability to captivity. In recent years, there has been a significant increase in the global production of gilthead sea bream, driven by growing demand and advancements in aquaculture technology (Mhalhel et al., 2023). Specifically, *S. aurata* is nowadays one of the most cultured marine fishes in Europe, peaking at about 40.5 % of total European marine fish production in 2021 (https://www.fao.org/fishery/en/fishstat). As a carnivorous fish, *S. aurata* makes efficient use of dietary protein for using amino acids as catabolic substrates to produce energy and the synthesis of new proteins. On the contrary, the supply of high carbohydrate-low protein diets markedly reduces growth performance due to the low ability of carnivorous fish to metabolize glucose (Metón et al., 1999; Fernández et al., 2007). However, we previously showed that *S. aurata* tolerates partial substitution of dietary protein by carbohydrates through a metabolic adaptation in the liver that involves enhanced glucose oxidation, decreased gluconeogenesis and weaker transamination capacity (Metón et al., 1999; Metón et al., 2000a; Metón et al., 2000b; Caseras et al., 2002; Fernández et al., 2007; Silva-Marrero et al., 2019). This metabolic flexibility allows for the formulation of cost-effective diets with lower protein content, thereby reducing production costs and environmental impacts associated with excessive protein intake (Pateiro et al., 2020).

However, despite its nutritional versatility, gilthead sea bream still faces challenges related to protein utilization and carbohydrate metabolism. Inefficient utilization of dietary carbohydrates can lead to metabolic disorders and impaired growth performance in cultured fish. Therefore, optimizing the expression of key genes involved in carbohydrate metabolism is essential for maximizing the use of dietary carbohydrates and minimizing protein requirements in gilthead sea bream aquaculture (Feidantsis et al., 2018).

1.1.3. Environmental Impacts of Aquaculture

Aquaculture, while offering significant benefits in terms of food production and economic development, also poses various environmental challenges. The expansion of aquaculture operations can lead to environmental degradation, including habitat destruction,

pollution, and the introduction of invasive species. Studying the relationship between the substances excreted by cultured fish and water pollution is an important topic in environmental biology. For example, nitrogenous waste produced from the supply of high-protein diets to carnivorous fish in culture can lead to water eutrophication and promote excessive growth of microorganisms such as microbes and algae, which in turn can cause hypoxic waters and subsequent impairment of marine ecosystems. Furthermore, chemicals used in the fish farming process can also act as water pollutants. For instance, aquaculture operations can contribute to water pollution through the discharge of excess nutrients, antibiotics, and chemical additives into aquatic ecosystems. Intensive fish farming can result in the accumulation of organic waste and uneaten feed, leading to eutrophication and algal blooms. Additionally, the use of antibiotics and pesticides in aquaculture can pose risks to aquatic organisms and human health through the development of antibiotic-resistant bacteria and the contamination of water sources (Fernández et al., 2007; Ahmad et al., 2022).

Another primary environmental impact of aquaculture is habitat modification and loss. The conversion of natural ecosystems, such as mangroves, wetlands, and estuaries, into aquaculture facilities can disrupt fragile habitats and alter ecosystem dynamics. Clearing mangrove forests for shrimp farming, for example, not only destroys vital breeding grounds for marine species but also increases vulnerability to coastal erosion and storm damage (Talijančić et al., 2019).

In addition, a significant part of total fish production is derived to produce fishmeal and fish oil, which constitute highly nutritious and digestible ingredients for aquafeeds. Therefore, steady growing of the aquculture sector and the subsequent need for aquafeed production results in unsustainable pressure on capture fisheries, which in turn are often overexploited (Naylor et al., 2009).

The escape of farmed fish into the wild can have significant ecological consequences, particularly for native fish populations. Intermixing between escaped farmed fish and wild populations can lead to genetic dilution, loss of genetic diversity, and potential disruption of local adaptation. Furthermore, escaped farmed fish may compete with native species for resources and habitat, further exacerbating biodiversity loss and ecosystem disruption (Alvanou et al., 2023).

The translocation of non-native species for aquaculture purposes can introduce invasive species into new environments, posing threats to native biodiversity and ecosystem stability.

Invasive species may outcompete native organisms, alter food webs, and disrupt ecosystem functions, leading to cascading effects on ecosystem health and resilience (Arechavala-Lopez et al., 2018).

To mitigate the environmental impacts of aquaculture, effective effluent management and waste treatment strategies are essential. Implementing best management practices, such as site selection, waste recycling, and nutrient management, can minimize nutrient loading and reduce the risk of water pollution. Additionally, the development of innovative wastewater treatment technologies, such as biofiltration and constructed wetlands, can help mitigate the environmental footprint of aquaculture operations (Bradbury et al., 2020).

1.1.4. Advances in Aquaculture Technology

Aquaculture technology has undergone significant advancements in recent decades, revolutionizing the industry and enhancing its efficiency, productivity, and sustainability. From innovative breeding techniques to precision aquafeed formulations, these technological developments have played a crucial role in meeting the growing global demand for seafood while minimizing environmental impacts.

Selective Breeding and Genetic Improvement

One of the most significant advances in aquaculture technology is the application of selective breeding and genetic improvement programs to enhance the performance and resilience of cultured species. By selectively breeding individuals with desirable traits such as fast growth, disease resistance, and efficient feed conversion, aquaculture producers can develop genetically superior strains that outperform their wild counterparts. Furthermore, the use of molecular tools, such as marker-assisted selection and genomic selection, has accelerated the pace of genetic improvement, allowing for more precise and targeted breeding strategies (Shah & Mraz, 2020).

Precision Nutrition and Feed Management

Advances in aquafeed formulation and feed management have revolutionized the way fish nutrition is optimized in aquaculture. Precision nutrition approaches take into account the specific nutrient requirements of different life stages and physiological conditions of cultured

species, allowing for tailored feed formulations that maximize growth performance and minimize environmental impacts. Additionally, the integration of alternative feed ingredients, such as plant-based proteins and single-cell proteins, reduces reliance on marine resources and enhances the sustainability of aquafeed production (Kumar et al., 2018).

Environmental Monitoring and Management

Technological innovations in environmental monitoring and management have improved the sustainability and resilience of aquaculture operations. Real-time monitoring systems allow for continuous assessment of water quality parameters, such as temperature, dissolved oxygen, and nutrient levels, enabling prompt response to environmental fluctuations and potential hazards. Furthermore, the use of predictive modeling and remote sensing technologies facilitates proactive decision-making and risk management, optimizing production efficiency while minimizing environmental impacts (Antonucci & Costa, 2020).

Integrated Aquaculture Systems

Integrated aquaculture systems, such as aquaponics and integrated multitrophic aquaculture (IMTA), capitalize on synergies between different aquaculture species and production systems to maximize resource utilization and minimize waste generation. In aquaponic systems, fish farming and hydroponic crop production are integrated in a symbiotic relationship, where fish waste serves as nutrient-rich fertilizer for plant growth, and plants help purify the water for fish. Similarly, IMTA systems combine the cultivation of multiple species, such as finfish, shellfish, and seaweeds, to create a balanced ecosystem that enhances overall productivity and environmental sustainability (Iber & Kasan, 2021).

1.1.5. Challenges and Opportunities in Aquaculture

Aquaculture faces a myriad of challenges and opportunities as it strives to meet the growing global demand for seafood while ensuring sustainability, environmental stewardship, and economic viability. From disease management to market volatility, these challenges present complex obstacles that require innovative solutions. However, amidst these challenges lie opportunities for technological innovation, scientific advancement, and strategic collaboration that can propel the aquaculture industry forward.

Aquaculture operations are subject to various regulatory requirements and standards aimed at ensuring food safety, animal welfare, and environmental protection. Compliance with regulatory frameworks, certification schemes, and international standards can be complex and costly, particularly for small-scale producers and developing countries. Streamlining regulatory processes, enhancing transparency, and fostering international cooperation are crucial for facilitating sustainable aquaculture development (Ottinger et al., 2018).

Challenges

Environmental Sustainability: Aquaculture can have adverse environmental impacts, including habitat degradation, water pollution, and biodiversity loss. Balancing the need for increased food production with environmental sustainability requires innovative approaches to minimize resource use, waste generation, and ecosystem disruption. Implementing best management practices, adopting eco-friendly production systems, and promoting responsible aquaculture practices are essential for achieving environmental sustainability goals (Kurniawan et al., 2021). Research in the field of aquaculture has highlighted the necessity of enhancing our understanding of the molecular and metabolic characteristics of cultured fish, particularly carnivorous species. This knowledge is crucial for optimizing diet formulation and maximizing the efficient utilization of dietary nutrients. In recent years, there has been growing interest in the molecular mechanisms underlying nutrient metabolism and utilization in fish species raised in aquaculture systems. This interest stems from the need to develop sustainable aquafeeds that promote optimal growth, health, and nutrient utilization while minimizing environmental impacts. Several studies have emphasized the importance of investigating the specific dietary requirements and metabolic pathways of carnivorous fish species. Understanding the molecular basis of nutrient metabolism is essential for formulating diets that meet specific nutritional requirements and support the physiological functions. Furthermore, advances in omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, have provided valuable tools for studying the molecular and metabolic responses of cultured fish to dietary interventions. These approaches enable researchers to identify key genes, proteins, and metabolic pathways involved in nutrient digestion, absorption, transport, and utilization, thereby facilitating the development of targeted nutritional strategies for improving feed efficiency and fish performance (Babaei et al., 2016; Silva-Marrero et al., 2019).

Overall, continued research into the molecular and metabolic features of cultured fish,

with a focus on carnivorous species, is essential for advancing our understanding of fish nutrition and aquafeed formulation. By elucidating the intricate mechanisms governing nutrient utilization in these species, we can enhance the sustainability and profitability of aquaculture operations while minimizing environmental impacts.

Disease Management: Disease outbreaks pose a significant threat to aquaculture production, leading to massive economic losses and environmental degradation. Common diseases affecting aquaculture species include viral, bacterial, and parasitic infections. Developing effective disease prevention and control measures, such as vaccination, biosecurity protocols, and genetic resistance breeding, is essential for mitigating disease risks and ensuring the long-term sustainability of aquaculture operations (Aich et al., 2020).

Market Volatility: Aquaculture markets are subject to fluctuating demand, price volatility, and trade uncertainties, posing challenges for producers, investors, and stakeholders. Adapting to changing market conditions, diversifying product portfolios, and exploring niche markets can help mitigate the risks associated with market volatility and enhance the resilience of aquaculture enterprises (Sievers et al., 2022).

Opportunities

Technological Innovation: Advances in aquaculture technology, such as selective breeding, precision nutrition, and environmental monitoring, offer opportunities to improve production efficiency, reduce environmental impacts, and enhance product quality. Investing in research and development, fostering collaboration between academia, industry, and government, and adopting innovative technologies can unlock new opportunities for sustainable aquaculture growth (López-Pedrouso et al., 2020).

Market Expansion: Growing consumer demand for sustainable and nutritious seafood presents opportunities for aquaculture producers to expand their market reach and diversify product offerings. Investing in value-added products, branding, and marketing strategies that emphasize sustainability, traceability, and quality can help differentiate aquaculture products in the global marketplace and capture new market segments (Karim et al., 2020).

Collaboration and Partnerships: Collaborative initiatives and partnerships between governments, industry stakeholders, non-governmental organizations, and research institutions can drive collective action towards addressing common challenges and achieving shared goals in aquaculture development. Establishing platforms for knowledge sharing, capacity building,

and technology transfer can foster innovation, facilitate information exchange, and promote sustainable aquaculture practices worldwide (Tan et al., 2023).

Sustainable Investment: Increasing investment in sustainable aquaculture projects and initiatives can stimulate economic growth, create employment opportunities, and alleviate poverty in rural and coastal communities. Encouraging responsible investment practices, promoting public-private partnerships, and leveraging innovative financing mechanisms, such as impact investing and green bonds, can mobilize capital towards environmentally and socially responsible aquaculture development (Ateweberhan et al., 2018). Overall, aquaculture represents a vital component of the global food system, providing nutritious seafood, generating income and employment, and supporting livelihoods in coastal communities. By adopting sustainable practices and embracing technological advancements, aquaculture can continue to thrive while minimizing its environmental footprint and contributing to food security and economic development (Bandara, 2018).

1.2. Carbohydrate Metabolism in Fish

1.2.1. Carbohydrate Metabolism in Carnivorous Fish

Carbohydrate metabolism plays a crucial role in the nutrition and growth of fish species, including *S. aurata*, which is of great interest in aquaculture due to its economic importance. Carnivorous fish, such as *S. aurata*, use more efficiently amino acids than carbohydrates to produce energy. Indeed, dietary carbohydrates and free sugars are metabolized markedly slower than in mammals, and give rise to prolonged hyperglycemia (Polakof et al., 2012). Dysregulation of rate-limiting enzymes in glycolysis-gluconeogenesis that control the hepatic flux through the glucose/glucose-6-phosphate (Glu-6-P) substrate cycle, glucokinase (GCK) and glucose-6-phosphatase (G6PC), was hypothesized to play a major role in glucose intolerance of carnivorous fish. In this regard, *S. aurata* GCK exhibits lower affinity for glucose than rat GCK and delayed postprandial stimulation of GCK mRNA levels in the liver (Caseras et al., 2000). On the other hand, the content of dietary carbohydrate fails to regulate the hepatic expression of G6PC, which in turn is poorly repressed by insulin (Panserat et al., 2000; Panserat et al., 2001; Caseras et al., 2002).

Unlike terrestrial animals, fish exhibit a wide range of strategies for carbohydrate utilization due to their diverse habitats and dietary preferences. The process of carbohydrate digestion begins in the fish's digestive tract, where enzymes such as amylase break down

complex carbohydrates into simpler sugars like glucose, which can be absorbed through the intestinal wall. Once absorbed, glucose can be oxidized for energy production or stored as glycogen in the liver and muscles for later use (Phan et al., 2021). Additionally, some fish species have the ability to convert excess glucose into lipids through de *novo* lipogenesis, which serves as an energy reserve during periods of fasting or low carbohydrate availability (Wang et al., 2019). The efficiency of carbohydrate digestion and utilization in fish varies depending on factors such as species, diet composition, and environmental conditions, highlighting the adaptability of these organisms to diverse ecological niches. Understanding the mechanisms underlying carbohydrate metabolism in fish is essential for optimizing their nutrition and enhancing their productivity in aquaculture settings (Lu et al., 2018). Although fishes exhibit glucose intolerance, particularly carnivorous fish, they possess various enzymatic mechanisms for digesting dietary carbohydrates, including α-amylase and other carbohydrases, allowing utilization of dietary carbohydrates for energy and metabolic processes. Different tissues in fish, such as liver, muscle, and adipose tissue, exhibit varying metabolic pathways for utilizing carbohydrates. Better knowledge of tissue-specific mechanisms is essential for optimizing dietary formulations in aquaculture. Key genes involved in carbohydrate metabolism, such as those encoding for enzymes in glycolysis, gluconeogenesis, and glycogen synthesis pathways, are subject to regulation based on dietary carbohydrate levels and other environmental factors (Younus et al., 2020).

1.2.2. Carbohydrate Digestion and Absorption in Fish

Carbohydrates are essential macronutrients for fish, providing energy for various physiological functions, including growth, reproduction, and locomotion. In fish, carbohydrate digestion begins in the oral cavity with mechanical breakdown by chewing and salivary amylase secretion. Upon reaching the stomach, carbohydrates encounter acidic conditions that partially hydrolyze complex polysaccharides. The main site of carbohydrate digestion in fish is the intestine, where pancreatic amylase and intestinal carbohydrases break down carbohydrates into absorbable monosaccharides (Moraes & de Almeida, 2020).

Once carbohydrates are broken down into monosaccharides such as glucose, fructose, and galactose, they are absorbed across the intestinal epithelium into the bloodstream. Absorption occurs via specific transport mechanisms, including sodium-dependent glucose transporters (SGLTs) and facilitative glucose transporters (GLUTs), ensuring efficient uptake of glucose for energy metabolism and other cellular processes (Zhou et al., 2022).

Several factors influence carbohydrate digestion and absorption in fish, including diet composition, feeding frequency, water temperature, and intestinal health. High dietary fiber content, for example, may decrease carbohydrate digestibility and absorption, while optimal water temperature can enhance enzymatic activity and nutrient uptake efficiency (Moraes & de Almeida, 2020).

Gilthead sea bream, like many marine teleosts, have evolved specialized adaptations for carbohydrate digestion and absorption. Their short and relatively simple digestive tract, coupled with efficient carbohydrase production, enables rapid digestion and absorption of carbohydrates from various dietary sources, including plant-based ingredients (Talijančić et al., 2019). Understanding the mechanisms of carbohydrate digestion and absorption in gilthead sea bream is essential for formulating aquafeeds that maximize nutrient utilization and minimize environmental impacts. By optimizing dietary carbohydrate sources and processing techniques, aquaculture practitioners can enhance the growth performance and overall health of gilthead sea bream while reducing production costs and ecological footprint (Arechavala-Lopez et al., 2018).

1.2.3. Importance for Aquaculture Sustainability

Carbohydrate metabolism is a critical aspect of fish physiology and nutrition, particularly in species cultivated for aquaculture purposes (Basto-Silva et al., 2022), such as gilthead sea bream. . Carbohydrates are a significant source of energy for fish and play a crucial role in supporting various metabolic processes, including growth, reproduction, and immune function. In aquaculture, the efficient utilization of dietary carbohydrates can significantly impact feed formulation, production costs, and environmental sustainability (Zhang et al., 2020). Species like gilthead sea bream have unique metabolic requirements and dietary preferences, particularly regarding carbohydrate utilization. These species have evolved specialized mechanisms to digest, absorb, and metabolize carbohydrates efficiently, making it imperative to tailor aquafeeds to meet their specific nutritional needs (Thomas et al., 2021). By understanding the molecular mechanisms underlying carbohydrate metabolism in species like gilthead sea bream, aquaculture practitioners can develop targeted strategies to optimize carbohydrate utilization. This includes identifying key genes involved in carbohydrate metabolism pathways and manipulating their expression to enhance the efficiency of carbohydrate utilization while sparing protein (Phan et al., 2021). Optimizing the expression of key genes involved in carbohydrate metabolism can have significant implications for

aquaculture sustainability. By sparing protein utilization and increasing the use of dietary carbohydrates, aquaculture operations can reduce reliance on expensive protein sources, such as fishmeal, leading to improved economic and environmental sustainability (Maulu et al., 2021). Efficient utilization of dietary carbohydrates not only improves production efficiency but also contributes to aquaculture sustainability. By reducing reliance on expensive protein sources such as fishmeal and minimizing environmental impacts associated with excess nitrogen excretion, optimized carbohydrate utilization can enhance the economic and ecological sustainability of aquaculture operations (Maulu et al., 2021).

1.2.4. Tissue Utilization of Carbohydrates in Fish

Liver Metabolism

The liver plays a central role in carbohydrate metabolism in fish, serving as a hub for glucose homeostasis and glycogen storage. Upon ingestion of dietary carbohydrates, the liver uptakes glucose from the bloodstream and regulates its utilization through glycolysis, gluconeogenesis, and glycogen synthesis pathways. During periods of high energy demand, the liver can mobilize glycogen stores to supply glucose to other tissues.

Among the macronutrients, acetyl-CoA derived from carbohydrates typically plays a significant role in *de novo* lipogenesis (DNL) within the liver (Figure 1). Upon entering the cytosol, glucose undergoes phosphorylation by GCK to form Glu-6-P. Glu-6-P serves as the initial substrate for various metabolic pathways including glycogenesis, glycolysis, and the pentose phosphate pathway (PPP). The oxidation of glucose through glycolysis and PPP results in the production of pyruvate, which can be further metabolized into acetyl-CoA and NADPH, both of which are pivotal for DNL.

In glycolysis, key rate-limiting steps are mediated by enzymes such as GCK, which initiates phosphorylation of glucose, 6-phosphofructo-1-kinase (PFK), responsible for converting hepatic fructose-6-phosphate (Fru-6-P) into fructose-1,6-bisphosphate, and pyruvate kinase L/R (PK), facilitating the conversion of phosphoenolpyruvate (PEP) into pyruvate. Conversely, G6PC and fructose-1,6-bisphosphatase (FBP) catalyze reactions opposite to those catalyzed by GCK and PFK, respectively (Feidantsis et al., 2018).

The enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1) plays a crucial role in regulating glycolysis by converting Fru-6-P into fructose 2,6-bisphosphate (Fru-

2,6-P_2), a potent allosteric activator of PFK and inhibitor of FBP.

Pyruvate, produced by glycolysis in the cytosol, is transported into the mitochondria where it is converted into acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA, upon conversion into citrate, can undergo further oxidation in the Krebs cycle. Key rate-limiting reactions in the Krebs cycle are mediated by enzymes such as citrate synthase, isocitrate dehydrogenase (IDH), and oxoglutarate dehydrogenase (OGDH) (Wang et al., 2019).

Additionally, citrate formed in the mitochondria can be transported back into the cytosol, where it is converted into acetyl-CoA to contribute to DNL. In the PPP, Glu-6-P is acted upon by two pivotal enzymes, glucose-6-phosphate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD), which generate NADPH, an essential cofactor for DNL (Feidantsis et al., 2018).

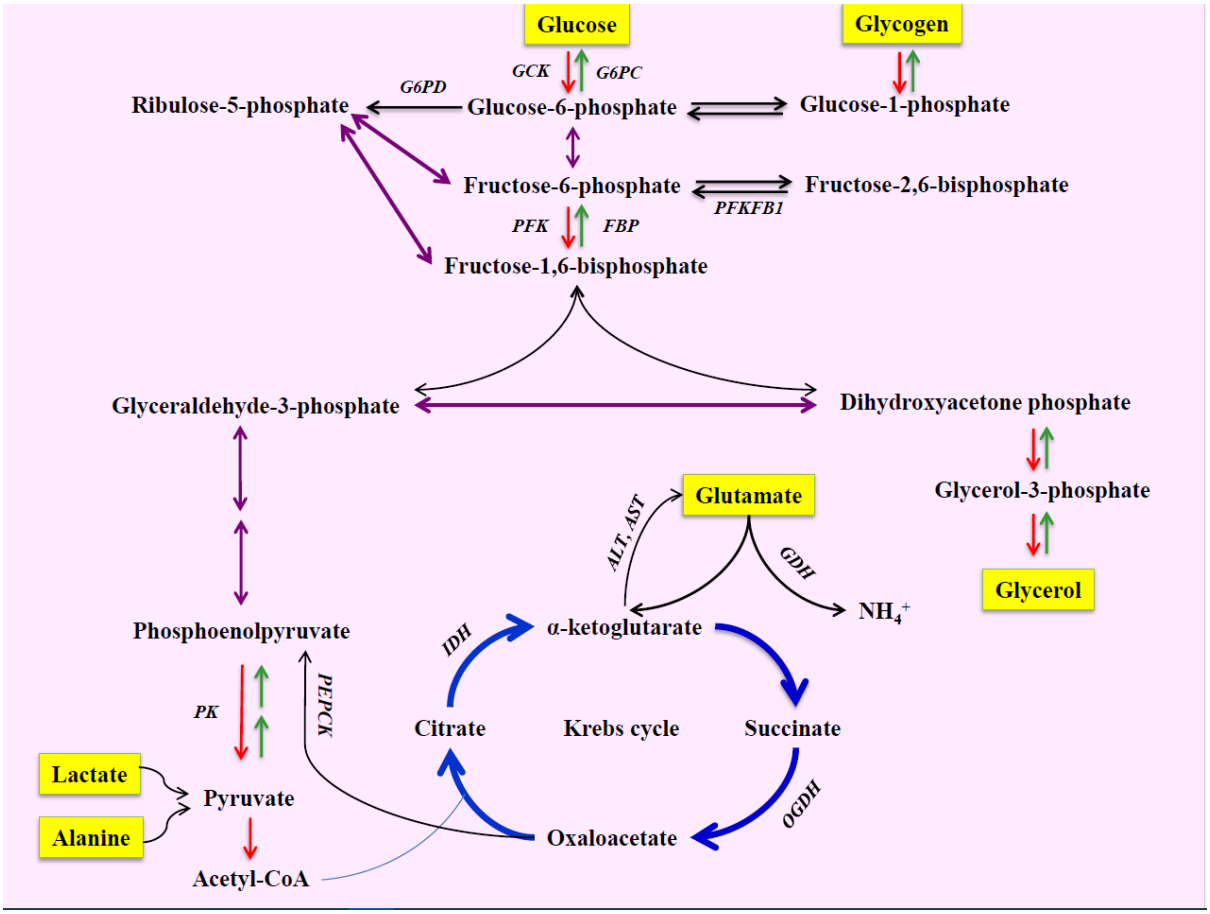


Figure 1. Schematic overview of hepatic glycolysis, gluconeogenesis and the Krebs cycle. GCK, glucokinase; G6PC, glucose-6-phosphatase, catalytic subunit 1; G6PD, glucose-6-phosphate dehydrogenase; PGD, 6-phosphogluconate dehydrogenase; PFKFB1, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; FBP, fructose 1,6-bisphosphatase; PFK, phosphofructokinase,

liver type; PCK1, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase L/R; ALT, alanine transaminase; AST, aspartate transaminase; OGDH, α-ketoglutarate dehydrogenase; IDH, isocitrate dehydrogenase.

Muscle Energy Production

Muscle tissue in fish utilizes carbohydrates primarily for energy production during swimming and locomotion. Glycolysis, the breakdown of glucose into pyruvate, generates ATP to fuel muscle contraction. Additionally, fish muscle can store small amounts of glycogen for immediate energy needs, although it relies predominantly on aerobic metabolism to sustain prolonged activity (Soengas et al., 2019).

Adipose Tissue Function

While adipose tissue in fish is relatively less prominent compared to mammals, it also participates in carbohydrate metabolism by storing excess energy in the form of lipids. During periods of carbohydrate abundance, adipose tissue can convert surplus glucose into fatty acids through de novo lipogenesis, contributing to energy storage and body condition (Salmerón, 2018).

Regulation of Carbohydrate Utilization

The tissue-specific utilization of carbohydrates in fish is tightly regulated by hormonal and nutritional signals, including insulin, glucagon, and dietary carbohydrate availability. Hormones such as insulin promote glucose uptake and utilization in insulin-sensitive tissues like liver and muscle, whereas glucagon stimulates glycogen breakdown and gluconeogenesis during fasting or low-carbohydrate conditions (Song et al., 2019).

1.2.5. Regulation of Carbohydrate Metabolism Genes in Gilthead Sea Bream

The regulation of carbohydrate metabolism genes plays a critical role in determining the utilization of dietary carbohydrates in fish. Through a comprehensive analysis of genomic and transcriptomic data, several candidate genes have emerged as central players in the regulation of carbohydrate metabolism pathways in *S. aurata*. One such gene is G6PD, which catalyzes the first and rate-limiting step of the PPP. This pathway plays a crucial role in generating NADPH, an essential cofactor in numerous biosynthetic processes and antioxidant

defense mechanisms. The expression of G6PD is tightly regulated to meet the dynamic metabolic demands of the organism. Another noteworthy gene is PFK, a key enzyme in glycolysis that catalyzes the conversion of Fru-6-P to fructose-1,6-bisphosphate. PFK is known to be allosterically regulated by various metabolites, hormones, and signaling pathways, thereby serving as a central node for coordinating glycolytic flux with cellular energy status and metabolic signaling. Additionally, GCK, an enzyme responsible for phosphorylating glucose to Glu-6-P in the liver and pancreatic β-cells, emerges as a critical regulator of glucose homeostasis. GCK activity is tightly regulated by glucose concentration and allosteric modulators, enabling precise control of glucose uptake and utilization in response to changing metabolic demands.

Furthermore, PK, the enzyme catalyzing the final step of glycolysis, has been identified as a key regulator of carbohydrate metabolism. PK exists in multiple isoforms with tissue-specific expression patterns, allowing fine-tuning of glycolytic flux and energy production according to the metabolic requirements of different tissues (Metón et al., 2000; Caseras et al., 2002; Metón et al., 2004).

The expression of carbohydrate metabolism genes in gilthead sea bream is tightly regulated at the transcriptional level by various transcription factors and regulatory elements. For example, transcription factors such as sterol regulatory element-binding proteins (SREBPs) and carbohydrate-responsive element-binding protein (ChREBP) can directly bind to gene promoters and modulate their expression in response to dietary carbohydrate levels (Xie et al., 2021).

Dietary carbohydrates play a crucial role in regulating the expression of carbohydrate metabolism genes in gilthead sea bream. High-carbohydrate diets can up-regulate the expression of genes involved in glycolysis and glycogen synthesis, while low-carbohydrate diets may stimulate gluconeogenic gene expression to maintain blood glucose levels. Additionally, other dietary components such as fatty acids and amino acids can also influence the expression of carbohydrate metabolism genes through complex metabolic interactions (Zhang et al., 2020). Environmental factors such as water temperature, salinity, and photoperiod can impact the expression of carbohydrate metabolism genes in gilthead sea bream by influencing metabolic rates and energy requirements. Furthermore, hormonal signals such as insulin, glucagon, and cortisol play crucial roles in coordinating carbohydrate metabolism gene expression in response to physiological states and external stimuli (Xie et al., 2021).

1.2.6. Effects of Dietary Carbohydrates on Gilthead Sea Bream Production

Dietary carbohydrates are essential macronutrients for fish, providing energy for growth, metabolism, and various physiological functions.

The inclusion of dietary carbohydrates in gilthead sea bream feeds can significantly influence growth performance parameters, including feed conversion ratio (FCR), specific growth rate (SGR), and final body weight. Studies have shown that partial replacement of dietary protein by carbohydrates keeping dietary carbohydrates below 20 % can promote optimal or suboptimal growth and development in gilthead sea bream and reduce nitrogen losses to the ambient waters, leading to more sustainable and efficient production. However, high levels of dietary carbohydrates have been associated with reduced growth rates and feed efficiency in some cases, potentially due to the limited ability of fish to efficiently utilize carbohydrates. Conversely, moderate levels of dietary carbohydrates, particularly those derived from digestible sources such as starches, have been shown to support optimal growth performance in gilthead sea bream. Furthermore, the interaction between dietary carbohydrates and other nutrients, such as proteins and lipids, can also influence growth performance. For instance, balanced ratios of carbohydrates to proteins have been linked to improved growth rates and feed utilization efficiency. Additionally, the inclusion of certain types of carbohydrates, such as prebiotic fibers, may exert positive effects on gut health and nutrient absorption, further enhancing growth performance (Metón et al., 1999; Fernández et al., 2007; Ashry et al., 2021).

Nutrient utilization efficiency plays a pivotal role in determining the growth performance and overall production of gilthead sea bream. Carbohydrates, as a major dietary component, significantly influence the utilization of other nutrients in fish diets. Studies have shown that the inclusion of carbohydrates in the diet of gilthead sea bream can affect various aspects of nutrient utilization efficiency, including protein sparing, lipid metabolism, and energy utilization. Dietary carbohydrates contribute to overall nutrient utilization efficiency in gilthead sea bream by providing readily available energy substrates for metabolic processes. Carbohydrate-rich diets can spare protein utilization for growth and tissue maintenance, leading to improved protein efficiency and reduced nitrogen excretion in aquaculture systems (Metón et al., 2006; Egea et al., 2008; Basto-Silva et al., 2022).

Carbohydrates play a multifaceted role in the metabolism of these fish, influencing

various biochemical pathways that ultimately determine growth, energy utilization, and overall performance. One significant aspect is the interplay between dietary carbohydrates and the glycolytic pathway. Carbohydrates serve as a primary energy source, undergoing glycolysis to produce pyruvate, a key metabolite. The regulation of enzymes involved in glycolysis, such as GCK and PFK, is crucial for optimizing energy production and utilization in sea bream. Moreover, the carbohydrate metabolism in fish involves intricate interactions with the PPP and the citric acid cycle. These pathways not only contribute to energy generation but also play essential roles in biosynthetic processes, including the synthesis of nucleotides, amino acids, and lipids. Increasing knowledge about how dietary carbohydrates influence the flux through these pathways can provide insights into enhancing growth and nutrient utilization efficiency. Additionally, dietary carbohydrates influence the hepatic glycogen synthesis and gluconeogenesis pathways in sea bream. The balance between glycogen storage and mobilization is vital for maintaining blood glucose levels and meeting the energy demands of various physiological processes. Modulating these pathways through dietary interventions can impact glucose homeostasis and metabolic flexibility in response to changing environmental conditions (Rito et al., 2018; Salgado et al., 2004). Furthermore, dietary carbohydrates can affect lipid metabolism in gilthead sea bream, influencing pathways such as lipogenesis, lipolysis, and fatty acid oxidation. The availability of carbohydrates can alter the carbon flux towards lipid synthesis or oxidation, thereby influencing lipid deposition and composition in the tissues. Understanding the interplay between carbohydrate and lipid metabolism is crucial for optimizing feed formulations to achieve desirable growth performance and flesh quality (Anemaet et al., 2008).

Hepatic and Muscle Glycogen Storage

The hepatic and muscle glycogen storage play crucial roles in the energy metabolism of *S. aurata* and are significantly influenced by dietary carbohydrate composition. Hepatic glycogen serves as a readily mobilizable energy source during periods of increased metabolic demand, while muscle glycogen primarily supports locomotor activity and sustained swimming performance. Variations in dietary carbohydrate levels and sources can profoundly impact the glycogen storage capacity of both liver and muscle tissues in gilthead sea bream. High dietary carbohydrate levels often lead to elevated hepatic glycogen reserves, facilitating rapid energy release, while moderate carbohydrate diets may optimize muscle glycogen deposition, promoting enhanced endurance and growth performance. Unraveling the interplay

between dietary carbohydrates and glycogen metabolism is essential for optimizing the production efficiency and physiological well-being of gilthead sea bream in aquaculture systems (Salgado et al., 2012; González et al., 2012).

1.3. Lipid Metabolism in Fish

Lipid metabolism plays a crucial role in energy storage, membrane structure, and cellular signaling in fish. Regulation of lipid metabolism is an essential process for optimizing dietary nutrient utilization and improving production outcomes in aquaculture species like *S. aurata*.

1.3.1. Lipid Digestion and Absorption

Lipid digestion and absorption are fundamental processes in the utilization of dietary lipids by fish (Ayisi et al., 2018). Lipid digestion in fish begins in the stomach, where gastric lipases hydrolyze dietary triglycerides into free fatty acids and monoglycerides. These lipolytic enzymes are secreted by gastric glands and act on the ester bonds of triglycerides, releasing fatty acids that are more readily absorbed by the intestinal epithelium. Gastric lipolysis is particularly important in carnivorous fish species like gilthead sea bream, which consume lipid-rich diets consisting of fish meal and fish oil (Xie et al., 2020). Following gastric lipolysis, lipid digestion continues in the intestine, where bile salts secreted by the gallbladder emulsify lipid droplets into smaller micelles. This emulsification process increases the surface area of lipids, facilitating the action of pancreatic lipases and enhancing lipid hydrolysis. The emulsified lipids, along with bile salts, form mixed micelles that transport lipid digestion products to the surface of enterocytes for absorption (Guo et al., 2019).

Once emulsified, lipid digestion products are absorbed by enterocytes lining the intestinal epithelium. Free fatty acids, monoglycerides, and other lipid digestion products diffuse across the apical membrane of enterocytes and are re-esterified into triglycerides within the cell. These newly synthesized triglycerides, along with cholesterol and other lipids, are packaged into chylomicrons, which are released into the lymphatic system and transported to the bloodstream (Chen et al., 2020). Chylomicrons, large lipoprotein particles containing dietary lipids, are transported via the lymphatic system and eventually enter the bloodstream, where they deliver lipids to peripheral tissues for utilization or storage. In fish, lipids serve as a crucial energy source for metabolic processes, including growth, reproduction, and

swimming activity. Additionally, lipids are incorporated into cell membranes, used as precursors for hormone synthesis, and stored as energy reserves in adipose tissue and liver (Sun et al., 2020c).

Several factors can influence lipid digestion and absorption in fish, including diet composition, feeding frequency, and fish size. High-fat diets containing lipid sources with different fatty acid compositions can affect the efficiency of lipid digestion and absorption in fish. Additionally, environmental factors such as water temperature can influence the activity of digestive enzymes and the rate of lipid metabolism in fish (Lai et al., 2021).

1.3.2. Lipid Transport and Storage

In fish, lipid transport and storage mechanisms are essential for regulating energy balance and maintaining physiological functions. Lipoproteins, such as chylomicrons, very-low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs), serve as vehicles for transporting lipids, including triglycerides, cholesterol, and phospholipids, to peripheral tissues. These lipoprotein particles vary in size, density, and lipid composition, reflecting their different roles in lipid metabolism and homeostasis (Wang et al., 2018).

Chylomicrons, synthesized in the intestine, transport dietary lipids from the intestine to peripheral tissues, such as liver, muscle, and adipose tissue. Once chylomicrons deliver their lipid cargo to tissues, they undergo lipolysis by lipoprotein lipase (LPL), releasing free fatty acids for cellular uptake. Meanwhile, the liver synthesizes VLDLs, which transport endogenous lipids, primarily triglycerides synthesized from dietary carbohydrates, to peripheral tissues. Similar to chylomicrons, VLDLs are metabolized by LPL, releasing fatty acids for energy production or storage (Ayisi et al., 2018).

Adipose tissue and liver are major sites of lipid storage in fish, serving as reservoirs of energy for periods of fasting or high-energy demands. Adipose tissue stores excess dietary lipids in the form of triglycerides within adipocytes, while the liver stores both exogenous and endogenous lipids in hepatocytes. Lipid uptake by adipose tissue and liver is regulated by hormonal signals, such as insulin and glucagon, as well as by nutritional status and metabolic demands (Cao et al., 2019).

Once taken up by adipose tissue and liver, lipids are stored as triglycerides in lipid

droplets or lipid vacuoles within cells. These intracellular lipid stores serve as a reservoir of energy for cellular metabolism and physiological functions. During periods of energy deficit, lipids stored in adipose tissue and liver are mobilized through lipolysis and beta-oxidation, providing a source of fatty acids for energy production in mitochondria (Zhang et al., 2018).

The transport and storage of lipids in fish are tightly regulated by hormonal signals, including insulin, glucagon, and adipokines, as well as by nutrient availability and metabolic demands. Hormones such as insulin promote lipid storage by stimulating lipogenesis and inhibiting lipolysis, whereas glucagon and adipokines promote lipid mobilization and oxidation by activating lipolysis and beta-oxidation pathways (Chen et al., 2018).

1.3.3. Lipid Oxidation and Energy Production

Lipid oxidation is a fundamental process in fish metabolism, essential for energy production, maintaining metabolic homeostasis, and supporting various physiological functions. The primary pathway for lipid oxidation in fish is beta-oxidation, which occurs in the mitochondria and peroxisomes of cells. During beta-oxidation, fatty acids are sequentially cleaved into two-carbon units forming part of acetyl-CoA. This process involves a series of enzymatic reactions, including oxidation, hydration, dehydrogenation, and thiolysis, resulting in the production of acetyl-CoA, NADH, and $FADH_2$. These metabolic intermediates enter the citric acid cycle (Krebs cycle) to generate ATP, the primary energy currency of cells (Hematyar et al., 2019).

Lipid oxidation and beta-oxidation pathways in fish are tightly regulated by various factors, including hormonal signals, nutrient availability, and metabolic demands. Hormones such as glucagon and catecholamines promote lipid mobilization and oxidation by activating lipolytic enzymes and increasing fatty acid transport into mitochondria. Conversely, hormones such as insulin and leptin inhibit lipid oxidation by promoting lipid storage and suppressing lipolytic pathways (Yin et al., 2019b).

Fish exhibit metabolic flexibility in their substrate preferences for energy production, depending on dietary nutrient composition and physiological conditions. Under normal feeding conditions, fish predominantly utilize lipids as a source of energy, sparing protein for growth and tissue maintenance. However, during periods of fasting or high metabolic demand, fish may switch to alternative energy substrates, such as carbohydrates or amino acids, to meet their

energy needs (Shi et al., 2018).

Optimal lipid oxidation is essential for supporting growth performance and production outcomes in aquaculture. Efficient lipid oxidation ensures the utilization of dietary lipids as an energy source, promoting growth and protein sparing. Imbalances in lipid oxidation, such as impaired beta-oxidation or excessive lipid accumulation, can lead to metabolic disorders, reduced growth rates, and compromised production efficiency in fish (Guo et al., 2019). Environmental factors, such as water temperature, oxygen levels, and stocking density, can influence lipid oxidation rates and metabolic rates in fish. Additionally, dietary factors, including lipid content, fatty acid composition, and carbohydrate-to-lipid ratio, can affect lipid metabolism and energy utilization in fish. Optimizing environmental conditions and dietary nutrient profiles can enhance lipid oxidation efficiency and improve growth performance in aquaculture (Abeyrathne et al., 2021).

1.3.4. Lipid Synthesis and Regulation

Lipid synthesis and regulation processes are vital for the maintenance of energy balance and growth in fish. Lipogenesis is the process of endogenous lipid synthesis, which begins with the synthesis of fatty acids in the mitochondria. Acetyl-CoA, derived from the oxidative decarboxylation of pyruvate in carbohydrate metabolism or from the oxidative degradation of some amino acids, is the carbon source required to initiate the fatty acid synthesis process. Fatty acid synthesis requires the formation of malonyl-CoA, the product of the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA is subsequently condensed with another molecule of acetyl-CoA by the action of fatty acid synthase (FASN), a macroprotein complex that allows the successive addition of 2 carbons to a growing chain, which through this pathway can reach up to 18 carbon atoms in length (Xie et al., 2021).

The process of fatty acid formation is a sequential process in which, at each of its steps, NADPH is utilized to condense a molecule of malonyl-CoA and extend the forming chain by two carbon atoms. The pathways through which the necessary NADPH for FASN to function and elongate the chain can be generated include the PPP, controlled by G6PD and 6-phosphogluconate dehydrogenase (6PGD), and the enzyme malic or malate dehydrogenase (MDH). In fish, it has been described that the main source of NADPH generation is the PPP and particularly the enzyme G6PD (Ayisi et al., 2018).

In trout and tilapia, the main lipogenic organ is the liver, tissue from which lipids are

exported to other tissues where they are stored or utilized. However, the importance of the lipogenic process in metabolism largely depends on the species and their dietary habits, so studies not only focus on the liver but have also been conducted in other tissues such as adipose or skeletal muscle. It is important to consider that dietary habits regulate lipid synthesis in different ways. For example, alanine is the main carbon source used in trout for fatty acid synthesis, so the incorporation of protein into the diet has a lipogenic effect, while a higher proportion of lipids relative to proteins tends to decrease lipid synthesis (Monroig et al., 2018).

Fish, like other animals, have the ability to elongate and add unsaturations to saturated fatty acids of 16 and 18 carbons synthesized by the action of FASN. The first step of this process involves the production of palmitoleic acid (16:1*n*-7) and oleic acid (18:1*n*-9) in an aerobic process, which requires NADPH and O_2 and occurs in the endoplasmic reticulum through a complex system involving NADPH-cytochrome b5 reductase, cytochrome b5, and desaturases such as stearoyl Δ9 fatty acid desaturase (SCD), whose activity and transcriptional modulation have been described in various studies conducted in fish. The conventional microsomal elongation pathway, which acts on the products of the desaturation process, is capable of generating several species of longer fatty acids, highlighting fatty acids 18:1*n*-7, 20:1*n*-9, 22:1*n*-9, and 24:1*n*-9 (Wang et al., 2023).

However, through endogenous molecular mechanisms, vertebrates cannot efficiently introduce additional double bonds to oleic acid to produce linoleic acid (18:2*n*-6) and alpha-linolenic acid (18:3*n*-3), which are produced by Δ12 and Δ15 desaturases in other organisms. Therefore, these fatty acids, which in turn are precursors of long-chain (LC) ω-6 and ω-3 polyunsaturated fatty acids (PUFA), such as arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are essential for vertebrates, including fish, and must be consumed in the diet. The process through LC-PUFA, with important functions in animal metabolism, are formed, occurs thanks to the action of Δ6 and Δ5 desaturases, and elongation processes in which several elongases with affinity for different substrates act (Figure 2). However, vertebrates, including bony fish, can only convert a small portion of linoleic acid and alpha-linolenic acid to LC-PUFA, such as EPA and DHA, and at insufficient rates to cover physiological demands. Nevertheless, the process of adding double bonds and elongating LC-PUFAs seems strongly influenced by animals' adaptation processes to the diet they consume. Freshwater fish and salmonids have greater capacity of converting *n*-3 and *n*-6 C_{18} PUFA into highly unsaturated LC-PUFA than marine fish. The high content of LC-PUFA in microalgae and other organisms in the natural diet of marine fish, the trophic level in the food chain and

diadromy may have determined divergent evolution and limited ability to biosynthesize LC-PUFA (Castro et al., 2016; Vagner & Santigosa, 2011). Marine fish and seafood are the main source of *n*-3 LC-PUFA, such as EPA and DHA in the human diet (Tocher et al., 2019; Osmond & Colombo, 2019), so understanding this process is of great interest to aquaculture production. Multiple studies have been conducted to understand the effectiveness of this process in various species of fish for human consumption and the possibility of substituting fish oil with other sources of essential fatty acids in the diet (Torres et al., 2020).

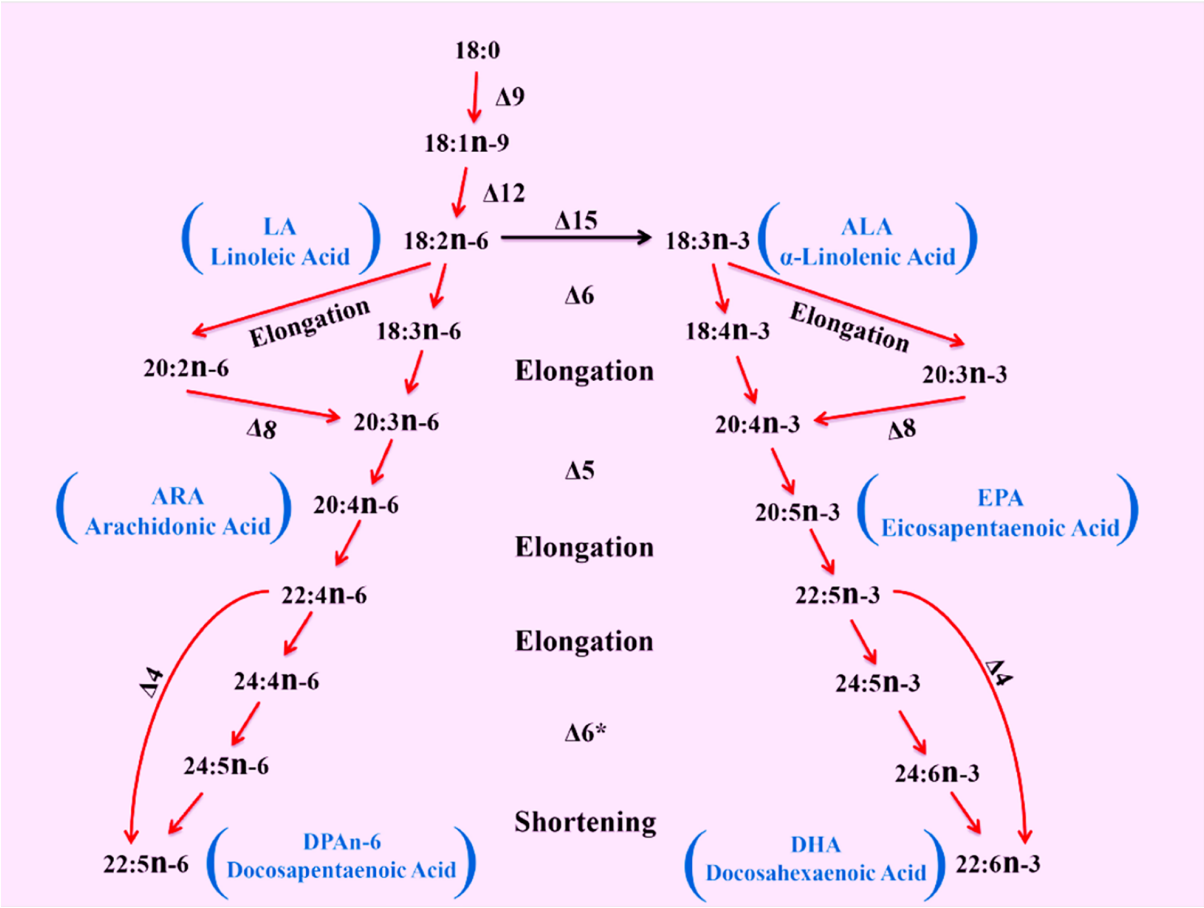


Figure 2. Synthetic pathway of C_{20} and C_{22} polyunsaturated fatty acids from *n*-3 and *n*-6 18-carbon precursors. Desaturase activities: Δ4, Δ5, Δ6, Δ6*, Δ8, Δ9, Δ12, and Δ15. The Δ6* desaturase enzyme, which acts on 24-carbon fatty acids, may or may not be the same enzyme (Δ6) that acts on 18-carbon fatty acids depending on the species.

The synthesis process of other lipid metabolism components, such as cholesterol,

sphingolipids, or phospholipids, is poorly studied in fish. However, thanks to the use of new technologies such as microarrays to analyze gene expression patterns, there are studies that reveal the presence and modulation of transcription of enzymes involved in lipid metabolism pathways in fish. The *de novo* synthesis process of phospholipids is very similar to that of terrestrial mammals, although certain evidence suggests that some fish larvae have a limited capacity for their synthesis, which could be related to the abundance of these components in the natural diets of fish (Balbuena-Pecino et al., 2019).

The formation of triglycerides involves the sequential esterification of two fatty acids with glycerol-3-phosphate, the cleavage of a phosphate group, and the esterification of a third fatty acid through the sequential action of the enzymes glycerol-3-phosphate acyltransferase (GPAT), 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT), phosphatidate phosphatase, and diacylglycerol acyltransferase (DGAT). During feeding, excess fatty acids are exported from the liver forming part of VLDLs, accumulating as triglycerides in storage tissues such as adipose tissue, muscle, and between the skin and muscle. Additionally, the process of triglyceride accumulation in the liver may be relevant in some fish species (Ofori-Mensah et al., 2020).

Regulation of Lipogenesis

Lipogenesis is tightly regulated by hormonal signals, nutrient availability, and gene expression. Insulin, a hormone secreted by the pancreas in response to elevated blood glucose levels, promotes lipogenesis by activating key enzymes such as ACC and FAS. Conversely, glucagon and catecholamines inhibit lipogenesis and promote lipid mobilization by activating lipolytic pathways. Nutrient availability, particularly the availability of dietary carbohydrates and fatty acids, also influences the rate of lipogenesis in fish (Feidantsis et al., 2018).

The expression of key genes involved in lipogenesis is regulated at the transcriptional level by a number of transcription factors and co-regulators including sterol regulatory element-binding proteins (SREBPs), which are considered major transcription factors in the regulation of the expression of lipogenic genes (Eberlé et al., 2004), the nuclear receptor family of peroxisome proliferator activated receptors (PPARs) and the liver X receptor (LXR) (Sánchez-Gurmaches et al., 2013).

SREBPs comprise a family of transcription factors with a pivotal role in the transcriptional regulation of genes controlling cellular lipid biosynthesis (DeBose-Boyd & Ye,

2018; Bertolio et al., 2019). Newly synthesized SREBP precursors remain anchored to the membrane of the endoplasmic reticulum, where the C-terminus of the protein associates with a sterol sensor, the SREBP-cleavage activating protein (Scap), which in turn interacts with the insulin-induced gene protein (Insig) (Edwards et al., 2000; Inoue & Sato, 2013). Decreased cellular levels of sterol drives the SREBP/Scap complex to the Golgi apparatus, where site-1 and site-2 proteases release the N-terminus of SREBPs, which constitute transcriptionally active basic-helix-loop-helix leucine zippers (bHLH-Zip) that dimerize, translocate to the nucleus and transactivate target genes by binding to sterol regulatory elements (SRE) (Brown & Goldstein, 1997; Horton et al., 2003; Torno et al., 2018). Alternate promoters in the srebf1 gene generate SREBP1a and SREBP1c in mammals, while srebf2 encodes for SREBP2 (Shimano et al., 2017). SREBP1c primarily transactivates genes required for the biosynthesis of fatty acids and triglycerides, while SREBP2 transactivate genes associated to cholesterol biosynthesis (Shimano et al., 2017; Horton et al., 2002). Compared to SREBP1c, the presence of a longer N-terminal transactivation domain in SREBP1a confers to this protein stronger transcriptional activity, being a potent activator of all SREBP-responsive genes (Pai et al., 1998; Shimano et al., 1997; Horton et al., 1998). In addition to the effects of SREBP1 isoforms on the control of lipid biosynthesis (DeBose-Boyd & Ye, 2018; Bertolio et al., 2019; Shimano et al., 2017), SREBP1 is also involved in the transcriptional activation of glucose metabolism related-genes. Thus, SREBP1c mediates insulin-dependent up-regulation of hexokinase II and GCK by transactivating both gene promoters in the mammalian liver (Foretz et al., 1999; Gosmain et al., 2004; Kim et al., 2004; Gosmain et al., 2005). Moreover, SREBP1a overexpression decreases gluconeogenesis by suppressing PCK1 and G6PC transcription mediated by hepatocyte nuclear factor-4 in the liver of transgenic mice (Yamamoto et al., 2004). Similarly as in mammals, SREBP1 up-regulates the transcription of GCK and PFKFB1 by binding to SRE boxes located in their respective gene promoters in *S. aurata* (Metón et al., 2006; Egea et al., 2008).

Dietary Influence on Lipid Synthesis

The composition of the diet, particularly the ratio of carbohydrates to lipids, can influence the rate of lipid synthesis in fish. High-carbohydrate diets stimulate lipogenic gene expression and promote fatty acid synthesis, whereas high-lipid diets suppress lipogenesis and promote lipid oxidation. Additionally, dietary fatty acid composition, particularly the presence of essential fatty acids such as omega-3 and omega-6 fatty acids, can influence the rate of lipid

synthesis and tissue lipid composition in fish (Sánchez-Nuño et al., 2018). By controlling the expression of key genes involved in lipogenesis, researchers can manipulate the partitioning of dietary nutrients towards lipid synthesis or oxidation, thereby optimizing energy utilization and growth efficiency in fish (Carvalho et al., 2021).

1.3.5. Lipid Metabolism and Growth Performance

Lipid metabolism plays a crucial role in the growth performance of fish, including *S. aurata*, in aquaculture settings. Lipids serve as a dense energy source in fish, providing more than twice the energy content per gram compared to proteins and carbohydrates. Efficient lipid metabolism enables fish to store excess energy in adipose tissue and liver during periods of abundance and mobilize stored lipids for energy production during periods of fasting or high metabolic demand. Optimal lipid metabolism ensures a continuous energy supply for growth and physiological functions, contributing to improved growth performance in fish (Li et al., 2019).

Fish growth performance is closely linked to feed conversion efficiency, which is influenced by the utilization of dietary nutrients, including lipids. Efficient lipid metabolism allows fish to utilize dietary lipids for growth rather than energy production, thereby improving feed conversion ratios and growth rates. Conversely, imbalances in lipid metabolism, such as excessive lipid deposition or impaired lipid utilization, can lead to reduced growth efficiency and increased feed wastage in aquaculture systems (Liu et al., 2018).

The composition of dietary lipids can influence tissue development and growth performance in fish. Essential fatty acids, such as omega-3 and omega-6 fatty acids, play critical roles in membrane structure, cellular signaling, and tissue growth. Balanced dietary lipid profiles containing optimal levels of essential fatty acids promote tissue development and growth in fish, leading to improved growth performance and overall health (Guo et al., 2019).

Hormonal signals, such as insulin-like growth factors (IGFs) and insulin, play key roles in regulating both growth and lipid metabolism in fish. IGFs and GH promote growth by stimulating protein synthesis and cell proliferation, while insulin regulates nutrient uptake and energy metabolism, including lipid synthesis and storage. Hormonal dysregulation, resulting from genetic factors or environmental stressors, can impair growth performance and lipid metabolism in fish (Chen et al., 2020).

Lipid metabolism is a complex and tightly regulated process that plays a critical role in energy homeostasis, growth regulation, and production performance in fish. Optimizing lipid metabolism in fish has significant implications for aquaculture production, including improved growth performance, feed efficiency, and production sustainability. By controlling the expression of key genes involved in lipid metabolism, researchers can develop strategies to enhance growth performance and minimize environmental impacts, aligning with the goals of sustainable aquaculture production (Silva-Marrero et al., 2019).

1.4. Amino Acid Metabolism in Fish

1.4.1. Amino Acid Absorption and Transport

Amino acid absorption and transport are fundamental processes in amino acid metabolism in fish, directly influencing protein synthesis and growth performance (Teodósio et al., 2022). Amino acid absorption in fish primarily occurs in the intestine, where dietary proteins are hydrolyzed into amino acids by digestive enzymes. Amino acids are then transported across the intestinal epithelium into the bloodstream, where they are distributed to various tissues for protein synthesis and other metabolic processes. The efficiency of amino acid absorption depends on factors such as the composition of the diet, intestinal morphology, and the activity of transporters on the enterocyte membrane (Fabrikov et al., 2020). Amino acid transport across the intestinal epithelium is mediated by specific transporters located on the apical and basolateral membranes of enterocytes. These transporters facilitate the uptake of amino acids from the intestinal lumen into enterocytes and their release into the bloodstream. The two main families of amino acid transporters in fish are the sodium-dependent neutral amino acid transporters (SNATs) and the proton-coupled amino acid transporters (PATs), which play key roles in amino acid absorption and distribution (Iaconisi et al., 2019).

The expression and activity of amino acid transporters in the intestine are regulated by various factors, including dietary amino acid levels, hormonal signals, and metabolic demands. Hormones such as insulin and glucagon, as well as amino acids themselves, can modulate the expression and function of amino acid transporters, thereby regulating the rate of amino acid absorption. Additionally, nutrient-sensing pathways such as the mechanistic target of rapamycin (mTOR) pathway may also influence amino acid transporter activity in response to changes in nutritional status (Kaya Öztürk et al., 2020).

Efficient amino acid absorption and transport are essential for supporting protein

synthesis and growth in fish. Optimal amino acid utilization ensures that dietary protein is efficiently utilized for tissue growth and maintenance, leading to improved growth performance and feed efficiency. By controlling the expression of key genes involved in amino acid absorption and transport, researchers can enhance nutrient utilization and growth outcomes in aquaculture (Paulino et al., 2022).

1.4.2. Protein Synthesis and Turnover

Protein synthesis is the process by which amino acids are assembled into polypeptide chains according to the genetic code encoded in mRNA molecules. Protein synthesis occurs in ribosomes, where amino acids are linked together in a sequence dictated by the mRNA template. The initiation of protein synthesis involves the binding of initiator tRNA to the start codon on the mRNA molecule, followed by the elongation and termination phases, which involve the addition of amino acids to the growing polypeptide chain and the release of the completed protein, respectively (Sáez-Arteaga et al., 2022).

Protein synthesis is tightly regulated by various factors, including nutrient availability, hormonal signals, and cellular energy status. Hormones such as insulin and IGFs play key roles in stimulating protein synthesis by activating signaling pathways such as the mTOR pathway, which promotes ribosome biogenesis and translation initiation. Conversely, conditions of nutrient deprivation or energy deficiency can suppress protein synthesis to conserve resources and maintain cellular homeostasis (Feidantsis et al., 2018).

Protein turnover encompasses the processes of protein degradation and renewal, which are essential for maintaining cellular integrity and function. In fish, protein turnover occurs via proteolytic pathways such as the ubiquitin-proteasome system and the autophagy-lysosome pathway. These pathways target damaged or misfolded proteins for degradation, allowing for the recycling of amino acids and the synthesis of new proteins to replace damaged ones. Protein turnover rates can vary depending on factors such as tissue type, growth stage, and environmental conditions (Blasco et al., 2021).

Optimal protein synthesis and turnover are crucial for supporting growth performance and nutrient utilization in fish. Efficient protein synthesis ensures that dietary amino acids are utilized for tissue growth and maintenance, while protein turnover allows for the removal of damaged proteins and the recycling of amino acids for protein synthesis. By modulating the expression of key genes involved in protein synthesis and turnover, researchers can enhance

growth rates and production outcomes in aquaculture.

1.4.3. Amino Acid Catabolism and Energy Production

Amino acid catabolism is a vital process in amino acid metabolism in fish, playing a significant role in energy production and metabolic homeostasis. Amino acid catabolism involves the breakdown of amino acids into intermediates that can be used for energy production or converted into other metabolites. The main pathways of amino acid catabolism in fish include transamination, deamination, and oxidative decarboxylation. Transamination reactions transfer amino groups from amino acids to α-keto acids, giving rise to the formation of new amino acids and keto acids. Deamination reactions remove amino groups from amino acids, forming ammonia or ammonium ions, which are then excreted or converted into less toxic compounds. Oxidative decarboxylation reactions oxidize amino acids to produce acetyl-CoA or intermediates of the Krebs cycle for energy production (Fabrikov et al., 2020).

Amino acid catabolism contributes to energy production in fish by generating ATP through the oxidation of amino acid carbon skeletons. Amino acids can be converted into acetyl-CoA, which enters the Krebs cycle to produce reducing equivalents (NADH and $FADH_2$) and ATP through oxidative phosphorylation. Additionally, amino acids can be converted into intermediates of the glycolytic pathway or the PPP, providing alternative routes for ATP synthesis. The efficiency of energy production from amino acid catabolism depends on factors such as the availability of oxygen, the metabolic state of the organism, and the specific amino acids being catabolized (Fabrikov et al., 2020).

The catabolism of amino acids is tightly regulated to maintain metabolic homeostasis and prevent the accumulation of toxic intermediates. Glucagon and cortisol stimulate amino acid catabolism during fasting or stress conditions to provide energy for vital functions. Additionally, nutrient availability and metabolic demands influence the rate of amino acid catabolism, with high-energy demands leading to increased catabolic activity. Enzymes involved in amino acid catabolism are regulated by factors such as substrate availability, allosteric regulation, and post-translational modifications (Paulino et al., 2022).

Optimizing amino acid catabolism is essential for maximizing nutrient utilization and growth performance in aquaculture species as well as developing strategies to enhance energy production and metabolic efficiency in fish. Additionally, by controlling the expression of key genes involved in amino acid catabolism, aquaculture practitioners can modulate nutrient

partitioning and improve the utilization of alternative nutrients, such as carbohydrates, leading to improved growth outcomes and production efficiency infish.

1.4.4. Regulation of Amino Acid Metabolism

Hormones play a pivotal role in regulating amino acid metabolism in fish. Insulin, for example, acts as an anabolic hormone that stimulates amino acid uptake and protein synthesis in tissues, promoting growth and protein accretion. Conversely, catabolic hormones such as glucagon and cortisol enhance amino acid mobilization from tissues and stimulate amino acid catabolism during periods of fasting or stress. Hormonal regulation of amino acid metabolism ensures a dynamic balance between anabolic and catabolic processes to meet the metabolic demands of the organism (Pulido-Rodriguez et al., 2021).

Nutrient sensing pathways, such as the mTOR pathway, play a critical role in regulating amino acid metabolism in response to nutrient availability. Activation of the mTOR pathway promotes protein synthesis and inhibits protein degradation, thereby coordinating amino acid utilization with cellular growth and proliferation. Additionally, nutrient-sensing kinases such as AMP-activated protein kinase (AMPK) and sirtuins can modulate amino acid metabolism in response to changes in cellular energy status, ensuring metabolic flexibility and adaptation to environmental conditions (Basto-Silva et al., 2021).

Transcriptional regulation plays a central role in controlling the expression of genes involved in amino acid metabolism. Transcription factors such as activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein beta (C/EBPβ) can modulate the expression of genes encoding enzymes involved in amino acid synthesis, catabolism, and transport in response to cellular stress or nutrient availability. Additionally, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) can post-transcriptionally regulate the expression of genes involved in amino acid metabolism, fine-tuning metabolic responses to changing environmental conditions (Ramos-Pinto et al., 2021).

Post-translational modifications, such as phosphorylation, acetylation, and ubiquitination, can regulate the activity and stability of proteins involved in amino acid metabolism. Phosphorylation of enzymes involved in amino acid catabolism, for example, can modulate their activity in response to cellular signaling pathways. Similarly, ubiquitination targets proteins for degradation via the proteasome, controlling the turnover of enzymes and

regulatory proteins involved in amino acid metabolism (Sáez-Arteaga et al., 2022).

1.4.5. Implications for Growth Performance

Amino acids serve as the building blocks for protein synthesis, which is essential for tissue growth and repair in fish. Optimal amino acid metabolism ensures that dietary amino acids are efficiently utilized for protein synthesis, supporting muscle development, organ growth, and overall body composition. By controlling the expression of key genes involved in protein synthesis pathways, researchers can enhance growth performance and maximize protein accretion in aquaculture.

Amino acid metabolism also contributes to energy production in fish, providing ATP through oxidative pathways such as the Krebs cycle and oxidative phosphorylation. Efficient amino acid catabolism ensures that energy is readily available for metabolic processes associated with growth and maintenance (Teles et al., 2020). The efficient utilization of dietary nutrients, including amino acids, is critical for maximizing feed efficiency and growth performance in aquaculture operations. Amino acid metabolism influences nutrient partitioning and utilization, determining the extent to which dietary nutrients are converted into biomass (Basto-Silva et al., 2022). Amino acid metabolism also contributes to the synthesis of non-protein biomolecules such as nucleotides, neurotransmitters, and hormones, which play essential roles in growth regulation and metabolic signaling. Understanding the metabolic pathways involved in amino acid biosynthesis and interconversion can provide insights into the nutrient requirements of fish and inform the formulation of balanced diets that support optimal growth performance and health (Krebs et al., 2023).

In summary, amino acid metabolism is a complex process that influences protein synthesis, energy production, and growth performance in fish. The implications of amino acid metabolism for growth performance have practical relevance for aquaculture practices, as they inform the development of feed formulations and feeding strategies that optimize nutrient utilization and promote efficient growth. By manipulating the expression of key genes involved in amino acid metabolism pathways, aquaculture researchers can tailor feeding regimes to meet the specific nutritional requirements of different fish species and production systems, ultimately enhancing growth performance and production efficiency, and contributing to the advancement of sustainable aquaculture practices (Bildik et al., 2019).

1.5. Use of Metformin in Aquaculture

1.5.1. Introduction to Metformin

Metformin, chemically known as dimethylbiguanide, belongs to the biguanide class of drugs and is commonly used as a first-line treatment for type 2 diabetes. It works primarily by reducing hepatic glucose production, increasing peripheral glucose uptake, and improving insulin sensitivity in target tissues. Metformin does not stimulate insulin secretion from the pancreas, making it distinct from other antidiabetic medications (Wei et al., 2020).

1.5.2. Application of Metformin in Aquaculture

The utilization of metformin in aquaculture systems aims to enhance the growth performance of aquatic organisms, thereby improving productivity and efficiency in fish farming operations. One of the primary mechanisms through which metformin exerts its growth-promoting effects is by modulating energy metabolism. Metformin acts as an activator of AMPK, a key regulator of cellular energy homeostasis. By activating AMPK, metformin stimulates glucose uptake and utilization, leading to increased energy production and improved metabolic efficiency in aquatic organisms. This enhanced energy metabolism facilitates enhanced growth rates and improved feed conversion efficiency in fish and other aquatic species (Zhou et al., 2019).

Moreover, metformin exhibits potential immunomodulatory effects in aquatic organisms, which can contribute to improved growth performance and overall health. Studies have suggested that metformin administration can enhance the immune response of fish by modulating inflammatory pathways and promoting the synthesis of antimicrobial peptides. By bolstering the immune system, metformin helps to mitigate the impact of stressors such as pathogens and environmental fluctuations, thereby promoting optimal growth and survival in aquaculture settings. Furthermore, the use of metformin in aquaculture offers potential environmental benefits. Unlike traditional growth-promoting agents such as antibiotics, metformin does not pose risks of antibiotic resistance or environmental contamination. Its favorable safety profile and low environmental impact make it an attractive option for sustainable aquaculture practices. Aquaculture environments can subject fish to various stressors, including handling, transportation, and environmental changes. Metformin's ability to regulate stress-responsive pathways, such as the hypothalamic-pituitary-adrenal axis, makes it a promising agent for alleviating stress in aquaculture. By reducing cortisol levels and

enhancing stress tolerance, metformin may help minimize the negative impacts of stress on growth and productivity in farmed fish (MacLaren et al., 2018; Xu et al., 2018).

Biofloc technology is gaining popularity in aquaculture for its potential to improve water quality, enhance nutrient utilization, and reduce environmental impact. Metformin's effects on nutrient metabolism and microbial communities make it a candidate for optimizing biofloc systems. Research exploring the interactions between metformin, microbial populations, and nutrient dynamics in biofloc reactors could inform strategies for maximizing the efficiency and sustainability of this technology in aquaculture production systems (MacLaren et al., 2018; Xu et al., 2018).

In conclusion, the application of metformin as a growth-promoting agent holds promise for improving the efficiency and sustainability of aquaculture operations. Through its effects on energy metabolism, immune function, and environmental safety, metformin offers an effective and environmentally friendly solution for enhancing the growth performance of aquatic organisms. Further research is warranted to elucidate the molecular effects driven by metformin in fish, the optimal dosing regimens and application strategies for maximizing the benefits of metformin in aquaculture. By modulating key metabolic pathways, such as glucose and lipid metabolism, metformin may enhance energy utilization and redirect nutrients towards growth and tissue development.

1.5.3. Gene Expression and Molecular Responses

Analyzing the effects of metformin on gene expression and molecular responses in fish can provide essential clues for further characterization of fish intermediary metabolism and unraveling its mechanisms of action and potential applications in aquaculture. Metformin regulates the expression of key metabolic genes involved in glucose and lipid metabolism, energy homeostasis, and growth regulation in fish. Through its activation of AMPK, metformin modulates the activity of transcription factors and coactivators that control the expression of metabolic genes. For example, metformin stimulates the expression of genes encoding GLUTs, glycolytic enzymes, and mitochondrial biogenesis factors, enhancing glucose uptake, glycolysis, and oxidative metabolism in fish tissues (Niemuth & Klaper, 2018). Similarly, metformin inhibits the expression of lipogenic genes such as FASN and SREBPs, leading to decreased fatty acid synthesis and lipid accumulation in fish. By altering the expression of metabolic genes, metformin promotes metabolic adaptation and energy conservation in

response to nutrient availability and energy status, thereby optimizing nutrient utilization and growth performance (Lee et al., 2019).

Molecular Signaling Pathways

In addition to its effects on metabolic gene expression, metformin influences various molecular signaling pathways involved in cellular growth, proliferation, and stress responses in fish. One of the key signaling pathways regulated by metformin is the mTOR pathway, which plays a central role in coordinating cell growth and protein synthesis in response to nutrient and energy signals (Zhou et al., 2019). Metformin inhibits mTOR signaling by activating AMPK and inhibiting the phosphorylation of downstream targets such as ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). This inhibition of mTOR signaling suppresses protein synthesis and cell growth, redirecting energy resources towards essential cellular functions and metabolic processes. In aquaculture species such as gilthead sea bream, metformin-mediated inhibition of mTOR signaling may contribute to the optimization of protein utilization and growth efficiency, particularly under conditions of dietary carbohydrate abundance (Elizalde-Velázquez et al., 2023).

Epigenetic Modifications

Emerging evidence suggests that metformin may also exert epigenetic effects on gene expression and cellular function in fish. Epigenetic modifications, such as DNA methylation and histone acetylation, can regulate gene expression patterns and phenotypic traits in response to environmental stimuli and metabolic signals. Metformin has been shown to modulate DNA methylation patterns and histone modifications in mammalian cells, influencing gene expression and cellular processes associated with metabolism, aging, and disease. In fish, the epigenetic effects of metformin on key metabolic genes and pathways remain to be fully elucidated. However, knowledge of the epigenetic mechanisms underlying metformin's effects on gene expression and metabolic regulation in fish could provide valuable insights into its therapeutic potential and applications in aquaculture (Wei et al., 2020).

1.6. Gene Therapy

Gene therapy has emerged as a promising approach in aquaculture for manipulating the

expression of key genes to optimize traits related to growth, health, and environmental sustainability. This section explores the potential of gene therapy techniques in revolutionizing aquaculture practices, focusing on the manipulation of gene expression to spare protein utilization and enhance the utilization of dietary carbohydrates in species such as gilthead sea bream.

1.6.1. Introduction to Gene Therapy in Aquaculture

Gene therapy involves the delivery of nucleic acids, such as DNA or RNA, into target cells to modulate gene expression and achieve specific phenotypic outcomes. In aquaculture, gene therapy offers a promising avenue for improving production efficiency, disease resistance, and nutrient utilization in farmed fish species. By precisely controlling the expression of key genes involved in metabolic pathways, researchers can develop strategies to enhance growth performance and reduce the environmental impact of aquaculture operations (Wu et al., 2024).

Several gene delivery methods are available for introducing therapeutic genes into fish cells. Viral vectors, such as adenoviruses and lentiviruses, have been used to efficiently deliver genes into target cells, but safety concerns and regulatory hurdles may limit their widespread use in aquaculture. Non-viral vectors, including liposomes and nanoparticles, offer alternative approaches for gene delivery with potentially fewer safety risks. Additionally, genome editing technologies like CRISPR-Cas9 may enable precise modifications to the fish genome, allowing for the targeted manipulation of specific genes related to nutrient utilization and metabolic pathways (Wang et al., 2021).

Gene therapy can be applied to modulate the expression of key genes involved in nutrient metabolism to optimize feed efficiency and nutrient utilization in cultured fish. Target genes may include those encoding enzymes and transcription factors involved in protein synthesis, carbohydrate metabolism, and energy production pathways. By enhancing the expression of genes related to carbohydrate utilization while sparing protein utilization, researchers can improve growth performance and metabolic efficiency in cultured fish (Mao et al., 2021).

Despite its potential benefits, gene therapy in aquaculture faces several challenges, including ethical concerns, regulatory complexities, and technological limitations. Issues such as public perception of genetically modified organisms (GMOs), environmental risks, and biosafety must be carefully considered in the development and implementation of gene therapy

strategies. Additionally, optimizing the efficiency, specificity, and safety of gene delivery methods remains a key research priority in aquaculture genetics (Al-Nimry et al., 2021).

Future Directions

The future of gene therapy in aquaculture holds great promise for advancing sustainable aquaculture practices. Continued research efforts are needed to refine gene delivery techniques, enhance the specificity of genome editing technologies, and address safety and regulatory concerns. By leveraging the power of gene therapy to optimize nutrient utilization and metabolic pathways in farmed fish species, aquaculture practitioners can contribute to the development of more efficient and environmentally responsible aquaculture systems.

1.6.2. Techniques for Gene Delivery

When selecting gene delivery techniques for aquaculture applications, several factors must be considered, including safety, efficiency, scalability, and cost-effectiveness. The chosen method should ensure high transduction efficiency, minimal off-target effects, and long-term gene expression in target tissues without compromising fish health or welfare. Additionally, regulatory approval and public acceptance of gene therapy approaches in aquaculture must be carefully evaluated to facilitate their widespread adoption and commercialization.

Viral Vectors

Viral vectors are one of the most efficient gene delivery systems in aquaculture. Viruses such as adenoviruses, retroviruses, and lentiviruses can be engineered to carry therapeutic genes into fish cells. These vectors have high transduction efficiency and can deliver genes directly into the host genome, allowing for stable, long-term gene expression. However, safety concerns regarding potential viral pathogenicity and immune responses in fish must be carefully addressed.

Non-viral Vectors

Non-viral vectors offer alternative gene delivery methods with potentially fewer safety risks. Liposomes, nanoparticles, and polymer-based delivery systems can encapsulate and protect nucleic acids, facilitating their uptake by fish cells. Non-viral vectors are generally safer and easier to produce than viral vectors, but they often exhibit lower transfection efficiency

and transient gene expression compared to viral vectors (Sáez et al., 2018).

Chitosan

Nanotechnology-based approaches in the realm of gene delivery for fish hold promising prospects for advancing aquaculture and fisheries sciences. These techniques leverage nanoscale materials, such as nanoparticles and nanocarriers, to efficiently transport genetic material into fish cells. One prevalent method involves the encapsulation of DNA or RNA within biocompatible nanoparticles, enhancing their stability and cellular uptake. Additionally, surface modifications of these nanoparticles enable targeted delivery to specific tissues or cell types within fish organisms, minimizing off-target effects. Moreover, nanotechnology facilitates the development of non-viral vectors, offering a safer alternative to viral vectors commonly used in gene delivery. Through precise control over particle size, shape, and surface properties, nanotechnology enables optimized gene delivery systems tailored to the unique physiological characteristics of fish species (El-Naggar et al., 2021).

In recent years, chitosan has emerged as a non-viral vector and promising tool in the field of molecular biology, offering a method for transient modification of gene expression without the need for creating genetically modified organisms. Chitosan, a natural polysaccharide derived from chitin, has gained attention due to its biocompatibility, biodegradability, and low toxicity. One of the key advantages of utilizing chitosan in genetic studies is its ability to facilitate overexpression of exogenous genes and gene silencing through RNA interference (RNAi). By encapsulating small interference RNA (siRNA) or plasmid DNA within chitosan nanoparticles, researchers can efficiently deliver these genetic materials into target cells or tissues. The transient nature of chitosan-mediated gene expression modulation offers several benefits. Unlike traditional genetic engineering techniques that involve the permanent alteration of an organism's genome, chitosan-based methods allow for temporary modifications. This feature is particularly advantageous in applications where the desired genetic changes are only needed for a specific duration or where regulatory restrictions limit the use of GMOs, as it is the case in aquaculture. Furthermore, chitosan nanoparticles can be tailored to target specific cell types or tissues, enhancing the precision and efficiency of gene delivery. This targeted approach minimizes off-target effects and improves the overall safety profile of the technique. Overall, the use of chitosan as a vehicle for transient gene expression modulation represents a promising avenue for advancing molecular biology research and biotechnological applications. Its versatility, biocompatibility, and non-permanent nature make

it an attractive alternative to conventional genetic engineering methods, offering new opportunities for studying gene function, disease mechanisms, and therapeutic interventions (Silva-Marrero et al., 2019).

1.6.3. Target Genes for Nutrient Utilization

Glucose Metabolism Genes

Genes involved in glucose metabolism play a central role in regulating carbohydrate utilization and energy production in fish. Targeting genes encoding enzymes such as GCK, and PFK in the liver can enhance the efficiency of glycolysis and promote the utilization of dietary carbohydrates for energy production in cultured fish. Similar effects could be achieved by down-regulating the expression of gluconeogenic enzymes, such as G6PC. By modulating the expression of these genes, researchers can optimize carbohydrate utilization and reduce the reliance on dietary proteins for energy (Basto-Silva et al., 2021).

Insulin Signaling Pathway Genes and Growth Hormone/Insulin-like Growth Factor Axis Genes

The insulin signaling pathway regulates glucose uptake, glycogen synthesis, and lipid metabolism in fish. Targeting genes involved in insulin signaling, such as insulin receptor (IR), insulin-like growth factor 1 receptor (IGF-1R), and GLUT4, can enhance glucose uptake and utilization in target tissues. By promoting insulin sensitivity and glucose disposal, modulation of insulin signaling pathway genes can improve carbohydrate metabolism and growth performance (Xu et al., 2018).

The growth hormone (GH)/IGF axis regulates growth, metabolism, and nutrient partitioning in fish. Targeting genes involved in GH and IGF signaling pathways can modulate growth performance and nutrient utilization in cultured fish. Genes such as growth hormone receptor (GHR), IGF-1, and IGF-binding proteins (IGFBPs) play key roles in mediating the effects of GH and IGF on growth and metabolism. By manipulating the expression of these genes, nutrient partitioning and enhancement of growth efficiency may be optimized in farmed fish (Matejkova & Podhorec, 2019).

Lipid Metabolism Genes

SREBPs are key regulators of lipid homeostasis in cells, controlling the expression of genes involved in lipid synthesis and uptake. SREBP1 promotes the transcription of genes encoding enzymes required for fatty acid synthesis, such as ACC and fatty acid synthase. Additionally, it enhances the expression of genes involved in the synthesis of triglycerides and phospholipids. While SREBP2 primarily regulates cholesterol synthesis, SREBP1 also contributes to cholesterol homeostasis by regulating the expression of genes involved in cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase. SREBP1 activation leads to increased transcription of genes involved in fatty acid synthesis, resulting in elevated levels of fatty acids within cells. This is particularly important for cells with high lipid biosynthetic demands, such as hepatocytes and adipocytes. Enhanced SREBP1 activity promotes the synthesis of triglycerides, leading to lipid droplet formation and storage. This process is crucial for energy storage and adipocyte function. While SREBP2 is the primary regulator of cholesterol biosynthesis, SREBP1 also contributes to this process by inducing the expression of key enzymes involved in cholesterol synthesis. Dysregulation of SREBP1 activity has been implicated in insulin resistance and metabolic disorders such as obesity and type 2 diabetes. SREBP1 overexpression in liver and adipose tissue has been shown to impair insulin signaling and glucose metabolism.

Lipid metabolism genes also play a critical role in nutrient utilization and energy balance in fish. Targeting genes encoding enzymes involved in lipogenesis, lipolysis, and fatty acid oxidation can influence lipid storage, mobilization, and utilization in cultured fish. By modulating the expression of genes such as ACC, FAS, and carnitine palmitoyltransferase I (CPT1), lipid metabolism and energy production pathways may be regulated to spare protein and promote carbohydrate utilization (Droubogiannis & Katharios, 2022). The administration of chitosan-TPP-pSG5-SREBP1a significantly increased SREBP1a mRNA and protein levels in the liver of *S. aurata*. This overexpression of SREBP1a led to enhanced hepatic expression of key genes involved in glycolysis-gluconeogenesis (GCK and PFKFB1) and fatty acid synthesis (acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2)), while reducing the production of G6PD (Silva-Marrero et al., 2019).

Amino Acid Metabolism Genes

Amino acid metabolism genes contribute to protein synthesis, degradation, and recycling in fish. Targeting genes involved in amino acid transport, catabolism, and protein turnover can influence nitrogen utilization and protein sparing. Genes such as

aminotransferases, glutamate dehydrogenase (GDH), amino acid transporters (e.g., SLC7A5), amino acid degrading enzymes (e.g., alanine-glyoxylate aminotransferase), and proteolytic enzymes (e.g., proteasome subunits) are potential targets for modulating amino acid metabolism and optimizing protein utilization efficiency (Teles et al., 2018). In *S. aurata*, our research group previously examined the impact of cALT silencing in the liver of *S. aurata* 72 hours following intraperitoneal administration of chitosan-tripolyphosphate (TPP) nanoparticles loaded with a plasmid carrying a shRNA sequence aimed at reducing cALT expression (pCpG-si1sh1). In fish subjected to various protein-to-carbohydrate ratio diets and treated with chitosan-TPP-pCpG-si1sh1, levels of both cALT1 and cALT2 mRNA notably decreased, regardless of the dietary composition. Concurrently, liver ALT activity decreased in the treated specimens. Moreover, in the liver of *S. aurata* treated with chitosan-TPP-pCpG-si1sh1 nanoparticles, down-regulation of cALT expression led to increased activity of key enzymes involved in glycolysis (such as PFK and PK) (Gonzalez et al., 2016). In another study, *S. aurata* were examined 72 hours post-administration of TPP nanoparticles loaded with a plasmid containing a shRNA targeting GDH expression (pCpG-sh2GDH). Following this treatment, both the mRNA levels and detectable protein of GDH in the liver notably decreased, consequently leading to a reduction in GDH activity in both oxidative and reductive reactions to approximately 53-55 % of control levels. Silencing GDH also resulted in decreased activity of glutamate, glutamine, and aspartate aminotransferases, alongside an increase in 2-oxoglutarate content, OGDH activity, and PFK/FBP activity ratio (Gaspar et al., 2018). Taken together, these studies allowed us to conclude that chitosan-dependent decreased transdeamination in the liver of *S. aurata* is a promising strategy to induce carbohydrate utilization in carnivorous fish and promote protein sparing.

1.6.4. Implications for Aquaculture Sustainability

By controlling the expression of key genes involved in nutrient metabolism, gene therapy can enhance feed efficiency in farmed fish species. Manipulating genes related to protein sparing and carbohydrate utilization can reduce the reliance on expensive protein sources in aquafeeds, leading to cost savings for aquaculture producers and reduced pressure on wild fish stocks, while improving growth performance by promoting efficient conversion of dietary nutrients into biomass (Katz et al., 2019). Gene therapies that enable significant replacement of dietary protein by carbohydrates can contribute to the reduction of ammonia and nitrogenous waste, and the environmental pollution associated with aquaculture activities.

By minimizing the excretion of nitrogenous compounds and undigested nutrients, eutrophication of water bodies can be mitigated, minimizing the ecological footprint of aquaculture and promoting environmental sustainability and ecosystem health (Gratacap et al., 2020).

Gene therapy can also play a crucial role in enhancing disease resistance and health management in aquaculture. By modulating the expression of genes related to immune function and disease resistance, enhanced resilience to common pathogens and diseases can be achieved, in addition to reducing the need for antibiotics and chemical treatments in aquaculture systems (Polakof et al., 2012).

Despite its potential benefits, the widespread adoption of gene therapy in aquaculture faces regulatory and public acceptance challenges. Regulatory agencies must establish clear guidelines and safety standards for genetically modified fish strains, ensuring that they pose minimal risks to human health and the environment. Additionally, efforts to educate consumers and stakeholders about the safety and benefits of genetically modified aquaculture products are essential to gain public trust and acceptance (El-Nokrashy et al., 2021).

1.7. Review article; Chitosan-Based Drug Delivery System: Applications in Fish Biotechnology

polymers

MDPI

Review

Chitosan-Based Drug Delivery System: Applications in Fish Biotechnology

Yuanbing Wu [1,†], Ania Rashidpour [1,†], María Pilar Almajano [2] and Isidoro Metón [1,*]

[1] Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27–31, 08028 Barcelona, Spain; wuuanbing@gmail.com (Y.W.); aniyarashidpoor2017@gmail.com (A.R.)

[2] Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain; m.pilar.almajano@upc.edu

* Correspondence: imeton@ub.edu; Tel.: +34-93-4024521

† Both authors contributed equally to this work.

Received: 29 April 2020; Accepted: 19 May 2020; Published: 21 May 2020

Abstract: Chitosan is increasingly used for safe nucleic acid delivery in gene therapy studies, due to well-known properties such as bioadhesion, low toxicity, biodegradability and biocompatibility. Furthermore, chitosan derivatization can be easily performed to improve the solubility and stability of chitosan–nucleic acid polyplexes, and enhance efficient target cell drug delivery, cell uptake, intracellular endosomal escape, unpacking and nuclear import of expression plasmids. As in other fields, chitosan is a promising drug delivery vector with great potential for the fish farming industry. This review highlights state-of-the-art assays using chitosan-based methodologies for delivering nucleic acids into cells, and focuses attention on recent advances in chitosan-mediated gene delivery for fish biotechnology applications. The efficiency of chitosan for gene therapy studies in fish biotechnology is discussed in fields such as fish vaccination against bacterial and viral infection, control of gonadal development and gene overexpression and silencing for overcoming metabolic limitations, such as dependence on protein-rich diets and the low glucose tolerance of farmed fish. Finally, challenges and perspectives on the future developments of chitosan-based gene delivery in fish are also discussed.

Keywords: chitosan; gene delivery; gene overexpression; gene silencing; fish biotechnology

1. Introduction

Chitosan is a cationic polymer of β (1-4)-linked 2-amino-2-deoxy-D-glucose interspersed by residual 2-acetamido-2-deoxy-β-D-glucose, derived from chitin by deacetylation under alkaline conditions. Chitin is the second most abundant polysaccharide in nature, after cellulose, and it is obtained from the external skeleton and skin of arthropods and insects. Chitin is also found in some microorganisms, yeast and fungi. Mucoadhesion, low toxicity, biodegradability and biocompatibility, as well as antioxidant, antibacterial, antifungal, antitumor and anti-inflammatory properties led, in recent years, to the increasing use of chitosan in a wide variety of pharmaceutical, biomedical and biotechnological fields, including wound healing, tissue engineering, bone regeneration, gene therapy, food industry and agriculture [1–6].

Chitosan has many desirable biological properties that make it a highly suitable carrier to deliver nucleic acids for the development of gene therapy assays. The goal of gene therapy is to introduce exogenous genetic material into target cells, with the aim of modifying the expression of specific genes. The efficient delivery of plasmid DNA to express exogenous genes or siRNA to knockdown the expression of target genes must overcome systemic and cell barriers, depending on the target tissue

and nature of the molecular mechanism triggered by the gene therapy. Ideally, for safe nucleic acid delivery, the vector must establish a stable interaction with the cargo, protect it from the action of nucleases, reach target cells, enable crossing the cell membrane and, once inside the cell, facilitate escape from endosomes and lysosomes. Decomplexation from the carrier must allow plasmid DNA to cross the nuclear membrane and become transcribed, or in the case of siRNA, render the cargo in the cytosol [7–9].

Nucleic acid delivery into cells is facilitated by viral and non-viral vectors. The choice of the vector for gene therapy is a key step to properly reach target cells, confer protection from nucleases, cross the cell membrane, nucleic acid escape from endosomal vesicles, determine transient or permanent effects, allow transcription of delivered plasmid DNA and knockdown the expression of target genes by RNA interference (RNAi) [7,10].

Due to its high transfection efficiency, viral vectors are still used in most gene therapy assays. However, immunogenicity, acute inflammation and other unwanted effects, such as reversal of the wild-type phenotype associated with the use of viral vectors, have focused attention on the development of safer alternative gene delivery systems [9,11,12]. Non-viral vectors include lipid-based vectors and cationic polymers. Low transfection efficiency in vivo, reduced half-life of lipoplex circulation, cytotoxicity and other non-desired effects, such as complement activation, limit in vivo use of cationic lipids and lipid-based vectors [10,13–16]. Unlike viral vectors, cationic polymers, such as chitosan and its derivatives, exhibit increased ability to select target tissues, easy large-scale production, low toxicity and immunogenicity in vivo and biocompatibility [4,9,10]. In this review, we will summarize recent advances in chitosan-based formulations for delivering nucleic acids, and address current progress of the use of chitosan for fish biotechnology applications and gene therapy.

2. Chitosan as a Nucleic Acid Delivery Vector

The use of chitosan as a vector for nucleic acid delivery was proposed in 1995 [17]. A few years later, in 1998, in vivo administration of chitosan complexed with plasmid DNA to express a reporter gene in the upper small intestine and colon of rabbits was published [18]. It was in 2006 when chitosan nanoparticles encapsulating small interfering RNA (siRNA) were shown to be also effective for silencing the expression of target genes [19]. Since pioneering studies, much progress has been made in this area, and chitosan is considered, at present, one of the most effective non-viral gene delivery systems. Figure 1 shows Web of Science (Clarivate Analytics) citations, with the topics chitosan, fish and gene delivery until 2019.

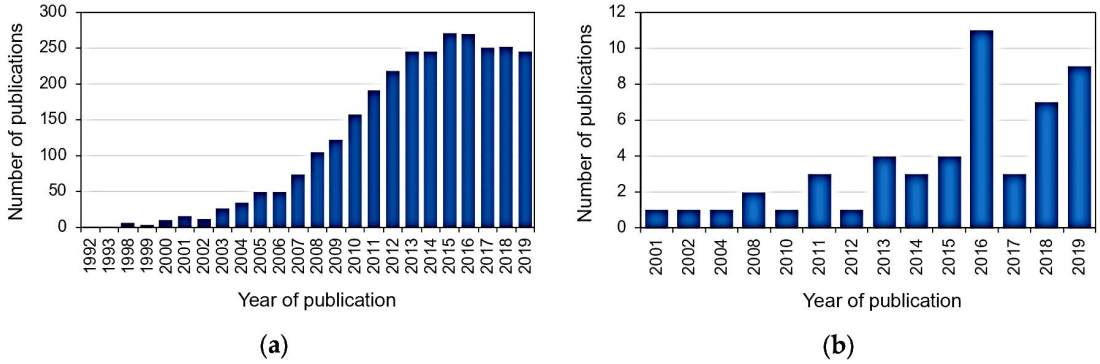


Figure 1. Web of Science (Clarivate Analytics) citations published until 2019 with the topics: (**a**) chitosan and gene therapy; (**b**) chitosan, fish and gene therapy.

The presence of numerous primary amine groups that are protonated at slightly acidic pH in chitosan allows electrostatic interaction with negatively charged nucleic acids. The stability of the complex formed between chitosan and nucleic acids allows oral, nasal, intravenous and intraperitoneal

administration of chitosan–DNA complexes, and prevents dissociation before reaching the intracellular compartment [20–22]. Oral delivery would mainly result in intestinal absorption of the product [22]. Biodistribution of radioiodinated chitosan fractions with different molecular mass, intravenously injected to rats, showed rapid plasma clearance (<15% in the blood 5 min following treatment) and localization in the liver of most of the chitosan with diameter size >10 kDa (>50% at 5 min following intravenous administration and >80% at 60 min post-treatment). However, low molecular weight chitosan (<5 kDa) was cleared more slowly from the circulation and significantly less retained in the liver at the short- and long-term [20].

2.1. Chitosan Derivatization

Derivatization can greatly influence biodistribution of chitosan complexes. An illustrative example was developed by Kang et al. to down-regulate Akt2 expression for treatment of colorectal liver metastases in mice [23]. To protect siRNA from gastrointestinal degradation, facilitate active transport into enterocytes and enhance transportation to the liver through the enterohepatic circulation, the authors first obtained gold nanoparticles conjugated with thiolated siRNA (AR). The resulting complex was subsequently complexed with glycol chitosan−taurocholic acid (GT) through electrostatic interaction to generate AR-GT nanoparticles. Derivatization with taurocholic acid successfully protected Akt2-siRNA from gastrointestinal degradation and favored targeting to the liver through the enterohepatic circulation. Chitosan derivatization with hydrophilic ethylene glycol (glycol chitosan) increases solubility in water at a neutral/acidic pH. In addition, the reactive functional groups of glycol chitosan facilitate chemical modifications and formation of different derivatives useful for targeting gene delivery [24]. In addition to the properties of chitosan derivatives, the efficient delivery of the cargo greatly depends on chitosan polyplex properties, such as pH, molecular weight, deacetylation degree and N/P ratio [7,9].

The molecular weight of chitosan is a major factor affecting polyplex formation, the stability of the chitosan/DNA complex, cell entry, DNA unpacking after endosomal escape and transfection efficiency. Furthermore, the average particle size is highly dependent on the molecular weight of chitosan [7,9,25]. Chitosan between ~20–150 kDa forms chitosan–plasmid DNA complexes with diameter size of ~155–200 nm. High molecular weight chitosan >150 kDa losses solubility and favors aggregate formation, whereas chitosan of molecular weight <20 kDa tends to form polyplexes with diameter size >200 nm [26]. The optimal molecular weight range for stable chitosan–siRNA nanoparticle formation and efficient transfection and silencing effect is considered to be ~65–170 kDa [27].

Chemical modification of chitosan can greatly improve desirable properties for gene delivery. Functional groups of chitosan include C_3-OH, C_6-OH, C_2-NH_2, acetyl amino and glycoside bonds [6,28]. Two of the functional groups, C_6-OH and C_2-NH_2, have chemical properties that make them of particular interest for derivatization (Figure 2).

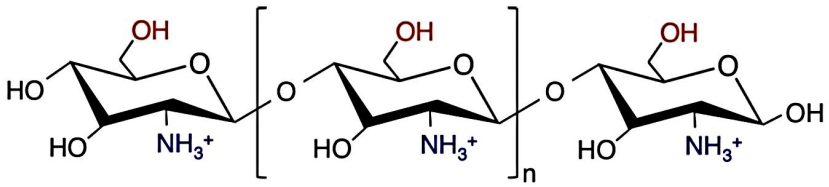


Figure 2. Schematic representation of chitosan. Functional groups C_2-NH_2 and C_6-OH and are represented in blue and red color, respectively.

2.2. Chitosan Solubility

The water solubility of chitosan is low due to the presence of highly crystalline intermolecular and intramolecular hydrogen bonds, and can be greatly influenced by the pH, molecular weight and deacetylation degree [6,9,29]. The solubility of chitosan has been improved by introducing a hydrophilic

group on amino or hydroxyl groups. Examples include: N-acylated chitosan derivatives, which exhibit enhanced biocompatibility, anticoagulability, blood compatibility and sustained drug release [6,30]; chitosan conjugation with saccharides through *N*-alkylation, such as glycosylation [3,31,32]; and the introduction of a quaternary ammonium salt group, which increases chargeability, mucoadhesion, crossing of mucus layers and binding to epithelial surfaces [6,33,34].

2.3. Stability of Chitosan Polyplexes

To increase the stability of chitosan-based formulations, a number of chitosan derivatives have been developed. Among them, PEGylation [35–37], glycosylation [3,38,39] and quaternization [39–42]. The choice of the method for preparing chitosan–nucleic acid complexes can also significantly affect stability of the complex and transfection efficiency. Katas and Alpar showed that for efficient siRNA-mediated silencing of the expression of target genes in CHO K1 and HEK 293 cells, nanoparticles produced by ionic gelation of tripolyphosphate (TPP) with chitosan were more efficient in delivering siRNA than chitosan–siRNA complexes and siRNA adsorbed onto chitosan–TPP nanoparticles. Chitosan–TPP-siRNA nanoparticles generated by ionic gelation presented higher binding capacity and loading efficiency [19]. During ionic gelation, TPP is a polyanion that crosslinks with positively charged chitosan through electrostatic interaction, avoiding the use of toxic reagents for chemical crosslinking, and allowing for the easy modulation of size and surface charge of the nanoparticles (Figure 3). The addition of TPP was shown to reduce the particle size and increase the stability of complexes in biological fluids [19,43–47]. The inclusion of hyaluronic acid in chitosan–siRNA polyplexes can be also a promising strategy to increase stability and targeting capacity, while lowering aggregation in the presence of serum proteins [48].

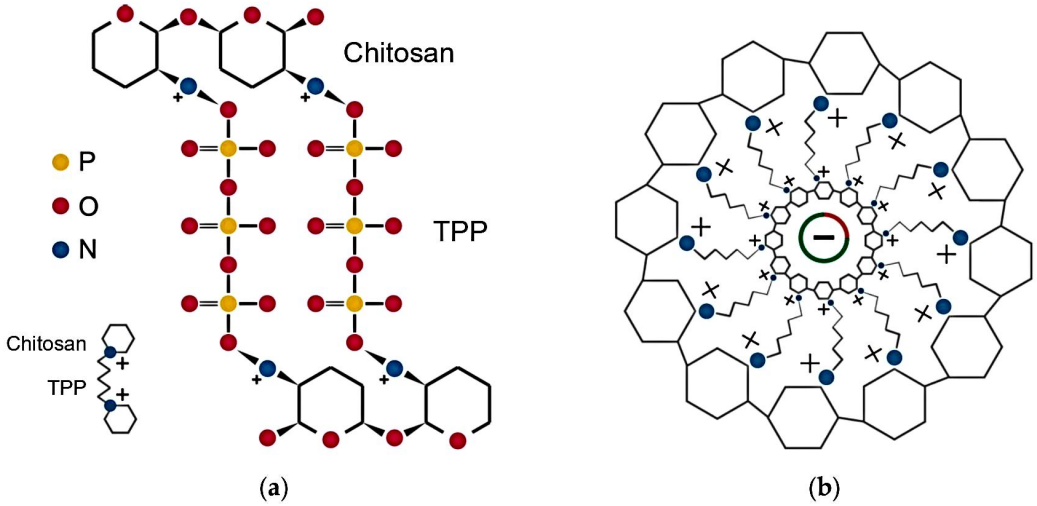


Figure 3. Molecular structure and electrostatic interactions of chitosan–tripolyphosphate (TPP) (**a**), and chitosan–TPP–plasmid DNA nanoparticles (**b**).

One major advantage of chitosan is that chitosan–DNA complexation protects DNA from DNase-mediated degradation, possibly as a result of modification of the DNA tertiary structure [20,49]. Cell penetration of chitosan-based gene delivery systems involves interaction between positively charged chitosan–nucleic acid polyplexes and negatively charged cell membrane components, such as heparan sulfate proteoglycans, enabling ATP-driven crossing of the cell membrane, or receptor-mediated endocytosis. In any case, chitosan polyplexes are internalized following the endocytic-lysosomal pathway [7].

2.4. Targeting Drug Delivery, Cellular Uptake and Intracellular Trafficking

Safe and effective therapies can be performed by using chitosan derivatives to improve target drug delivery. To this end, a variety of molecules can be conjugated to chitosan, such as proteins and peptides, polysaccharides, oligonucleotides and other molecules [4].

2.4.1. Targeting Drug Delivery with Chitosan Derivatives

A common strategy to target drug delivery is based on ligand-receptor specificity. Cell-target delivery drugs can be thus enhanced by conjugation of chitosan–nucleic acid complexes with ligands that enable binding to receptors specifically found in the target cell membrane. Examples of ligands conjugated to chitosan formulations include transferrin, galactose and mannose. For instance, transferrin can be used as a targeting ligand for delivery into tumor cells through binding to the transferrin receptor, whose expression is enhanced in tumor cells to provide iron as a necessary cofactor for DNA synthesis and rapid cell proliferation [50–52]. The presence of asialoglycoprotein receptors on the hepatocyte surface and selective binding of asialoglycoprotein receptors to galactose allow galactosylated chitosan to target hepatocytes [53,54]. Mannosylated chitosan takes advantage of mannose recognition by mannose receptors to target dendritic cells [55].

Chitosan derivatives generally achieve mucosal adhesion through hydrogen bonding or non-specific, non-covalent, electrostatic interactions. Thiolated chitosan increases mucoadhesion and enhances crossing capability trough the cell membrane and ophthalmic drug delivery [56–60]. The mucoadhesive properties of chitosan derivatives allow oral administration and nasal immunization to treat respiratory diseases [61]. Other examples include O-carboxymethyl chitosan, which can be used for intestine-targeted drug delivery [62], and acetylated low molecular weight chitosan, for targeting the kidneys [63].

2.4.2. Endosomal Escape, Unpacking and Nuclear Import of DNA

The proton sponge effect of chitosan gene delivery formulations allows endosomal escape before the maturation of early endosomes into late endosomes, and the ultimate fusion with lysosomes. The increasing acidification in early endosomes generated by the V-type ATPase proton pump results in progressive protonation of the amine groups of chitosan (pKa value of ~6.5), leading to the influx of water and chloride ions into the endosomes, increased osmotic swelling, endosome lysis and cytosolic release of the endosomal content [9,64]. The endosomal release of chitosan polyplexes can be enhanced by fusogenic peptides [65,66] and pH-sensitive neutral lipids [67]. Efficient transfection and endosomal escape of chitosan polyplexes can be also enhanced by chitosan–polyethylenimine (PEI) copolymeric delivery systems. PEI is a cationic polymer non-viral vector with high transfection efficiency and a strong buffering capacity, which may enhance the influx of chloride anions, osmotic swelling and endosomal lysis. However, PEI-dependent cytotoxic effects constitute a major concern when using PEI for gene delivery [7,68–70]. In contrast, chitosan–PEI complexes exhibit efficient uptake by target cells, high transfection efficiency and negligible toxicity [36,71–75].

Following endosomal escape into the cytosol, chitosan polyplexes carrying DNA must be unpacked, and the entrance of loaded DNA into the nucleus is needed for transfection. The molecular events that mediate DNA unpacking after endosomal release and translocation to the nucleus remain not fully understood. It is generally accepted that, in non-dividing cells, molecules smaller than ~40 kDa can passively diffuse through the nuclear pores, while larger molecules must carry nuclear localization signals for active transportation [68]. Sun et al. largely improved DNA unpacking from chitosan and transfection efficiency upon the conjugation of chitosan with small peptides that can be phosphorylated [76]. The phosphorylation of conjugated peptides mimics the process leading to genomic DNA release and the activation of transcription, mediated by histone phosphorylation. In addition, the introduction of negatively charged phosphate groups may result in electric repulsion between DNA and chitosan conjugated with phosphorylated peptides. Hence, further enhancement of

transfection was obtained by conjugating chitosan with small peptides carrying a nuclear localization signal, in addition to a potentially phosphorylatable serine residue [77]. Exogenous gene expression was improved through a mechanism that enabled DNA import into the nucleus, and enhanced unpacking by the action of nuclear histone kinases. Miao et al. improved endosomal escape and intracellular drug release in HepG2.2.15 cells by loading DNA into a redox-responsive chitosan oligosaccharide-SS-octadecylamine (CSSO) polymer. Intracellular reduction and cleavage of CSSO disulfide bonds '–SS-' by gluthation allowed rapid DNA release [78].

For strategies aiming RNAi on target genes, chitosan has been mostly complexed with siRNA, microRNA (miRNA) and plasmids expressing short hairpin RNA (shRNA). After unpacking, siRNA/miRNA associates with RNA-induced silencing complex (RISC) in the cytosol. The RNAi-guided complex hybridizes with target mRNA, leading to mRNA cleavage and/or translation repression, and subsequent inhibition of protein synthesis [9,10,48,79]. The use of shRNA expression plasmids allowing long lasting expression of siRNA may improve RNAi in vivo. Following plasmid DNA transcription in the nucleus, the transcribed shRNA is processed by Drosha, exported to the cytosol and processed by Dicer, leading to cleavage of double-stranded shRNA and the formation of specific siRNA [75,80–85].

Sequential events associated with three illustrative examples using chitosan to deliver nucleic acids are represented in Figure 4 (chitosan–TPP complexed with a plasmid construct, to express an exogenous protein), Figure 5 (chitosan–TPP complexed with a plasmid construct, to express a shRNA designed for target gene silencing) and Figure 6 (chitosan loading siRNA for target gene silencing).

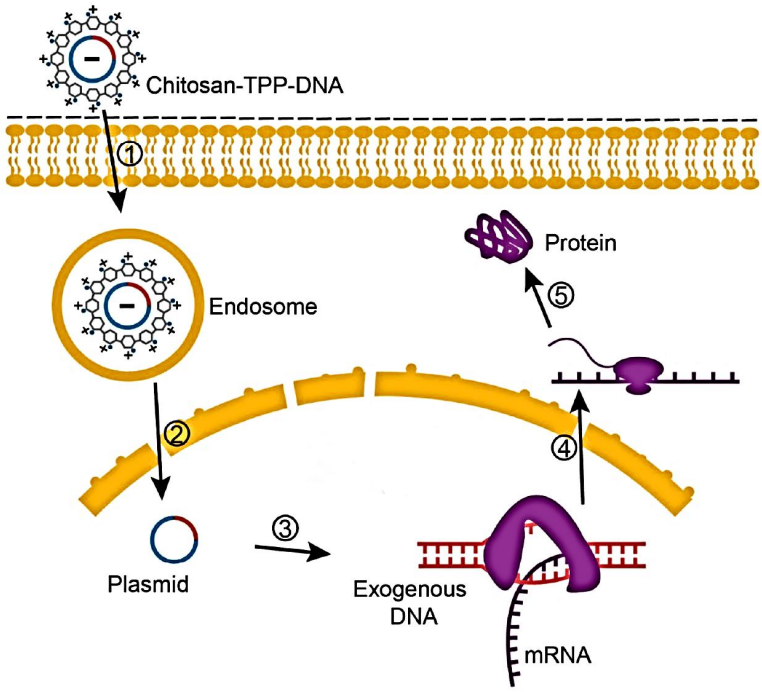


Figure 4. Cellular events associated with chitosan-based plasmid delivery for exogenous gene expression. 1, Cellular uptake of chitosan–DNA by endocytosis. 2, Endosomal escape of the chitosan–DNA complex, plasmid dissociation from chitosan and translocation to the nucleus. 3, Transcription of plasmid (exogenous DNA) in the nucleus and mRNA generation. 4, Translation of newly transcribed mRNA in the cytosol. 5, Exogenous protein assembly.

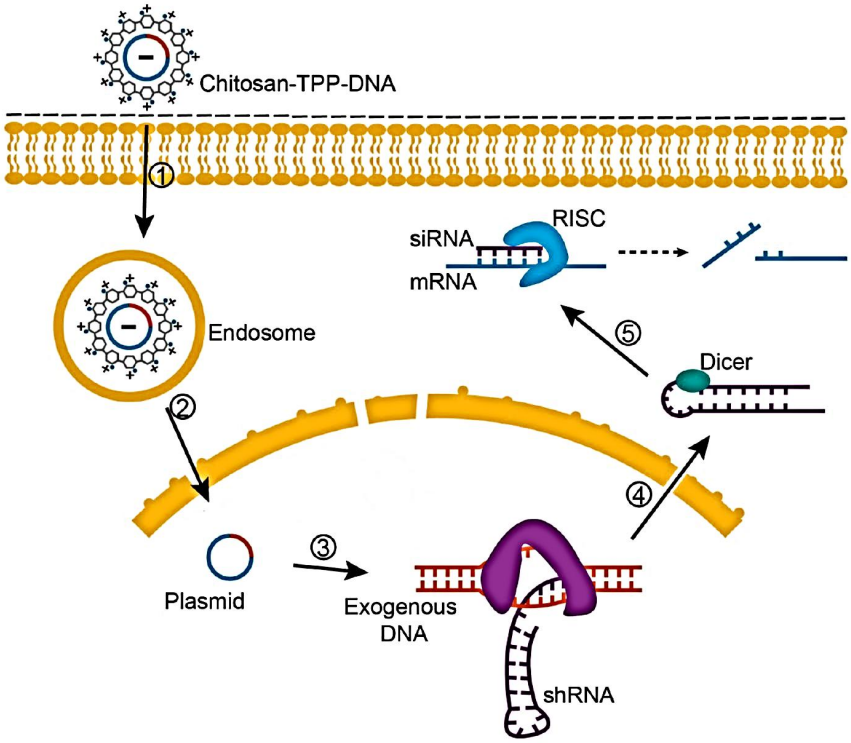


Figure 5. Cellular events associated with chitosan-based plasmid delivery for short hairpin RNA (shRNA) expression, siRNA formation and target gene silencing. 1, Cellular uptake of chitosan–DNA by endocytosis. 2, Endosomal escape of chitosan–DNA complex, plasmid dissociation from chitosan and translocation to the nucleus. 3, Transcription of plasmid (exogenous DNA) in the nucleus and generation of shRNA. 4, Transportation of shRNA to the cytosol and association with Dicer to generate siRNA. 5, siRNA association with RNA-induced silencing complex (RISC) and target mRNA by base pairing, resulting in mRNA cleavage and/or translation repression, and subsequent inhibition of protein synthesis.

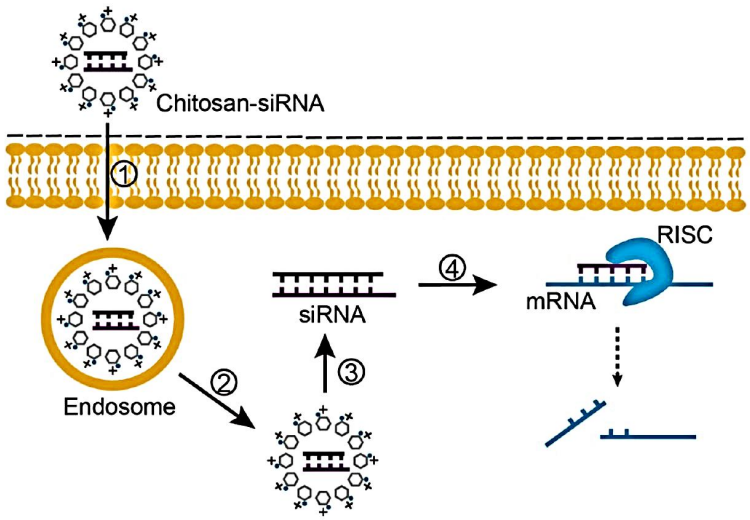


Figure 6. Cellular events associated with chitosan-based siRNA delivery for target gene silencing. 1, Cellular uptake of chitosan–siRNA by endocytosis. 2, Endosomal escape of chitosan–siRNA. 3, Dissociation of siRNA from chitosan. 4, siRNA association with RISC and target mRNA by base pairing, resulting in target mRNA cleavage and/or translation repression, and subsequent inhibition of protein synthesis.

3. Use of Chitosan in Fish Biotechnology

Chitosan and its derivatives are widely used in aquaculture. Low toxicity, biodegradability, biocompatibility, bioadhesion and immunomodulatory properties make chitosan and its derivatives of increasing interest for the fish farming industry as dietary additives, non-viral vectors enabling fish vaccination and protection against diseases, control of gonadal development and for the gene therapy-based modulation of fish metabolism.

3.1. Chitosan and Its Derivatives as Dietary Additives

Dietary supplementation with chitosan and its derivatives has been shown to improve fish growth performance, non-specific immunity and antioxidant effects [86,87]. However, the strategy for chitosan dietary supplementation in fish requires extensive investigation, according to the species and the growth stage of fish.

3.1.1. Dietary Supplementation with Chitosan

The inclusion of chitosan as feed additive for fish has been receiving attention since the 1980s [88]. Shiau et al. reported that inclusion of dietary levels of chitosan from 2% to 10% for 28 days decreases the weight gain and increases the feed conversion ratio (FCR) in hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) [89]. However, other studies performed in *Oreochromis niloticus* showed positive effects of chitosan on fish growth. Feed supplementation of tilapia with chitosan (0–8 g/kg dry diet) for 56 days led to the conclusion that 4 g/kg of chitosan was the optimal dose to promote the highest body weight gain (BWG) rate and specific growth rate (SGR) [90]. Similarly, chitosan supplementation at 5 g/kg diet for 60 days improved growth performance, BWG, SGR and FCR in tilapia [91]. The contradictory effects reported for chitosan on tilapia growth could be attributed to the fact that the studies were performed using different fish growth stages. Indeed, the initial weight of fish in the study by Shiau et al. was of 0.99 ± 0.01 g, while the latter two reports used a significantly higher initial body weight (50.1 ± 4.1 g and 39.3 ± 0.3 g, respectively).

In addition to the developmental stage and amount of dietary chitosan supplied, chitosan effects exerted on fish growth performance also seem to depend on the species [87]. According to the effect observed on SGR, the apparent digestibility coefficient of dry matter and the apparent digestibility coefficient of protein, 75 days of feeding on diets supplemented with 10–20 g chitosan/kg significantly reduced the growth performance of gibel carp (*Carassius gibelio*) (initial body weight, 4.80 ± 0.01 g) [92]. However, the supply of 0–0.2 g chitosan/kg diet caused a dose dependent increase of the average daily weight and SGR in post-larvae sea bass (*Dicentrarchus labrax*) [93]. Yan et al. also reported that dietary supplementation of 0%–5% chitosan improved growth performance by inducing dose dependent increases of BWG and SGR, while FCR decreased [94]. Similarly, 70 days of supplementation with 1–5 g chitosan/kg diet of loach fish (*Misgurnus anguillicadatus*) with an average body weight of 3.14 ± 0.05 g, significantly increased BWG, SGR and condition factor (CF), whereas it decreased FCR [95]. In contrast, Najafabad et al. found that Caspian kutum (*Rutilus kutum*) fingerlings (1.7 ± 0.15 g) supplied with 0–2 g chitosan/kg diet for 60 days showed no effect of final weight, SGR and condition factor [96].

The positive effect of chitosan on the growth performance of some fish species might result from its role in nonspecific immunity. Chitosan acts as an immunostimulary drug through induction of nonspecific immunity in fish. In loach fish, the dietary supplement of chitosan increased the serum levels of factors considered as immune boosters, such as the content of immunoglobulin M (IgM), complement component 3 (C3) levels, the activity of lysozyme, acid phosphatase and alkaline phosphatase, as well as increased the survival rate after being challenged by *Aeromonas hydrophila* [95]. In accordance with the immune boost, other investigations also showed immune reinforcement by chitosan, when fish were challenged by bacteria in regard to immunoglobulin content, serum lysozyme, bactericidal activity, immune-related gene expression, phagocytosis and respiratory burst activity [90,92,94,97]. Consistently, chitosan was shown to modify hematological parameters of fish,

which are also considered important indicators of immunostimulation. In Asian seabass (*Lates calcarifer*), chitosan supplement during 60 days at 5–20 g/kg diet increased red blood cells (RBC), white blood cells (WBC), total serum protein, albumin and globulin [98]. Supplementation with chitosan was reported also to increase RBC, WBC, haemoglobin, lymphocytes, monocytes, neutrophils and thrombocytes in mrigal carp (*Cirrhinus mrigala*) and kelp grouper (*Epinephelus bruneus*) [99–101].

Concomitant to the effects on immunity, chitosan also elevates antioxidant responses in fish. In loach fish, the activity of phenoloxidase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) increased after 12 weeks of chitosan supplementation [95]. Similarly, chitosan induced the activity of SOD and catalase (CAT) after 56 days of dietary supplementation in tilapia [90], and the mRNA levels of SOD, CAT, GPx and nuclear factor erythroid 2-related factor 2 [94]. The protective effect of chitosan from oxidative stress was also reported in olive flounder (*Paralichthys olivaceus*) challenged with H_2O_2 [97]. The authors observed that chitosan-coated diets significantly narrowed the increase of protein carbonyl formation and DNA damage in the plasma.

3.1.2. Dietary Supplementation with Chitosan Nanoparticles

Wang et al. reported that BWG significantly increased in tilapia (initial body weight, 23.6 ± 1.2 g) fed with chitosan nanoparticles (5 g/kg dry diet) [102]. Similar results were described by other authors. Chitosan nanoparticle intake increased final weight, weight gain, SGR and FCR in tilapia supplied for 45 days with 0–2 g/kg (initial body weight, 19.8 ± 0.6 g) and 70 days for 1–5 g/kg (initial body weight, 5.66 ± 0.02 g). In these reports, innate immunity was also enhanced and fish exhibited increased respiratory burst activity, lysozyme malondialdehyde, CAT and SOD activity, and hematological parameters such as RBC, hematocrit, hemoglobin, mean corpuscular volume, WBC and platelets [103,104]. Remarkably, optimal supplement of dietary chitosan nanoparticles to improve growth and immunity against pathogens may vary, according to parameters such as developmental growth stage and species.

Dietary supplementation of chitosan nanoparticles complexed with vitamin C and thymol is more effective in enhancing immunity than supplementation with the single additives. Dietary chitosan–vitamin C nanoparticles slightly improved growth performance of tilapia, while inducing the viscerosomatic index, therefore decreasing economic performance. However, when fish fed chitosan–vitamin C nanoparticles were challenged by imidacloprid-polluted water, chitosan–vitamin C supplementation significantly strengthened immunity and antioxidant activity, including the activity of lysozyme, glutathione reductase and CAT, C3 and immunoglobulins [105]. Growth effects of dietary supplementation with chitosan nanoparticles mixed with thymol, the most important phenolic compound in *Thymus vulgaris* essential oil, were evaluated on hematological parameters, and the liver and kidney function in tilapia [106]. The results showed that chitosan–thymol nanoparticle supplementation increased feed efficiency and protein efficiency ratio, while it had moderated effects on final weight, weight gain and SGR. Nevertheless, chitosan–thymol produced a synergistic effect on lymphocytes and monocyte leukocytes. The use of chitosan nanoparticles as feed additive is limited by the fact that it can exhibit toxic effects at high levels. In this regard, chitosan nanoparticles significantly decreased hatching rate and survival rate of zebrafish (*Danio rerio*) when the immersion concentration reached 20 and 30 μg/mL or higher [107,108].

3.1.3. Dietary Supplementation with Chitin and Chitooligosaccharide

Meanwhile the inclusion of chitin in the diet has no significant effects on fish growth performance [109–111], chitooligosaccharide (COS) enhances growth performance parameters such as BWG, hepatosomatic and intestosomatic index, SGR and FCR in a number of fish species, including juvenile largemouth bass (*Micropterus salmoides*) [112], striped catfish (*Pangasianodon hypophthalmus*) [113], Nile tilapia (*Oreochromis niloticus*) [114], tiger puffer (*Takifugu rubripes*) [115], koi (*Cyprinus carpio koi*) [116], and silverfish (*Trachinotus ovatus*) [117]. Similarly as in most fish species, dietary supplementation with low molecular weight and highly deacetylated COS

enhances growth performance, innate immunity and digestive enzyme activity in Pacific white shrimp (*Litopenaeus vannamei*) [118]. However, the effect of dietary COS may depend on the species. In this regard, dietary COS supplementation was reported to cause not significant effects on weight gain, FCR and the survival rate in hybrid tilapia (*Oreochromis niloticus*×*O. aureus*) [109]. Similar results were reported for rainbow trout (*Oncorhynchus mykiss*) [119]. Incomplete intestinal development in early developmental stages may contribute to the lack of COS effect on growth performance observed in several fish species.

A number of studies showed that both chitin and COS can be potentially utilized as immunostimulants in fish. Respiratory burst activity, phagocytic activity and lysozyme activity, which are considered indicators of non-specific immunity, have been shown to be significantly stimulated by chitin and COS in a number of fish species, including juvenile largemouth bass (*Micropterus salmoides*) [112], Nile tilapia (*Oreochromis niloticus*) [114], striped catfish (*Pangasianodon hypophthalmus*) [113] and mrigal carp (*Cirrhina mrigala*) [99]. Chitin and COS also induce other immunity parameters, such as nitric oxide production, inducible nitric oxide synthase (iNOS) activity and gene expression [112,120], leukocyte count [99,112,116] and complement activity [99,100].

3.2. Chitosan as a Carrier for Drug Delivery in Fish

Chitosan is nanoscale, biodegradable, biocompatible, hemocompatible, simple and mild for preparation conditions, and is highly efficient for drug loading. Therefore, chitosan has been used for loading a variety of bioactive compounds, such as vitamins, metal ions, inactivated pathogens for vaccines, proteins and nucleic acids in a variety of applications in fish farming. In addition, loading into chitosan can significantly boost the bioeffects of these compounds.

3.2.1. Chitosan Loading Chemical Compounds

The sustained release of compounds complexed with chitosan nanoparticles fulfills the requirements of artificial breeding in fish farming and enable delivery and cell uptake of compounds with low toxicity [121,122]. Chitosan nanoparticles loaded with vitamin C, an important but labile antioxidant, were proven to enhance sustained vitamin C release in the stomach, the intestine and in serum after oral administration in rainbow trout (*Oncorhynchus mykiss*) [123]. Chitosan–vitamin C nanoparticles exhibited a markedly high antioxidant activity and no toxicity up to 2.5 mg/mL in the culture medium of ZFL cells, a zebrafish liver-derived cell line. In addition, chitosan–vitamin C nanoparticles showed the capability to penetrate the intestinal epithelium of *Solea senegalensis* [124]. Several studies evaluated chitosan nanoparticles loading aromatase inhibitors and eurycomanone, compounds that promote gonadal development. Chitosan-mediated delivery of aromatase inhibitors and eurycomanone prolonged serum presence, improved testicular development with lack of testicular toxicity, and led to higher serum concentrations of reproductive hormones [125–128].

3.2.2. Chitosan Loading Metal Ions

Loading with chitosan facilitates delivery of metal ions that are micronutrients and antibacterial factors, such as selenium and silver, to fish in culture. Barakat et al. showed that chitosan–silver nanoparticles successfully treated European sea bass larvae infected with *Vibrio anguillarum*. Chitosan–silver nanoparticles significantly decreased the bacterial number and improved fish survival [129]. In addition, dietary supplementation with chitosan–silver nanoparticles were shown to altering gut morphometry and microbiota in zebrafish. Feeding with chitosan–silver nanoparticles increased *Fusobacteria* and *Bacteroidetes* phyla, goblet cell density and villi height, while upregulated the expression of immune-related genes [130]. Similarly, chitosan–selenium nanoparticles had immunostimulary roles and increased disease resistance in zebrafish and *Paramisgurnus dabryanus* by improving the activity of lysozyme, acid phosphatase and alkaline phosphatase, phagocytic respiratory burst and splenocyte-responses towards concanavalin A [131,132].

3.2.3. Chitosan Loading Inactivated Pathogens

Vaccines against pathogens is a major challenge in aquaculture. In this regard, chitosan can be used as proper carrier and adjuvant to enhance effectiveness of vaccination. A number of inactivated bacteria and virus have been evaluated with chitosan or its derivatives as adjuvant against infections in fish. Vaccines, such as inactivated *Edwardsiella ictaluri* and infectious spleen and kidney necrosis virus, have been tested with chitosan in yellow catfish (*Pelteobagrus fulvidraco*) and Chinese perch (*Siniperca chuasi*), respectively. Chitosan enhanced incorporation into the host cells and improved fish survival rate and immune response, increasing IgM content, lysozyme activity and mRNA levels of interleukin (IL)-1β, IL-2 and interferon (IFN)-γ2 [133,134]. A mixture of COS and inactivated *Vibrio anguillarum* vaccine significantly reduced zebrafish mortality against *Vibro anguillarum* [135], while COS combined with inactivated *Vibrio harveyi* also markedly increased survival rate, IgM and the expression of immune-related genes, such as IL-1β, IL-16, tumor necrosis factor-alpha (TNF-α) and major histocompatibility complex class I alpha (MHC-Iα), in the grouper ♀*Epinephelus fuscoguttatus*×♂*Epinephelus lanceolatus* [136]. Similarly, rainbow trout (*Oncorhynchus mykiss*) immunized against bacterial infection (*Lactococcus garvieae* and *Streptococcus iniae*) through chitosan–alginate coated vaccination exhibited a higher survival rate, immune-related gene expression, and antibody titer than fish submitted to non-coated vaccination [137].

Olive flounder (*Paralichthys olivaceus*) vaccinated against inactivated viral haemorrhagic septicaemia virus encapsulated with chitosan through oral and immersion routes showed effective immunization in the head kidney, which is considered as the primary organ responsible for the initiation of adaptive immunity in fish, skin and intestine, which are regarded as the main sites for antigen uptake and mucosal immunity. Additionally to upregulation of IgM, immunoglobulin T (IgT), polymeric Ig receptor (pIgR), MHC-I, major histocompatibility complex class II (MHC-II) and IFN-γ in the three tissues, caspase 3 was also highly induced 48 h post-challenge, suggesting cytotoxicity due to rapid T-cell response and impairment of viral proliferation [138].

Coating chitosan with membrane vesicles from pathogens such as *Piscirickettsia salmonis* was also shown to be an effective strategy to induce immune response in zebrafish (*Danio rerio*) and upregulation of CD 4, CD 8, MHC-I, macrophage-expressed 1, tandem duplicate 1 (Mpeg1.1), TNFα, IL-1β, IL-10, and IL-6 [139].

3.2.4. Chitosan Loading Proteins

Effectiveness of fish vaccination against infections can be also improved with antigenic proteins derived from bacteria and virus. For example, chitosan nanoparticles encapsulated with the recombinant outer membrane protein A of *Edwardsiella tarda* was used for oral vaccination of fringed-lipped peninsula carp (*Labeo fimbriatus*). Treated fish showed significant higher levels of post-vaccination antibody in circulation and survival rate against *Edwardsiella tarda* [140]. In another study, oral vaccination with alginate-chitosan microspheres encapsulating the recombinant protein serine-rich repeat (rSrr) of *Streptococcus iniae* were evaluated and the results showed that lysozyme activity and immune-related genes were induced, leading to a 60% increased survival rate of channel catfish (*Ictalurus punctatus*) against *Streptococcus iniae* infection [141]. In grass carp (*Ctenopharyngodon idella*), chitosan was also used for carrying the immunomodulatory factor IFN-γ2. Treatment with chitosan–*Ctenopharyngodon idella* IFN-γ2 highly upregulated inflammatory factors, leading to severe inflammatory damage in the intestine, hepatopancreas and decreased survival rate [142].

3.2.5. Chitosan Loading Nucleic Acids

Compared to chitosan-based gene delivery in other organisms, gene therapy methodologies using chitosan for improving desirable traits in farmed fish have great potential for development (Figure 1b). A number of studies addressed the characterization of factors that can influence the efficiency of chitosan loading and nucleic acid release, such as the average diameter, zeta potential

and encapsulation efficiency of chitosan–DNA microspheres or nanospheres. Table 1 summarizes chitosan–plasmid DNA encapsulation efficiency and changes in particle diameter and zeta potential before and after encapsulation for fish biotechnology studies. Existing data show that the diameter of chitosan nanospheres before loading DNA mostly ranged from ~30 to ~230 nm, while encapsulation with plasmid DNA led to ~40–190 nm diameter increase. The zeta potential indicates the surface charge on the particles. A higher positive zeta potential suggests higher stability of nanoparticles in the suspension [143]. The zeta potential before loading plasmid DNA were ~25–33 mV, which mostly tended to decrease to ~14–18 mV. The exception was reported by Rather et al., who found that zeta potential of chitosan nanospheres increased ~6 mV following DNA encapsulation [144]. DNA encapsulation efficiency was generally higher than 80%, which indicates that chitosan is capable to load a high mass of DNA, which in turn may benefit many applications in aquaculture.

Table 1. Characteristics of chitosan–plasmid DNA polyplexes for studies performed in fish.

Preloading Diameter (nm)	Postloading Diameter (nm)	Preloading Zeta Potential (mV)	Postloading Zeta Potential (mV)	Encapsulation Efficiency	References
-	<10,000	-	-	94.5%	[145]
30–60	-	-	-	-	[146]
-	200	-	-	91.5%	[147]
-	-	-	-	83.6%	[148]
193 ± 53 [1]	246 ± 74 [1]	32.0 ± 1.0 [1]	14.4 ± 1.3 [1]	-	[80]
-	146 ± 2 [2]	-	24.3 ± 0.5 [2]	92.8% ± 1.4% [2]	[149]
-	133	-	34.3	63%	[150]
-	50-200	-	-	97.5%	[151]
87	156	30.3	36.5	60%	[144]
-	743	-	-	98.6%	[152]
135	-	26.7	-	86%	[153]
-	-	-	-	84.2%	[154]
224 ± 62 [1]	Similar to preloading diameter	33.0 ± 1.2 [1]	14.4 ± 1.3 [1]	-	[81]
-	750–950	-	-	98.6%	[155]
116	306	24.7	18.0	-	[156]
231 ± 18 [2]	272 ± 36 [2]	31.2 ± 1.5 [2]	14.1 ± 2.3 [2]	-	[157]
-	267	-	27.1	87.4%	[158]

[1] Mean ± SD; [2] mean ± SEM.

Chitosan-encapsulated DNA is more stable in vivo, exhibit sustained-release and increased cell uptake than naked DNA. Taken together, these factors confer chitosan-delivered DNA a particular expression profile regarding tissue distribution, persistence of expression and abundance in fish. Sáez et al. found that intramuscular injection led to a restricted expression to adjacent tissues of both chitosan-encapsulated DNA and naked DNA, while the oral administration of chitosan-encapsulated DNA, largely used for fish vaccination studies, showed enhanced expression not only in the intestine, but also in the liver of gilthead sea bream (*Sparus aurata*) [152,155]. Furthermore, oral administration of chitosan nanoparticles loaded with pCMVβ, a plasmid encoding for *Escherichia coli* β-galactosidase, enabled sustained detection of the exogenous plasmid and bacterial β-galactosidase activity in the liver and the intestine of *Sparus aurata* juveniles up to 60 days posttreatment [152].

Through the immersion route, Rao et al. showed that chitosan-coated DNA was confined to the surface area of rohu (*Labeo rohita*), i.e., gill, intestine and skin-muscle, while no detection was observed in the kidney and the liver. Naked DNA was undetectable due to degradation [158]. Oral delivery seems to have a wider distribution of chitosan-encapsulated DNA, being found in the stomach, spleen, intestine, gill, muscle, liver, heart and kidney [148,154,159]. Chitosan-encapsulated DNA has longer and more abundant presence than naked DNA after administration. For example, Rajesh Kumar et al. showed that antibody in serum from fish immunized with a chitosan–DNA vaccine was 30% higher

than naked DNA after 21 days of oral immunity [160]. The presence of DNA vaccine was reported more than 90 days after oral administration of chitosan–DNA [145]. Additionally, Rather et al. reported that chitosan–DNA induced 2-fold longer and higher peak abundant expression of downstream genes than naked DNA [144].

3.3. Chitosan-Based Applications in Fish Biotechnology and Gene Therapy

In recent years, chitosan has been increasingly used for drug and gene delivery in fish biotechnology. Most of the studies used chitosan-based systems to improve oral vaccination, control of gonadal development, and the modification of fish intermediary metabolism.

3.3.1. Fish Vaccination

DNA vaccines delivered by chitosan significantly increase relative percent survival of fish at a range of 45%–82% against bacterial and viral infection [151,156]. Higher doses of chitosan–DNA vaccines resulted in concomitant increase of fish relative percent survival from ~47% to ~70% [154]. In addition, DNA vaccination with chitosan stimulated expression of immune-related genes. Zheng et al. reported upregulation of the expression of immune-related genes, such as interferon-induced GTP-binding protein Mx2 (MX2), IFN, chemokine receptor (CXCR), T-cell receptor (TCR), MHC-Iα and MHC-IIα, 7 days after oral vaccination against reddish body iridovirus in turbot (*Scophthalmus maximus*). A 10-fold higher expression of TNF-α gene expression was found in the hindgut [149].

In addition to the short-term modification of the expression levels of immune-related genes, the administration of chitosan–DNA vaccines also promote a sustained effect after treatment. Valero et al. found that European sea bass (*Dicentrarchus labrax*) orally vaccinated with chitosan-encapsulated DNA against nodavirus failed to induce circulating IgM. However, the expression of genes involved in cell-mediated cytotoxicity (TCRβ and CD8α) and the interferon pathway (IFN, MX and IFN-γ) were upregulated. Three months following vaccination, challenged fish exhibited partial protection with retarded onset of fish death and lower cumulative mortality [151]. Kole et al. immunized rohu (*Labeo rohita*) with chitosan nanoparticles complexed with a bicistronic DNA plasmid encoding the antigen *Edwardsiella tarda* glyceraldehyde 3-phosphate dehydrogenase and the immune adjuvant gene *Labeo rohita* IFN-γ [156]. Follow-up of the expression of immune-related genes in the the kidney, liver and spleen showed maximal upregulation of IgHC (IgM heavy chain), iNOS, toll like receptor 22 (TLR22), nucleotide binding and oligomerization domain-1 (NOD1) and IL-1β at 14 days post immunization. The authors also confirmed that oral and immersion vaccination with chitosan–DNA nanoparticles enhances the fish immune response to a greater extent than intramuscular injection of naked DNA. In another study, the oral vaccination of rainbow trout fry with chitosan–TPP nanoparticles complexed with pcDNA3.1-VP2, showed that the expression of genes related with innate immune response, IFN-1 and MX, reached maximal values at 3 days postvaccination and 7 days after boosting (22 days postvaccination), while, with regard to genes involved in the adaptative immune response, CD4 peaked at 15 days postvaccination and IgM and IgT at 30 days postvaccination [154].

3.3.2. Control of Gonadal Development

Chitosan nanoparticles have been used for drug delivery in studies aiming proper gonadal development in fish farming. Bhat et al. administered chitosan conjugated with salmon luteinizing hormone-releasing hormone (sLHRH) into walking catfish (*Clarias batrachus*) to promote gonadal development. Chitosan-conjugated sLHRH and naked sLHRH exerted similar effects: upregulation of Sox9 expression in the gonads and increase of circulating steroid hormonal levels, testosterone and 11-ketotestosterone in males and testosterone and 17β-estradiol in females. However, sLHRH conjugation with chitosan induced sustained and controlled release of the hormones with maximal levels observed in the last sampling point of the experiment (36 h posttreatment), while naked sLHRH peaked circulating steroid hormones at 12 h posttreatment [150]. Similarly, compared to the administration of naked kisspeptin-10, intramuscular injection of chitosan-encapsulated kisspeptin-10

in immature female *Catla catla* caused a delayed but greater increase of gonadotropin-releasing hormone, luteinizing hormone and follicle-stimulating hormone expression, as well as circulating levels of 11-ketotestosterone and 17β-estradiol [144].

With the ultimate goal of controlling gonadal development in fish, chitosan was also assayed for gene delivery. In walking catfish (*Clarias batrachus*), intramuscular administration of chitosan nanoparticles conjugated with an expression plasmid encoding steroidogenic acute regulatory protein (StAR), a major regulator of steroidogenesis, also resulted in long-lasting stimulatory effects than administration of the naked plasmid construct on the expression of key genes in reproduction, cytochrome P450 (CYP) 11A1, CYP17A1, CYP19A1, 3β-hydroxysteroid dehydrogenase and 173β-hydroxysteroid dehydrogenase [153].

3.3.3. Control of Fish Metabolism

Chitosan has been used for enhancing fish digestibility, the absorption of food constituents and increasing the utilization of dietary carbohydrate in carnivorous fish. To supplement exogenous proteolytic enzymes and thus facilitate protein digestion and amino acid absorption, Kumari et al. orally administered chitosan–TPP nanoparticles encapsulating trypsin to *Labeo rohita* over 45 days. Treatment with chitosan–TPP–trypsin enhanced nutrient digestibility, intestinal protease activity and transamination activity, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in the liver and the muscle [161].

The substitution of dietary protein by cheaper nutrients with reduced environmental impact in farmed fish is a challenging trend for sustainable aquaculture [162]. However, the metabolic features of fish, particularly carnivorous fish, constrain the replacement of dietary protein by other nutrients in aquafeeds. Carnivorous fish exhibit a preferential use of amino acids as fuel and gluconeogenic substrates, and thus require high levels of dietary protein for optimal growth. Instead, carbohydrates are metabolized markedly slower than in mammals, and give rise to prolonged hyperglycemia [163,164]. The essential role of the liver in controlling the intermediary metabolism makes this organ an ideal target for investigating and modifying the glucose tolerance of farmed fish.

To overcome metabolic limitations of carnivorous fish, in recent years we synthesized chitosan–TPP nanoparticles, complexed with plasmid DNA, to induce in vivo transient overexpression and the silencing of target genes in the liver of gilthead sea bream (*Sparus aurata*). With the aim of decreasing the use of amino acids for gluconeogenic purposes and improving carbohydrate metabolism in the liver, chitosan–TPP nanoparticles complexed with a plasmid overexpressing a shRNA designed to silence the expression of cytosolic ALT (cALT) were intraperitoneally administered to *Sparus aurata* juveniles. Seventy-two hours posttreatment, a significant decrease in cALT1 mRNA levels, immunodetectable ALT and ALT activity was observed in the liver of treated fish. Knockdown of cALT expression to ~63%–70% of the values observed in control fish significantly increased the hepatic activity of key enzymes in glycolysis, 6-phosphofructo 1-kinase (PFK1) and pyruvate kinase, and protein metabolism, glutamate dehydrogenase (GDH). In addition to showing efficient gene silencing after administration of chitosan–TPP–DNA nanoparticles, the findings supported evidence that the downregulation of liver transamination increased the use of dietary carbohydrates to obtain energy, and thus made it possible to spare protein in carnivorous fish [80].

Following the same methodology, we showed that the shRNA-mediated knockdown of GDH significantly decreased GDH mRNA and immunodetectable levels in the liver, which, in turn, reduced GDH activity to ~53%. Downregulation of GDH decreased liver glutamate, glutamine and 2-oxoglutarate, as well as the hepatic activity of AST, while it increased 2-oxoglutarate dehydrogenase activity and the PFK1/fructose-1,6-bisphosphatase (FBP1) activity ratio. Therefore, by reducing hepatic transdeamination and gluconeogenesis, the knockdown of GDH could impair the use of amino acids as gluconeogenic substrates and facilitate the metabolic use of dietary carbohydrates [81].

With the aim of inducing a multigenic action leading to a stronger protein-sparing effect, *Sparus aurata* were intraperitoneally injected with chitosan–TPP nanoparticles complexed with a

plasmid expressing the N-terminal nuclear fragment of hamster SREBP1a, a transcription factor that—in addition to exhibiting strong transactivating capacity of genes required for fatty acid, triglycerides and cholesterol synthesis—previous reports showed can also transactivate the promoter of genes encoding key enzymes in hepatic glycolysis, glucokinase (GK) and 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase (PFKFB1) in fish [165,166]. Overexpression of exogenous SREBP1a in the liver of *Sparus aurata* enhanced the expression of glycolytic enzymes GK and PFKFB1, decreased the activity of the gluconeogenic enzyme FBP1 and increased the mRNA levels of key enzymes in fatty acid synthesis, elongation and desaturation (acetyl-CoA carboxylase 1, acetyl-CoA carboxylase 2, elongation of very long chain fatty acids protein 5, fatty acid desaturase 2), as well as induced NADPH formation (glucose 6-phophate dehydrogenase) and cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A reductase). As a result, chitosan-mediated SREBP1a overexpression caused a multigenic action that enabled the conversion of dietary carbohydrates into lipids (Figure 7), leading to increased circulating levels of triglycerides and cholesterol in carnivorous fish [157].

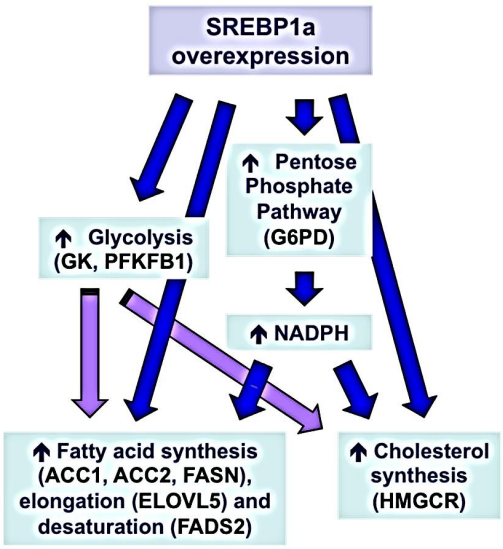


Figure 7. Multigenic action and metabolic effects in the liver of *Sparus aurata* after intraperitoneal administration of chitosan–TPP–DNA nanoparticles to overexpress exogenous SREBP1a [157]. ACC1, acetyl-CoA carboxylase 1; ACC2, acetyl-CoA carboxylase 2; ELOVL5, elongation of very long chain fatty acids protein 5; FADS2, fatty acid desaturase 2; G6PD, glucose 6-phophate dehydrogenase; GK, glucokinase; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; PFKFB1, 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase.

4. Conclusions

Characteristics such as nanoscale, low-toxicity, biodegradability, biocompatibility, derivatization, immunomodulatory effects, and easily affordable preparation conditions make chitosan a strong candidate for drug delivery into fish. Therefore, the use of chitosan in fish biotechnology has received growing attention in recent years. However, applications based on novel chitosan-based gene therapy methodologies to improve desirable traits in farmed fish have enormous potential for development. Most remarkable advances in the field addressed fish immunization, the control of reproduction for broodstock management and the modulation of gene expression to spare protein and overcome metabolic limitations of farmed fish. Further studies are needed for a better understanding of the extracellular and intracellular process, following chitosan-mediated gene delivery into fish. In addition, future trends in fish farming may greatly benefit from improved and more efficient chitosan formulations for enhancing gene delivery targeting and intracellular traffic in farmed fish.

Author Contributions: Conceptualization, I.M. and M.P.A.; writing—Original draft preparation, Y.W., A.R., M.P.A. and I.M.; writing—Review and editing, M.P.A. and I.M.; supervision, I.M.; project administration, I.M.; funding acquisition, I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministerio de Economía, Industria y Competitividad (Spain), grant number AGL2016-78124-R, co-funded by the European Regional Development Fund (EC).

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

References

1. Mahdy Samar, M.; El-Kalyoubi, M.H.; Khalaf, M.M.; Abd El-Razik, M.M. Physicochemical, functional, antioxidant and antibacterial properties of chitosan extracted from shrimp wastes by microwave technique. *Ann. Agric. Sci.* **2013**, *58*, 33–41. [CrossRef]
2. Sun, M.; Wang, T.; Pang, J.; Chen, X.; Liu, Y. Hydroxybutyl chitosan centered biocomposites for potential curative applications: A critical review. *Biomacromolecules* **2020**, *21*, 1351–1367. [CrossRef] [PubMed]
3. Sacco, P.; Cok, M.; Scognamiglio, F.; Pizzolitto, C.; Vecchies, F.; Marfoglia, A.; Marsich, E.; Donati, I. Glycosylated-chitosan derivatives: A systematic review. *Molecules* **2020**, *25*, 1534. [CrossRef] [PubMed]
4. Chuan, D.; Jin, T.; Fan, R.; Zhou, L.; Guo, G. Chitosan for gene delivery: Methods for improvement and applications. *Adv. Colloid Interface Sci.* **2019**, *268*, 25–38. [CrossRef]
5. Ivanova, D.G.; Yaneva, Z.L. Antioxidant properties and redox-modulating activity of chitosan and its derivatives: Biomaterials with application in cancer therapy. *Biores. Open Access* **2020**, *9*, 64–72. [CrossRef]
6. Wang, W.; Meng, Q.; Li, Q.; Liu, J.; Zhou, M.; Jin, Z.; Zhao, K. Chitosan derivatives and their application in biomedicine. *Int. J. Mol. Sci.* **2020**, *21*, 487. [CrossRef]
7. Raftery, R.; O'Brien, F.J.; Cryan, S.-A. Chitosan for gene delivery and orthopedic tissue engineering applications. *Molecules* **2013**, *18*, 5611–5647. [CrossRef]
8. Lostalé-Seijo, I.; Montenegro, J. Synthetic materials at the forefront of gene delivery. *Nat. Rev. Chem.* **2018**, *2*, 258–277. [CrossRef]
9. Cao, Y.; Tan, Y.F.; Wong, Y.S.; Liew, M.W.J.; Venkatraman, S. Recent Advances in Chitosan-Based Carriers for Gene Delivery. *Mar. Drugs* **2019**, *17*, 381. [CrossRef]
10. Santos-Carballal, B.; Fernández Fernández, E.; Goycoolea, F. Chitosan in non-viral gene delivery: Role of structure, characterization methods, and insights in cancer and rare diseases therapies. *Polymers* **2018**, *10*, 444. [CrossRef]
11. Ginn, S.L.; Amaya, A.K.; Alexander, I.E.; Edelstein, M.; Abedi, M.R. Gene therapy clinical trials worldwide to 2017: An update. *J. Gene Med.* **2018**, *20*, e3015. [CrossRef] [PubMed]
12. Picanço-Castro, V.; Pereira, C.G.; Covas, D.T.; Porto, G.S.; Athanassiadou, A.; Figueiredo, M.L. Emerging patent landscape for non-viral vectors used for gene therapy. *Nat. Biotechnol.* **2020**, *38*, 151–157. [CrossRef] [PubMed]
13. Simões, S.; Filipe, A.; Faneca, H.; Mano, M.; Penacho, N.; Düzgünes, N.; de Lima, M.P. Cationic liposomes for gene delivery. *Expert Opin. Drug Deliv.* **2005**, *2*, 237–254. [CrossRef] [PubMed]
14. Saffari, M.; Moghimi, H.; Dass, C. Barriers to liposomal gene delivery: From application site to the target. *Iran. J. Pharm. Res.* **2016**, *15*, 3–17.
15. Ramamoorth, M.; Narvekar, A. Non viral vectors in gene therapy- an overview. *J. Clin. Diagn. Res.* **2015**, *9*, GE01–GE06. [CrossRef]
16. Patil, S.; Gao, Y.G.; Lin, X.; Li, Y.; Dang, K.; Tian, Y.; Zhang, W.J.; Jiang, S.F.; Qadir, A.; Qian, A.R. The development of functional non-viral vectors for gene delivery. *Int. J. Mol. Sci.* **2019**, *20*, 5491. [CrossRef]
17. Mumper, R.; Wang, J.; Claspell, J.; Rolland, A. Novel polymeric condensing carriers for gene delivery. *Proc. Int. Symp. Control. Release Bioact. Mater.* **1995**, *22*, 178–179.
18. MacLaughlin, F.C.; Mumper, R.J.; Wang, J.; Tagliaferri, J.M.; Gill, I.; Hinchcliffe, M.; Rolland, A.P. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *J. Control. Release* **1998**, *56*, 259–272. [CrossRef]
19. Katas, H.; Alpar, H.O. Development and characterisation of chitosan nanoparticles for siRNA delivery. *J. Control. Release* **2006**, *115*, 216–225. [CrossRef]

20. Richardson, S.C.; Kolbe, H.V.; Duncan, R. Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *Int. J. Pharm.* **1999**, *178*, 231–243. [CrossRef]
21. Xu, W.; Shen, Y.; Jiang, Z.; Wang, Y.; Chu, Y.; Xiong, S. Intranasal delivery of chitosan-DNA vaccine generates mucosal SIgA and anti-CVB3 protection. *Vaccine* **2004**, *22*, 3603–3612. [CrossRef] [PubMed]
22. Sun, C.-J.; Pan, S.-P.; Xie, Q.-X.; Xiao, L.-J. Preparation of chitosan-plasmid DNA nanoparticles encoding zona pellucida glycoprotein-3alpha and its expression in mouse. *Mol. Reprod. Dev.* **2004**, *68*, 182–188. [CrossRef] [PubMed]
23. Kang, S.H.; Revuri, V.; Lee, S.-J.; Cho, S.; Park, I.-K.; Cho, K.J.; Bae, W.K.; Lee, Y. Oral siRNA delivery to treat colorectal liver metastases. *ACS Nano* **2017**, *11*, 10417–10429. [CrossRef] [PubMed]
24. Lin, F.; Jia, H.-R.; Wu, F.-G. Glycol chitosan: A water-soluble polymer for cell imaging and drug delivery. *Molecules* **2019**, *24*, 4371. [CrossRef] [PubMed]
25. Huang, M.; Fong, C.-W.; Khor, E.; Lim, L.-Y. Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *J. Control. Release* **2005**, *106*, 391–406. [CrossRef] [PubMed]
26. Huang, M.; Khor, E.; Lim, L.-Y. Uptake and cytotoxicity of chitosan molecules and nanoparticles: Effects of molecular weight and degree of deacetylation. *Pharm. Res.* **2004**, *21*, 344–353. [CrossRef] [PubMed]
27. Liu, X.; Howard, K.A.; Dong, M.; Andersen, M.Ø.; Rahbek, U.L.; Johnsen, M.G.; Hansen, O.C.; Besenbacher, F.; Kjems, J. The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. *Biomaterials* **2007**, *28*, 1280–1288. [CrossRef]
28. Razmi, F.A.; Ngadi, N.; Wong, S.; Inuwa, I.M.; Opotu, L.A. Kinetics, thermodynamics, isotherm and regeneration analysis of chitosan modified pandan adsorbent. *J. Clean. Prod.* **2019**, *231*, 98–109. [CrossRef]
29. Alameh, M.; Lavertu, M.; Tran-Khanh, N.; Chang, C.-Y.; Lesage, F.; Bail, M.; Darras, V.; Chevrier, A.; Buschmann, M.D. siRNA delivery with chitosan: Influence of chitosan molecular weight, degree of deacetylation, and amine to phosphate ratio on in vitro silencing efficiency, hemocompatibility, biodistribution, and in vivo efficacy. *Biomacromolecules* **2018**, *19*, 112–131. [CrossRef]
30. Al-Remawi, M. Application of N-hexoyl chitosan derivatives with high degree of substitution in the preparation of super-disintegrating pharmaceutical matrices. *J. Drug Deliv. Sci. Technol.* **2015**, *29*, 31–41. [CrossRef]
31. Chung, Y.-C.; Kuo, C.-L.; Chen, C.-C. Preparation and important functional properties of water-soluble chitosan produced through Maillard reaction. *Bioresour. Technol.* **2005**, *96*, 1473–1482. [CrossRef] [PubMed]
32. Gullón, B.; Montenegro, M.I.; Ruiz-Matute, A.I.; Cardelle-Cobas, A.; Corzo, N.; Pintado, M.E. Synthesis, optimization and structural characterization of a chitosan-glucose derivative obtained by the Maillard reaction. *Carbohydr. Polym.* **2016**, *137*, 382–389. [CrossRef] [PubMed]
33. Uccello-Barretta, G.; Balzano, F.; Aiello, F.; Senatore, A.; Fabiano, A.; Zambito, Y. Mucoadhesivity and release properties of quaternary ammonium-chitosan conjugates and their nanoparticulate supramolecular aggregates: An NMR investigation. *Int. J. Pharm.* **2014**, *461*, 489–494. [CrossRef] [PubMed]
34. Li, H.; Zhang, Z.; Bao, X.; Xu, G.; Yao, P. Fatty acid and quaternary ammonium modified chitosan nanoparticles for insulin delivery. *Colloids Surf. B. Biointerfaces* **2018**, *170*, 136–143. [CrossRef]
35. Jiang, X.; Dai, H.; Leong, K.W.; Goh, S.-H.; Mao, H.-Q.; Yang, Y.-Y. Chitosan-g-PEG/DNA complexes deliver gene to the rat liver via intrabiliary and intraportal infusions. *J. Gene Med.* **2006**, *8*, 477–487. [CrossRef]
36. Ping, Y.; Liu, C.; Zhang, Z.; Liu, K.L.; Chen, J.; Li, J. Chitosan-graft-(PEI-β-cyclodextrin) copolymers and their supramolecular PEGylation for DNA and siRNA delivery. *Biomaterials* **2011**, *32*, 8328–8341. [CrossRef]
37. Lee, H.; Jeong, J.H.; Park, T.G. PEG grafted polylysine with fusogenic peptide for gene delivery: High transfection efficiency with low cytotoxicity. *J. Control. Release* **2002**, *79*, 283–291. [CrossRef]
38. Strand, S.P.; Issa, M.M.; Christensen, B.E.; Vårum, K.M.; Artursson, P. Tailoring of chitosans for gene delivery: Novel self-branched glycosylated chitosan oligomers with improved functional properties. *Biomacromolecules* **2008**, *9*, 3268–3276. [CrossRef]
39. Thanou, M.; Florea, B.I.; Geldof, M.; Junginger, H.E.; Borchard, G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials* **2002**, *23*, 153–159. [CrossRef]
40. Kean, T.; Roth, S.; Thanou, M. Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency. *J. Control. Release* **2005**, *103*, 643–653. [CrossRef]

41. Ren, Y.; Zhao, X.; Liang, X.; Ma, P.X.; Guo, B. Injectable hydrogel based on quaternized chitosan, gelatin and dopamine as localized drug delivery system to treat Parkinson's disease. *Int. J. Biol. Macromol.* **2017**, *105*, 1079–1087. [CrossRef]
42. Raik, S.V.; Andranovitš, S.; Petrova, V.A.; Xu, Y.; Lam, J.K.-W.; Morris, G.A.; Brodskaia, A.V.; Casettari, L.; Kritchenkov, A.S.; Skorik, Y.A. Comparative study of diethylaminoethyl-chitosan and methylglycol-chitosan as potential non-viral vectors for gene therapy. *Polymers* **2018**, *10*, 442. [CrossRef] [PubMed]
43. Calvo, P.; Remuñán-López, C.; Vila-Jato, J.L.; Alonso, M.J. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *J. Appl. Polym. Sci.* **1997**, *63*, 125–132. [CrossRef]
44. Gan, Q.; Wang, T.; Cochrane, C.; McCarron, P. Modulation of surface charge, particle size and morphological properties of chitosan–TPP nanoparticles intended for gene delivery. *Colloids Surf. B Biointerfaces* **2005**, *44*, 65–73. [CrossRef] [PubMed]
45. Raja, M.A.G.; Katas, H.; Jing Wen, T. Stability, intracellular delivery, and release of siRNA from chitosan nanoparticles using different cross-linkers. *PLoS ONE* **2015**, *10*, e0128963.
46. Vimal, S.; Abdul Majeed, S.; Taju, G.; Nambi, K.S.N.; Sundar Raj, N.; Madan, N.; Farook, M.A.; Rajkumar, T.; Gopinath, D.; Sahul Hameed, A.S. Chitosan tripolyphosphate (CS/TPP) nanoparticles: Preparation, characterization and application for gene delivery in shrimp. *Acta Trop.* **2013**, *128*, 486–493. [CrossRef]
47. Fàbregas, A.; Miñarro, M.; García-Montoya, E.; Pérez-Lozano, P.; Carrillo, C.; Sarrate, R.; Sánchez, N.; Ticó, J.R.; Suñé-Negre, J.M. Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan-tripolyphosphate nanoparticles. *Int. J. Pharm.* **2013**, *446*, 199–204. [CrossRef]
48. Serrano-Sevilla, I.; Artiga, Á.; Mitchell, S.G.; De Matteis, L.; de la Fuente, J.M. Natural polysaccharides for siRNA delivery: Nanocarriers based on chitosan, hyaluronic acid, and their derivatives. *Molecules* **2019**, *24*, 2570. [CrossRef]
49. Köping-Höggård, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Vårum, K.; Artursson, P. Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Ther.* **2001**, *8*, 1108–1121. [CrossRef]
50. Mao, H.Q.; Roy, K.; Troung-Le, V.L.; Janes, K.A.; Lin, K.Y.; Wang, Y.; August, J.T.; Leong, K.W. Chitosan-DNA nanoparticles as gene carriers: Synthesis, characterization and transfection efficiency. *J. Control. Release* **2001**, *70*, 399–421. [CrossRef]
51. Chan, P.; Kurisawa, M.; Chung, J.E.; Yang, Y.-Y. Synthesis and characterization of chitosan-g-poly(ethylene glycol)-folate as a non-viral carrier for tumor-targeted gene delivery. *Biomaterials* **2007**, *28*, 540–549. [CrossRef] [PubMed]
52. Jhaveri, A.; Deshpande, P.; Pattni, B.; Torchilin, V. Transferrin-targeted, resveratrol-loaded liposomes for the treatment of glioblastoma. *J. Control. Release* **2018**, *277*, 89–101. [CrossRef] [PubMed]
53. Gao, S.; Chen, J.; Xu, X.; Ding, Z.; Yang, Y.-H.; Hua, Z.; Zhang, J. Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-targeting. *Int. J. Pharm.* **2003**, *255*, 57–68. [CrossRef]
54. Park, I.-K.; Yang, J.; Jeong, H.-J.; Bom, H.-S.; Harada, I.; Akaike, T.; Kim, S.-I.; Cho, C.-S. Galactosylated chitosan as a synthetic extracellular matrix for hepatocytes attachment. *Biomaterials* **2003**, *24*, 2331–2337. [CrossRef]
55. Kim, T.H.; Nah, J.W.; Cho, M.-H.; Park, T.G.; Cho, C.S. Receptor-mediated gene delivery into antigen presenting cells using mannosylated chitosan/DNA nanoparticles. *J. Nanosci. Nanotechnol.* **2006**, *6*, 2796–2803. [CrossRef] [PubMed]
56. Negm, N.A.; Hefni, H.H.H.; Abd-Elaal, A.A.A.; Badr, E.A.; Abou Kana, M.T.H. Advancement on modification of chitosan biopolymer and its potential applications. *Int. J. Biol. Macromol.* **2020**, *152*, 681–702. [CrossRef] [PubMed]
57. Shastri, D.H. Thiolated chitosan: A boon to ocular delivery of therapeutics. *MOJ Bioequivalence Bioavailab.* **2017**, *3*, 34–37. [CrossRef]
58. Mahmood, A.; Lanthaler, M.; Laffleur, F.; Huck, C.W.; Bernkop-Schnürch, A. Thiolated chitosan micelles: Highly mucoadhesive drug carriers. *Carbohydr. Polym.* **2017**, *167*, 250–258. [CrossRef]
59. Boateng, J.S.; Ayensu, I. Preparation and characterization of laminated thiolated chitosan-based freeze-dried wafers for potential buccal delivery of macromolecules. *Drug Dev. Ind. Pharm.* **2014**, *40*, 611–618. [CrossRef]
60. Boateng, J.; Mitchell, J.; Pawar, H.; Ayensu, I. Functional characterisation and permeation studies of lyophilised thiolated chitosan xerogels for buccal delivery of insulin. *Protein Pept. Lett.* **2014**, *21*, 1163–1175. [CrossRef]

61. Liu, Q.; Zhang, C.; Zheng, X.; Shao, X.; Zhang, X.; Zhang, Q.; Jiang, X. Preparation and evaluation of antigen/N-trimethylaminoethylmethacrylate chitosan conjugates for nasal immunization. *Vaccine* **2014**, *32*, 2582–2590. [CrossRef] [PubMed]
62. Huang, G.-Q.; Zhang, Z.-K.; Cheng, L.-Y.; Xiao, J.-X. Intestine-targeted delivery potency of O-carboxymethyl chitosan-coated layer-by-layer microcapsules: An in vitro and in vivo evaluation. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2019**, *105*, 110129. [CrossRef] [PubMed]
63. Zhou, P.; Sun, X.; Zhang, Z. Kidney-targeted drug delivery systems. *Acta Pharm. Sin. B* **2014**, *4*, 37–42. [CrossRef] [PubMed]
64. Vasanthakumar, T.; Rubinstein, J.L. Structure and roles of V-type ATPases. *Trends Biochem. Sci.* **2020**, *45*, 295–307. [CrossRef]
65. Li, W.; Nicol, F.; Szoka, F.C. GALA: A designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv. Drug Deliv. Rev.* **2004**, *56*, 967–985. [CrossRef]
66. El Ouahabi, A.; Thiry, M.; Pector, V.; Fuks, R.; Ruysschaert, J.M.; Vandenbranden, M. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett.* **1997**, *414*, 187–192.
67. Ma, Z.; Yang, C.; Song, W.; Wang, Q.; Kjems, J.; Gao, S. Chitosan Hydrogel as siRNA vector for prolonged gene silencing. *J. Nanobiotechnol.* **2014**, *12*, 23. [CrossRef]
68. Shi, B.; Zheng, M.; Tao, W.; Chung, R.; Jin, D.; Ghaffari, D.; Farokhzad, O.C. Challenges in DNA delivery and recent advances in multifunctional polymeric DNA delivery systems. *Biomacromolecules* **2017**, *18*, 2231–2246. [CrossRef]
69. Molinaro, R.; Wolfram, J.; Federico, C.; Cilurzo, F.; Di Marzio, L.; Ventura, C.A.; Carafa, M.; Celia, C.; Fresta, M. Polyethylenimine and chitosan carriers for the delivery of RNA interference effectors. *Expert Opin. Drug Deliv.* **2013**, *10*, 1653–1668. [CrossRef]
70. Boussif, O.; Lezoualc'h, F.; Zanta, M.A.; Mergny, M.D.; Scherman, D.; Demeneix, B.; Behr, J.P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301. [CrossRef]
71. Bae, Y.; Lee, Y.H.; Lee, S.; Han, J.; Ko, K.S.; Choi, J.S. Characterization of glycol chitosan grafted with low molecular weight polyethylenimine as a gene carrier for human adipose-derived mesenchymal stem cells. *Carbohydr. Polym.* **2016**, *153*, 379–390. [CrossRef] [PubMed]
72. Chen, H.; Cui, S.; Zhao, Y.; Zhang, C.; Zhang, S.; Peng, X. Grafting chitosan with polyethylenimine in an ionic liquid for efficient gene delivery. *PLoS ONE* **2015**, *10*, e0121817. [CrossRef] [PubMed]
73. Liu, Q.; Jin, Z.; Huang, W.; Sheng, Y.; Wang, Z.; Guo, S. Tailor-made ternary nanopolyplexes of thiolated trimethylated chitosan with pDNA and folate conjugated cis-aconitic amide-polyethylenimine for efficient gene delivery. *Int. J. Biol. Macromol.* **2020**, *152*, 948–956. [CrossRef]
74. Lee, Y.H.; Park, H.I.; Choi, J.S. Novel glycol chitosan-based polymeric gene carrier synthesized by a Michael addition reaction with low molecular weight polyethylenimine. *Carbohydr. Polym.* **2016**, *137*, 669–677. [CrossRef]
75. Javan, B.; Atyabi, F.; Shahbazi, M. Hypoxia-inducible bidirectional shRNA expression vector delivery using PEI/chitosan-TBA copolymers for colorectal Cancer gene therapy. *Life Sci.* **2018**, *202*, 140–151. [CrossRef]
76. Sun, B.; Zhao, R.; Kong, F.; Ren, Y.; Zuo, A.; Liang, D.; Zhang, J. Phosphorylatable short peptide conjugation for facilitating transfection efficacy of CS/DNA complex. *Int. J. Pharm.* **2010**, *397*, 206–210. [CrossRef]
77. Zhao, R.; Sun, B.; Liu, T.; Liu, Y.; Zhou, S.; Zuo, A.; Liang, D. Optimize nuclear localization and intra-nucleus disassociation of the exogene for facilitating transfection efficacy of the chitosan. *Int. J. Pharm.* **2011**, *413*, 254–259. [CrossRef]
78. Miao, J.; Yang, X.; Gao, Z.; Li, Q.; Meng, T.; Wu, J.; Yuan, H.; Hu, F. Redox-responsive chitosan oligosaccharide-SS-Octadecylamine polymeric carrier for efficient anti-Hepatitis B Virus gene therapy. *Carbohydr. Polym.* **2019**, *212*, 215–221. [CrossRef]
79. Cryan, S.-A.; McKiernan, P.; Cunningham, C.M.; Greene, C. Targeting miRNA-based medicines to cystic fibrosis airway epithelial cells using nanotechnology. *Int. J. Nanomed.* **2013**, *8*, 3907. [CrossRef]
80. González, J.D.; Silva-Marrero, J.I.; Metón, I.; Caballero-Solares, A.; Viegas, I.; Fernández, F.; Miñarro, M.; Fàbregas, A.; Ticó, J.R.; Jones, J.G.; et al. Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. *Mar. Biotechnol.* **2016**, *18*, 85–97. [CrossRef]

81. Gaspar, C.; Silva-Marrero, J.I.; Fàbregas, A.; Miñarro, M.; Ticó, J.R.; Baanante, I.V.; Metón, I. Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. *J. Biotechnol.* **2018**, *286*, 5–13. [CrossRef] [PubMed]
82. Acharya, R. The recent progresses in shRNA-nanoparticle conjugate as a therapeutic approach. *Mater. Sci. Eng. C* **2019**, *104*, 109928. [CrossRef] [PubMed]
83. Zheng, H.; Tang, C.; Yin, C. Oral delivery of shRNA based on amino acid modified chitosan for improved antitumor efficacy. *Biomaterials* **2015**, *70*, 126–137. [CrossRef] [PubMed]
84. Wang, S.-L.; Yao, H.-H.; Guo, L.-L.; Dong, L.; Li, S.-G.; Gu, Y.-P.; Qin, Z.-H. Selection of optimal sites for TGFB1 gene silencing by chitosan-TPP nanoparticle-mediated delivery of shRNA. *Cancer Genet. Cytogenet.* **2009**, *190*, 8–14. [CrossRef] [PubMed]
85. Karimi, M.; Avci, P.; Ahi, M.; Gazori, T.; Hamblin, M.R.; Naderi-Manesh, H. Evaluation of chitosan-tripolyphosphate nanoparticles as a p-shRNA delivery vector: Formulation, optimization and cellular uptake study. *J. Nanopharm. Drug Deliv.* **2013**, *1*, 266–278. [CrossRef] [PubMed]
86. Ahmed, F.; Soliman, F.M.; Adly, M.A.; Soliman, H.A.M.; El-Matbouli, M.; Saleh, M. Recent progress in biomedical applications of chitosan and its nanocomposites in aquaculture: A review. *Res. Vet. Sci.* **2019**, *126*, 68–82. [CrossRef]
87. Abdel-Ghany, H.M.; Salem, M.E. Effects of dietary chitosan supplementation on farmed fish; a review. *Rev. Aquac.* **2020**, *12*, 438–452. [CrossRef]
88. Kono, M.; Matsui, T.; Shimizu, C. Effect of chitin, chitosan, and cellulose as deit supplements on the growth of cultured fish. *Bull. Jpn. Soc. Sci. Fish.* **1987**, *53*, 125–129. [CrossRef]
89. Shiau, S.-Y.; Yu, Y.-P. Dietary supplementation of chitin and chitosan depresses growth in tilapia, Oreochromis niloticus×O. aureus. *Aquaculture* **1999**, *179*, 439–446. [CrossRef]
90. Wu, S. The growth performance, body composition and nonspecific immunity of Tilapia (Oreochromis niloticus) affected by chitosan. *Int. J. Biol. Macromol.* **2020**, *145*, 682–685. [CrossRef]
91. Fadl, S.E.; El-Gammal, G.A.; Abdo, W.S.; Barakat, M.; Sakr, O.A.; Nassef, E.; Gad, D.M.; El-Sheshtawy, H.S. Evaluation of dietary chitosan effects on growth performance, immunity, body composition and histopathology of Nile tilapia (*Oreochromis niloticus*) as well as the resistance to *Streptococcus agalactiae* infection. *Aquac. Res.* **2020**, *51*, 1120–1132. [CrossRef]
92. Chen, Y.; Zhu, X.; Yang, Y.; Han, D.; Jin, J.; Xie, S. Effect of dietary chitosan on growth performance, haematology, immune response, intestine morphology, intestine microbiota and disease resistance in gibel carp (*Carassius auratus gibelio*). *Aquac. Nutr.* **2014**, *20*, 532–546. [CrossRef]
93. El-Sayed, H.S.; Barakat, K.M. Effect of dietary chitosan on challenged Dicentrarchus labrax post larvae with Aeromonas hydrophila. *Russ. J. Mar. Biol.* **2016**, *42*, 501–508. [CrossRef]
94. Yan, J.; Guo, C.; Dawood, M.A.O.; Gao, J. Effects of dietary chitosan on growth, lipid metabolism, immune response and antioxidant-related gene expression in *Misgurnus anguillicaudatus*. *Benef. Microbes* **2017**, *8*, 439–449. [CrossRef] [PubMed]
95. Chen, J.; Chen, L. Effects of chitosan-supplemented diets on the growth performance, nonspecific immunity and health of loach fish (Misgurnus anguillicadatus). *Carbohydr. Polym.* **2019**, *225*, 115227. [CrossRef] [PubMed]
96. Kamali Najafabad, M.; Imanpoor, M.R.; Taghizadeh, V.; Alishahi, A. Effect of dietary chitosan on growth performance, hematological parameters, intestinal histology and stress resistance of Caspian kutum (Rutilus frisii kutum Kamenskii, 1901) fingerlings. *Fish Physiol. Biochem.* **2016**, *42*, 1063–1071. [CrossRef] [PubMed]
97. Samarakoon, K.W.; Cha, S.-H.; Lee, J.-H.; Jeon, Y.-J. The growth, innate immunity and protection against H_2O_2-induced oxidative damage of a chitosan-coated diet in the olive flounder Paralichthys olivaceus. *Fish. Aquat. Sci.* **2013**, *16*, 149–158. [CrossRef]
98. Ranjan, R.; Prasad, K.P.; Vani, T.; Kumar, R. Effect of dietary chitosan on haematology, innate immunity and disease resistance of Asian seabass *Lates calcarifer* (Bloch). *Aquac. Res.* **2014**, *45*, 983–993. [CrossRef]
99. Shanthi Mari, L.S.; Jagruthi, C.; Anbazahan, S.M.; Yogeshwari, G.; Thirumurugan, R.; Arockiaraj, J.; Mariappan, P.; Balasundaram, C.; Harikrishnan, R. Protective effect of chitin and chitosan enriched diets on immunity and disease resistance in Cirrhina mrigala against Aphanomyces invadans. *Fish Shellfish Immunol.* **2014**, *39*, 378–385. [CrossRef]

100. Harikrishnan, R.; Kim, J.-S.; Balasundaram, C.; Heo, M.-S. Immunomodulatory effects of chitin and chitosan enriched diets in Epinephelus bruneus against Vibrio alginolyticus infection. *Aquaculture* **2012**, *326–329*, 46–52. [CrossRef]
101. Harikrishnan, R.; Kim, J.-S.; Balasundaram, C.; Heo, M.-S. Dietary supplementation with chitin and chitosan on haematology and innate immune response in Epinephelus bruneus against Philasterides dicentrarchi. *Exp. Parasitol.* **2012**, *131*, 116–124. [CrossRef] [PubMed]
102. Wang, Y.; Li, J. Effects of chitosan nanoparticles on survival, growth and meat quality of tilapia, Oreochromis nilotica. *Nanotoxicology* **2011**, *5*, 425–431. [CrossRef] [PubMed]
103. Abdel-Tawwab, M.; Razek, N.A.; Abdel-Rahman, A.M. Immunostimulatory effect of dietary chitosan nanoparticles on the performance of Nile tilapia, Oreochromis niloticus (L.). *Fish Shellfish Immunol.* **2019**, *88*, 254–258. [CrossRef] [PubMed]
104. Abd El-Naby, F.S.; Naiel, M.A.E.; Al-Sagheer, A.A.; Negm, S.S. Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in Oreochromis niloticus. *Aquaculture* **2019**, *501*, 82–89. [CrossRef]
105. Naiel, M.A.E.; Ismael, N.E.M.; Abd El-hameed, S.A.A.; Amer, M.S. The antioxidative and immunity roles of chitosan nanoparticle and vitamin C-supplemented diets against imidacloprid toxicity on Oreochromis niloticus. *Aquaculture* **2020**, *523*, 735219. [CrossRef]
106. Abd El-Naby, A.S.; Al-Sagheer, A.A.; Negm, S.S.; Naiel, M.A.E. Dietary combination of chitosan nanoparticle and thymol affects feed utilization, digestive enzymes, antioxidant status, and intestinal morphology of Oreochromis niloticus. *Aquaculture* **2020**, *515*, 734577. [CrossRef]
107. Gao, J.-Q.; Hu, Y.L.; Wang, Q.; Han, F.; Shao, J.Z. Toxicity evaluation of biodegradable chitosan nanoparticles using a zebrafish embryo model. *Int. J. Nanomed.* **2011**, *6*, 3351–3359. [CrossRef]
108. Nikapitiya, C.; Dananjaya, S.H.S.; De Silva, B.C.J.; Heo, G.-J.; Oh, C.; De Zoysa, M.; Lee, J. Chitosan nanoparticles: A positive immune response modulator as display in zebrafish larvae against Aeromonas hydrophila infection. *Fish Shellfish Immunol.* **2018**, *76*, 240–246. [CrossRef]
109. Qin, C.; Zhang, Y.; Liu, W.; Xu, L.; Yang, Y.; Zhou, Z. Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia Oreochromis niloticus ♀× Oreochromis aureus ♂. *Fish Shellfish Immunol.* **2014**, *40*, 267–274. [CrossRef]
110. Gopalakannan, A.; Arul, V. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of Cyprinus carpio and control of Aeromonas hydrophila infection in ponds. *Aquaculture* **2006**, *255*, 179–187. [CrossRef]
111. Karlsen, Ø.; Amlund, H.; Berg, A.; Olsen, R.E. The effect of dietary chitin on growth and nutrient digestibility in farmed Atlantic cod, Atlantic salmon and Atlantic halibut. *Aquac. Res.* **2017**, *48*, 123–133. [CrossRef]
112. Lin, S.-M.; Jiang, Y.; Chen, Y.-J.; Luo, L.; Doolgindachbaporn, S.; Yuangsoi, B. Effects of Astragalus polysaccharides (APS) and chitooligosaccharides (COS) on growth, immune response and disease resistance of juvenile largemouth bass, Micropterus salmoides. *Fish Shellfish Immunol.* **2017**, *70*, 40–47. [CrossRef] [PubMed]
113. Nguyen, N.D.; Van Dang, P.; Le, A.Q.; Nguyen, T.K.L.; Pham, D.H.; Van Nguyen, N.; Nguyen, Q.H. Effect of oligochitosan and oligo-β-glucan supplementation on growth, innate immunity, and disease resistance of striped catfish (Pangasianodon hypophthalmus). *Biotechnol. Appl. Biochem.* **2017**, *64*, 564–571. [CrossRef]
114. Meng, X.; Wang, J.; Wan, W.; Xu, M.; Wang, T. Influence of low molecular weight chitooligosaccharides on growth performance and non-specific immune response in Nile tilapia Oreochromis niloticus. *Aquac. Int.* **2017**, *25*, 1265–1277. [CrossRef]
115. Su, P.; Han, Y.; Jiang, C.; Ma, Y.; Pan, J.; Liu, S.; Zhang, T. Effects of chitosan-oligosaccharides on growth performance, digestive enzyme and intestinal bacterial flora of tiger puffer (*Takifugu rubripes* Temminck et Schlegel, 1850). *J. Appl. Ichthyol.* **2017**, *33*, 458–467. [CrossRef]
116. Lin, S.; Mao, S.; Guan, Y.; Luo, L.; Luo, L.; Pan, Y. Effects of dietary chitosan oligosaccharides and Bacillus coagulans on the growth, innate immunity and resistance of koi (Cyprinus carpio koi). *Aquaculture* **2012**, *342–343*, 36–41.
117. Lin, S.; Mao, S.; Guan, Y.; Lin, X.; Luo, L. Dietary administration of chitooligosaccharides to enhance growth, innate immune response and disease resistance of Trachinotus ovatus. *Fish Shellfish Immunol.* **2012**, *32*, 909–913. [CrossRef]

118. Liu, Y.; Xing, R.; Liu, S.; Qin, Y.; Li, K.; Yu, H.; Li, P. Effects of chitooligosaccharides supplementation with different dosages, molecular weights and degrees of deacetylation on growth performance, innate immunity and hepatopancreas morphology in Pacific white shrimp (Litopenaeus vannamei). *Carbohydr. Polym.* **2019**, *226*, 115254. [CrossRef]
119. Luo, L.; Cai, X.; He, C.; Xue, M.; Wu, X.; Cao, H. Immune response, stress resistance and bacterial challenge in juvenile rainbow trouts Oncorhynchus mykiss fed diets containing chitosan-oligosaccharides. *Curr. Zool.* **2009**, *55*, 416–422. [CrossRef]
120. Liu, L.; Zhou, Y.; Zhao, X.; Wang, H.; Wang, L.; Yuan, G.; Asim, M.; Wang, W.; Zeng, L.; Liu, X.; et al. Oligochitosan stimulated phagocytic activity of macrophages from blunt snout bream (Megalobrama amblycephala) associated with respiratory burst coupled with nitric oxide production. *Dev. Comp. Immunol.* **2014**, *47*, 17–24. [CrossRef]
121. Fernández-Díaz, C.; Coste, O.; Malta, E. Polymer chitosan nanoparticles functionalized with Ulva ohnoi extracts boost in vitro ulvan immunostimulant effect in Solea senegalensis macrophages. *Algal Res.* **2017**, *26*, 135–142. [CrossRef]
122. Wisdom, K.S.; Bhat, I.A.; Chanu, T.I.; Kumar, P.; Pathakota, G.-B.; Nayak, S.K.; Walke, P.; Sharma, R. Chitosan grafting onto single-walled carbon nanotubes increased their stability and reduced the toxicity in vivo (catfish) model. *Int. J. Biol. Macromol.* **2020**, *155*, 697–707. [CrossRef] [PubMed]
123. Alishahi, A.; Mirvaghefi, A.; Tehrani, M.R.; Farahmand, H.; Koshio, S.; Dorkoosh, F.A.; Elsabee, M.Z. Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (Oncorhynchus mykiss). *Carbohydr. Polym.* **2011**, *86*, 142–146. [CrossRef]
124. Jiménez-Fernández, E.; Ruyra, A.; Roher, N.; Zuasti, E.; Infante, C.; Fernández-Díaz, C. Nanoparticles as a novel delivery system for vitamin C administration in aquaculture. *Aquaculture* **2014**, *432*, 426–433. [CrossRef]
125. Bhat, I.A.; Nazir, M.I.; Ahmad, I.; Pathakota, G.-B.; Chanu, T.I.; Goswami, M.; Sundaray, J.K.; Sharma, R. Fabrication and characterization of chitosan conjugated eurycomanone nanoparticles: In vivo evaluation of the biodistribution and toxicity in fish. *Int. J. Biol. Macromol.* **2018**, *112*, 1093–1103. [CrossRef]
126. Wisdom, K.S.; Bhat, I.A.; Kumar, P.; Pathan, M.K.; Chanu, T.I.; Walke, P.; Sharma, R. Fabrication of chitosan nanoparticles loaded with aromatase inhibitors for the advancement of gonadal development in Clarias magur (Hamilton, 1822). *Aquaculture* **2018**, *497*, 125–133. [CrossRef]
127. Bhat, I.A.; Ahmad, I.; Mir, I.N.; Yousf, D.J.; Ganie, P.A.; Bhat, R.A.H.; Gireesh-Babu, P.; Sharma, R. Evaluation of the in vivo effect of chitosan conjugated eurycomanone nanoparticles on the reproductive response in female fish model. *Aquaculture* **2019**, *510*, 392–399. [CrossRef]
128. Bhat, I.A.; Ahmad, I.; Mir, I.N.; Bhat, R.A.H.; P, G.-B.; Goswami, M.; Sundaray, J.K.; Sharma, R. Chitosan-eurycomanone nanoformulation acts on steroidogenesis pathway genes to increase the reproduction rate in fish. *J. Steroid Biochem. Mol. Biol.* **2019**, *185*, 237–247. [CrossRef]
129. Barakat, K.M.; El-Sayed, H.S.; Gohar, Y.M. Protective effect of squilla chitosan–silver nanoparticles for Dicentrarchus labrax larvae infected with Vibrio anguillarum. *Int. Aquat. Res.* **2016**, *8*, 179–189. [CrossRef]
130. Udayangani, R.M.C.; Dananjaya, S.H.S.; Nikapitiya, C.; Heo, G.-J.; Lee, J.; De Zoysa, M. Metagenomics analysis of gut microbiota and immune modulation in zebrafish (Danio rerio) fed chitosan silver nanocomposites. *Fish Shellfish Immunol.* **2017**, *66*, 173–184. [CrossRef]
131. Xia, I.F.; Cheung, J.S.; Wu, M.; Wong, K.-S.; Kong, H.-K.; Zheng, X.-T.; Wong, K.-H.; Kwok, K.W. Dietary chitosan-selenium nanoparticle (CTS-SeNP) enhance immunity and disease resistance in zebrafish. *Fish Shellfish Immunol.* **2019**, *87*, 449–459. [CrossRef] [PubMed]
132. Victor, H.; Zhao, B.; Mu, Y.; Dai, X.; Wen, Z.; Gao, Y.; Chu, Z. Effects of Se-chitosan on the growth performance and intestinal health of the loach Paramisgurnus dabryanus (Sauvage). *Aquaculture* **2019**, *498*, 263–270. [CrossRef]
133. Zhang, J.; Fu, X.; Zhang, Y.; Zhu, W.; Zhou, Y.; Yuan, G.; Liu, X.; Ai, T.; Zeng, L.; Su, J. Chitosan and anisodamine improve the immune efficacy of inactivated infectious spleen and kidney necrosis virus vaccine in Siniperca chuatsi. *Fish Shellfish Immunol.* **2019**, *89*, 52–60. [CrossRef]
134. Zhu, W.; Zhang, Y.; Zhang, J.; Yuan, G.; Liu, X.; Ai, T.; Su, J. Astragalus polysaccharides, chitosan and poly(I:C) obviously enhance inactivated Edwardsiella ictaluri vaccine potency in yellow catfish Pelteobagrus fulvidraco. *Fish Shellfish Immunol.* **2019**, *87*, 379–385. [CrossRef]

135. Liu, X.; Zhang, H.; Gao, Y.; Zhang, Y.; Wu, H.; Zhang, Y. Efficacy of chitosan oligosaccharide as aquatic adjuvant administrated with a formalin-inactivated Vibrio anguillarum vaccine. *Fish Shellfish Immunol.* **2015**, *47*, 855–860. [CrossRef]
136. Wei, G.; Cai, S.; Wu, Y.; Ma, S.; Huang, Y. Immune effect of Vibrio harveyi formalin-killed cells vaccine combined with chitosan oligosaccharide and astragalus polysaccharides in ♀Epinephelus fuscoguttatus×♂Epinephelus lanceolatus. *Fish Shellfish Immunol.* **2020**, *98*, 186–192. [CrossRef]
137. Halimi, M.; Alishahi, M.; Abbaspour, M.R.; Ghorbanpoor, M.; Tabandeh, M.R. Valuable method for production of oral vaccine by using alginate and chitosan against Lactococcus garvieae/Streptococcus iniae in rainbow trout (Oncorhynchus mykiss). *Fish Shellfish Immunol.* **2019**, *90*, 431–439. [CrossRef]
138. Kole, S.; Qadiri, S.S.N.; Shin, S.-M.; Kim, W.-S.; Lee, J.; Jung, S.-J. Nanoencapsulation of inactivated-viral vaccine using chitosan nanoparticles: Evaluation of its protective efficacy and immune modulatory effects in olive flounder (Paralichthys olivaceus) against viral haemorrhagic septicaemia virus (VHSV) infection. *Fish Shellfish Immunol.* **2019**, *91*, 136–147. [CrossRef]
139. Tandberg, J.; Lagos, L.; Ropstad, E.; Smistad, G.; Hiorth, M.; Winther-Larsen, H.C. The use of chitosan-coated membrane vesicles for immunization against salmonid rickettsial septicemia in an adult zebrafish model. *Zebrafish* **2018**, *15*, 372–381. [CrossRef]
140. Dubey, S.; Avadhani, K.; Mutalik, S.; Sivadasan, S.; Maiti, B.; Girisha, S.; Venugopal, M.; Mutoloki, S.; Evensen, Ø.; Karunasagar, I.; et al. Edwardsiella tarda OmpA encapsulated in chitosan nanoparticles shows superior protection over inactivated whole cell vaccine in orally vaccinated fringed-lipped peninsula carp (Labeo fimbriatus). *Vaccines* **2016**, *4*, 40. [CrossRef]
141. Wang, E.; Wang, X.; Wang, K.; He, J.; Zhu, L.; He, Y.; Chen, D.; Ouyang, P.; Geng, Y.; Huang, X.; et al. Preparation, characterization and evaluation of the immune effect of alginate/chitosan composite microspheres encapsulating recombinant protein of Streptococcus iniae designed for fish oral vaccination. *Fish Shellfish Immunol.* **2018**, *73*, 262–271. [CrossRef] [PubMed]
142. Chen, T.; Hu, Y.; Zhou, J.; Hu, S.; Xiao, X.; Liu, X.; Su, J.; Yuan, G. Chitosan reduces the protective effects of IFN-γ2 on grass carp (Ctenopharyngodon idella) against Flavobacterium columnare infection due to excessive inflammation. *Fish Shellfish Immunol.* **2019**, *95*, 305–313. [CrossRef] [PubMed]
143. Sharma, D.; Maheshwari, D.; Philip, G.; Rana, R.; Bhatia, S.; Singh, M.; Gabrani, R.; Sharma, S.K.; Ali, J.; Sharma, R.K.; et al. Formulation and optimization of polymeric nanoparticles for intranasal delivery of lorazepam using Box-Behnken design: In vitro and in vivo evaluation. *Biomed Res. Int.* **2014**, *2014*, 156010. [CrossRef]
144. Rather, M.A.; Bhat, I.A.; Gireesh-Babu, P.; Chaudhari, A.; Sundaray, J.K.; Sharma, R. Molecular characterization of kisspeptin gene and effect of nano-encapsulted kisspeptin-10 on reproductive maturation in Catla catla. *Domest. Anim. Endocrinol.* **2016**, *56*, 36–47. [CrossRef] [PubMed]
145. Tian, J.; Yu, J.; Sun, X. Chitosan microspheres as candidate plasmid vaccine carrier for oral immunisation of Japanese flounder (Paralichthys olivaceus). *Vet. Immunol. Immunopathol.* **2008**, *126*, 220–229. [CrossRef]
146. Vimal, S.; Taju, G.; Nambi, K.S.N.; Abdul Majeed, S.; Sarath Babu, V.; Ravi, M.; Sahul Hameed, A.S. Synthesis and characterization of CS/TPP nanoparticles for oral delivery of gene in fish. *Aquaculture* **2012**, *358–359*, 14–22. [CrossRef]
147. Li, L.; Lin, S.-L.; Deng, L.; Liu, Z.-G. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in black seabream Acanthopagrus schlegelii Bleeker to protect from Vibrio parahaemolyticus. *J. Fish Dis.* **2013**, *36*, 987–995. [CrossRef]
148. Vimal, S.; Abdul Majeed, S.; Nambi, K.S.N.; Madan, N.; Farook, M.A.; Venkatesan, C.; Taju, G.; Venu, S.; Subburaj, R.; Thirunavukkarasu, A.R.; et al. Delivery of DNA vaccine using chitosan–tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, Lates calcarifer (Bloch, 1790) for protection against nodavirus infection. *Aquaculture* **2014**, *420–421*, 240–246. [CrossRef]
149. Zheng, F.; Liu, H.; Sun, X.; Zhang, Y.; Zhang, B.; Teng, Z.; Hou, Y.; Wang, B. Development of oral DNA vaccine based on chitosan nanoparticles for the immunization against reddish body iridovirus in turbots (Scophthalmus maximus). *Aquaculture* **2016**, *452*, 263–271. [CrossRef]
150. Bhat, I.A.; Rather, M.A.; Saha, R.; Pathakota, G.-B.; Pavan-Kumar, A.; Sharma, R. Expression analysis of Sox9 genes during annual reproductive cycles in gonads and after nanodelivery of LHRH in Clarias batrachus. *Res. Vet. Sci.* **2016**, *106*, 100–106. [CrossRef]
151. Valero, Y.; Awad, E.; Buonocore, F.; Arizcun, M.; Esteban, M.Á.; Meseguer, J.; Chaves-Pozo, E.; Cuesta, A. An oral chitosan DNA vaccine against nodavirus improves transcription of cell-mediated cytotoxicity and

interferon genes in the European sea bass juveniles gut and survival upon infection. *Dev. Comp. Immunol.* **2016**, *65*, 64–72. [CrossRef] [PubMed]
152. Sáez, M.I.; Vizcaíno, A.J.; Alarcón, F.J.; Martínez, T.F. Comparison of lacZ reporter gene expression in gilthead sea bream (Sparus aurata) following oral or intramuscular administration of plasmid DNA in chitosan nanoparticles. *Aquaculture* **2017**, *474*, 1–10. [CrossRef]
153. Rathor, P.K.; Bhat, I.A.; Rather, M.A.; Gireesh-Babu, P.; Kumar, K.; Purayil, S.B.P.; Sharma, R. Steroidogenic acute regulatory protein (StAR) gene expression construct: Development, nanodelivery and effect on reproduction in air-breathing catfish, Clarias batrachus. *Int. J. Biol. Macromol.* **2017**, *104*, 1082–1090. [CrossRef] [PubMed]
154. Ahmadivand, S.; Soltani, M.; Behdani, M.; Evensen, Ø.; Alirahimi, E.; Hassanzadeh, R.; Soltani, E. Oral DNA vaccines based on CS-TPP nanoparticles and alginate microparticles confer high protection against infectious pancreatic necrosis virus (IPNV) infection in trout. *Dev. Comp. Immunol.* **2017**, *74*, 178–189. [CrossRef] [PubMed]
155. Sáez, M.I.; Vizcaíno, A.J.; Alarcón, F.J.; Martínez, T.F. Feed pellets containing chitosan nanoparticles as plasmid DNA oral delivery system for fish: In vivo assessment in gilthead sea bream (Sparus aurata) juveniles. *Fish Shellfish Immunol.* **2018**, *80*, 458–466. [CrossRef]
156. Kole, S.; Kumari, R.; Anand, D.; Kumar, S.; Sharma, R.; Tripathi, G.; Makesh, M.; Rajendran, K.V.; Bedekar, M.K. Nanoconjugation of bicistronic DNA vaccine against Edwardsiella tarda using chitosan nanoparticles: Evaluation of its protective efficacy and immune modulatory effects in Labeo rohita vaccinated by different delivery routes. *Vaccine* **2018**, *36*, 2155–2165. [CrossRef]
157. Silva-Marrero, J.I.; Villasante, J.; Rashidpour, A.; Palma, M.; Fàbregas, A.; Almajano, M.P.; Viegas, I.; Jones, J.G.; Miñarro, M.; Ticó, J.R.; et al. The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of Sparus aurata. *Biomolecules* **2019**, *9*, 297. [CrossRef]
158. Rao, B.M.; Kole, S.; Gireesh-Babu, P.; Sharma, R.; Tripathi, G.; Bedekar, M.K. Evaluation of persistence, bio-distribution and environmental transmission of chitosan/PLGA/pDNA vaccine complex against Edwardsiella tarda in Labeo rohita. *Aquaculture* **2019**, *500*, 385–392. [CrossRef]
159. Ramos, E.A.; Relucio, J.L.V.; Torres-Villanueva, C.A.T. Gene expression in tilapia following oral delivery of chitosan-encapsulated plasmid DNA incorporated into fish feeds. *Mar. Biotechnol.* **2005**, *7*, 89–94. [CrossRef]
160. Rajesh Kumar, S.; Ishaq Ahmed, V.P.; Parameswaran, V.; Sudhakaran, R.; Sarath Babu, V.; Sahul Hameed, A.S. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (Lates calcarifer) to protect from Vibrio (Listonella) anguillarum. *Fish Shellfish Immunol.* **2008**, *25*, 47–56. [CrossRef]
161. Kumari, R.; Gupta, S.; Singh, A.R.; Ferosekhan, S.; Kothari, D.C.; Pal, A.K.; Jadhao, S.B. Chitosan nanoencapsulated exogenous trypsin biomimics zymogen-like enzyme in fish gastrointestinal tract. *PLoS ONE* **2013**, *8*, e74743. [CrossRef] [PubMed]
162. Naylor, R.L.; Hardy, R.W.; Bureau, D.P.; Chiu, A.; Elliott, M.; Farrell, A.P.; Forster, I.; Gatlin, D.M.; Goldburg, R.J.; Hua, K.; et al. Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15103–15110. [CrossRef] [PubMed]
163. Polakof, S.; Panserat, S.; Soengas, J.L.; Moon, T.W. Glucose metabolism in fish: A review. *J. Comp. Physiol. B.* **2012**, *182*, 1015–1045. [CrossRef]
164. Rashidpour, A.; Silva-Marrero, J.I.; Seguí, L.; Baanante, I.V.; Metón, I. Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (*Sparus aurata*). *Am. J. Physiol. Integr. Comp. Physiol.* **2019**, *316*, R265–R273. [CrossRef]
165. Metón, I.; Egea, M.; Anemaet, I.G.; Fernández, F.; Baanante, I.V. Sterol regulatory element binding protein-1a transactivates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoter. *Endocrinology* **2006**, *147*, 3446–3456. [CrossRef]
166. Egea, M.; Metón, I.; Córdoba, M.; Fernández, F.; Baanante, I.V. Role of Sp1 and SREBP-1a in the insulin-mediated regulation of glucokinase transcription in the liver of gilthead sea bream (Sparus aurata). *Gen. Comp. Endocrinol.* **2008**, *155*, 359–367. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

2. Objectives

2.1. General objective

S. aurata is one of the most cultured marine fishes in Spain and Europe. Despite the commercial interest of *S. aurata* production, the molecular mechanisms involving dietary nutrients and regulation of the intermediary metabolism in this species are not fully understood. As most fishes with aquaculture interest in Europe, *S. aurata* is a carnivorous ectothermic species. Carnivorous fish use efficiently amino acids for growth and to obtain energy. However, carnivorous fish have low ability to utilize dietary carbohydrates and exhibit prolonged hyperglycemia after a glucose load or intake of high-carbohydrate diets. Thus, optimal growth of teleost fish requires elevated levels of dietary protein. Fishmeal is the main protein source in aquafeeds, due to its high protein content, adequate amino acid profile, high palatability and lack of antinutrients.

Although aquaculture produces undeniable benefits such as the provision of good quality and accessible food for populations and the generation of millions of related jobs, fish farming raises important concerns in terms of environmental welfare and sustainability, including capture of wild fish for rearing, and local eutrophication and nitrification. Since a significant proportion of wild fish captures is processed into fishmeal for aquafeeds, a reduction in the amount of protein in aquafeeds would alleviate dependence on wild fisheries (often overexploited) and the environmental impact of aquaculture. Indeed, excess of dietary protein in aquafeeds increases amino acid oxidation, ammonia release and the eutrophication of effluent receiving ecosystems. In addition to reduce the environmental impact of aquaculture, reduction of fishmeal in aquafeeds and substitution of dietary protein by cheaper nutrients would reduce the production cost, contributing to a more sustainable aquaculture.

In this context, our research endeavors to broaden the comprehension of the intermediary metabolism and the control of glucose homeostasis in carnivorous fish, particularly *S. aurata*. To this end, we studied whether metformin, a widely employed drug for managing diabetes in humans, may improve glucose homeostasis in *S. aurata* at the molecular level. Furthermore, previous studies of our research group pointed to the N-terminal nuclear fragment of hamster SREBP1a as a key exogenous factor to induce a protein-sparing effect in cultured *S. aurata*. In addition to strongly transactivate the expression of genes required for fatty acid and cholesterol synthesis, we showed that the transcription factor SREBP1a also binds and transactivates the gene promoters of GCK and PFKFB1, two key enzymes in the regulation of the glycolytic flux in the liver of *S. aurata*. Moreover, a single administration of chitosan-TPP-DNA nanoparticles

expressing the N-terminal nuclear fragment of hamster SREBP1a caused a multigenic effect in the liver of *S. aurata* 72 h post-treatment, allowing conversion of carbohydrates into lipids through enhanced expression of key enzymes in glycolysis, cholesterol synthesis and fatty acid synthesis, elongation and desaturation. Therefore, another important goal of this PhD thesis was to analyze the long-term effects of periodical administration of chitosan-TPP-DNA nanoparticles expressing the N-terminal nuclear fragment of hamster SREBP1a on the intermediary metabolism and growth performance of *S. aurata.*

2.2. Specific objectives

The **specific objectives** to address the main goal of this PhD thesis were:

Objective 1. To increase the current knowledge about glucose homeostasis and the metabolic characteristics of carnivorous fish by assessing the molecular mechanisms triggered by metformin on the intermediary metabolism and the expression of lipogenic factors in the liver of *S. aurata*.

Objective 2. To study long-term metabolic effects derived from periodical administration of chitosan-TPP nanoparticles complexed with a plasmid driving the expression of the N-terminal nuclear fragment of hamster SREBP-1a in the liver of *S. aurata*.

Objective 3. Determining the effect of periodical administration of chitosan-TPP nanoparticles complexed with a plasmid driving the expression of the N-terminal nuclear fragment of hamster SREBP-1a on growth performance of *S. aurata*.

3. Results

Article 1:

Metformin Counteracts Glucose-Dependent Lipogenesis and Impairs Transdeamination in the Liver of Gilthead Sea Bream (*Sparus aurata*)

Published in *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*

doi:10.1152/ajpregu.00216.2018.

January 2019

Ania Rashidpour*, Jonás I. Silva-Marrero*, Lidia Seguí, Isabel V. Baanante and Isidoro Metón

Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27, 08028 Barcelona, Spain

*Both authors had equal contribution to this work.

Am J Physiol Regul Integr Comp Physiol 316: R265–R273, 2019.
First published January 16, 2019; doi:10.1152/ajpregu.00216.2018.

RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (*Sparus aurata*)

Ania Rashidpour,* Jonás I. Silva-Marrero,* Lidia Seguí, Isabel V. Baanante, and Isidoro Metón

Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain

Submitted 16 July 2018; accepted in final form 14 January 2019

Rashidpour A, Silva-Marrero JI, Seguí L, Baanante IV, Metón I. Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (*Sparus aurata*). *Am J Physiol Regul Integr Comp Physiol* 316: R265–R273, 2019. First published January 16, 2019; doi:10.1152/ajpregu.00216.2018.— Metformin is an antidiabetic drug with a major impact on regulating blood glucose levels by decreasing hepatic gluconeogenesis, but also by affecting other pathways, including glucose transport and energy/lipid metabolism. Carnivorous fish are considered glucose intolerant, as they exhibit poor ability in using dietary carbohydrates. To increase the current knowledge about the molecular mechanisms by which metformin can improve glucose homeostasis in carnivorous fish, we addressed the effect of intraperitoneal administration of metformin, in the presence or absence of a glucose load, on metabolic rate-limiting enzymes and lipogenic factors in the liver of gilthead sea bream (*Sparus aurata*). Hyperglycemia markedly upregulated the expression of glycolytic enzymes (glucokinase and 6-phosphofructo-1-kinase, PFK1) 5 h following glucose administration, while at 24 h posttreatment, it increased isocitrate dehydrogenase (IDH) activity, a key enzyme of the tricarboxylic acid cycle, and the expression of lipogenic factors (PGC1β, Lpin1, and SREBP1). Metformin counteracted glucose-dependent effects, and downregulated glutamate dehydrogenase, alanine aminotransferase, and mammalian target of rapamycin 5 h posttreatment in the absence of a glucose load, leading to decreased long-term activity of PFK1 and IDH. The results of the present study suggest that hyperglycemia enhances lipogenesis in the liver of *S. aurata* and that metformin may exert specific metabolic effects in fish by decreasing hepatic transdeamination and suppressing the use of amino acids as gluconeogenic substrates. Our findings highlight the role of amino acid metabolism in the glucose-intolerant carnivorous fish model.

glutamate dehydrogenase; lipogenesis; liver; metformin; *Sparus aurata*

INTRODUCTION

Metformin (1,1-dimethylbiguanide hydrochloride) is an antidiabetic drug used for the treatment of Type 2 diabetes to enhance glucose homeostasis by improving the insulin sensitivity mainly in the liver and skeletal muscle (44). Metformin reduces the hepatic production of glucose by a mechanism involving inhibition of gluconeogenesis and glycogenolysis, increased insulin sensitivity and peripheral glucose uptake, and reduced intestinal glucose absorption (40, 42). Metformin-dependent reduction of hepatic glucose production involves transitory inhibition of complex I of the mitochondrial respiratory chain, leading to activation of adenosine monophosphate-activated protein kinase (AMPK), an energy sensor involved in glucose and lipid metabolism. AMPK activation stimulates glycolysis, while it downregulates the hepatic transcription of gluconeogenic genes (16, 23, 36). In addition, metformin represses lipogenesis and triglyceride accumulation in the liver through a mechanism that involves induced activation of AMPK and downregulation of sterol regulatory element-binding protein 1c (SREBP1c), a key transcription factor for de novo synthesis of lipids (19, 41, 47). Metformin also improves the glucose control via increasing insulin-stimulated glucose disposal, enhancing insulin receptor tyrosine kinase activity, increasing glycogen synthesis activity, and enhancing activity of glucose facilitative transporter type 4 (GLUT4) in skeletal muscle (6).

The molecular action of metformin has been mostly studied in rodents and human-derived cell lines. In fish, metformin reduces blood glucose levels when administered intraperitoneally, infused using osmotic pumps, or included in the food diet (15, 31, 33, 46). However, knowledge of the mechanisms underlying metformin action in fish remains limited. Carnivorous fish are considered glucose intolerant mainly because of prolonged hyperglycemia experienced after a glucose load or intake of high-carbohydrate diets (34). The molecular basis of glucose intolerance in fish has been mainly attributed to dysregulation of enzyme activities that control the rate of substrate cycling between glucose and glucose-6-phosphate in the liver, glucokinase (GK), and glucose-6-phosphatase (G6Pase). In this regard, lower glucose affinity and postprandial delayed induction of GK expression was reported for gilthead sea bream (*Sparus aurata*) (8). In addition, no significant modulation of G6Pase expression was reported in the liver of rainbow trout (*Oncorhynchus mykiss*), irrespective of the carbohydrate content of the diet (29, 30), whereas insulin hardly affected the promoter activity of the G6Pase catalytic subunit in the absence of glucose in *S. aurata* primary hepatocytes, suggesting that a reduced capacity of insulin-dependent repression of G6Pase may contribute to insulin resistance in fish (37).

In contrast to observations in mammals, although dietary metformin reduced postprandial glycemia in rainbow trout supplied with high-carbohydrate diets, unexpected induction of gluconeogenic and lipogenic gene expression by metformin was found in the liver (31). Indeed, metformin counteracts the effects of insulin after intraperitoneal administration of glucose

* A. Rashidpour and J. I. Silva-Marrero contributed equally to this work.

Address for reprint requests and other correspondence: I. Metón, Secció de Bioquímica i Biologia Molecular, Dept. de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain (e-mail: imeton@ub.edu).

0363-6119/19 Copyright © 2019 the American Physiological Society

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

in metformin-infused rainbow trout, especially in the muscle, which led the authors to conclude that metformin is unable to improve glucose homeostasis under hyperglycemic conditions in rainbow trout (35).

Since the effect of metformin on the intermediary metabolism of carnivorous fish remains limited to a few species and that the phylogenetic diversity of fish may determine specific metabolic adaptations, the purpose of the present study was to examine the metabolic effects of metformin in *S. aurata*. To this end, we analyzed the effect of intraperitoneal administration of metformin and glucose on the expression of key enzymes involved in hepatic glycolysis-gluconeogenesis: GK, 6-phosphofructo-1-kinase (PFK1), and fructose-1,6-bisphosphatase (FBPase1). Given the major role of amino acids as gluconeogenic substrates and fuel in carnivorous fish and the involvement of metformin on lipogenic gene expression (1, 31, 43), we also studied the effect of metformin on key enzymes of the tricarboxylic acid cycle (TCA) (isocitrate dehydrogenase, IDH; and α-ketoglutarate dehydrogenase, OGDH), amino acid metabolism (alanine aminotransferase, ALT; aspartate aminotransferase, AST; and glutamate dehydrogenase, GDH), nutrient-sensitive serine/threonine-protein kinase TOR (mammalian target of rapamycin, mTOR), and lipogenic factors (SREBP1; peroxisome proliferator-activated receptor γ coactivator 1-β, PGC1β; and Lpin1).

MATERIALS AND METHODS

Animals. Gilthead sea bream (*S. aurata*) juveniles obtained from Piscimar (Burriana, Castellón, Spain) were maintained at 20°C in 260-liter aquaria supplied with running seawater, as described previously (13). The diet, supplied at 30 g/kg body wt once a day (10:00 AM), contained 46% protein, 9.3% carbohydrates, 22% lipids, 10.6% ash, 12.1% moisture, and 21.1 kJ/g gross energy. To study the metabolic effects of metformin on *S. aurata*, four groups of fish were intraperitoneally administered 24 h after the last meal with a volume of 10 μl/g body wt containing saline (9 g/l NaCl; control group), glucose (2 g/kg body wt), metformin (150 mg/kg body wt), and glucose (2 g/kg body wt) + metformin (150 mg/kg body wt), respectively. At 5 and 24 h posttreatment, fish were euthanized by cervical section, blood was collected, and the liver was dissected out, frozen in liquid N_2 and kept at −80°C until use. To prevent stress, fish were anesthetized with MS-222 (1:12,500) before handling. The University of Barcelona's Animal Welfare Committee approved the experimental procedures in compliance with local and EU legislation.

Enzyme activity assays and metabolite determinations. To obtain liver crude extracts for determination of enzyme activities, powdered frozen tissue was homogenized (1:5, wt/vol) in 50 mM Tris·HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 250 mM sucrose using a PTA-7 Polytron (Kinematica, Littau-Luzern, Switzerland) (position 3, 30 s). After centrifugation at 20,000 *g* for 30 min at 4°C, the supernatant was collected and used to perform enzyme activity assays. PFK1 was assayed after the addition of 1 mM ATP to a 200-μl reaction mix containing 100 mM Tris·HCl pH 8.25, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM ammonium sulfate, 4 mM 2-mercaptoethanol, 0.15 mM NADH, 10 mM fructose 6-phosphate, 30 mM glucose 6-phosphate, 0.675 U/ml aldolase, 5 U/ml triose phosphate isomerase, 2 U/ml glycerol 3-phosphate dehydrogenase, and 4 μl of crude extract. FBPase1 was monitored in a final volume of 200 μl containing 85 mM imidazole-HCl pH 7.7, 5 mM $MgCl_2$, 0.5 mM NADP, 12 mM 2-mercaptoethanol, 0.05 mM fructose 1,6-bisphosphate, 2.5 U/ml phosphate glucose isomerase, 0.48 U/ml glucose 6-phosphate dehydrogenase, and 4 μl of extract. GDH was determined by monitoring NADH oxidation in a 250-μl mixture containing 50 mM imidazole-HCl (pH 7.4), 250 mM ammonium acetate, 5 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM ADP, and 4 μl crude extract. To assay IDH, $NADP^+$ reduction was assayed after the addition of 32 μM $NADP^+$ and 3.9 mM $MnSO_4$ to a final volume of 200 μl containing 80 mM triethanolamine buffer (pH 7.5), 42 mM NaCl, 3.7 mM isocitrate, and 4 μl crude extract. OGDH activity was determined after the addition of 0.12 mM coenzyme A to a final volume of 200 μl containing 50 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 0.6 mM thiamine pyrophosphate, 2 mM NAD^+, 10 mM α-ketoglutarate, 0.2 mM EGTA, 0.4 mM ADP and 4 μl crude extract. ALT and AST were assayed using commercial kits (Linear Chemicals, Montgat, Barcelona, Spain). All enzyme assays were performed at 30°C and monitored at 340 nm in a Cobas Mira S analyzer (Hoffman-La Roche, Basel, Switzerland). Enzyme activities were expressed per milligram of soluble protein (specific activity). One unit of enzyme activity was

Table 1. *Oligonucleotides used in the present study*

Primer	Sequence (5′ to 3′)	Gene, GenBank Accession No.
JYA01F1	TGTGTCAGCTCTCAACTCGACC	GK, AF169368
JYA02R1	AGGATCTGCTCTACCATGTGGAT	
JYA03F1	TGCTGGGGACAAAACGAACTCTTCC	PFK1, KF857580
JYA04R1	AAACCCTCCGACTACAAGCAGAGCT	
AE1305	CAGATGGTGAGCCGTGTGAGAAGGATG	FBPase1, AF427867
AE1306	GCCGTACAGAGCGTAACCAGCTGCC	
CG1543	GGTATTTCGGGGAGCTGCTGAG	GDH, MF459045
CG1544	CGCATCAGGGACGAGGACA	
AS1601	GGAGACTGTTTTGAGGTCGCC	mTOR, MH594580
AS1602	ACCTCCATCACCGTGTGGCA	
LS1703	ACCTCTTCTACCCCAACCAACAAC	Lpin1, MH594582
LS1704	TCCACCACCTCGCCCAG	
LS1705	GCATGGCTCGCGACGGC	PGC1β, MH594581
LS1706	GTGTTTTCAGTGGGCCATGGCATTG	
JS1406	CAGCAGCCCGAACACCTACA	SREBP1, JQ277709
JS1407	TTGTGGTCAGCCCTTGGAGTTG	
ASEF1Fw	CCCGCCTCTGTTGCCTTCG	EF1α, AF184170
ASEF1Rv	CAGCAGTGTGGTTCCGTTAGC	
JDRT18S	TTACGCCCATGTTGTCCTGAG	18s, AM490061
JDRT18AS	AGGATTCTGCATGATGGTCACC	
QBACTINF	CTGGCATCACACCTTCTACAACGAG	β-actin, X89920
QBACTINR	GCGGGGGTGTTGAAGGTCTC	

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

defined as the amount of enzyme required to transform 1 μmol of substrate per minute, except for PFK1 activity, which was defined as the amount of enzyme oxidizing 2 μmol of NADH per minute. The Bradford method (5) using BSA as a standard was adapted for automated determination of total protein in liver crude extracts, as described previously (27). Serum glucose, triglycerides, and cholesterol were measured with commercial kits (Linear Chemicals, Montgat, Barcelona, Spain).

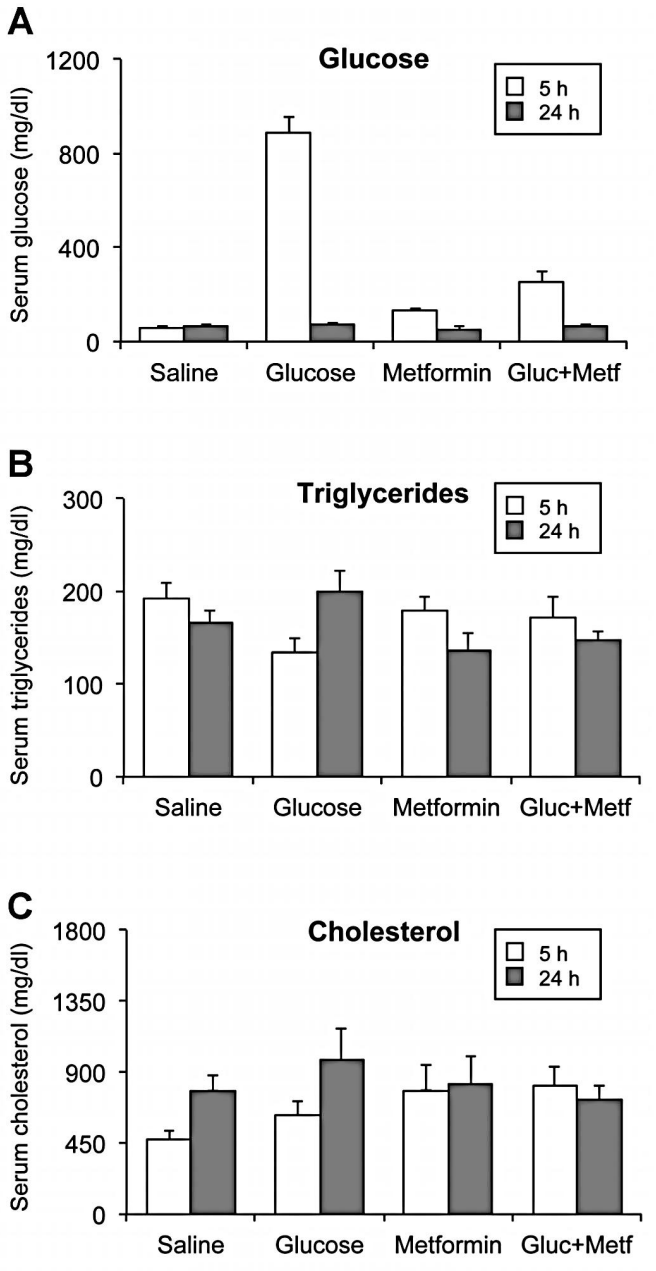
Quantitative real-time RT-PCR. One microgram of total RNA isolated from the liver of *S. aurata* was reverse-transcribed to cDNA using Moloney murine leukemia virus RT (Life Technologies, Carlsbad, CA) for 1 h at 37°C and random hexamer primers. *S. aurata* GK, PFK1, FBPase1, GDH, mTOR, SREBP1, PGC1β, and Lpin1 mRNA levels were determined in a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA) in a 20-μl mixture containing 0.4 μM of each primer (Table 1), 10 μl of SYBR Green (Applied Biosystems), and 1.6 μl of diluted cDNA. The temperature cycle protocol for amplification was 95°C for 10 min, followed by 40 cycles with 95°C for 15 s and 62°C for 1 min. A dissociation curve was applied after each experiment to confirm amplification of one product only. Specificity of the amplification was assayed by amplicon sequencing at least once for each gene. Standard curves were generated with serial dilutions of control cDNA to determine the efficiency of PCR reaction for each gene. Amplicon size was checked by agarose gel electrophoresis. The amount of mRNA for the gene of interest in each sample was normalized with *S. aurata* ribosomal subunit 18s, β-actin, and elongation factor 1 α (EF1α) as endogenous controls using primer pairs JDRT18S/JDRT18AS, QBACTINF/QBACTINR, and AS-EF1Fw/AS-EF1Rv, respectively (Table 1). Variations in gene expression were calculated by the standard $\Delta\Delta C_T$ method (32).

Statistics. Analyses were performed with SPSS software version 24 (IBM, Armonk, NY). Data were submitted to two-way ANOVA with time (5 h and 24 h) and treatment (saline, glucose, metformin, and glucose + metformin) as independent variables. Student-Newman-Keuls post hoc test was applied to determine differences among treatments ($P < 0.05$).

RESULTS

At 5 and 24 h after intraperitoneal administration with saline, glucose, metformin, and glucose plus metformin, serum metabolites and the expression of key enzymes, transcriptional coactivators, and transcription factors involved in the regulation of intermediary metabolism were analyzed in the liver of *S. aurata*. Data on serum glucose, triglycerides, and cholesterol are presented in Fig. 1. Plasma glucose levels were significantly affected by sampling time, treatment, and their interaction (Fig. 1*A*). Five hours after the administration of the glucose injection, plasma glucose levels increased from 3.3 mM in control animals (saline) to 49.0 mM. Thereafter, it decreased to the control values at 24 h posttreatment. At 5 h after the administration of metformin, glycemia reached 7.3 mM (2.2-fold over control values), while it promoted a slight hypoglycemia (2.9 mM) 24 h following the treatment. In combination with glucose, metformin prevented the increase in blood glucose levels, mostly at 5 h after treatment and completely at 24 h postadministration (Fig. 1*A*). No statistical differences were observed in serum triglycerides and cholesterol concerning the metformin effect. However, both triglycerides and cholesterol exhibited a similar trend to slightly increase as a result of glucose administration at 24 h after treatment. Such effect was totally prevented by the administration of metformin (Fig. 2, *B* and *C*).

The effect of sampling time and treatment on *S. aurata* liver mRNA levels for rate-limiting enzymes in glycolysis-gluco-



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment			
				Saline	Gluc	Metf	Gluc+Metf
Glucose	***	***	***	a	c	ab	b
Triglycerides	*	NS	NS	-	-	-	-
Cholesterol	NS	NS	NS	-	-	-	-

Fig. 1. Effect of glucose and metformin administration on serum metabolite levels in *Sparus aurata*. Twenty-four hours after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2 g/kg body wt glucose, 150 mg/kg body wt metformin, and 2 g/kg body wt glucose + 150 mg/kg body wt metformin, respectively. At 5 and 24 h posttreatment, blood was collected and serum was recovered. Serum levels of glucose (*A*), triglycerides (*B*), and cholesterol (*C*) are presented as means ± SE ($n = 6$ fish). *Bottom*: table shows statistical significance for independent variables (sampling time and treatment) as follows: $^*P < 0.05$; $^{***}P < 0.001$; NS, not significant. ^{a,b,c}Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

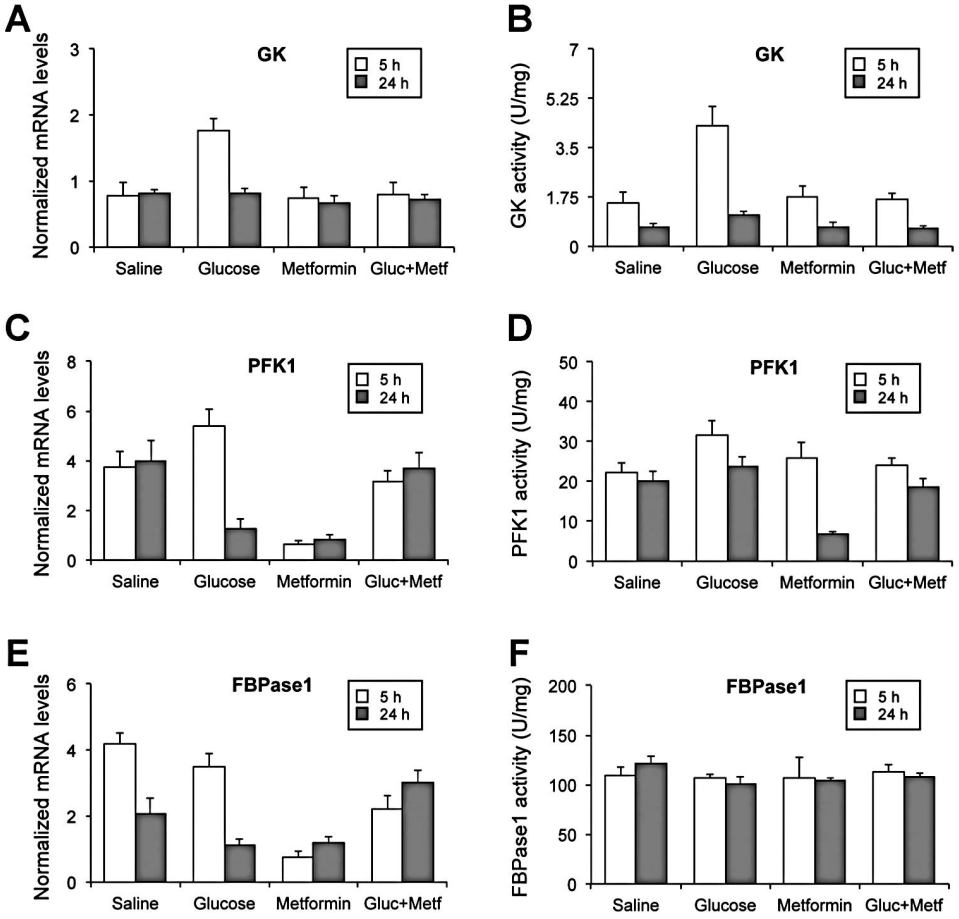


Fig. 2. Effect of glucose and metformin administration on the expression of key enzymes in glycolysis-gluconeogenesis in the liver of *S. aurata*. Twenty-four hours after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2 g/kg body wt glucose, 150 mg/kg body wt metformin, and 2 g/kg body wt glucose + 150 mg/kg body wt metformin, respectively. At 5 and 24 h posttreatment, the liver was collected and RNA was isolated. Hepatic mRNA levels and enzyme activity of glucokinase (GK; *A* and *B*), 6-phosphofructo-1-kinase (PFK1; *C* and *D*), and fructose-1,6-bisphosphatase (FBPase1; *E* and *F*) are presented as means ± SE (n = 6 fish). Expression levels for each gene were normalized using ribosomal subunit 18s, β-actin, and elongation factor 1α (EF1α) as housekeeping genes. *Bottom*: table shows statistical significance for independent variables (sampling time and treatment) as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant. [a,b]Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

Two-way ANOVA

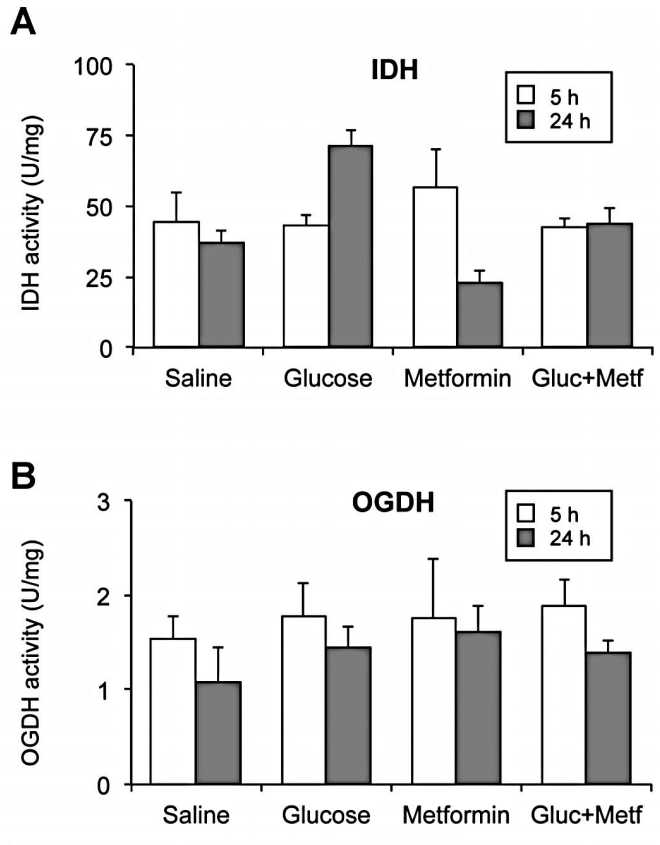
Dependent variable	Interaction	Time	Treatment	Treatment			
				Saline	Gluc	Metf	Gluc+Metf
GK mRNA	**	NS	***	a	b	a	a
GK activity	**	***	***	a	b	a	a
PFK1 mRNA	***	*	***	b	b	a	b
PFK1 activity	*	***	**	ab	b	a	ab
FBPase1 mRNA	***	**	***	b	b	a	b
FBPase1 activity	NS	NS	NS	-	-	-	-

neogenesis is shown in Fig. 2. Five hours after treatment, glucose injection significantly increased mRNA levels and enzyme activity for genes involved in glycolysis (GK and PFK1), GK being the most affected enzyme. The administration of metformin alone did not modulate GK expression, while downregulated PFK1 mRNA levels at 5 and 24 h posttreatment, as well as PFK1 activity at 24 h. When administrated with glucose, metformin totally prevented the effect of glucose administration on GK and PFK1 expression (Fig. 2, *A–D*). The mRNA levels of FBPase1 decreased as a result of metformin administration, while no significant effects were observed by injecting metformin combined with glucose. At the level of enzyme activity, no effects were found for FBPase1 in any of the treatments performed (Fig. 2, *E* and *F*).

For all treatments, we also analyzed the hepatic activity of two rate-limiting enzymes of the TCA: IDH, and OGDH. Glucose administration significantly increased twofold IDH activity compared with that in control fish at 24 h posttreatment. The opposite effects were observed after the administration of metformin alone, while metformin combined with glucose prevented the glucose-induced rise of IDH activity (Fig. 3*A*). In contrast to IDH, glucose and metformin did not affect OGDH activity significantly in the liver of *S. aurata* (Fig. 3*B*).

The effect of glucose and metformin in regard to key enzyme activities in amino acid metabolism in the liver is presented in Fig. 4. After the administered glucose injection, ALT activity significantly decreased at 24 h compared with control animals. Albeit not significant, a similar trend was observed for AST activity. Metformin prevented the glucose-dependent decrease in ALT activity 24 h posttreatment (Fig. 4, *A* and *B*). The effect of sampling time and treatment on mRNA levels and enzyme activity of GDH is shown in Fig. 4, *C* and *D*, respectively. Glucose injection did not affect GDH expression at any of the sampling times studied. However, metformin significantly downregulated both GDH mRNA levels (3.3-fold) and activity (2.9-fold) 5 h posttreat-

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment			
				Saline	Gluc	Metf	Gluc+Metf
IDH activity	**	NS	*	a	b	a	ab
OGDH activity	NS	*	NS	-	-	-	-

Fig. 3. Effect of glucose and metformin administration on the activity of key enzymes in the tricarboxylic acid cycle in the liver of *S. aurata*. Twenty-four hours after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2 g/kg body wt glucose, 150 mg/kg body wt metformin, and 2 g/kg body wt glucose + 150 mg/kg body wt metformin, respectively. At 5 and 24 h posttreatment, the liver was collected. Enzyme activity levels of isocitrate dehydrogenase (IDH; *A*) and α-ketoglutarate dehydrogenase (OGDH; *B*) are presented as means ± SE ($n = 6$ fish). *Bottom*: table shows statistical significance for independent variables (sampling time and treatment) as follows: $*P < 0.05$; $**P < 0.01$; NS, not significant. [a,b]Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

ment. Twenty-four hours after the treatment, GDH expression was not affected by metformin.

Given that the multiproteic complex mTORC1 is considered a sensor of nutrient availability and energy status of the cell (18), we analyzed the hepatic mRNA levels of mTOR, a cytosolic serine/threonine kinase included in the mTORC1 complex. A trend to decrease mTOR expression was observed 5 h after the administration of metformin. Glucose alone did not affect mTOR expression and reversed the effect observed with metformin (Fig. 5*A*). We also addressed the effect of metformin on transcriptional coactivators and transcription factors involved in the control of lipogenesis. Experimental treatments significantly affected PGC1β and SREBP1 mRNA levels. Five hours after glucose administration, SREBP1 expression increased 2.7-fold, whereas at 24 h posttreatment, glucose upregulated PGC1β and SREBP1 mRNA levels approximately twofold compared with control animals (saline), an effect that was at least partially reversed when glucose was administered in combination with metformin. At 5 h after the administration of metformin alone, a tendency to increase PGC1β and SREBP1 mRNA levels was found (Fig. 5, *B* and *C*). Sampling time also affected Lpin1 expression. At 5 h posttreatment, metformin upregulated Lpin1, whereas glucose in combination with metformin reversed this effect. At 24 h after the administration, the highest Lpin1 mRNA levels were observed in the group of fish injected with glucose (Fig. 5*D*).

DISCUSSION

Herein, we addressed the effect of metformin on key metabolic actors known to play important roles in the control of postprandial glycemia in the liver of *S. aurata*. Five hours after glucose administration, blood glucose levels markedly increased, but total recovery occurred 24 h posttreatment. Consistent with previous reports in *S. aurata* (9, 13, 27), our findings showed that hyperglycemia led to increased glycolytic rate by stimulating the expression of GK and PFK1, while hardly affecting the activity of the gluconeogenic enzyme, FBPase1. In the present study, metformin counteracted the activating effect of glucose on GK and PFK1 expression in the liver of *S. aurata*. When metformin was administered in the absence of glucose, it decreased both PFK1 and FBPase1 mRNA levels. However, only a significant decrease was observed in PFK1 activity 24 h posttreatment, possibly due to allosteric regulation of both PFK1 and FBPase1 activity. Similarly, the expression of GK, PFK1, G6Pase, FBPase1, and phosphoenolpyruvate carboxykinase (PEPCK) decreased in the liver of metformin-infused rainbow trout 6 h after intraperitoneal administration of glucose (35). However, in the same species, no effect of dietary metformin supplied to fish fed high-carbohydrate diets was observed on the activity of glycolytic enzymes in the liver and white muscle, while it paradoxically increased the expression of FBPase1 and other gluconeogenic enzymes (31). Differences in the experimental design, as well as metformin dosage and administration, may explain the results obtained in rainbow trout.

Considering rate-limiting enzymes of the TCA in the liver of *S. aurata*, IDH activity, which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, appeared to be more responsive than OGDH to experimental treatments: glucose stimulated IDH activity while metformin decreased the enzyme activity. However, OGDH activity was not significantly affected. Possibly, increased availability of fuel in the liver after glucose administration and glucose-dependent enhancement of glycolysis may be responsible for long-term enhancement of IDH activity and, therefore, enable glucose oxidation in the liver for ATP production. Similarly, as in *S. aurata*, metformin inhibits oxidative reaction of IDH in human-derived cancer cells (3, 17).

Given that a tendency to increase triglycerides and cholesterol blood levels was observed 24 h after glucose administration, our findings support that hepatic lipogenesis seems an efficient metabolic pathway for long-term transformation of excess glucose to lipids in *S. aurata*. Consistent with this hypothesis, glucose administration upregulated the expression of SREBP1, a transcription factor that plays a major role in the

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

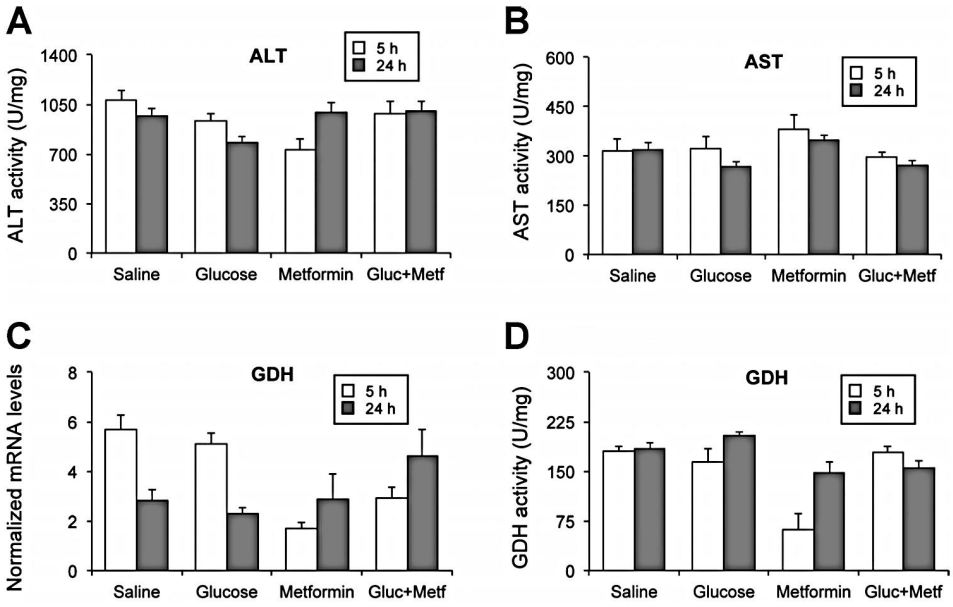


Fig. 4. Effect of glucose and metformin administration on key enzymes in amino acid metabolism in the liver of *S. aurata*. Twenty-four hours after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2 g/kg body wt glucose, 150 mg/kg body wt metformin, and 2 g/kg body wt glucose + 150 mg/kg body wt metformin, respectively. At 5 and 24 h posttreatment, the liver was collected and RNA was isolated. Enzyme activity values of alanine aminotransferase (ALT; *A*), aspartate aminotransferase (AST; *B*), and mRNA levels, as well as enzyme activity of glutamate dehydrogenase (GDH; *C* and *D*) are presented as means ± SE ($n = 6$ fish). Expression levels for GDH were normalized using ribosomal subunit 18s, β-actin, and elongation factor 1α (EF1α) as housekeeping genes. *Bottom*: table shows statistical significance for independent variables (sampling time and treatment) as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant. [a,b]Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment			
				Saline	Gluc	Metf	Gluc+Metf
ALT activity	*	NS	*	b	a	ab	ab
AST activity	NS	NS	*	ab	ab	b	a
GDH mRNA	***	NS	*	b	ab	a	ab
GDH activity	**	*	***	b	b	a	b

transcriptional regulation of genes involved in fatty acid and cholesterol synthesis (39), and, at long-term, Lpin1 and the transcriptional coactivator PGC1β, which are also involved in lipogenesis (10, 12). We previously showed that although *S. aurata* is a carnivorous fish, it tolerates partial replacement of dietary protein by carbohydrates through a mechanism that involves modulation of glycolysis in the liver (8, 13, 25–27). Conceivably, glucose-dependent effects on SREBP1, Lpin1, and PGC1β mRNA levels, which lasted 24 h after the treatment, may exert a major role in lipid synthesis from dietary carbohydrates in the liver when treated long-term.

The administration of metformin prevented glucose-dependent hyperglycemia and the effects on other serum metabolites, the activity of key enzymes in glycolysis and TCA cycle, as well as in the expression of SREBP1, Lpin1, and PGC1β. In the absence of a glucose load, a trend toward increasing SREBP1 and PGC1β mRNA levels was found in the liver of *S. aurata* 5 h after the administration of metformin. However, the fact that *S. aurata* treated with metformin plus glucose presented lower SREBP1, Lpin1, and PGC1β mRNA levels than glucose-treated fish, points to downregulation of hepatic lipogenesis as a result of metformin action to reduce blood glucose levels in the hyperglycemic state. Metformin-dependent downregulation of SREBP1 expression and lipogenesis is consistent with previous observations in mammals (19, 28, 41, 47), but contrasts with results reported for rainbow trout, which indicated that dietary metformin increases expression of lipogenic enzymes in the liver when rainbow trout were fed high-carbohydrate diets (31). Indeed, the administration of metformin together with insulin upregulated SREBP1 expression in the liver of rainbow trout fed high-carbohydrate diets, while metformin alone did not affect SREBP1 mRNA levels (33). Different metabolic responses to metformin in *S. aurata* and rainbow trout may result from the fact that although both species are carnivores, they belong to phylogenetically distant orders (*Spariformes* and *Salmoniformes*, respectively).

Remarkably, at 5 h posttreatment, metformin administration in the absence of a glucose load downregulated GDH, which plays a major role in amino acid catabolism by catalyzing reversible oxidative deamination of L-glutamate into α-ketoglutarate, and ALT activity. Carnivorous fish exhibit preferential use of amino acids as gluconeogenic substrates and fuel (1, 43). Indeed, optimal growth of fish requires high levels of dietary protein, and amino acids are the most potent insulin secretagogues in fish (34). Therefore, transdeamination, which involves transferring of amino groups from amino acids to α-ketoglutarate to produce glutamate and subsequent glutamate deamination by GDH, is very important in the fish liver for entering the carbon skeleton of amino acids into the TCA cycle to obtain energy and for biosynthetic purposes (7, 14, 20, 22, 38). The fact that metformin markedly decreased GDH expression, and to a lesser extent ALT activity, suggests reduced amino acid deamination and transamination capacity in the liver of *S. aurata*, and, therefore, limited use of amino acids for gluconeogenic purposes, which, in turn, may be essential for the long-term hypoglycemic effect of metformin in the liver of *S. aurata*. In contrast to ALT, AST activity remained unaffected by metformin. Indeed, although ALT and AST are quantitatively the most important aminotransferases in the fish liver, previous studies indicated greater sensibility of ALT to changes in the nutritional status than AST in the liver of *S. aurata* (2, 13, 27). In addition to GDH and ALT,

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

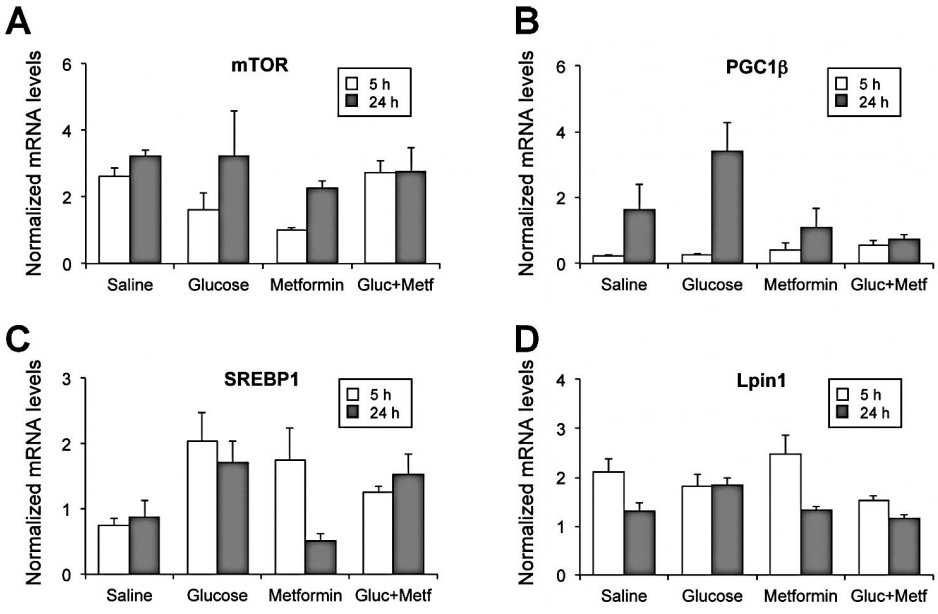


Fig. 5. Effect of glucose and signaling metformin administration on the mRNA levels of signaling proteins involved in the control of intermediary metabolism in the liver of *S. aurata*. Twenty-four hours after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2 g/kg body wt glucose, 150 mg/kg body wt metformin, and 2 g/kg body wt glucose + 150 mg/kg body wt metformin, respectively. At 5 and 24 h after treatment, the liver was collected and RNA isolated. The mRNA levels of mammalian target of rapamycin (mTOR; *A*), PGC1β (*B*), sterol regulatory element-binding protein (SREBP1; *C*), and Lpin1 (*D*) are presented as means ± SE (n = 6 fish). Expression levels for each gene were normalized using ribosomal subunit 18s, β-actin, and elongation factor 1α (EF1α) as housekeeping genes. *Bottom*: table shows statistical significance for independent variables (sampling time and treatment) as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant. [a,b]Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment			
				Saline	Gluc	Metf	Gluc+Metf
mTOR mRNA	NS	NS	NS	-	-	-	-
PGC1β mRNA	*	***	*	ab	b	a	a
SREBP1 mRNA	NS	NS	**	a	b	a	ab
Lpin1 mRNA	NS	**	NS	-	-	-	-

metformin downregulated mTOR expression at 5 h post-treatment in the liver of *S. aurata*. Consistently, metformin supplementation suppresses upregulation of hepatic mTOR mRNA levels when feeding the herbivorous cyprinid *Megalobrama amblycephala* with high-carbohydrate diets (45). The mTOR pathway is considered a major signaling pathway in fish and, as in mammals, is sensitive to the dietary protein to carbohydrate ratio (4). As in fish, metformin inhibits mTORC1 signaling in humans (24). Bearing in mind that mTOR is part of the amino acid sensor in the mTORC1 complex, knockdown of GDH1 inhibits mTORC1 activity, and leucine requires GDH1 for promoting mTORC1 activity in human cells (21), our results suggest that metformin may inhibit mTORC1 signaling by decreasing GDH expression in the liver of *S. aurata*. Conceivably, metformin-dependent reduced transdeamination activity in the liver of *S. aurata* would decrease availability of amino acid carbon skeletons as fuel and determine the low levels of PFK1 and IDH activity observed 24 h after metformin administration in the absence of a glucose load. In this regard, it was proposed that inhibition of mTORC1 improves glucose tolerance by inhibiting hepatic gluconeogenesis in rainbow trout (11).

Perspectives and Significance

The present study addressed for the first time the effect of acute metformin treatment on the intermediary metabolism of *S. aurata*. Our findings suggest that hyperglycemia enhances lipogenesis in the liver and that metformin may improve glucose homeostasis by counteracting the activating effects of glucose on the activity of rate-limiting enzymes in glycolysis and TCA, as well as the expression of lipogenic factors. In addition, the present study provides evidence that metformin may reduce the gluconeogenic rate by decreasing hepatic transdeamination and the entrance of amino acids into the TCA cycle in fish. Further studies are needed to better understand the link between metformin action, GDH expression, and the use of amino acids as gluconeogenic substrates in carnivorous fish.

ACKNOWLEDGMENTS

The authors thank Piscimar (Burriana, Castellón, Spain) for providing *S. aurata* juveniles and Aquarium of Barcelona (Barcelona, Spain) for supplying filtered seawater.

GRANTS

This work was supported by the AGL2016-78124-R grant (MEC, Spain; cofunded by the European Regional Development Fund, EC).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.M., I.V.B. and J.I.S.-M. conceived and designed research; A.R., L.S. and J.I.S.-M. performed experiments; I.M., J.I.S.-M. and A.R. analyzed data and interpreted results of experiments; A.R. and I.M. prepared figures and drafted manuscript; A.R., J.I.S.-M., L.S., I.M. and I.V.B. edited and revised manuscript, and approved final version of manuscript.

REFERENCES

1. **Andoh T.** Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (*Verasper moseri*). *Gen Comp Endocrinol* 151: 308–317, 2007. doi:10.1016/j.ygcen.2007.01.015.
2. **Anemaet IG, Metón I, Salgado MC, Fernández F, Baanante IV.** A novel alternatively spliced transcript of cytosolic alanine aminotransferase gene associated with enhanced gluconeogenesis in liver of *Sparus aurata*.

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

Int J Biochem Cell Biol 40: 2833–2844, 2008. doi:10.1016/j.biocel.2008.05.018.

3. **Bai M, Yang L, Liao H, Liang X, Xie B, Xiong J, Tao X, Chen X, Cheng Y, Chen X, Feng Y, Zhang Z, Zheng W.** Metformin sensitizes endometrial cancer cells to chemotherapy through IDH1-induced Nrf2 expression via an epigenetic mechanism. *Oncogene* 37: 5666–5681, 2018. doi:10.1038/s41388-018-0360-7.
4. **Borges P, Valente LMP, Véron V, Dias K, Panserat S, Médale F.** High dietary lipid level is associated with persistent hyperglycaemia and down-regulation of muscle Akt-mTOR pathway in Senegalese sole (*Solea senegalensis*). *PLoS One* 9: e102196, 2014. doi:10.1371/journal.pone.0102196.
5. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976. doi:10.1016/0003-2697(76)90527-3.
6. **Buse JB, DeFronzo RA, Rosenstock J, Kim T, Burns C, Skare S, Baron A, Fineman M.** The primary glucose-lowering effect of metformin resides in the gut, not the circulation: results from short-term pharmacokinetic and 12-week dose-ranging studies. *Diabetes Care* 39: 198–205, 2016. doi:10.2337/dc15-0488.
7. **Caballero-Solares A, Viegas I, Salgado MC, Siles AM, Sáez A, Metón I, Baanante IV, Fernández F.** Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead sea bream (*Sparus aurata*) juveniles. *Aquaculture* 444: 79–87, 2015. doi:10.1016/j.aquaculture.2015.03.025.
8. **Caseras A, Metón I, Fernández F, Baanante IV.** Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). *Biochim Biophys Acta* 1493: 135–141, 2000. doi:10.1016/S0167-4781(00)00173-1.
9. **Caseras A, Metón I, Vives C, Egea M, Fernández F, Baanante IV.** Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). *Br J Nutr* 88: 607–614, 2002. doi:10.1079/BJN2002701.
10. **Chen Y, Rui B-B, Tang L-Y, Hu C-M.** Lipin family proteins—key regulators in lipid metabolism. *Ann Nutr Metab* 66: 10–18, 2015. doi:10.1159/000368661.
11. **Dai W, Panserat S, Terrier F, Seiliez I, Skiba-Cassy S.** Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 307: R1231–R1238, 2014. doi:10.1152/ajpregu.00166.2014.
12. **Ducheix S, Vegliante MC, Villani G, Napoli N, Sabbà C, Moschetta A.** Is hepatic lipogenesis fundamental for NAFLD/NASH? A focus on the nuclear receptor coactivator PGC-1β. *Cell Mol Life Sci* 73: 3809–3822, 2016. doi:10.1007/s00018-016-2331-x.
13. **Fernández F, Miquel AG, Cordoba M, Varas M, Metón I, Caseras A, Baanante IV.** Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J Exp Mar Biol Ecol* 343: 1–10, 2007. doi:10.1016/j.jembe.2006.10.057.
14. **Gaspar C, Silva-Marrero JI, Salgado MC, Baanante IV, Metón I.** Role of upstream stimulatory factor 2 in glutamate dehydrogenase gene transcription. *J Mol Endocrinol* 60: 247–259, 2018. doi:10.1530/JME-17-0142.
15. **Hertz Y, Epstein N, Abraham M, Madar Z, Hepher B, Gertler A.** Effects of metformin on plasma insulin, glucose metabolism and protein synthesis in the common carp (*Cyprinus carpio* L.). *Aquaculture* 80: 175–187, 1989. doi:10.1016/0044-8486(89)90283-4.
16. **Hostalek U, Gwilt M, Hildemann S.** Therapeutic use of metformin in prediabetes and diabetes prevention. *Drugs* 75: 1071–1094, 2015. doi:10.1007/s40265-015-0416-8.
17. **Keibler MA, Wasylenko TM, Kelleher JK, Iliopoulos O, Vander Heiden MG, Stephanopoulos G.** Metabolic requirements for cancer cell proliferation. *Cancer Metab* 4: 16, 2016. doi:10.1186/s40170-016-0156-6.
18. **Laplante M, Sabatini DM.** mTOR signaling in growth control and disease. *Cell* 149: 274–293, 2012. doi:10.1016/j.cell.2012.03.017.
19. **Liu Z-Q, Song X-M, Chen Q-T, Liu T, Teng J-T, Zhou K, Luo D-Q.** Effect of metformin on global gene expression in liver of KKAy mice. *Pharmacol Rep* 68: 1332–1338, 2016. doi:10.1016/j.pharep.2016.09.004.
20. **Liu Z, Zhou Y, Liu S, Zhong H, Zhang C, kang X, Liu Y.** Characterization and dietary regulation of glutamate dehydrogenase in different ploidy fishes. *Amino Acids* 43: 2339–2348, 2012. doi:10.1007/s00726-012-1313-z.
21. **Lorin S, Tol MJ, Bauvy C, Strijland A, Poüs C, Verhoeven AJ, Codogno P, Meijer AJ.** Glutamate dehydrogenase contributes to leucine sensing in the regulation of autophagy. *Autophagy* 9: 850–860, 2013. doi:10.4161/auto.24083.
22. **Lushchak VI, Husak VV, Storey KB.** Regulation of AMP-deaminase activity from white muscle of common carp *Cyprinus carpio*. *Comp Biochem Physiol B Biochem Mol Biol* 149: 362–369, 2008. doi:10.1016/j.cbpb.2007.10.008.
23. **Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, Gomes AP, Ward TM, Minor RK, Blouin M-J, Schwab M, Pollak M, Zhang Y, Yu Y, Becker KG, Bohr VA, Ingram DK, Sinclair DA, Wolf NS, Spindler SR, Bernier M, de Cabo R.** Metformin improves healthspan and lifespan in mice. *Nat Commun* 4: 2192, 2013. doi:10.1038/ncomms3192.
24. **Melnik BC, Schmitz G.** Metformin: an inhibitor of mTORC1 signaling. *J Endocrinol Diabetes Obes* 2: 1029, 2014.
25. **Metón I, Caseras A, Fernández F, Baanante IV.** 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, *Sparus aurata*. *Biochim Biophys Acta* 1491: 220–228, 2000. doi:10.1016/S0167-4781(00)00040-3.
26. **Metón I, Fernández F, Baanante IV.** Short- and long-term effects of refeeding on key enzyme activities in glycolysis–gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*). *Aquaculture* 225: 99–107, 2003. doi:10.1016/S0044-8486(03)00281-3.
27. **Metón I, Mediavilla D, Caseras A, Cantó E, Fernández F, Baanante IV.** Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). *Br J Nutr* 82: 223–232, 1999.
28. **de Oliveira Santana KN, Lelis DF, Mendes KL, Lula JF, Paraíso AF, Andrade JMO, Feltenberger JD, Cota J, da Costa DV, de Paula AMB, Guimarães ALS, Santos SHS.** Metformin reduces lipogenesis markers in obese mice fed a low-carbohydrate and high-fat diet. *Lipids* 51: 1375–1384, 2016. doi:10.1007/s11745-016-4209-y.
29. **Panserat S, Capilla E, Gutierrez J, Frappart PO, Vachot C, Plagnes-Juan E, Aguirre P, Brèque J, Kaushik S.** Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. *Comp Biochem Physiol B Biochem Mol Biol* 128: 275–283, 2001. doi:10.1016/S1096-4959(00)00322-5.
30. **Panserat S, Médale F, Brèque J, Plagnes-Juan E, Kaushik S.** Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J Nutr Biochem* 11: 22–29, 2000. doi:10.1016/S0955-2863(99)00067-4.
31. **Panserat S, Skiba-Cassy S, Seiliez I, Lansard M, Plagnes-Juan E, Vachot C, Aguirre P, Larroquet L, Chavernac G, Medale F, Corraze G, Kaushik S, Moon TW.** Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *Am J Physiol Regul Integr Comp Physiol* 297: R707–R715, 2009. doi:10.1152/ajpregu.00120.2009.
32. **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001. doi:10.1093/nar/29.9.e45.
33. **Polakof S, Moon TW, Aguirre P, Skiba-Cassy S, Panserat S.** Glucose homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin interact in a tissue-dependent manner. *Am J Physiol Regul Integr Comp Physiol* 300: R166–R174, 2011. doi:10.1152/ajpregu.00619.2010.
34. **Polakof S, Panserat S, Soengas JL, Moon TW.** Glucose metabolism in fish: a review. *J Comp Physiol B* 182: 1015–1045, 2012. doi:10.1007/s00360-012-0658-7.
35. **Polakof S, Skiba-Cassy S, Panserat S.** Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 297: R1769–R1776, 2009. doi:10.1152/ajpregu.00369.2009.
36. **Pryor R, Cabreiro F.** Repurposing metformin: an old drug with new tricks in its binding pockets. *Biochem J* 471: 307–322, 2015. doi:10.1042/BJ20150497.
37. **Salgado MC, Metón I, Egea M, Baanante IV.** Transcriptional regulation of glucose-6-phosphatase catalytic subunit promoter by insulin and glucose in the carnivorous fish, *Sparus aurata*. *J Mol Endocrinol* 33: 783–795, 2004. doi:10.1677/jme.1.01552.

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

38. **Sánchez-Muros MJ, García-Rejón L, García-Salguero L, de la Higuera M, Lupiáñez JA.** Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diet on glutamate dehydrogenase and alanine aminotransferase kinetics. *Int J Biochem Cell Biol* 30: 55–63, 1998. doi:10.1016/S1357-2725(97)00100-3.
39. **Shimano H, Sato R.** SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology. *Nat Rev Endocrinol* 13: 710–730, 2017. doi:10.1038/nrendo.2017.91.
40. **van Stee MF, de Graaf AA, Groen AK.** Actions of metformin and statins on lipid and glucose metabolism and possible benefit of combination therapy. *Cardiovasc Diabetol* 17: 94, 2018. doi:10.1186/s12933-018-0738-4.
41. **Tang X, Li J, Xiang W, Cui Y, Xie B, Wang X, Xu Z, Gan L.** Metformin increases hepatic leptin receptor and decreases steatosis in mice. *J Endocrinol* 230: 227–237, 2016. doi:10.1530/JOE-16-0142.
42. **Ursini F, Russo E, Pellino G, D'Angelo S, Chiaravalloti A, De Sarro G, Manfredini R, De Giorgio R.** Metformin and autoimmunity: a "new deal" of an old drug. *Front Immunol* 9: 1236, 2018. doi:10.3389/fimmu.2018.01236.
43. **Vilhelmsson OT, Martin SAM, Médale F, Kaushik SJ, Houlihan DF.** Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* 92: 71–80, 2004. doi:10.1079/BJN20041176.
44. **Wang Z, Lai S-T, Xie L, Zhao J-D, Ma N-Y, Zhu J, Ren Z-G, Jiang G-L.** Metformin is associated with reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. *Diabetes Res Clin Pract* 106: 19–26, 2014. doi:10.1016/j.diabres.2014.04.007.
45. **Xu C, Liu W-B, Zhang D-D, Cao X-F, Shi H-J, Li X-F.** Interactions between dietary carbohydrate and metformin. Implications on energy sensing, insulin signaling pathway, glycolipid metabolism and glucose tolerance in blunt snout bream *Megalobrama amblycephala*. *Aquaculture* 483: 183–195, 2018. doi:10.1016/j.aquaculture.2017.10.022.
46. **Zang L, Shimada Y, Nishimura N.** Development of a novel zebrafish model for type 2 diabetes mellitus. *Sci Rep* 7: 1461, 2017. doi:10.1038/s41598-017-01432-w.
47. **Zhu X, Yan H, Xia M, Chang X, Xu X, Wang L, Sun X, Lu Y, Bian H, Li X, Gao X.** Metformin attenuates triglyceride accumulation in HepG2 cells through decreasing stearyl-coenzyme A desaturase 1 expression. *Lipids Health Dis* 17: 114, 2018. doi:10.1186/s12944-018-0762-0.

Downloaded from journals.physiology.org/journal/ajpregu (005.235.020.092) on November 28, 2021.

Article 2:

Chitosan-Based Sustained Expression of SREBP1a Stimulates Hepatic Glucose Oxidation and Growth in *Sparus aurata*

Published in *Marine Drugs*

https://doi.org/10.3390/md21110562

October 2023

Ania Rashidpour [1], Yuanbing Wu [1], María P. Almajano [2], Anna Fàbregas [3] and Isidoro Metón [1]

[1] Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain

[2] Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain

[3] Departament de Farmàcia i Tecnologia Farmacèutica, i Fisicoquímica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain

marine drugs

MDPI

Article

Chitosan-Based Sustained Expression of Sterol Regulatory Element-Binding Protein 1a Stimulates Hepatic Glucose Oxidation and Growth in *Sparus aurata*

Ania Rashidpour [1], Yuanbing Wu [1], María Pilar Almajano [2], Anna Fàbregas [3] and Isidoro Metón [1,*]

[1] Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain; aniyarashidpoor2017@gmail.com (A.R.); wuuanbing@gmail.com (Y.W.)
[2] Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain; m.pilar.almajano@upc.edu
[3] Departament de Farmàcia i Tecnologia Farmacèutica, i Fisicoquímica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain; anna5791@gmail.com
* Correspondence: imeton@ub.edu; Tel.: +34-934024521

Abstract: The administration of a single dose of chitosan nanoparticles driving the expression of sterol regulatory element-binding protein 1a (SREBP1a) was recently associated with the enhanced conversion of carbohydrates into lipids. To address the effects of the long-lasting expression of SREBP1a on the growth and liver intermediary metabolism of carnivorous fish, chitosan-tripolyphosphate (TPP) nanoparticles complexed with a plasmid expressing the N terminal active domain of hamster SREBP1a (pSG5-SREBP1a) were injected intraperitoneally every 4 weeks (three doses in total) to gilthead sea bream (*Sparus aurata*) fed high-protein–low-carbohydrate and low-protein–high-carbohydrate diets. Following 70 days of treatment, chitosan-TPP-pSG5-SREBP1a nanoparticles led to the sustained upregulation of SREBP1a in the liver of *S. aurata*. Independently of the diet, SREBP1a overexpression significantly increased their weight gain, specific growth rate, and protein efficiency ratio but decreased their feed conversion ratio. In agreement with an improved conversion of dietary carbohydrates into lipids, SREBP1a expression increased serum triglycerides and cholesterol as well as hepatic glucose oxidation via glycolysis and the pentose phosphate pathway, while not affecting gluconeogenesis and transamination. Our findings support that the periodical administration of chitosan-TPP-DNA nanoparticles to overexpress SREBP1a in the liver enhanced the growth performance of *S. aurata* through a mechanism that enabled protein sparing by enhancing dietary carbohydrate metabolisation.

Keywords: chitosan; SREBP1; gene delivery; liver; growth; *Sparus aurata*

check for updates

Citation: Rashidpour, A.; Wu, Y.; Almajano, M.P.; Fàbregas, A.; Metón, I. Chitosan-Based Sustained Expression of Sterol Regulatory Element-Binding Protein 1a Stimulates Hepatic Glucose Oxidation and Growth in *Sparus aurata*. *Mar. Drugs* **2023**, *21*, 562. https://doi.org/10.3390/md21110562

Academic Editor: Hitoshi Sashiwa

Received: 6 October 2023
Revised: 18 October 2023
Accepted: 25 October 2023
Published: 26 October 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Chitosan is a linear cationic polymer of β (1-4)-linked 2-amino-2-deoxy-D-glucose interspersed by residual 2-acetamido-2-deoxy-β-D-glucose, produced by the deacetylation of chitin under alkaline conditions [1–3]. The interaction between positively charged chitosan and negatively charged nucleic acids results in the spontaneous formation of polyplexes. This ability together with bioadhsesion, low immunogenicity, biodegradability, and biocompatibility, prompted recent exploration into the use of chitosan as a vector for nucleic acid delivery in fish biotechnology, as non-viral vectors enabling fish vaccination, the control of gonadal development, and the gene therapy-based modulation of fish metabolism [4]. We previously showed that a single intraperitoneal administration of chitosan-tripolyphosphate (TPP) nanoparticles complexed with plasmids to induce gene silencing or the transient expression of exogenous proteins is an efficient method to produce an acute modification of intermediary metabolism in the liver of gilthead sea bream (*Sparus*

aurata). Thus, chitosan-TPP nanoparticles complexed with a plasmid encoding a short hairpin RNA (shRNA) designed to silence the expression of cytosolic alanine aminotransferase (cALT) significantly downregulated liver transamination but increased the hepatic activity of key enzymes in glycolysis [5]. The same methodology was used to demonstrate that the shRNA-mediated knockdown of glutamate dehydrogenase (*gdh*) reduced hepatic transdeamination and gluconeogenesis from amino acids but increased the metabolic use of dietary carbohydrates in the liver through the stimulation of the 6-phosphofructo-1-kinase (PFKL)/fructose-1,6-bisphosphatase (FBP1) enzyme activity ratio [6].

More recently, chitosan-TPP nanoparticles complexed with a plasmid expressing the N-terminus of hamster sterol regulatory element-binding protein 1a (SREBP1a) induced a multigenic action in the liver of *S. aurata* 72 h post-treatment, leading to the enhanced conversion of dietary carbohydrates into lipids and increased the blood levels of triglycerides and cholesterol through a mechanism involving the upregulation of the hepatic expression of genes encoding key enzymes in fatty acid and cholesterol biosynthesis, NADPH formation (glucose-6-phosphate dehydrogenase, *g6pd*), and glycolysis (glucokinase, *gck*; 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, *pfkfb1*), while decreasing the activity of the gluconeogenic enzyme FBP1 [7].

SREBP1a belongs to the SREBP family of transcription factors. SREBPs are synthesised as inactive precursors anchored to the endoplasmic reticulum membrane. The C-terminus of SREBP precursors associates with the SREBP-cleavage activating protein (Scap), a sterol sensor, and the insulin-induced gene (Insig) protein [8,9]. Decreased cellular levels of sterol drive the SREBP/Scap complex to the Golgi apparatus, where the N-terminus of SREBPs is released after a two-step cleavage by site-1 and site-2 proteases. The N-termini of SREBPs are active basic-helix–loop–helix leucine zipper transcription factors that, after dimerisation, translocate to the nucleus and activate transcription by binding to sterol regulatory elements (SREs) in the promoter of target genes. Alternate promoters in the *srebf1* gene generate SREBP1a and SREBP1c [10–13]. The presence of a long N-terminal transactivation domain confers strong transcriptional activity to SREBP1a, which is a potent activator of all SREBP-responsive genes [14–16].

Given that *S. aurata* and carnivorous fish in general have low abilities to metabolise dietary carbohydrates and exhibit prolonged hyperglycaemia after a glucose load or being fed high carbohydrate diets [17,18], and the fact that a single dose of chitosan nanoparticles complexed with a plasmid encoding the N terminus of hamster SREBP1a stimulated a short-term metabolic change in the liver of *S. aurata* that enabled carbohydrate conversion into lipids [7], the aim of the present study was to study the effects of long-lasting, sustained expression of transcriptionally active SREBP1a on growth and liver intermediary metabolism in *S. aurata*. To this end, chitosan-TPP nanoparticles complexed with a plasmid expressing the N terminal active domain of hamster SREBP1a were periodically administered to *S. aurata* fed diets differing in macronutrient composition.

2. Results

2.1. Effect of Periodical Administration of Chitosan-TPP-SREBP1a on SREBP1a mRNA Levels in the Liver of S. aurata

To address the effect of hepatic SREBP1a overexpression on growth and liver intermediary metabolism in *S. aurata*, chitosan-TPP nanoparticles complexed with empty pSG5 (control) and pSG5-SREBP1a were prepared by ionic gelation, as previously reported [7]. The characterisation of naked chitosan-TPP and chitosan-TPP complexed with plasmids (pSG5 and pSG5-SREBP1a) by dynamic light scattering showed no differences in particle mean diameter size. However, laser Doppler electrophoresis allowed us to show that the incorporation of pSG5 and pSG5-SREBP1a into chitosan-TPP significantly reduced the positivity of the *Z* potential to 54–58% of the values observed in naked chitosan-TPP (Figure 1).

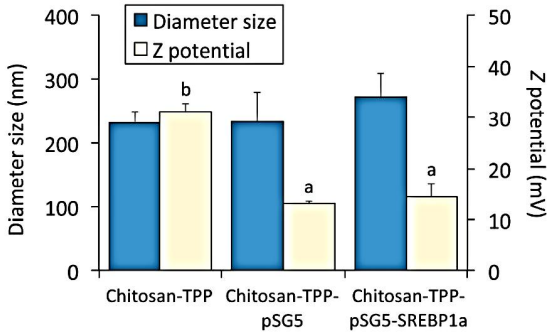
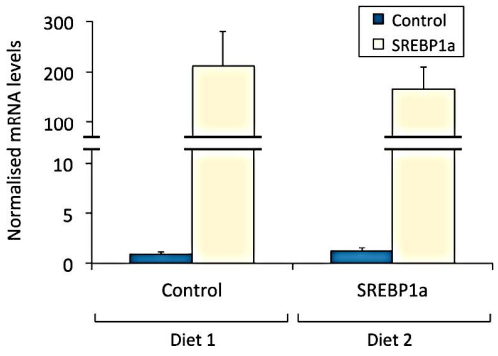


Figure 1. Diameter size and Z potential of chitosan-TPP-DNA. Particle size and Z potential values of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were determined by dynamic light scattering and laser Doppler electrophoresis. The values are expressed as mean ± SEM (n = 3). Different letters indicate significant differences between conditions ($p < 0.05$).

To assess the effect of the long-term, sustained expression of SREBP1a, intraperitoneal injections of chitosan-TPP nanoparticles complexed with pSG5 or pSG5-SREBP1a were periodically administered every 4 weeks to *S. aurata*. The fish received a total of three doses of the nanoparticles (each consisting of 10 μg of the corresponding plasmid per g of body weight, BW). Sampling proceeded 14 days after the last injection, which corresponded to a total treatment period of 70 days. The dosage schedule was based on the persistent hepatic expression of exogenous enzyme activity after the chitosan-based delivery of plasmid DNA in *S. aurata* [19]. The effect of periodical administration of nanoparticles was studied in fish fed two diets differing in macronutrient composition: Diet 1 (high-protein–low-carbohydrate commercial diet) and Diet 2 (low-protein–high-carbohydrate diet).

Reverse transcription coupled with quantitative PCR (RT-qPCR) revealed a huge expression level of SREBP1a in the liver of treated fish irrespective of diet. In the liver of *S. aurata* subjected to the periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles, the mRNA levels of SREBP1a reached 239-fold (Diet 1) and 136-fold (Diet 2) significantly higher values than in control fish (Figure 2).



Two-way ANOVA			
Dependent variable	Diet	SREBP1a	Interaction
SREBP1a mRNA	NS	***	NS

Figure 2. Effect of periodical administration of chitosan-TPP-DNA nanoparticles on SREBP1a mRNA levels in the liver of *S. aurata*. Three doses of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were periodically administered every 4 weeks by intraperitoneal injection to *S. aurata* fed Diets 1 and 2. After 70 days of treatment and 14 days following the last injection, liver samples were obtained and subjected to RNA isolation and subsequent RT-qPCR assays. The mRNA levels of SREBP1a were normalised with the geometrical mean of *S. aurata* β-actin (*actb*), elongation factor 1 alpha (*eef1a*) and ribosomal subunit 18S (*18s*). The values are expressed as mean ± SEM (n = 5). Statistical significance for independent variables (diet and treatment with SREBP1a nanoparticles) are indicated as follows: *** $p < 0.001$; NS, not significant.

2.2. Effect of SREBP1a Expression on Growth and Whole-Body Composition in S. aurata

Consistent with previous results, the supply of a low-protein–high-carbohydrate diet (Diet 2) significantly decreased final BW (to 86.7% of the levels found in fish fed Diet 1), weight gain (to 83.4%), and specific growth rate (SGR) (to 92.7%). In addition, higher lipid retention (LR) (2.47-fold) and protein efficiency ratio (PER) (1.14-fold) values were found in fish fed Diet 2. Independently of the diet supplied, long-term treatment with periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles significantly increased weight gain (13.8% for fish fed Diet 1 and 9.2% for fish fed Diet 2), SGR (14.6% for fish fed Diet 1 and 3.6% for fish fed Diet 2), and PER (7.9% for fish fed Diet 1 and 20.6% for fish fed Diet 2) but decreased feed conversion ratio (FCR) (to 6.4% and 14.7% of control values in fish fed Diet 1 and Diet 2, respectively). Neither diet nor nanoparticle administration caused significant effects on hepatosomatic index (HSI), protein retention (PR), moisture, ash, protein, and lipid body composition (Table 1).

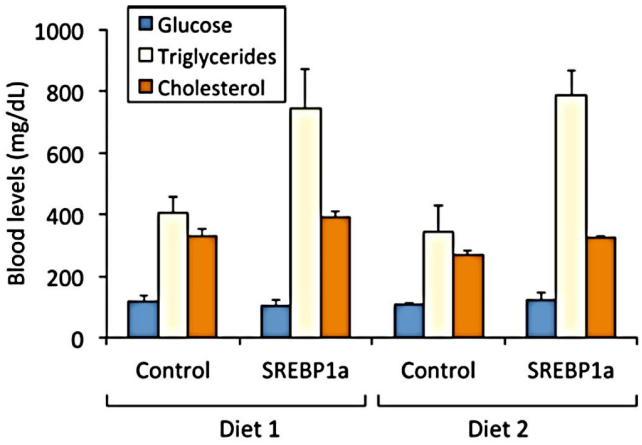
Table 1. Growth performance, nutrient retention, and whole-body composition of *S. aurata* fed diets differing in macronutrient composition and after periodical intraperitoneal administration of chitosan-TPP complexed with empty vector (pSG5, control) and pSG5-SREBP1a.

	Diet 1		**Diet 2**		**2-Way ANOVA**		
	Control	**SREBP1a**	**Control**	**SREBP1a**	**Treatment**	**Diet**	**Interaction**
Initial BW (g)	9.97 ± 0.69	10.6 ± 0.8	9.41 ± 0.74	9.34 ± 0.80	NS	NS	NS
Final BW (g)	34.6 ± 1.0	38.7 ± 1.8	30.0 ± 1.5	31.8 ± 3.4	NS	*	NS
Weight gain (g)	24.7 ± 0.5	28.1 ± 0.9	20.6 ± 0.9	22.5 ± 2.7	*	***	NS
SGR (%)	1.78 ± 0.05	2.04 ± 0.04	1.65 ± 0.06	1.71 ± 0.06	*	**	NS
FCR	1.40 ± 0.03	1.31 ± 0.04	1.57 ± 0.08	1.34 ± 0.12	*	NS	NS
HSI (%)	1.55 ± 0.12	1.36 ± 0.05	1.48 ± 0.14	1.79 ± 0.10	NS	NS	NS
PR (%)	25.0 ± 3.2	19.6 ± 0.8	28.6 ± 2.7	25.5 ± 2.0	NS	NS	NS
LR (%)	24.6 ± 5.6	20.4 ± 3.4	60.8 ± 13.6	46.6 ± 4.1	NS	**	NS
PER	1.40 ± 0.03	1.51 ± 0.05	1.60 ± 0.07	1.93 ± 0.20	*	***	NS
Moisture (%)	71.3 ± 0.7	69.0 ± 2.5	71.2 ± 0.7	71.9 ± 0.3	NS	NS	NS
Ash (%)	13.7 ± 0.9	13.6 ± 0.3	13.9 ± 0.2	14.5 ± 0.5	NS	NS	NS
Protein (%)	62.2 ± 2.2	59.4 ± 2.9	61.1 ± 1.7	61.5 ± 1.1	NS	NS	NS
Lipid (%)	27.3 ± 1.4	27.6 ± 1.1	28.8 ± 1.8	27.2 ± 0.6	NS	NS	NS

Data are expressed as mean ± SEM ($n = 3$). The effect of periodical administration of chitosan-TPP complexed with pSG5 or pSG5-SREBP1a (Treatment) and diet composition (Diet) was subjected to 2-way ANOVA. Statistical significance for independent variables (treatment and diet) and the interaction between independent variables are expressed as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS: not significant.

2.3. Effect of SREBP1a Expression on Serum Metabolites in S. aurata

Serum glucose, triglycerides, and cholesterol were determined in 70-day treated *S. aurata* (Figure 3). Blood glucose levels remained unchanged irrespective of the treatments assayed (diet and nanoparticle administration). In contrast, the nanoparticle-mediated expression of SREBP1a in the liver of *S. aurata* significantly increased blood triglycerides and cholesterol independently of the diet supplied. Chitosan-TPP-pSG5-SREBP1a administration increased the amount of triglycerides and cholesterol by 1.8- and 1.2-fold, respectively, in fish fed Diet 1 and by 2.3- and 1.2-fold in fish fed Diet 2. In addition, total cholesterol was affected by diet, leading to 1.2-fold higher values in fish fed Diet 1.

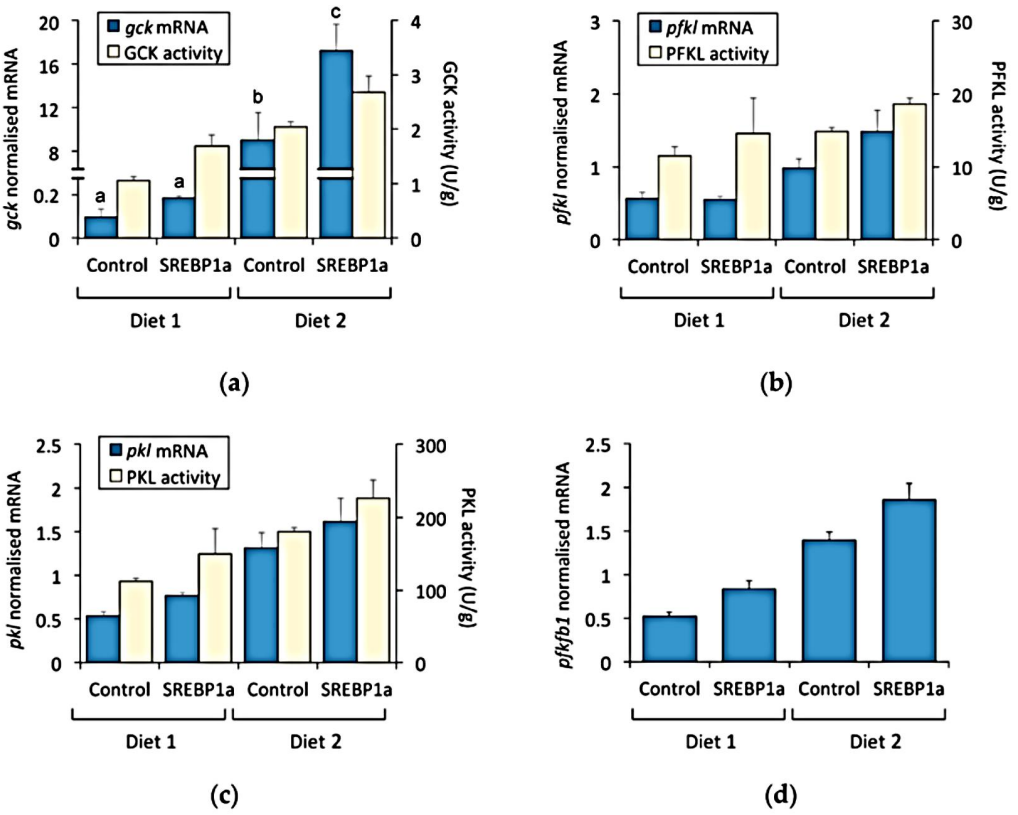


Two-way ANOVA			
Dependent variable	Diet	SREBP1a	Interaction
Glycemia	NS	NS	NS
Triglycerides	NS	**	NS
Cholesterol	**	*	NS

Figure 3. Effect of periodical administration of chitosan-TPP-DNA nanoparticles on serum glucose, triglycerides, and cholesterol in *S. aurata*. Three doses of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were periodically administered every 4 weeks by intraperitoneal injection to *S. aurata* fed Diets 1 and 2. After 70 days of treatment and 14 days following the last injection, blood was collected. Serum levels of glucose, triglycerides, and cholesterol are expressed as mean ± SEM (n = 4–7). Statistical significance for independent variables (diet and treatment with SREBP1a nanoparticles) and the interaction between independent variables are indicated as follows: * $p < 0.05$; ** $p < 0.01$; NS, not significant.

2.4. Effect of SREBP1a Expression on the Intermediary Metabolism of *S. aurata*

The effect of the sustained expression of SREBP1a on the liver intermediary metabolism of *S. aurata* was addressed by analysing the gene expression and activity levels of key enzymes in liver glycolysis, gluconeogenesis, the pentose phosphate pathway, and amino acid metabolism. Concerning glycolysis, in addition to the gene expression of rate-limiting enzymes that control glycolytic substrate cycles, *gck*, *pfkl*, and pyruvate kinase (*pkl*) (Figure 4a–c), the activity of these enzymes was also determined given the well-known importance of their allosteric regulation. Since *pfkfb1* encodes a bifunctional enzyme that catalyses the synthesis and degradation of fru-2,6-P_2, which in turn is a major regulator of glycolysis–gluconeogenesis [20], the mRNA levels of *pfkfb1* were also measured (Figure 4d). Dietary macronutrient composition strongly affected the gene expression and activity of glycolytic enzymes. The consumption of Diet 2 (low-protein–high-carbohydrate) significantly upregulated the expression of *gck* (94-fold), *pfkfb1* (2.6-fold), *pfkl* (1.8-fold), and *pkl* (2.5-fold), as well as the enzyme activity of GCK (2.0-fold), PFKL (1.3-fold), and PKL (1.6-fold). Treatment with chitosan-TPP-pSG5-SREBP1a significantly increased the mRNA levels of *gck* (1.9-fold for Diet 2) and *pfkfb1* (Diet 1: 1.6-fold, Diet 2: 1.3-fold) in the liver of *S. aurata*. Consistent with the rise in mRNA abundance, SREBP1a expression also produced a significant increase in GCK activity (Diet 1: 1.5-fold, Diet 2: 1.2-fold). In contrast to *gck* and *pfkfb1*, the mRNA levels of *pfkl* and *pkl* were not significantly affected by chitosan-TPP-pSG5-SREBP1a nanoparticles. However, the hepatic expression of SREBP1a significantly increased the activity of PFKL and PKL in fish fed Diet 1 and Diet 2 by 1.3-fold.



Two-way ANOVA

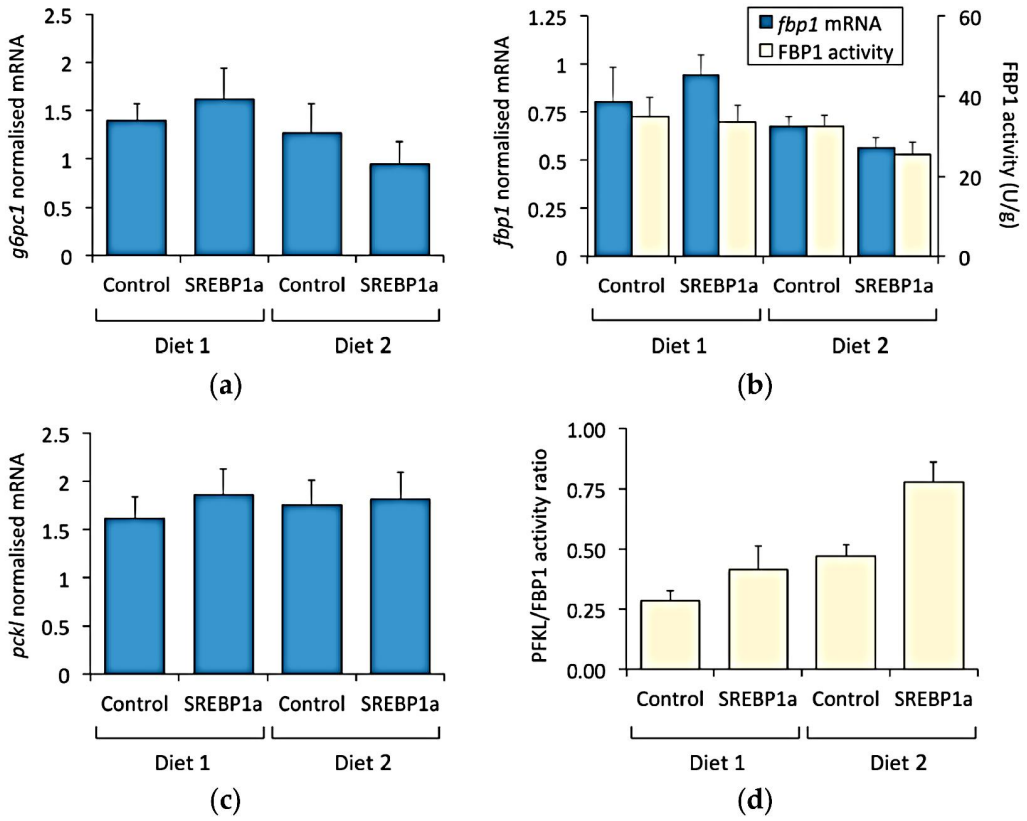
Dependent variable	Diet	SREBP1a	Interaction
gck mRNA	***	*	*
GCK activity	***	**	NS
pfkl mRNA	**	NS	NS
PFKL activity	*	*	NS
pkl mRNA	***	NS	NS
PKL activity	**	*	NS
pfkfb1 mRNA	***	**	NS

(e)

Figure 4. Effect of periodical administration of chitosan-TPP-DNA nanoparticles on key enzymes of liver glycolysis. Three doses of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were periodically administered every 4 weeks by intraperitoneal injection to *S. aurata* fed Diets 1 and 2. After 70 days of treatment and 14 days following the last injection, liver samples were obtained and subjected to RNA and crude extract isolation for subsequent RT-qPCR and enzyme activity assays. Hepatic expression of of *gck* (**a**), *pfkl* (**b**), *pkl* (**c**), and *pfkfb1* (**d**). The mRNA levels were normalised with the geometrical mean of *S. aurata actb*, *eef1a*, and *18s*. The mRNA and activity values are expressed as mean ± SEM (n = 5–6). (**e**) Statistical significance for independent variables (diet and treatment with SREBP1a nanoparticles) and the interaction between independent variables are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant. For independent variables presenting significant interaction, different letters (a, b, c) indicate homogeneous subsets ($p < 0.05$).

The effect of periodical administration of chitosan-TPP-DNA on the expression of the *gck*, *pfkl*, and *pkl* counterparts in gluconeogenesis (*g6pc1*, *fbp1*, and *pckl*) was also studied. While diet composition only affected the mRNA levels of *fbp1* (1.2-fold increase in fish fed Diet 1), the chitosan-mediated expression of SREBP1a did not affect the mRNA levels of *g6pc1*, *fbp1*, and *pckl* nor FBP1 activity (Figure 5a–c). Since the fru-6-P/fru-1,6-P_2 substrate

cycle exerts a pivotal role in the control of the flux through glycolysis–gluconeogenesis, the PFKL/FBP1 activity ratio was also measured (Figure 5d). Both diet composition and SREBP1a expression significantly affected the PFKL/FBP1 activity ratio in the liver of *S. aurata*. Feeding with Diet 2 increased the PFKL/FBP1 activity ratio by 1.7-fold. Irrespective of the diet supplied, treatment with chitosan-TPP-pSG5-SREBP1a also stimulated the PFKL/FBP1 activity ratio (Diet 1: 1.5-fold; Diet 2: 1.7-fold).



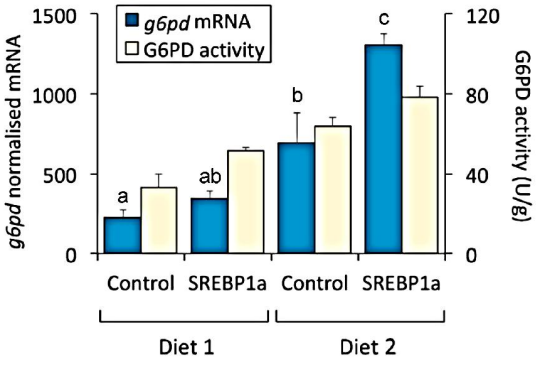
Two-way ANOVA

Dependent variable	Diet	SREBP1a	Interaction
g6pc1 mRNA	NS	NS	NS
fbp1 mRNA	*	NS	NS
FBP1 activity	NS	NS	NS
pckl mRNA	NS	NS	NS
PFKL/FBP1 activity ratio	**	**	NS

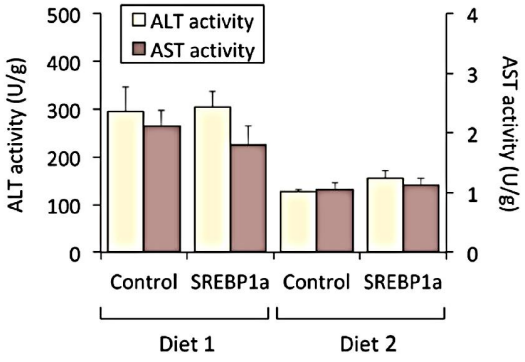
(e)

Figure 5. Effect of periodical administration of chitosan-TPP-DNA nanoparticles on key enzymes of liver gluconeogenesis and PFKL/FBP1 activity ratio. Three doses of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were periodically administered every 4 weeks by intraperitoneal injection to *S. aurata* fed Diets 1 and 2. After 70 days of treatment and 14 days following the last injection, liver samples were obtained and subjected to RNA and crude extract isolation for subsequent RT-qPCR and enzyme activity assays. Hepatic expression of *g6pc1* (**a**), *fbp1* (**b**), and *pckl* (**c**). The mRNA levels were normalised with the geometrical mean of *S. aurata actb*, *eef1a*, and *18s*. (**d**) PFKL/FBP1 enzyme activity ratio. The values are expressed as mean ± SEM (n = 5–6). (**e**) Statistical significance for independent variables (diet and treatment with SREBP1a nanoparticles) and the interaction between independent variables are indicated as follows: * $p < 0.05$; ** $p < 0.01$; NS, not significant.

In addition to glycolysis and gluconeogenesis, we also explored the effect of sustained SREBP1a expression on key hepatic enzymes in the pentose phosphate pathway and transamination. Diet composition and SREBP1a expression significantly affected the mRNA levels and enzyme activity of G6PD, a rate-limiting enzyme of the oxidative phase of the pentose phosphate pathway. Feeding with Diet 2 increased *g6pd* mRNA (3.1-fold) and G6PD activity (1.9-fold), while the administration of chitosan-TPP-pSG5-SREBP1a nanoparticles significantly stimulated *g6pd* at mRNA (Diet 2: 1.9-fold) and activity (Diet 1: 1.6-fold; Diet 2: 1.2-fold) levels (Figure 6a). Diet 1 significantly increased the enzyme activity of two major liver transaminases: ALT (2.3-fold) and aspartate aminotransferase (AST) (2.0-fold). However, nanoparticle administration had no effects on the hepatic activity of ALT and AST (Figure 6b).



(a)



(b)

Two-way ANOVA

Dependent variable	Diet	SREBP1a	Interaction
g6pd mRNA	***	**	*
G6PD activity	***	*	NS
ALT activity	***	NS	NS
AST activity	***	NS	NS

(c)

Figure 6. Effect of periodical administration of chitosan-TPP-DNA nanoparticles on key enzymes of liver pentose phosphate pathway and transaminases. Three doses of chitosan-TPP nanoparticles complexed with pSG5 and pSG5-SREBP1a were periodically administered every 4 weeks by intraperitoneal injection to *S. aurata* fed Diets 1 and 2. After 70 days of treatment and 14 days following the last injection, liver samples were obtained and subjected to RNA and crude extract isolation for subsequent RT-qPCR and enzyme activity assays. (**a**) Hepatic expression of *g6pd*. The mRNA levels were normalised with the geometrical mean of *S. aurata actb*, *eef1a*, and *18s*. (**b**) Liver enzyme activity of ALT and AST. The mRNA and activity values are expressed as mean ± SEM (n = 5–6). (**c**) Statistical significance for independent variables (diet and treatment with SREBP1a nanoparticles) and the interaction between independent variables are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant. For independent variables presenting significant interaction, different letters (a, b, c) indicate homogeneous subsets ($p < 0.05$).

3. Discussion

The formulation of sustainable aquafeeds through the partial substitution of fishmeal by microalgae and a combination of plant- and animal-derived protein, such as soybean and poultry by-product meals, is the subject of intense research [21–25]. However, the substitution of fishmeal and dietary protein with cheaper nutrients with less environmental impact is a challenging issue due to the metabolic features of fish, particularly carnivorous fish, such as *S. aurata*. Carnivorous fish preferentially use amino acids as fuel and

gluconeogenic substrates and therefore require high levels of dietary protein for optimal growth. Instead, carbohydrates are metabolised less efficiently than in mammals and give rise to prolonged hyperglycemia [17,18,26,27]. Nevertheless, and similarly to that previously reported [28,29], the supply of a low-protein–high-carbohydrate diet (Diet 2) induced, in the present study, metabolic adaptation in the liver of *S. aurata*, leading to the increased gene expression and activity of rate-limiting enzymes in glycolysis (GCK, PFKL, and PKL) and the oxidative phase of the pentose phosphate pathway (G6PD) but decreased the expression of the gluconeogenic gene *fbp1*.

We previously reported that the single intraperitoneal administration of chitosan-TPP nanoparticles complexed with a plasmid encoding the N-terminal active domain of hamster SREBP1a significantly upregulated SREBP1a mRNA and protein levels in the liver of *S. aurata* at 72 h post-treatment, which in turn led to increased blood triglycerides and cholesterol as a result of the enhanced hepatic upregulation of genes encoding key enzymes in fatty acid synthesis (acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2), elongation (elongation of very long chain fatty acids protein 5), and desaturation (fatty acid desaturase 2), as well as NADPH production (*g6pd*) and cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A reductase). In addition to the increased expression of genes involved in lipid biosynthesis, SREBP1a also favoured the glycolytic pathway by increasing the expressions of *gck* and *pfkfb1*, and the PFKL/FBP1 enzyme activity ratio [7]. These results led us to conclude that SREBP1a overexpression in the liver of *S. aurata* enabled dietary carbohydrate conversion into fatty acids and cholesterol.

With the aim of exploring the long-term effect of sustained expression of SREBP1a on fish growth performance and the hepatic intermediary metabolism, particularly on glycolysis–gluconeogenesis, in the present study, we periodically administered three doses of chitosan-TPP complexed with a plasmid driving the expression of the N-terminus of hamster SREBP1a (or empty plasmid as a control) to *S. aurata* fed two diets differing in macronutrient composition. After 70 days of treatment, a huge amount of SREBP1a mRNA levels were observed in the liver of fish administered with chitosan-TPP-pSG5-SREBP1a independently of the diet supplied. In agreement with previously reported persistence and stability of exogenous enzyme expression in the liver of *S. aurata* after oral delivery of chitosan-plasmid DNA polyplexes [19,30], the hepatic expression of hamster SREBP1a achieved herein after three periodical doses of nanoparticles greatly exceeded (one order of magnitude) the levels previously reported after single-dose administration [7]. Therefore, the periodical administration of chitosan-TPP-DNA nanoparticles behaved as a highly efficient nucleic acid vector to achieve long-lasting expression of an exogenous protein, following cellular uptake by endocytosis, intracellular endosomal scape of polyplexes, plasmid unpacking from chitosan, and translocation to the nucleus in fish hepatocytes.

The long-lasting expression of SREBP1a in the liver of *S. aurata* exerted a major impact on the hepatic intermediary metabolism, leading to increased circulating levels of triglycerides and cholesterol. Similarly to that for SREBP1a mRNA levels, the periodical administration of SREBP1a nanoparticles produced a greater effect on blood triglycerides, about 2-fold increase, than a single administration, which was reported to induce a 1.3-fold increase [7]. However, the sustained expression of SREBP1a did not increase the effect of a single nanoparticle dose on blood cholesterol. The rise in circulating lipids could be a direct result from the well-known transactivating effect of SREBP1 on the expression of genes involved in the biosynthesis of fatty acid and cholesterol, as it was previously demonstrated in mammals [10,13,31,32] and particularly in the liver of *S. aurata* administered with a single dose of chitosan-TPP-pSG5-SREBP1a [7].

In addition to the effect of SREBP1a on lipid metabolism, we previously showed that SREBP1a transactivates *gck* and *pfkfb1* by binding to SRE boxes in both gene promoters [33,34], while chitosan-TPP-pSG5-SREBP1a nanoparticles upregulate *gck* and *pfkfb1* mRNA levels and the PFKL/FBP1 enzyme activity ratio in the liver of *S. aurata* 72 h after nanoparticle administration [7]. The results of the present study confirm the activating effect of SREBP1a on hepatic glycolysis in *S. aurata*. The sustained expression of SREBP1a

stimulated the transcriptional activation of *gck* expression, which in turn increased the intracellular content of glu-6-P and the subsequent oxidation of glu-6-P via glycolysis and the pentose phosphate pathway [35].

Further activation of the glycolytic pathway would result from SREBP1a-dependent enhanced *pfkfb1* mRNA levels. The *pfkfb1* gene encodes a bifunctional enzyme that catalyses the synthesis and degradation of fru-2,6-P_2, which exerts a pivotal role in both glycolysis and gluconeogenesis through the allosteric activation of PFKL and the inhibition of FBP1, the enzymes that control the flux through the fru-6-P/fru-1,6-P_2 substrate cycle [20,36,37]. Hence, the upregulation of *pfkfb1* expression and fru-2,6-P_2 levels in the liver of *S. aurata* is associated with nutritional and physiological conditions favouring glycolysis, such as starved-to-fed transition, the supply of low-protein–high-carbohydrate diets, or treatment with glucose and insulin [28,33,38]. In the present study, the transcriptional activity of other key enzymes in glycolysis such as *pfkl* and *pkl* was not significantly affected by SREBP1a treatment. However, increased fru-2,6-P_2 levels could be responsible for the allosteric activation of PFKL and the increase in PFKL/FBP1 activity ratio that were observed under conditions with sustained SREBP1a expression. The subsequent increase in fru-1,6-P_2 production, which is a known allosteric activator of PKL, suggests that SREBP1a could indirectly activate PKL activity.

Similarly to that in *S. aurata*, SREBP1 isoforms have been also involved in the transcriptional activity of genes associated with glucose metabolism, favouring flux through the glycolytic pathway in the liver. For instance, SREBP1c upregulates the insulin-dependent transcription of hexokinase II and *gck* in mammals [39–42], and SREBP family members are required for the elevation of glycolysis in NK cells [43]. Furthermore, SREBP-1c silencing augmented the glucose production of HepG2 cells by upregulating the expression of *pckl* and *g6pc1* [44]. Similarly, SREBP-1 knockdown increased *pck1* expression and decreased glycogen deposition in the liver of fed mice [45], while SREBP1a transgenesis reduced gluconeogenesis in mouse liver due to the suppression of *pckl* and *g6pc1* transcription mediated by hepatocyte nuclear factor-4α [46].

The fact that the periodical administration of SREBP1a nanoparticles did not significantly affect the expression of genes encoding rate-limiting enzymes that participate in the control of key substrate cycles (*g6pc1*, *fbp1*, and *pckl*) nor did FBP1 activity support that, in contrast to glycolysis, sustained SREBP1a expression did not modulate hepatic gluconeogenesis in *S. aurata*. In regard to other important enzymes for hepatic intermediary metabolism, the present study also confirms the SREBP1a-dependent upregulation of *g6pd* expression and enzyme activity. The increased availability of glu-6-P and G6PD activity suggests an enhanced production of NADPH in the hepatocytes. Considered altogether, although *S. aurata* is a carnivorous fish with a low ability to metabolise carbohydrates, sustained SREBP1a expression in the liver seemed to trigger a long-term complex effect that favoured glucose oxidation via glycolysis and the oxidative phase of the pentose phosphate pathway to provide pyruvate, as a carbon backbone; NADPH; and ATP for hepatic lipid biosynthesis and the consequent rise in blood triglycerides and cholesterol.

Consistent with previous studies, *S. aurata* fed a low-protein–high-carbohydrate diet (Diet 2) exhibited poor growth performance [29,47]. Interestingly, in the present study, the sustained expression of SREBP1a in the liver improved the growth performance in *S. aurata* irrespective of the dietary macronutrient composition. The periodical administration of SREBP1a nanoparticles increased weight gain and SGR. Furthermore, it reduced FCR, which also indicates higher efficiency in converting feed into fish weight gain [48]. Indeed, the weight gain, SGR, and FCR values in fish treated with chitosan-TPP-pSG5-SREBP1a fed a low-protein–high-carbohydrate diet (Diet 2), were similar to those observed in the control fish (not treated with SREBP1a) fed a high-protein–low-carbohydrate diet (Diet 1). In other words, our findings support that treatment with SREBP1a compensated the loss in growth performance resulting from feeding a carnivorous fish a low-protein–high-carbohydrate diet. The fact that SREBP1a increased blood triglycerides and cholesterol while keeping the total body lipids unaffected suggests that the SREBP1a-dependent increased circulating

lipids would mostly be used for growth-related structural and energy purposes rather than fat accumulation.

Treatment with SREBP1a increased PER independently of the diet supplied, which suggests that the hepatic expression of SREBP1a improved the amount of dietary protein resulting into body weight gain. Therefore, the growth-promoting effect of SREBP1a on *S. aurata* may rely on the fact that the enhanced SREBP1a-dependent metabolisation of dietary carbohydrates and subsequent conversion into lipids may favour a protein-sparing effect that enabled the use of dietary amino acids for protein biosynthesis and growth rather than as gluconeogenic substrates. Indeed, treatment with SREBP1a deeply impacted hepatic glucose metabolism without altering ALT and AST activity, which indicates that the transamination capacity of the liver remained unaffected. Consistently with the important role of SREBP1 on growth, lipogenic regulation by SREBP1 has been shown to be responsible for modulating growth in cancer cells [49–52], and SREBP proteins were reported to control the cytokine-induced growth and proliferation of natural killer cells by a mechanism involving elevated glycolysis and oxidative phosphorylation [43].

4. Materials and Methods

4.1. Experimental Design

Juvenile gilthead seabream (*S. aurata*) (7.4 g ± 0.2, mean weight ± SEM) were acquired from Piscicultura Marina Mediterranea (AVRAMAR Group, Burriana, Spain). Upon arrival to the aquatic animal facility of the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB), the fish were maintained at 20 °C in 250 L aquaria supplied with running seawater, as described [53]. For acclimation, for two weeks, the fish were fed twice daily (9:00 and 17:00) at 5% BW with Diet 1 (Dibaq Microbaq 165, Dibaq, Segovia, Spain), which is a commercial diet containing 52% protein, 18% lipids, 12% carbohydrates, 10% ash, and 21.3 kJ/g gross energy. Two weeks before nanoparticle administration and until the end of the experimental procedure, the fish were divided into 2 groups according to the experimental diet received: Diet 1 or Diet 2 (experimental low-protein–high-carbohydrate diet containing 38.6% protein, 12.1% lipids, 37.1% carbohydrates, 10.5% ash, and 20.1 kJ/g gross energy). The experimental diets were supplied at 3% BW. To study the effect of sustained SREBP1a expression on growth and intermediary metabolism, 3 intraperitoneal injections of chitosan-TPP nanoparticles complexed with pSG5-SREBP1a or empty pSG5 (each dose consisting of 10 μg plasmid per gram BW) were periodically administered every 4 weeks. Fourteen days following the last injection and 24 h after the last meal, the fish were sacrificed by cervical section; their blood was collected; and their liver was dissected out, frozen in liquid N_2 and kept at −80 °C until use. To prevent stress, the fish were anesthetised with tricaine methanesulfonate (MS-222; 1:12,500).

4.2. Preparation and Characterisation of Chitosan-TPP-DNA Nanoparticles

The ionic gelation method was used to encapsulate chitosan-TPP nanoparticles with either pSG5-SREBP1a, an expression plasmid encoding the N-terminus of hamster SREBP1a [33], or pSG5 (Agilent Technologies, Palo Alto, CA, USA), which was used as a negative control (empty vector). Based on previous reports [7], 1 mg of plasmid was first mixed with 4 mL of 0.84 mg/mL TPP (Sigma-Aldrich, St. Louis, MO, USA). TPP-DNA solutions were added dropwise to 10 mL of 2 mg/mL low-molecular-weight chitosan (Sigma-Aldrich, St. Louis, MO, USA)-acetate buffer (chitosan:TPP ratio, 1:0.4). Chitosan-TPP-DNA nanoparticles were sedimented by centrifugation at 36,000× *g* for 20 min at 15 °C, rinsed twice with ultrapure water, and resuspended in 2 mL of 2% *w*/*v* mannitol (cryoprotector during lyophilisation). After a freeze–dry cycle at −47 °C, the particle size and *Z* potential of chitosan-TPP-DNA nanoparticles were determined by dynamic light scattering and laser Doppler electrophoresis, respectively, using Zetasizer Nano ZS fitted with a 633 nm laser (Malvern Instruments, Malvern, UK). Chitosan-TPP-DNA nanoparticles were resuspended in 0.9% NaCl before being administered to the *S. aurata*.

4.3. Growth Parameters

Weight gain is defined as the difference between final and initial body fresh weight. SGR, FCR, HSI, PR, LR, and PER were calculated according to Equations (1), (2), (3), (4), (5) and (6), respectively.

$$\text{SGR} = ((\ln W_f - \ln W_i) \times 100)/T, \text{ where } W_f \text{ and } W_i \text{ are mean final and initial body fresh weight in g and T is time in days,} \quad (1)$$

$$\text{FCR} = \text{g dry feed intake/g wet weight gain,} \quad (2)$$

$$\text{HSI} = (\text{g liver fresh weight} \times 100)/\text{g fish body weight,} \quad (3)$$

$$\text{PR} = (\text{g body protein gain} \times 100)/\text{g protein intake,} \quad (4)$$

$$\text{LR} = (\text{g body lipid gain} \times 100)/\text{g lipid intake,} \quad (5)$$

$$\text{PER} = \text{g weight gain/g feed protein provided,} \quad (6)$$

4.4. Whole-Body Composition

Fish were dried at 85 °C until constant weight was reached to determine moisture, which was calculated according to Equation (7) [54,55].

$$\text{Moisture} = (\text{g wet weight} - \text{g dry weight}) \times 100/\text{g wet weight,} \quad (7)$$

Dried fish were also used for assaying nitrogen (N), lipid, and ash. N content was determined using FlashEA 1112 analyser (Thermo Fisher Scientific, Waltham, MA, USA) and was multiplied by 6.25 to estimate crude protein. A Soxhlet extractor and petroleum ether were used to extract crude lipid. To determine the ash content, sample incineration was performed in a Hobersal 12PR/300 muffle furnace (Hobersal, Caldes de Montbui, Spain) at 550 °C for 12 h [54,55]. Crude protein, lipid, and ash are expressed as the percentage of dry weight.

4.5. Reverse Transcription Coupled to Quantitative PCR

In total, 1 µg of total RNA isolated from the powdered liver of *S. aurata* was reverse-transcribed to cDNA using random hexamer primers and Moloney murine leukaemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 °C. A QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), was used to determine the mRNA levels of the genes listed in Table 2.

Table 2. Primer sequences used for RT-qPCR in the present study.

Gene	Forward Sequences (5′ to 3′)	Reverse Sequences (5′ to 3′)	GenBank Accession
actb	CTGGCATCACACCTTCTACAACGAG	GCGGGGGTGTTGAAGGTCTC	X89920
eef1a	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	AF184170
fbp1	CAGATGGTGAGCCGTGTGAGAAGGATG	GCCGTACAGAGCGTAACCAGCTGCC	AF427867
gck	TGTGTCAGCTCTCAACTCGACC	AGGATCTGCTCTACCATGTGGAT	AF169368
g6pc1	GCGTATTGGTGGCTGAGGTCG	AAGGAGAGGGTGGTGTGGAAG	AF151718
g6pd	TGATGATCCAACAGTTCCTA	GCTCGTTCCTGACACACTGA	JX073711
pck1	CAGCGATGGAGGAGTGTGGTGGGA	GCCCATCCCAATTCCCGCTTCTGTGCT CCGGCTGGTCAGTGT	AF427868
pfkfb1	TGCTGATGGTGGGACTGCCG	CTCGGCGTTGTCGGCTCTGAAG	U84724
pfk1	TGCTGGGGACAAAACGAACTCTTCC	AAACCCTCCGACTACAAGCAGAGCT	KF857580
pklr	CAAAGTGGAAAGCCGGCAAGGG	GTCGCCCCTGGCAACCATAAC	KF857579
srebp1a	CCTCCTGCCTCCGAGTTTCC	GAAGGAAGGCTAGAATACCCC	U09103
18s	TTACGCCCATGTTGTCCTGAG	AGGATTCTGCATGATGGTCACC	AM490061

The 10 µL reaction mixtures contained 0.4 µM of each primer (Table 2), 5 µL of SYBR Green (Applied Biosystems, Foster City, CA, USA), and 0.8 µL of diluted cDNA. The temperature cycle protocol for amplification was 95 °C for 10 min, followed by 40 cycles

with 95 °C for 15 s and 62 °C for 1 min. For each gene, the efficiency of the amplification reaction was determined by generating standard curves from serial dilutions of the control cDNA. Dissociation curves were run after each experiment to confirm single product amplification, and amplicon size was confirmed by agarose gel electrophoresis. To normalise gene expression, *S. aurata actb*, *eef1a*, and *18s* were used as endogenous controls. Variations in gene expression were calculated using the standard $\Delta\Delta C_T$ method [56].

4.6. Metabolite Determinations and Enzyme Activity Assays

Glucose, triglycerides, and cholesterol in serum were measured with commercial kits (Linear Chemicals, Montgat, Spain). The liver crude extracts to assay PFKL, PKL, FBP1, G6PD, ALT, and AST were obtained from homogenisation of powdered frozen tissue in a buffer (1:5, *w*/*v*) containing 50 mM Tris-HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 250 mM sucrose. For GCK activity, the homogenisation buffer additionally contained 100 mM KCl. A PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland) was used for homogenising the samples (30 sec at 4 °C). The homogenised samples were centrifuged at 10,000× *g* for 30 min at 4 °C, and the supernatants were collected for assaying the enzyme activity. The reaction mixtures for GCK, PFKL, PK, FBP1, ALT, AST, and total protein were as previously described [57,58]. The enzyme activities were expressed as specific activity (U/g protein). One unit of GCK and PFKL activity was considered the amount of enzyme needed to consume 2 µmol of $NADP^+$ or NADH, respectively, per min. One unit of PKL, FBP1, G6PD, ALT, and AST activity was defined as the amount of enzyme necessary for transforming 1 µmol of substrate per min. The metabolites and enzyme activity assays were spectrophotometrically determined at 30 °C in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

4.7. Statistics

Statistical analyses were performed with SPSS Version 25 software (IBM, Armonk, NY, USA). One-way analysis of variance followed by the Scheffe post hoc test was used to analyse the nanoparticle diameter size and *Z* potential. To identify significant differences due to diet composition and nanoparticle administration with pSG5-SREBP1a or an empty vector, pSG5 (control), the experimental data were submitted to a two-way analysis of variance followed by the Scheffe post hoc test when the independent variables significantly interacted.

5. Conclusions

The periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles allowed for the long-standing expression of an exogenous transcription factor in the liver of *S. aurata*. Sustained SREBP1a expression increased the hepatic glucose oxidation and circulating levels of triglycerides and cholesterol. The metabolic changes triggered by SREBP1a enhanced the metabolisation of dietary carbohydrates, leading to improved growth performance in a carnivorous fish species even when feeding low-protein–high-carbohydrate diets.

Author Contributions: Conceptualisation, I.M.; methodology, A.R., Y.W., A.F. and I.M.; validation, A.R., Y.W., M.P.A. and I.M.; formal analysis, A.R., Y.W. and I.M.; investigation, A.R., Y.W., M.P.A., A.F. and I.M.; resources, M.P.A. and I.M.; writing—original draft preparation, A.R. and I.M.; writing—review and editing, A.R., Y.W., M.P.A., A.F. and I.M.; supervision, M.P.A. and I.M.; project administration, I.M.; funding acquisition, I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministerio de Ciencia e Innovación (Spain) and co-funded by the European Regional Development Fund (European Comission), grant number PID2021-125642OB-I00.

Institutional Review Board Statement: The animal study protocol was approved by the University of Barcelona's Animal Welfare Committee and Generalitat de Catalunya (proceeding #10811; date of approval: 14 April 2020).

Data Availability Statement: The data presented in this study are available from the corresponding author upon request.

Acknowledgments: The authors thank Piscicultura Marina Mediterranea (AVRAMAR Group, Burriana, Spain) for providing *S. aurata* juveniles and Eurocoyal S.L. (Sant Cugat del Vallès, Spain) for supplying diet components.

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

References

1. Chuan, D.; Jin, T.; Fan, R.; Zhou, L.; Guo, G. Chitosan for gene delivery: Methods for improvement and applications. *Adv. Colloid Interface Sci.* **2019**, *268*, 25–38. [CrossRef]
2. Thambiliyagodage, C.; Jayanetti, M.; Mendis, A.; Ekanayake, G.; Liyanaarachchi, H.; Vigneswaran, S. Recent Advances in Chitosan-Based Applications—A Review. *Materials* **2023**, *16*, 2073. [CrossRef]
3. Gao, Y.; Wu, Y. Recent advances of chitosan-based nanoparticles for biomedical and biotechnological applications. *Int. J. Biol. Macromol.* **2022**, *203*, 379–388. [CrossRef] [PubMed]
4. Wu, Y.; Rashidpour, A.; Almajano, M.P.; Metón, I. Chitosan-based drug delivery system: Applications in fish biotechnology. *Polymers* **2020**, *12*, 1177. [CrossRef] [PubMed]
5. González, J.D.; Silva-Marrero, J.I.; Metón, I.; Caballero-Solares, A.; Viegas, I.; Fernández, F.; Miñarro, M.; Fàbregas, A.; Ticó, J.R.; Jones, J.G.; et al. Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. *Mar. Biotechnol.* **2016**, *18*, 85–97. [CrossRef] [PubMed]
6. Gaspar, C.; Silva-Marrero, J.I.; Fàbregas, A.; Miñarro, M.; Ticó, J.R.; Baanante, I.V.; Metón, I. Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. *J. Biotechnol.* **2018**, *286*, 5–13. [CrossRef]
7. Silva-Marrero, J.I.; Villasante, J.; Rashidpour, A.; Palma, M.; Fàbregas, A.; Almajano, M.P.; Viegas, I.; Jones, J.G.; Miñarro, M.; Ticó, J.R.; et al. The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of *Sparus aurata*. *Biomolecules* **2019**, *9*, 297. [CrossRef]
8. Edwards, P.A.; Tabor, D.; Kast, H.R.; Venkateswaran, A. Regulation of gene expression by SREBP and SCAP. *Biochim. Biophys. Acta-Mol. Cell Biol. Lipids* **2000**, *1529*, 103–113. [CrossRef]
9. Inoue, J.; Sato, R. New insights into the activation of sterol regulatory element-binding proteins by proteolytic processing. *Biomol. Concepts* **2013**, *4*, 417–423. [CrossRef]
10. Shimano, H.; Sato, R. SREBP-regulated lipid metabolism: Convergent physiology—Divergent pathophysiology. *Nat. Rev. Endocrinol.* **2017**, *13*, 710–730. [CrossRef]
11. Harada, N.; Yonemoto, H.; Yoshida, M.; Yamamoto, H.; Yin, Y.; Miyamoto, A.; Hattori, A.; Wu, Q.; Nakagawa, T.; Nakano, M.; et al. Alternative splicing produces a constitutively active form of human SREBP-1. *Biochem. Biophys. Res. Commun.* **2008**, *368*, 820–826. [CrossRef] [PubMed]
12. Felder, T.K.; Klein, K.; Patsch, W.; Oberkofler, H. A novel SREBP-1 splice variant: Tissue abundance and transactivation potency. *Biochim. Biophys. Acta-Gene Struct. Expr.* **2005**, *1731*, 41–47. [CrossRef] [PubMed]
13. Raghow, R.; Dong, Q.; Elam, M.B. Phosphorylation dependent proteostasis of sterol regulatory element binding proteins. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2019**, *1864*, 1145–1156. [CrossRef] [PubMed]
14. Pai, J.T.; Guryev, O.; Brown, M.S.; Goldstein, J.L. Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *J. Biol. Chem.* **1998**, *273*, 26138–26148. [CrossRef]
15. Shimano, H.; Horton, J.D.; Shimomura, I.; Hammer, R.E.; Brown, M.S.; Goldstein, J.L. Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J. Clin. Investig.* **1997**, *99*, 846–854. [CrossRef]
16. Horton, J.D.; Bashmakov, Y.; Shimomura, I.; Shimano, H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5987–5992. [CrossRef]
17. Polakof, S.; Panserat, S.; Soengas, J.L.; Moon, T.W. Glucose metabolism in fish: A review. *J. Comp. Physiol. B* **2012**, *182*, 1015–1045. [CrossRef]
18. Forbes, J.; Kostyniuk, D.; Mennigen, J.; Weber, J. Unexpected effect of insulin on glucose disposal explains glucose intolerance of rainbow trout. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2019**, *316*, R387–R394. [CrossRef]
19. Sáez, M.I.; Vizcaíno, A.J.; Alarcón, F.J.; Martínez, T.F. Comparison of lacZ reporter gene expression in gilthead sea bream (*Sparus aurata*) following oral or intramuscular administration of plasmid DNA in chitosan nanoparticles. *Aquaculture* **2017**, *474*, 1–10. [CrossRef]

20. Houddane, A.; Bultot, L.; Novellasdemunt, L.; Johanns, M.; Gueuning, M.-A.; Vertommen, D.; Coulie, P.G.; Bartrons, R.; Hue, L.; Rider, M.H. Role of Akt/PKB and PFKFB isoenzymes in the control of glycolysis, cell proliferation and protein synthesis in mitogen-stimulated thymocytes. *Cell. Signal.* **2017**, *34*, 23–37. [CrossRef]
21. Salin, K.R.; Arun, V.V.; Mohanakumaran Nair, C.; Tidwell, J.H. Sustainable aquafeed. In *Sustainable Aquaculture, Applied Environmental Science and Engineering for a Sustainable Future*; Hai, F.I., Visvanathan, C., Boopathy, R., Eds.; Springer International Publishing: Cham, Switzerland, 2018; pp. 123–151.
22. Sánchez-Moya, A.; García-Meilán, I.; Riera-Heredia, N.; Vélez, E.J.; Lutfi, E.; Fontanillas, R.; Gutiérrez, J.; Capilla, E.; Navarro, I. Effects of different dietary vegetable oils on growth and intestinal performance, lipid metabolism and flesh quality in gilthead sea bream. *Aquaculture* **2020**, *519*, 734881. [CrossRef]
23. Xu, H.; Turkmen, S.; Rimoldi, S.; Terova, G.; Zamorano, M.J.; Afonso, J.M.; Sarih, S.; Fernández-Palacios, H.; Izquierdo, M. Nutritional intervention through dietary vegetable proteins and lipids to gilthead sea bream (*Sparus aurata*) broodstock affects the offspring utilization of a low fishmeal/fish oil diet. *Aquaculture* **2019**, *513*, 734402. [CrossRef]
24. Basto-Silva, C.; Enes, P.; Oliva-Teles, A.; Balbuena-Pecino, S.; Navarro, I.; Capilla, E.; Guerreiro, I. Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*). *Aquaculture* **2021**, *533*, 736142. [CrossRef]
25. Reis, B.; Ramos-Pinto, L.; Martos-Sitcha, J.A.; Machado, M.; Azeredo, R.; Fernández-Boo, S.; Engrola, S.; Unamunzaga, C.; Calduch-Giner, J.; Conceição, L.E.C.; et al. Health status in gilthead seabream (*Sparus aurata*) juveniles fed diets devoid of fishmeal and supplemented with Phaeodactylum tricornutum. *J. Appl. Phycol.* **2021**, *33*, 979–996. [CrossRef]
26. Naylor, R.L.; Hardy, R.W.; Bureau, D.P.; Chiu, A.; Elliott, M.; Farrell, A.P.; Forster, I.; Gatlin, D.M.; Goldburg, R.J.; Hua, K.; et al. Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15103–15110. [CrossRef] [PubMed]
27. Takase, K.; Kakuta, I. Oral administration of wild plant-derived minerals and red ginseng ameliorates insulin resistance in fish through different pathways. *Physiol. Rep.* **2023**, *11*, e15667. [CrossRef]
28. Metón, I.; Caseras, A.; Fernández, F.; Baanante, I.V. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, *Sparus aurata*. *Biochim. Biophys. Acta-Gene Struct. Expr.* **2000**, *1491*, 220–228. [CrossRef]
29. Fernández, F.; Miquel, A.G.; Cordoba, M.; Varas, M.; Metón, I.; Caseras, A.; Baanante, I.V. Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J. Exp. Mar. Bio. Ecol.* **2007**, *343*, 1–10. [CrossRef]
30. Sáez, M.I.; Vizcaíno, A.J.; Alarcón, F.J.; Martínez, T.F. Feed pellets containing chitosan nanoparticles as plasmid DNA oral delivery system for fish: In vivo assessment in gilthead sea bream (*Sparus aurata*) juveniles. *Fish Shellfish Immunol.* **2018**, *80*, 458–466. [CrossRef]
31. DeBose-Boyd, R.A.; Ye, J. SREBPs in lipid metabolism, insulin signaling, and beyond. *Trends Biochem. Sci.* **2018**, *43*, 358–368. [CrossRef]
32. Bertolio, R.; Napoletano, F.; Mano, M.; Maurer-Stroh, S.; Fantuz, M.; Zannini, A.; Bicciato, S.; Sorrentino, G.; Del Sal, G. Sterol regulatory element binding protein 1 couples mechanical cues and lipid metabolism. *Nat. Commun.* **2019**, *10*, 1326. [CrossRef] [PubMed]
33. Metón, I.; Egea, M.; Anemaet, I.G.; Fernández, F.; Baanante, I.V. Sterol regulatory element binding protein-1a transactivates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoter. *Endocrinology* **2006**, *147*, 3446–3456. [CrossRef] [PubMed]
34. Egea, M.; Metón, I.; Córdoba, M.; Fernández, F.; Baanante, I.V. Role of Sp1 and SREBP-1a in the insulin-mediated regulation of glucokinase transcription in the liver of gilthead sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol.* **2008**, *155*, 359–367. [CrossRef] [PubMed]
35. Baker, S.A.; Rutter, J. Metabolites as signalling molecules. *Nat. Rev. Mol. Cell Biol.* **2023**, *24*, 355–374. [CrossRef]
36. Okar, D.A.; Wu, C.; Lange, A.J. Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Adv. Enzym. Regul.* **2004**, *44*, 123–154. [CrossRef] [PubMed]
37. Bartrons, R.; Simon-Molas, H.; Rodríguez-García, A.; Castaño, E.; Navarro-Sabaté, À.; Manzano, A.; Martinez-Outschoorn, U.E. Fructose 2,6-bisphosphate in cancer cell metabolism. *Front. Oncol.* **2018**, *8*, 331. [CrossRef]
38. Metón, I.; Caseras, A.; Mediavilla, D.; Fernández, F.; Baanante, I.V. Molecular cloning of a cDNA encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from liver of *Sparus aurata*: Nutritional regulation of enzyme expression. *Biochim. Biophys. Acta* **1999**, *1444*, 153–165. [CrossRef]
39. Foretz, M.; Guichard, C.; Ferre, P.; Foufelle, F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12737–12742. [CrossRef]
40. Gosmain, Y.; Dif, N.; Berbe, V.; Loizon, E.; Rieusset, J.; Vidal, H.; Lefai, E. Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J. Lipid Res.* **2005**, *46*, 697–705. [CrossRef]
41. Gosmain, Y.; Lefai, E.; Ryser, S.; Roques, M.; Vidal, H. Sterol regulatory element-binding protein-1 mediates the effect of insulin on hexokinase II gene expression in human muscle cells. *Diabetes* **2004**, *53*, 321–329. [CrossRef]
42. Kim, S.Y.; Kim, H.I.; Kim, T.H.; Im, S.S.; Park, S.K.; Lee, I.K.; Kim, K.S.; Ahn, Y.H. SREBP-1c mediates the insulin-dependent hepatic glucokinase expression. *J. Biol. Chem.* **2004**, *279*, 30823–30829. [CrossRef]

43. Assmann, N.; O'Brien, K.L.; Donnelly, R.P.; Dyck, L.; Zaiatz-Bittencourt, V.; Loftus, R.M.; Heinrich, P.; Oefner, P.J.; Lynch, L.; Gardiner, C.M.; et al. Srebp-controlled glucose metabolism is essential for NK cell functional responses. *Nat. Immunol.* **2017**, *18*, 1197–1206. [CrossRef]
44. Bai, X.P.; Dong, F.; Yang, G.H.; Zhang, L. Influences of sterol regulatory element binding protein-1c silencing on glucose production in HepG2 cells treated with free fatty acid. *Lipids Health Dis.* **2019**, *18*, 89. [CrossRef]
45. Ruiz, R.; Jideonwo, V.; Ahn, M.; Surendran, S.; Tagliabracci, V.S.; Hou, Y.; Gamble, A.; Kerner, J.; Irimia-Dominguez, J.M.; Puchowicz, M.A.; et al. Sterol regulatory element-binding protein-1 (SREBP-1) is required to regulate glycogen synthesis and gluconeogenic gene expression in mouse liver. *J. Biol. Chem.* **2014**, *289*, 5510–5517. [CrossRef]
46. Yamamoto, T.; Shimano, H.; Nakagawa, Y.; Ide, T.; Yahagi, N.; Matsuzaka, T.; Nakakuki, M.; Takahashi, A.; Suzuki, H.; Sone, H.; et al. SREBP-1 interacts with hepatocyte nuclear factor-4 alpha and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *J. Biol. Chem.* **2004**, *279*, 12027–12035. [CrossRef]
47. Sáez-Arteaga, A.; Wu, Y.; Silva-Marrero, J.I.; Rashidpour, A.; Almajano, M.P.; Fernández, F.; Baanante, I.V.; Metón, I. Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (*Sparus aurata*). *Aquaculture* **2022**, *555*, 738221. [CrossRef]
48. Charles Bai, S.; Hardy, R.W.; Hamidoghli, A. Diet analysis and evaluation. In *Fish Nutrition*; Academic Press: Cambridge, MA, USA, 2022; pp. 709–743.
49. Sun, Q.; Yu, X.; Peng, C.; Liu, N.; Chen, W.; Xu, H.; Wei, H.; Fang, K.; Dong, Z.; Fu, C.; et al. Activation of SREBP-1c alters lipogenesis and promotes tumor growth and metastasis in gastric cancer. *Biomed. Pharmacother.* **2020**, *128*, 110274. [CrossRef]
50. Huang, W.C.; Li, X.; Liu, J.; Lin, J.; Chung, L.W.K. Activation of androgen receptor, lipogenesis, and oxidative stress converged by SREBP-1 is responsible for regulating growth and progression of prostate cancer cells. *Mol. Cancer Res.* **2012**, *10*, 133–142. [CrossRef]
51. Cho, S.; Chun, Y.; He, L.; Ramirez, C.B.; Ganesh, K.S.; Jeong, K.; Song, J.; Cheong, J.G.; Li, Z.; Choi, J.; et al. FAM120A couples SREBP-dependent transcription and splicing of lipogenesis enzymes downstream of mTORC1. *Mol. Cell* **2023**, *83*, 3010–3026. [CrossRef]
52. Cheng, C.; Geng, F.; Li, Z.; Zhong, Y.; Wang, H.; Cheng, X.; Zhao, Y.; Mo, X.; Horbinski, C.; Duan, W.; et al. Ammonia stimulates SCAP/Insig dissociation and SREBP-1 activation to promote lipogenesis and tumour growth. *Nat. Metab.* **2022**, *4*, 575–588. [CrossRef]
53. Silva-Marrero, J.I.; Sáez, A.; Caballero-Solares, A.; Viegas, I.; Almajano, M.P.; Fernández, F.; Baanante, I.V.; Metón, I. A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). *BMC Genom.* **2017**, *18*, 768. [CrossRef]
54. Busacker, G.P.; Adelman, I.R.; Goolish, E.M. Growth. In *Methods for Fish Biology*; Schreck, C.B., Moyle, P.B., Eds.; American Fisheries Society: Bethesda, MD, USA, 1990; pp. 363–387.
55. Lucas, A. Bioenergetics of Organisms: Methods. In *Bioenergetics of Aquatic Animals*; Priede, I.G., Ed.; Taylor & Francis: London, UK, 1996; pp. 65–81.
56. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef]
57. Metón, I.; Mediavilla, D.; Caseras, A.; Cantó, E.; Fernández, F.; Baanante, I.V. Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* **1999**, *82*, 223–232. [CrossRef]
58. Caseras, A.; Metón, I.; Fernández, F.; Baanante, I.V. Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). *Biochim. Biophys. Acta-Gene Struct. Expr.* **2000**, *1493*, 135–141. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

4. Discussion

4.1. Metabolic Effects of Metformin on the Hepatic Metabolism of *S. aurata*

Previous research on the effects of metformin in different animal models, particularly mammals, has largely focused on its anti-diabetic properties, particularly its ability to improve insulin sensitivity, decrease hepatic glucose production, and enhance peripheral glucose uptake (Wei et al., 2020). While the molecular mechanisms underlying these effects have been extensively studied in mammals, comparatively less is known about its effects in non-mammalian species, especially carnivorous fish. In mammals, metformin's primary mechanism of action involves the activation of AMPK, leading to inhibition of hepatic gluconeogenesis and stimulation of glucose uptake in peripheral tissues such as muscle and adipose tissue (Agius et al., 2020). Metformin has been shown to modulate various metabolic pathways, including lipid metabolism and mitochondrial function, contributing to its overall glucose-lowering effects. In addition to its effects on glucose metabolism, metformin influences lipid metabolism in fish by promoting lipid oxidation and reducing lipid accumulation in tissues. By increasing fatty acid oxidation and mitochondrial function, metformin enhances energy expenditure and reduces lipid deposition, leading to improvements in body composition and growth efficiency (Madiraju et al., 2018). Metformin supplementation in aquaculture is generally regarded as beneficial for improving glucose homeostasis, growth performance and antioxidant, anti-inflammatory and immunomodulatory capacity (Wei et al., 2020; Wang et al., 2024; Zhou et al., 2024). However, the molecular mechanisms triggered by metformin on fish metabolism remain unclear and gave rise to paradoxical results.

Given that metformin may further enhance glucose uptake and utilization in glucose-intolerant carnivorous fish, thereby promoting growth and reducing reliance on protein-derived energy (Xu et al., 2018; Wang et al., 2024), we addressed the effect of metformin, a widely used anti-diabetic drug for humans, on the intermediary metabolism in the liver of *S. aurata*. The findings shed light on how metformin administration, both with and without a glucose load, impacts the activity and expression of key enzymes involved in the control of the intermediary metabolism and the mRNA levels of lipogenic factors in this species. Consistent with limited control of postprandial glycemia in carnivorous fish, blood glucose levels showed a sharped increase at 5 hours following glucose administration. Glycemia was totally recovered at 24 hours post-treatment. In agreement with previous reports in *S. aurata* (Metón et al., 1999; Caseras et al., 2002; Fernández et al., 2007), hyperglycemia strongly up-regulated the activity and mRNA levels of GCK, a rate-limiting enzyme in hepatic glycolysis, at 5 hours following intraperitoneal administration of glucose. In contrast, the activity of FBP, a key gluconeogenic

enzyme, was not affected. Metformin counteracted glucose-dependent activation of GCK expression in the liver of *S. aurata*. When administered alone, metformin significantly down-regulated the expression of PFK and FBP at both 5 hours and 24 hours post-treatment. However, at the enzyme activity level only a decrease could be observed for PFK at 24 hours post-treatment, possibly because of allosteric regulation of PFK and FBP. The effect of metformin on glycolytic and gluconeogenic enzymes in fish seems to be species-dependent. Similarly as in *S. aurata*, metformin down-regulated the expression of key enzymes in glycolysis (GCK, PFK) and gluconeogenesis (G6PC, FBP, and PEPCK) in the liver of rainbow trout 6 hours after intraperitoneal administration of glucose (Polakof et al., 2009). In contrast, metformin up-regulated GCK and PK, while down-regulated G6PC, FBP and PEPCK in the liver of *Megalobrama amblycephala* (Xu et al., 2018). Metformin also increased the hepatic expression of ADP-dependent glucokinase, PFK and PK, and decreased that of G6PC in *Micropterus salmoides* fed a high carbohydrate diet (Liu et al., 2023; Wang et al., 2024).

The effect of glucose and metformin administration was also evaluated in the hepatic activity of two rate-limiting enzymes of the tricarboxylic acid cycle (TCA): IDH and OGDH. At 24 hours post-treatment of *S. aurata* juveniles, the glucose load notably increased the hepatic activity of IDH, a crucial enzyme in the TCA. IDH activity, which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, was stimulated by glucose and inhibited by metformin. In contrast, OGDH remained unaffected. Increased fuel availability after glucose administration and glucose-dependent activation of glycolysis may stimulate IDH activity and, therefore, favor hepatic glucose oxidation and ATP synthesis 24 hours after the glucose load. As in *S. aurata*, metformin inhibits IDH activity in human-derived cancer cells (Keibler et al., 2016; Bai et al., 2018).

Albeit not significant, a trend to increase serum triglycerides and cholesterol was found 24 hours after glucose administration. This suggests enhanced hepatic lipogenesis and transformation of excess glucose into lipids. Consistently, treatment with glucose up-regulated SREBP1, a transcription factor with a pivotal role in transactivation of genes required for the synthesis of fatty acids, triglycerides and cholesterol (Shimano and Sato, 2017). The same trend was observed 24 hours following glucose administration in the expression of other lipogenic factors such as PGC1β and LPIN1. Even though *S. aurata* is a carnivorous fish, we previously reported that it tolerates partial substitution of dietary protein by carbohydrates through metabolic adaptation involving modulation of the glycolytic flux in the liver (Caseras et al., 2000; Metón et al., 2000b; Fernández et al., 2007). Similarly as for GCK (glycolysis) and IDH

(TCA), metformin prevented, at least partially, glucose-dependent up-regulation of lipogenic factors, particularly at 24 hours post-treatment. Metformin-dependent inhibition of lipogenesis prevents the accumulation of lipid deposits, which are often associated with insulin resistance and metabolic disorders in fish (Liu et al., 2023). By reducing lipid accumulation and promoting lean tissue deposition, metformin can improve the fillet yield, texture, and nutritional quality of cultured fish, enhancing its market value and consumer acceptance. This optimization of body composition aligns with consumer preferences for healthier and higher-quality seafood products, further supporting the economic viability of aquaculture.

Metformin-dependent down-regulation of SREBP1 expression and lipogenesis is consistent with previous observations in mammals (Liu et al., 2016; de Oliveira Santana et al., 2016). However, as for glycolysis and gluconeogenesis, the effect of metformin on lipogenesis in fish remains controversial. In agreement with our findings in *S. aurata*, metformin decreased the expression of SREBP1 and FASN in primary hepatocytes from *Megalobrama amblycephala* by promoting AMPK activation, leading to decreased fatty acid synthesis and increased fatty acid oxidation (Zhou et al., 2019). In constrast, dietary metformin supplementation increased the expression of lipogenic enzymes in the liver of *Oncorhynchus mykiss* fed high-carbohydrate diets (Panserat et al., 2009), while in another study, the administration of metformin plus insulin up-regulated SREBP1 in the liver of *Oncorhynchus mykiss* fed high-carbohydrate diets, while metformin alone did not affect SREBP1 expression (Polakof et al., 2011). Metformin also up-regulated SREBP1, ACC and FASN in the liver of *Megalobrama amblycephala*, while decreased plasma triglycerides (Xu et al., 2018). In *Micropterus salmoides*, metformin stimulated blood triglycerides and the hepatic expression of lipogenic FASN (Liu et al., 2023). In addition to belong to phylogenetically distant orders, differences in habitat and feeding habits of fish species may determine different metabolic responses to metformin in the piscine liver. Divergent methodological approaches, such as dosage and duration of the treatment may also result in striking differences among fish species.

Carnivorous fish use efficiently amino acids as gluconeogenic substrates, being the liver the main site of amino acid catabolism (Andoh, 2007). Indeed, optimal growth of teleost fish requires elevated levels of dietary protein (Moon, 2001; Polakof et al., 2012), and amino acids are considered as strong insulin secretagogues in fish (Polakof et al., 2012). Thus, in addition to provide building blocks for proteins and backbones for glucogenic and ketogenic purposes, a major function of amino acids in fish, is to produce energy by entering catabolic pathways. Several metabolic features favor a preferential use of amino acids as fuel and gluconeogenic

substrates in fish. For instance, fishes display efficient conversion of dietary protein into tissue protein up to 20-fold higher than in mammals and birds. Moreover, ammonia excretion across the fish gills consumes less enery than urea formation in urotelic organisms (Kaushik and Seiliez, 2010). In the liver of *S. aurata*, and in the absence of a glucose load, we observed that 5 hours post-treatment metformin down-regulated the expression of GDH, which exerts a pivotal role in amino acid metabolism through the catalysis of reversible oxidative deamination of L-glutamate into α-ketoglutarate. Meanwhile AST remained unaffected by metformin, a similar trend to that of GDH was also found for ALT activity, a transaminase that exhibit greater sensibility than AST to changes in the nutritional status and diet composition in *S. aurata* (Fernández et al., 2007; Anemaet et al., 2008). Metformin-dependent down-regulation of GDH and to a lesser extent reduced ALT activity suggest that metformin decreased amino acid transdeamination in the liver. Transdeamination, a process that involves the transfer of amino groups from amino acids to α-ketoglutarate to produce glutamate and the subsequent GDH-mediated deamination of glutamate, is a pivotal pathway in the fish liver to enable incorporation of carbon backbones of amino acids into the TCA to obtain energy and biosynthesize macromolecules (Liu et al., 2012; Caballero-Solares et al., 2015; Gaspar et al., 2018). Consequently, our findings indicated that metformin administration decreased incorporation of amino acid carbon backbones into the TCA and its use as gluconeogenic substrates. Likewise, metformin supplementation decreased AST activity in serum of *Megalobrama amblycephala* (Wang et al., 2024). Metformin-dependent inhibition of hepatic transdeamination may determine the long-term hypoglycemic effect caused by metformin in the liver of *S. aurata*.

Similarly as for GDH and ALT, a trend to decrease mTOR expression was found in the liver of *S. aurata* 5 hours following treatment with metformin. Consistently with our findings, metformin was reported to suppress up-regulation of mTOR in the liver of the cyprinid *Megalobrama amblycephala* fed high-carbohydrate diets (Xu et al., 2018). The mTOR pathway is a key regulator of cellular metabolism that is highly sensitive to dietary protein (Borges et al., 2014). Since mTOR is part of the amino acid sensor in the mTORC1 complex, the results of the present study suggest that metformin-dependent down-regulation of GDH expression could inhibit mTORC1 signaling in the liver of *S. aurata*. In favor of this hypothesis, metformin inhibits mTORC1 signaling in humans (Melnik et al., 2014), and knock down of GDH1 also decreases mTORC1 activity, while leucine requires GDH1 to up-regulate mTORC1 in human cells (Lorin et al., 2013). Metformin-dependent decreased use of amino

acids as gluconeogenic substrates in the liver of *S. aurata* could, in turn, determine the low levels of FBP observed 5 hours following metformin administration. Similarly, it was proposed that inhibition of mTORC1 improves glucose tolerance by inhibiting hepatic gluconeogenesis in rainbow trout (Dai et al., 2014). This mechanism highlights the intricate interplay between amino acid metabolism and glucose regulation in carnivorous fish (Liu et al., 2023). Overall, our findings suggest that metformin may exert specific metabolic effects in carnivorous fish by mitigating hyperglycemia-induced lipogenesis and suppressing the use of amino acids as gluconeogenic substrates. The findings underscored the importance of understanding amino acid metabolism in the context of glucose intolerance in carnivorous fish, highlighting the potential of metformin as a therapeutic intervention in managing metabolic disorders in aquatic species.

Carnivorous fish present a unique challenge in understanding glucose metabolism due to their inherent glucose intolerance and limited capacity to utilize dietary carbohydrates. Investigating the role of glucose intolerance in these species holds significant scientific value, shedding light on fundamental metabolic processes and potential therapeutic interventions. Firstly, knowledge of glucose metabolism in carnivorous fish is crucial due to their physiological reliance on amino acids and lipids as primary energy sources, rather than carbohydrates. Their evolutionary adaptation to a high-protein diet necessitates a deeper exploration of how glucose homeostasis is regulated in these organisms. Moreover, elucidating the molecular mechanisms underlying glucose intolerance in carnivorous fish offers insights into metabolic diseases such as diabetes. By studying how carnivorous fish cope with glucose challenges, researchers can uncover novel therapeutic targets and strategies applicable to both aquatic species and terrestrial organisms. The study of glucose metabolism in carnivorous fish also underscores the importance of comprehending interspecies variations in metabolic pathways. Insights gained from studying carnivorous fish can contribute to a broader comprehension of metabolic diversity across vertebrates, enhancing our knowledge of evolutionary adaptations and metabolic regulation. In summary, investigating glucose metabolism in carnivorous fish not only addresses fundamental questions in biology but also holds implications for human health and aquaculture. Furthermore, metformin supplementation can improve growth parameters such as weight gain, feed intake, and feed efficiency in various fish species, indicating its potential as a growth-promoting agent in aquaculture (Wang et al., 2024). However, deeper knowledge of the molecular events triggered by metformin in fish is an absolute need before developing applications in aquaculture, particularly in terms of dosage

and timing given that chronic exposure to high concentrations of metformin in water bodies has been associated with increase in reactive oxygen species and the generation of free radicals, leading to oxidative stress, lipid peroxidation and DNA damage in fish (Tavlo et al., 2022; Sibiya et al., 2023).

Our findings indicate that metformin administration counteracted hyperglycemia-induced up-regulation of glycolytic enzymes and lipogenic factors in the liver, suggesting a potential role for metformin in improving glucose homeostasis in *S. aurata*. While there are similarities in the metabolic effects of metformin between carnivorous fish and mammals, such as its ability to suppress hepatic gluconeogenesis, there are also notable differences. For instance, the present study highlights the role of amino acid metabolism in the response to metformin in *S. aurata*, suggesting that metformin may exert specific effects on hepatic transdeamination and the utilization of amino acids as gluconeogenic substrates in this species. Knowledge of the fundamental mechanisms of metformin action across different animal models can provide valuable insights into the evolutionarily conserved and divergent pathways regulating glucose homeostasis and metabolic adaptation across species.

In summary, the results of the present study suggest that meanwhile hyperglycemia stimulated lipogenesis in the liver of *S. aurata*, metformin exerted profound effects on glucose homeostasis by targeting various metabolic pathways. Metformin may improve glucose homeostasis by counteracting glucose-mediated activation of key enzymes in glycolysis and TCA, and the expression of lipogenic factors. In addition, we provide evidence that metformin may reduce hepatic gluconeogenesis through a mechanism involving reduced transdeamination and amino acid incorporation into the TCA. By targeting key pathways, enzymes and transcription factors, metformin represents a promising therapeutic intervention for improving glucose intolerance in carnivorous fish.

4.2. Effect of Periodical Administration of Chitosan-TPP Nanoparticles Complexed With a Plasmid Expressing SREBP1a on Hepatic Metabolism and Growth of *S. aurata*

Carnivorous fish, such as *S. aurata*, primarily use amino acids as gluconeogenic substrates to obtain energy and for biosynthesis. In contrast, these animals present low ability to metabolize carbohydrates and exhibit prolonged hyperglycemia after a glucose load and deficient growth performance when feeding high-carbohydrate diets. As a consequence, high-protein/low-carbohydrate diets lead to optimal growth conditions in carnivorous fish. In this

context, one of the major challenges for the aquaculture sector is to reduce fishmeal and dietary protein in aquafeeds to reduce the environmental impact and the production cost of fish farming (Naylor et al., 2009; Polakof et al., 2012; Forbes et al., 2019; Takase et al., 2023).

Deacetylation of chitin under alkaline conditions produces chitosan, which is a linear cationic polymer of β (1-4)-linked 2-amino-2-deoxy-D-glucose interspersed by residual 2-acetamido-2-deoxy-β-D-glucose (Chuan et al., 2019; Gao et al., 2022; Picos-Corrales et al., 2023). Chitosan's fundamental characteristics, such as bioadhesion, low toxicity, biodegradability, and biocompatibility laid the groundwork for its application in gene therapy (Harugade et al., 2023; Thambiliyagodage et al., 2023). The remarkable versatility of chitosan-mediated gene delivery, leveraging chitosan's unique properties for safe and effective nucleic acid delivery, leads to increasing use in the field of fish biotechnology applications, including vaccination against bacterial and viral infections, modulation of gonadal development, and control of the expression of target genes (Chuan et al., 2019; Sun et al., 2020a; Wu et al. 2020; Aranaz et al., 2021).

Our research group was a pioneer in using chitosan-based nanoparticles to induce transient modulation of the expression of target genes with the aim to overcome metabolic and genetic limitations inherent to carnivorous fish and improve glucose tolerance in these animals without producing genetically modified organisms. Although *S. aurata* is a carnivorous fish, it tolerates partial substitution of dietary protein by carbohydrates through a metabolic adaptation in the liver that involves increased glucose oxidation and decreased gluconeogenesis and transamination (Metón et al., 1999; Metón et al., 2000b; Caseras et al., 2002; Fernández et al., 2007). Therefore, we hypothesized that chitosan nanoparticles complexed with plasmids enabling control of the expression of key genes leading to increased glycolysis, decreased gluconeogenesis and/or reduced transdeamination in the liver could improve dietary carbohydrate metabolism and spare protein while maintaining an optimal growth rate of fish in culture. In recent years, we developed a methodology based on ionic gelation to synthesize chitosan-TPP nanoparticles complexed with plasmid DNA to induce *in vivo* transient overexpression and silencing of target genes in the liver of *S. aurata*. Firstly, we analyzed the effect of a single dose of chitosan-TPP nanoparticles complexed with a plasmid expressing a short hairpin RNA (shRNA) designed to knock down the expression of cytosolic ALT (cALT) in the liver of *S. aurata*. Seventy-two hours following intraperitoneal administration of the nanoparticles to *S. aurata*, this methodolody successfully allowed us to decrease the hepatic expression of cALT, which in turn stimulated the activity of key enzymes in hepatic glycolysis,

suggesting that decreased availability of amino acids as gluconeogenic substrates leads to improved carbohydrate metabolism in the liver of *S. aurata* (González et al. 2016). Following the same methodology, administration a single dose of chitosan-TPP-DNA nanoparticles and analysis at 72 hours post-treatment, we also showed that shRNA-mediated knock down of GDH impaired transdeamination and decreased gluconeogenesis in the liver *S. aurata* (Gaspar et al., 2018).

Although the metabolic effects of silencing cALT and GDH were promising, a multigenic action could be possibly more suitable to produce a more robust protein-sparing effect. Therefore, we administered a single dose of *S. aurata* with chitosan-TPP nanoparticles complexed with a plasmid expressing the N-terminus of hamster SREBP1a. SREBP1a is a transcription factor that in addition to exhibit strong transactivating capacity of genes required for fatty acid, triglycerides and cholesterol synthesis (Chandrasekaran & Weiskirchen, 2024), we previously showed, by transient transfection of HepG2 cells, that it can bind to and transactivate the gene promoters of key enzymes from *S. aurata* involved in glycolysis, GCK and PFKFB1 (Metón et al., 2006; Egea et al., 2008). Consistently, 72 hours post-treatment, the rise in the expression levels of exogenous SREBP1a in the liver of *S. aurata* led to up-regulation of the expression of GCK and PFKFB1. In addition, SREBP1a decreased the activity of the gluconeogenic enzyme FBP, while increased the mRNA levels of key enzymes in fatty acid synthesis, elongation and desaturation, as well as induced NADPH formation and cholesterol synthesis. As a result, we concluded that a single dose of nanoparticles driving the expression of SREBP1a caused a multigenic short-term effect that could enable lipid biosynthesis from carbohydrates in the liver and increased circulating levels of triglycerides and cholesterol in *S. aurata* (Silva-Marrero et al., 2019).

Considering the previous results obtained by our research group and with the aim to improve the metabolization of dietary carbohydrates and eventually enhance growth performance in carnivorous fish, herein we addressed long-term sustained expression of SREBP1a in the liver of *S. aurata*. To this end, over a total period of 70 days, chitosan-TPP nanoparticles complexed with a plasmid driving the expression of the N-terminus of hamster SREBP1a (pSG5-SREBP1a) were periodically administered by intraperitoneal injection (3 doses in total, each of 10 μg DNA/g body weight and administered every 4 weeks) to facilitate continuous overexpression of the transcription factor in the liver of *S. aurata*. Two different diets were supplied to discriminate whether long-term effects of SREBP1a on the intermediary metabolism and growth could depend on the macronutrient composition of the diet.

The dosing schedule of chitosan-TPP-DNA nanoparticles was based on preliminary studies of our group showing sustained and stable release of gene-encoding plasmids from chitosan-TPP in *S. aurata*. Indeed, 28 days following the administration of a single dose of chitosan-TPP-DNA (10 μg DNA/g body weight), the expression levels of exogenous genes in the liver were even higher (between 2.4- to 3.5-fold) than at 72 hours post-treatment (Wu et al., 2024). In fact, nanoparticles produced by ionic gelation of TPP with chitosan were shown to increase the stability of complexes in biological fluids (Wu et al 2020). Persistence of chitosan-mediated hepatic expression of exogenous proteins in *S. aurata* is consistent with previous reports of other groups (Sáez et al., 2017). The cellular events associated with chitosan-TPP-DNA include nanoparticle endocytosis, proton sponge effect allowing endosomal escape of the chitosan-TPP-DNA complex before the maturation of early endosomes into late endosomes and the ultimate fusion with lysosomes, plasmid dissociation from chitosan-TPP, translocation to the nucleus, followed by nuclear transcription of the plasmid into mRNA, translation of newly transcribed mRNA in the cytosol, and exogenous protein assembly (Wu et al., 2020).

After 70 days of treatment, a huge amount of SREBP1a mRNA levels were observed in the liver of *S. aurata* administered with chitosan-TPP-pSG5-SREBP1a independently of the diet supplied. Periodical administration of 3 doses of nanoparticles exceeded 1 order of magnitude the hepatic expression levels of hamster SREBP1a previously observed after the administration a single dose (Silva-Marrero et al., 2019). Our findings are consistent with the stability of exogenous enzyme expression in the liver of *S. aurata* after oral and intramuscular delivery of chitosan-DNA polyplexes, which was shown to last at least 60 days after the administration (Sáez et al., 2017). On the other hand, in another study we showed that biodistribution of chitosan-TPP-DNA nanoparticles intraperitoneally administered to *S. aurata* allowed high expressional levels of exogenous proteins in the liver, mild expression in the intestine and barely detectable levels in the skeletal muscle and brain (Wu et al., 2024). Discontinuous endothelia of the intestine facilitate chitosan-TPP-DNA absorption and portal transportation to the liver (Hagens et al., 2007). However, the tight morphology of the capillary endothelium in muscle and brain seem to hinder nanoparticle transfer in these tissues (Kooij et al., 2005).

Sustained expression of SREBP1a in the liver of *S. aurata* led to increased circulating levels of triglycerides and cholesterol. As it was observed for SREBP1a expression values, periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles had a greater effect

on blood triglycerides (2-fold increase) than a single administration of nanoparticles (1.3-fold increase) (Silva-Marrero et al., 2019). Similarly as in *S. aurata* treated with a single dose of chitosan-TPP-pSG5-SREBP1a (Silva-Marrero et al., 2019), the rise in blood triglycerides and cholesterol may result from SREBP1-dependent transactivation of genes involved in the synthesis of fatty acids and cholesterol. In agreement with the observations in *S. aurata*, high-carbohydrate diets stimulated the hepatic expression of SREBP1 and increased blood and liver triglycerides in *Carassius gibelio* (Gong et al., 2023), and inhibition of SREBP1 by fatostatin, a diarylthiazole derivative that specifically inhibits of SREBPs activation, attenuated the Cu-induced increase of triglycerides accumulation in the liver of zebrafish, *Danio rerio* (Pan et al. 2019). Furthermore, SREBPs are required for the biosynthesis of triglycerides and cholesterol in mammals (Bertolio et al., 2019). Indeed, down-regulation of SREBP1c led to decreased blood triglycerides in rats supplemented with ethanol extract of peanut sprout (Ha et al., 2015), and knock down of SREBP1 expression significantly diminished triglyceride accumulation along with the expression of genes encoding key enzymes in glycolysis and glycogen synthesis, and up-regulated gluconeogenic enzymes in the liver of *db/db* mice (Ruiz et al., 2014).

We previously showed that 72 hours after the administration of a single dose of chitosan-TPP-pSG5-SREBP1a nanoparticles, up-regulation of SREBP1a in the liver of *S. aurata* enhanced hepatic up-regulation of the expression levels of key enzymes in fatty acid synthesis, elongation and desaturation, NADPH production (G6PD) and cholesterol synthesis, leading to increased blood triglycerides and cholesterol. In addition, SREBP1a also stimulated glycolysis, particularly GCK and PFKFB1 expression, and the PFK/FBP enzyme activity ratio (Silva-Marrero et al., 2019). The results of the present study confirm SREBP1a-mediated activation of glycolysis in the liver of *S. aurata*. Conceivably, SREBP1a-dependent transactivation of GCK would increase the intracellular pool of Glu-6-P and its subsequent oxidation through glycolysis and the PPP (Baker et al., 2023). Further activation of glycolysis involves SREBP1a-dependent PFKFB1 transactivation. PFKFB1 is a bifunctional enzyme with a major role in the control of the flux through the Fru-6-P/Fru-1,6-P_2 substrate cycle by catalysizing both synthesis and degradation of Fru-2,6-P_2, a strong allosteric activator of PFK (glycolysis) and inhibitor of FBP (gluconeogenesis) (Okar et al., 2004; Bartrons et al., 2018). Conditions that enhance glycolysis, such as the starved-to-fed transition, feeding with low protein-high carbohydrate diets and treatment with glucose and insulin, up-regulate PFKFB1 and increase Fru-2,6-P_2 levels in the liver of *S. aurata* (Metón et al., 1999; Metón et al., 2000b). The expression of SREBP1a did not significantly affect the expression of other key enzymes in glycolysis, such

as PFK and PK. However, the rise in Fru-2,6-P_2 levels in conditions with sustained SREBP1a expression led to allosteric activation of PFK and increased PFK/FBP activity ratio. The subsequent increase in Fru-1,6-P_2 production, which is an allosteric activator of PK, suggests that SREBP1a could indirectly activate PK activity.

Consistent with a transactivating role of SREB1a on GCK and PFKFB1 in *S. aurata*, SREBP1 has been also involved in the expressional control of glycolytic and gluconeogenic genes in the liver of other species. Thus, inhibition of SREBP1 with betulin, a terpenoid that inhibits the cleavage and activation of SREBP1 by direct binding to SCAP, reduces glycolytic activity in human hepatocellular carcinoma cells (Yin et al., 2019a), and SREBP family members are needed for elevation of glycolysis in natural killer cells (Assmann et al., 2017). Moreover, SREBP1c up-regulates insulin-dependent transcription of hexokinase II and GCK in mammals (Foretz et al., 1999), while SREBP1 silencing leads to decreased expression levels of GCK in the liver of fasted mice (Jideonwo et al., 2018). By inducing GCK activity, SREBP1c exert a complementary role with ChREBP in the activation of hepatic glycolysis and lipogenesis in mammals. The GCK-dependent rise of intracellular Glu-6-P activates ChREBP, which in turn contribute to transactivation of glycolytic genes (Ferré et al., 2021). A similar mechanism was described for the omnivorous freshwater fish *Carassius gibelio* after 8 weeks of feeding with a high-carbohydrate diet, which induced lipid deposition in the liver via increased hepatic expression of SREBP1 and ChREBP, leading to Glu-6-P accumulation and up-regulation of GLUT2, glycolytic genes (GCK and PK) and lipogenesis. Paradoxically, the expression levels of G6PC and PEPCK were also found increased (Gong et al., 2023).

In addition to diminish PK expression, knock down of SREBP1 improved glucose production by up-regulating the expression of gluconeogenic enzymes (G6PC, PEPCK) in the liver of *db/db* mice and in HepG2 cells (Ruiz et al., 2014; Bai et al., 2019). SREBP1c seems to amplify, in reciprocate manner with atypical protein kinase C, mechanisms leading to increased expression of G6PC and PEPCK in mice liver (Sajan et al., 2018). Accordingly, silencing of SREBP1 expression up-regulated PEPCK, while reduced hepatic deposition of glycogen in the liver of fed mice (Ruiz et al., 2014). Indeed, SREBP1a transgenesis reduced gluconeogenesis in mouse liver through suppression of PEPCK and G6PC transcription mediated by hepatocyte nuclear factor-4α (Yamamoto et al., 2004). However, in contrast to glycolysis, periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles did not significantly modulate gluconeogenesis in the liver of *S. aurata*.

Long-lasting overexpression of SREBP1a in the liver of *S. aurata* also stimulated the mRNA and activity of G6PD, a rate-limiting enzyme of the oxidative phase of the PPP that plays a pivotal role in generating NADPH for facilitating biosynthetic reactions and antioxidant defenses, and therefore supporting cellular growth and proliferation (Silva-Marrero et al., 2019). Periodical administration of nanoparticles leading to SREBP1a overexpression may couple up-regulation of GCK and subsequent increased availability of Glu-6-P with enhanced G6PD activity, leading to increased production of NADPH in the hepatocytes. Although *S. aurata* is a carnivorous fish with low ability to metabolize carbohydrates, sustained expression of SREBP1a in the liver could trigger a long-term complex effect enabling glucose oxidation via glycolysis and the oxidative phase of the PPP to provide pyruvate (carbon backbone), ATP and NADPH for lipid biosynthesis and the consequent rise in serum triglycerides and cholesterol. Increased hepatic glucose oxidation via glycolysis and the PPP in *S. aurata* overexpressing SREBP1a suggests a shift towards utilizing carbohydrates for energy production and lipid synthesis rather than for gluconeogenesis or transamination processes. Consistently with the activating role of SREBP1 on G6PD activity, inhibition of SREBP1 by fatostatin attenuated the Cu-induced hepatic up-regulation of G6PD and genes involved in fatty acid synthesis (ACC, FASN) in *Danio rerio* (Pan et al., 2019). Similarly as in fish species, decreased expression of SREBP-1c in the liver of rats supplied with ethanol extract of peanut sprout also leads to reduced G6PD and FASN mRNA levels (Ha et al., 2015), while L-cysteine down-regulates the transcription of SREBP1c and its target genes, including G6PD, FASN and stearoyl-coenzyme A desaturase 1 in HepG2 cells (Bettzieche et al., 2008).

The supply of low-protein/high-carbohydrate diets to *S. aurata* results in poor growth performance (Fernández et al., 2007; Sáez-Arteaga et al., 2022). In contrast, in the present study, sustained hepatic expression of SREBP1a improved growth performance in *S. aurata* regardless of diet composition. Periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles enhanced weight gain and SGR. Consistently, SREBP1a overexpression reduced FCR, which also supports higher efficiency in converting feed into weight gain (Charles Bai et al., 2022). Remarkably, fish treated with SREBP1a nanoparticles and fed a low-protein/high-carbohydrate diet (Diet 2) and control fish (not treated with SREBP1a) fed a high-protein/low-carbohydrate diet (Diet 1) presented similar weight gain, SGR and FCR values. Therefore, the effects of SREBP1a compensated the loss in growth performance resulting from feeding carnivorous fish with low-protein/high-carbohydrate diets. Since hepatic overexpression of SREBP1a led to increased serum triglycerides and cholesterol keeping total body lipids

unaffected, in this group of animals circulating lipids would be mainly used for growth-related structures and energy production rather than fat accumulation. Increased efficiency in converting carbohydrates into lipids reduces the reliance on dietary protein for energy and growth, enabling the fish to allocate more ingested protein towards essential physiological processes such as tissue repair and maintenance. Irrespective of the diet, hepatic overexpression of SREBP1a increased PER. This suggests that treatment with SREBP1a improved the amount of dietary protein led to body weight gain. Therefore, the growth promoting actions of SREBP1a in *S. aurata* may result from enhanced SREBP1a-dependent metabolization of dietary carbohydrates and subsequent conversion into lipids, which in turn may enable protein sparing and the use of dietary amino acids for protein synthesis and growth rather than as gluconeogenic substrates.

The fact that ALT and AST activities were not significantly affected by treatment with SREBP1a suggests that liver transamination remained unaltered. Lack of significant effects of SREBP1a on gluconeogenesis and transamination indicates that *S. aurata* can maintain glucose homeostasis and amino acid synthesis pathways while prioritizing the use of carbohydrates for lipid synthesis. In agreement with a pivotal role of SREBP1 on growth, SREBPs control cytokine-induced growth and proliferation of natural killer cells by a mechanism involving enhanced glycolysis and oxidative phosphorylation (Assmann et al., 2017), and SREBP1-mediated lipogenic regulation is considered essential for tumor growth (Sun et al., 2020b; Cheng et al., 2022).

In summary, SREBP1a is recognized as a master transcriptional regulator of lipid metabolism, orchestrating the expression of genes involved in fatty acid and cholesterol biosynthesis. The present study extends this understanding by highlighting the impact of SREBP1a overexpression on glucose metabolism, emphasizing its role in promoting carbohydrate utilization for lipid synthesis and growth promotion. The periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles to *S. aurata* allowed long-lasting high expression levels of the transcription factor in the liver, where SREBP1a exerts a multifaceted influence on metabolic pathways, enhancing glycolysis, the oxidative phase of the PPP and lipogenesis, facilitating lipid synthesis from carbohydrates and the rise of serum triglycerides and cholesterol. These findings underscore the potential of chitosan to induce a sustained gene therapy without producing genetically modified organisms, and of SREBP1a as a molecular target to enhance metabolic efficiency and growth in aquaculture.

5. Conclusions

1. The intraperitoneal administration of glucose led to marked hyperglycemia 5 hours post-treatment in the liver of *S. aurata*, an effect that was greatly diminished by metformin. Twenty-four hours following glucose administration, glycemia returned to control values.

2. Twenty-four hours after glucose administration, the blood levels of triglycerides and cholesterol showed a trend to increase, while treatment with metformin did not significantly affect serum triglycerides and cholesterol.

3. Five hours after a glucose load, the hepatic mRNA levels and enzyme activity for genes involved in glycolysis (GCK and PFK) significantly increased, remarkably those of GCK. Metformin alone did not alter GCK expression, while down-regulated PFK mRNA levels at 5 hours and both mRNA and activity at 24 hours post-treatment. When administered with glucose, metformin totally prevented the effect of glucose administration on GCK and PFK expression.

4. Treatment with metformin decreased the mRNA levels of the gluconeogenic enzyme FBP in the liver of *S. aurata*, particularly at 5 hours post-treatment. No significant effects were observed by injecting metformin combined with glucose. Any of the treatments assayed significantly affected hepatic FBP activity.

5. In regard of the hepatic activity of two rate-limiting enzymes of the TCA: IDH, and OGDH, glucose administration significantly increased IDH at 24 hours post-treatment. Metformin alone caused the opposite effects on IDH, while metformin combined with glucose prevented the glucose-induced rise of IDH activity. In contrast, neither glucose nor metformin significantly affected OGDH in the liver of *S. aurata*.

6. The effect of glucose and metformin was also assessed on key enzyme activities of the amino acid metabolism in the liver of *S. aurata*. Glucose administration significantly decreased ALT activity at 24 hours, and a similar trend was found in AST activity. Metformin prevented the glucose-dependent decrease in ALT activity. Glucose did not affect GDH expression. However, metformin significantly down-regulated GDH mRNA levels and enzyme activity 5 hours post-treatment.

7. Treatment with glucose up-regulated the hepatic expression of lipogenic factors such as PGC1β and SREBP1, an effect that was at least partially reversed by the combined administration of glucose and metformin. At 5 hours post-administration of metformin

alone, a trend to increase PGC1β and SREBP1 mRNA levels was observed. A similar effect was found in LPIN1 expression, which in turn was reversed by combining glucose and metformin. At 24 hours post-treatment, the highest LPIN1 mRNA levels were observed in the liver of *S. aurata* administered with glucose.

8. Chitosan-TPP nanoparticles complexed with pSG5 (control) and pSG5-SREBP1a were obtained by ionic gelation. Dynamic light scattering showed no differences in particle mean diameter size of naked chitosan-TPP and chitosan-TPP complexed with plasmids (pSG5 and pSG5-SREBP1a). Laser Doppler electrophoresis showed that plasmid incorporation into chitosan-TPP significantly reduced the positivity of the *Z* potential to 54–58 % of the values observed in naked chitosan-TPP.

9. The intraperitoneal administration of 3 doses of chitosan-TPP-pSG5-SREBP1a nanoparticles (each dose administered every 4 weeks, over a total period of 70 days, and consisting of 10 μg of plasmid per g of body weight), caused a huge up-regulation of hamster SREBP1a mRNA levels (up to 239-fold increase) in the liver of treated *S. aurata*. Chitosan-based up-regulation of SREBP1a expression was not significantly affected by the macronutrient composition of the diet supplied to *S. aurata* during the experimental period.

10. Blood glucose was not affected by any of the treatments assayed (diet and nanoparticle administration). However, chitosan-TPP-pSG5-SREBP1a administration increased blood triglycerides and cholesterol by 1.8- and 1.2-fold, respectively, in fish fed a high-protein/low-carbohydrate diet and by 2.3- and 1.2-fold in fish fed a low-protein/high-carbohydrate diet.

11. Dietary macronutrient composition strongly affected the gene expression and activity of glycolytic enzymes in the liver of *S. aurata*. The supply of a low-protein/high-carbohydrate diet significantly up-regulated the expression of GCK, PFKFB1, PFK, and PK, as well as the enzyme activity of GCK, PFK, and PK. The administration of chitosan-TPP-pSG5-SREBP1a nanoparticles significantly up-regulated GCK and PFKFB1 irrespective of diet composition. Nanoparticle-mediated expression of SREBP1a significantly increased the activity of PFK and PK, while not affecting their mRNA levels.

12. Concerning hepatic gluconeogenesis, diet composition only affected the mRNA levels of FBP, which were found up-regulated in fish fed the high-protein/low-carbohydrate

commercial diet. Chitosan-mediated expression of SREBP1a did not affect the mRNA levels of G6PC1, FBP, and PCKL, nor FBP activity. Considering the importance of the Fru-6-P/Fru-1,6-P_2 substrate cycle in the control of the flux through glycolysis–gluconeogenesis, the effect of diet composition and SREBP1a expression on the PFK/FBP1 activity ratio in the liver of *S. aurata* was also assessed. The supply of the low-protein/high-carbohydrate diet increased the PFK/FBP1 activity ratio. Independently of the diet supplied, treatment with chitosan-TPP-pSG5-SREBP1a also stimulated the PFK/FBP1 activity ratio.

13. In regard of the PPP, both feeding with the low-protein/high-carbohydrate diet and the administration of chitosan-TPP-pSG5-SREBP1a nanoparticles significantly increased G6PD mRNA and G6PD activity in the liver of *S. aurata*.

14. The supply of a high-protein/low-carbohydrate diet significantly increased the enzyme activity of two major liver transaminases in *S. aurata*: ALT and AST. However, nanoparticle administration did not affect the hepatic activity of ALT and AST.

15. The supply of a low-protein/high-carbohydrate diet significantly decreased final body weight, weight gain, and SGR, while increased LR and PER. In *S. aurata* fed either with a high-protein/low-carbohydrate or a low-protein/high-carbohydrate diet, periodical administration of chitosan-TPP-pSG5-SREBP1a nanoparticles significantly increased weight gain (13.8 % and 9.2 %, respectively), SGR (14.6 % and 3.6 %), and PER (7.9 % and 20.6 %), while decreased FCR (to 6.4 % and 14.7 % of control values, respectively). Neither diet nor nanoparticle administration significantly affected HSI, PR, moisture, ash, protein, and lipid body composition.

References

Abdel-Ghany, H. M., & Salem, M. E. S. (2020). Effects of dietary chitosan supplementation on farmed fish; a review. *Reviews in Aquaculture*, 12(1), 438-452.

Abeyrathne, E. D. N. S., Nam, K., & Ahn, D. U. (2021). Analytical methods for lipid oxidation and antioxidant capacity in food systems. *Antioxidants*, 10(10), 1587.

Agius, L., Ford, B. E., & Chachra, S. S. (2020). The metformin mechanism on gluconeogenesis and AMPK activation: the metabolite perspective. *International Journal of Molecular Sciences*, 21(9), 3240.

Ahmad, A. L., Chin, J. Y., Harun, M. H. Z. M., & Low, S. C. (2022). Environmental impacts and imperative technologies towards sustainable treatment of aquaculture wastewater: A review. *Journal of Water Process Engineering*, 46, 102553.

Aich, N., Nama, S., Biswal, A., & Paul, T. (2020). A review on recirculating aquaculture systems: Challenges and opportunities for sustainable aquaculture. *Innovative Farming*, 5(1), 17-24.

Al-Nimry, S., Dayah, A. A., Hasan, I., & Daghmash, R. (2021). Cosmetic, biomedical and pharmaceutical applications of fish gelatin/hydrolysates. *Marine Drugs*, 19(3), 145.

Alvanou, M. V., Gkagkavouzis, K., Karaiskou, N., Feidantsis, K., Lattos, A., Michaelidis, B., & Giantsis, I. A. (2023). Mediterranean aquaculture and genetic pollution: a review combined with data from a fish farm evaluating the ecological risks of finfish escapes. *Journal of Marine Science and Engineering*, 11(7), 1405.

Andoh, T. (2007). Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (Verasper moseri). *General and Comparative Endocrinology*, 151, 308–317.

Anemaet, I. G., Metón, I., Salgado, M. C., Fernández, F., & Baanante, I. V. (2008). A novel alternatively spliced transcript of cytosolic alanine aminotransferase gene associated with enhanced gluconeogenesis in liver of Sparus aurata. *International Journal of Biochemistry & Cell Biology*, 40(12), 2833-2844.

Antonucci, F., & Costa, C. (2020). Precision aquaculture: a short review on engineering innovations. *Aquaculture International*, 28(1), 41-57.

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras Caballero, A., & Acosta, N. (2021). Chitosan: An overview of its properties and applications. *Polymers*, 13(19), 3256.

Arechavala-Lopez, P., Toledo-Guedes, K., Izquierdo-Gomez, D., Šegvić-Bubić, T., & Sanchez-Jerez, P. (2018). Implications of sea bream and sea bass escapes for sustainable aquaculture management: a review of interactions, risks and consequences. *Reviews in Fisheries Science & Aquaculture*, 26(2), 214-234.

Ashry, A. M., Hassan, A. M., Habiba, M. M., El-Zayat, A., El-Sharnouby, M. E., Sewilam, H., & Dawood, M. A. (2021). The impact of dietary curcumin on the growth performance, intestinal antibacterial capacity, and haemato-biochemical parameters of gilthead seabream (Sparus aurata). *Animals*, 11(6), 1779.

Assmann, N., O'Brien, K. L., Donnelly, R. P., Dyck, L., Zaiatz-Bittencourt, V., Loftus, R. M., Heinrich, P., Oefner, P. J., Lynch, L., Gardiner, C. M., Dettmer, K., & Finlay, D. K. (2017). Srebp-controlled glucose metabolism is essential for NK cell functional responses. *Nature Immunology*, 18, 1197–1206.

Ateweberhan, M., Hudson, J., Rougier, A., Jiddawi, N. S., Msuya, F. E., Stead, S. M., & Harris, A. (2018). Community based aquaculture in the western Indian Ocean: challenges and

opportunities for developing sustainable coastal livelihoods. *Ecology and Society*, 23(4), 17.

Ayisi, C. L., Yamei, C., & Zhao, J. L. (2018). Genes, transcription factors and enzymes involved in lipid metabolism in fin fish. *Agri Gene*, 7, 7-14.

Babaei, S., Abedian Kenari, A., Hedayati, M., Yazdani Sadati, M. A., & Metón, I. (2016). Effect of diet composition on growth performance, hepatic metabolism and antioxidant activities in Siberian sturgeon (Acipenser baerii, Brandt, 1869) submitted to starvation and refeeding. *Fish Physiology and Biochemistry*, 42, 1509-1520.

Bai, M., Yang, L., Liao, H., Liang, X., Xie, B., Xiong, J., Tao, X., Chen, X., Cheng, Y., Chen, X., Feng, Y., Zhang, Z., & Zheng, W. (2018). Metformin sensitizes endometrial cancer cells to chemotherapy through IDH1-induced Nrf2 expression via an epigenetic mechanism. *Oncogene*, 37(42), 5666-5681.

Bai, X.P., Dong, F., Yang, G.H., & Zhang, L. (2019). Influences of sterol regulatory element binding protein-1c silencing on glucose production in HepG2 cells treated with free fatty acid. *Lipids in Health and Disease*, 18, 89.

Baker, S.A., & Rutter, J. (2023). Metabolites as signalling molecules. *Nature Reviews Molecular Cell Biology*, 24, 355–374.

Balbuena-Pecino, S., Riera-Heredia, N., Vélez, E. J., Gutiérrez, J., Navarro, I., Riera-Codina, M., & Capilla, E. (2019). Temperature affects musculoskeletal development and muscle lipid metabolism of gilthead sea bream (Sparus aurata). *Frontiers in Endocrinology*, 10, 173.

Bandara, T. (2018). Alternative feed ingredients in aquaculture: Opportunities and challenges. *Journal of Entomology and Zoology Studies*, 6(2), 3087-3094.

Bartrons, R., Simon-Molas, H., Rodríguez-García, A., Castaño, E., Navarro-Sabaté, À., Manzano, A., & Martinez-Outschoorn, U.E. (2018). Fructose 2,6-bisphosphate in cancer cell metabolism. *Frontiers in Oncology*, 8, 331.

Basto-Silva, C., Enes, P., Oliva-Teles, A., Balbuena-Pecino, S., Navarro, I., Capilla, E., & Guerreiro, I. (2021). Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (Sparus aurata). *Aquaculture*, 533, 736142.

Basto-Silva, C., Enes, P., Oliva-Teles, A., Capilla, E., & Guerreiro, I. (2022). Dietary protein/carbohydrate ratio and feeding frequency affect feed utilization, intermediary metabolism, and economic efficiency of gilthead seabream (Sparus aurata) juveniles. *Aquaculture*, 554, 738182.

Bertolio, R., Napoletano, F., Mano, M., Maurer-Stroh, S., Fantuz, M., Zannini, A., & Del Sal, G. (2019). Sterol regulatory element binding protein 1 couples mechanical cues and lipid metabolism. *Nature Communications*, 10(1), 1326.

Bettzieche, A., Brandsch, C., Hirche, F., Eder, K., & Stangl, G. I. (2008). L -Cysteine Down-Regulates SREBP-1c-Regulated Lipogenic Enzymes Expression via Glutathione in HepG2 Cells. *Annals of Nutrition and Metabolism*, 52, 196–203.

Bhat, I. A., Nazir, M. I., Ahmad, I., Pathakota, G. B., Chanu, T. I., Goswami, M., & Sharma, R. (2018). Fabrication and characterization of chitosan conjugated eurycomanone nanoparticles: In vivo evaluation of the biodistribution and toxicity in fish. *International Journal of Biological Macromolecules*, 112, 1093-1103.

Bildik, A., Asıcı Ekren, G., Akdeniz, G., & Kıral, F. (2019). Effect of enviromental temperature on heat

shock proteins (HSP30, HSP70, HSP90) and IGF-I mRNA expression in Sparus aurata. *Iranian Journal of Fisheries Sciences*, 18, 4.

Blasco, J., Vélez, E. J., Perelló-Amorós, M., Azizi, S., Capilla, E., Fernández-Borràs, J., & Gutiérrez, J. (2021). Recombinant bovine growth hormone-induced metabolic remodelling enhances growth of gilthead sea-bream (Sparus aurata): insights from stable isotopes composition and proteomics. *International Journal of Molecular Sciences*, 22(23), 13107.

Borges, P., Valente, L. M. P., Véron, V., Dias, K., Panserat, S., & Médale, F. (2014) High dietary lipid level is associated with persistent hyperglycaemia and downregulation of muscle Akt-mTOR pathway in Senegalese sole (Solea senegalensis). *PLoS One*, 9, e102196.

Bradbury, I. R., Burgetz, I., Coulson, M. W., Verspoor, E., Gilbey, J., Lehnert, S. J., & McGinnity, P. (2020). Beyond hybridization: the genetic impacts of nonreproductive ecological interactions of salmon aquaculture on wild populations. *Aquaculture Environment Interactions*, 12, 429-445.

Brown, M. S., & Goldstein, J. L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, 89(3), 331-340.

Caballero-Solares, A., Viegas, I., Salgado, M. C., Siles, A. M., Sáez, A., Metón, I., Baanante, I. V., & Fernández, F. (2015). Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (Sparus aurata) juveniles. *Aquaculture*, 444, 79–87.

Cao, X. F., Dai, Y. J., Liu, M. Y., Yuan, X. Y., Wang, C. C., Huang, Y. Y., & Jiang, G. Z. (2019). High-fat diet induces aberrant hepatic lipid secretion in blunt snout bream by activating endoplasmic reticulum stress-associated IRE1/XBP1 pathway. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1864(3), 213-223.

Carvalho, M., Montero, D., Torrecillas, S., Castro, P., Zamorano, M. J., & Izquierdo, M. (2021). Hepatic biochemical, morphological and molecular effects of feeding microalgae and poultry oils to gilthead sea bream (Sparus aurata). *Aquaculture*, 532, 736073.

Caseras, A., Metón, I., Fernández, F., & Baanante, I. V. (2000). Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (Sparus aurata). *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1493(1-2), 135-141.

Caseras, A., Metón, I., Vives, C., Egea, M., Fernández, F., & Baanante, A. I. (2002). Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). *British Journal of Nutrition*, 88(6), 607-614.

Castro, L. F. C., Tocher, D. R., & Monroig, O. (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. *Progress in Lipid Research*, 62, 25-40.

Chandrasekaran, P., & Weiskirchen, R. (2024). The Role of SCAP/SREBP as Central Regulators of Lipid Metabolism in Hepatic Steatosis. *International Journal of Molecular Sciences*, 25(2), 1109.

Charles Bai, S., Hardy, R.W., & Hamidoghli, A. (2022) Diet analysis and evaluation. In: Fish Nutrition, Elsevier, pp 709–743.

Chen, G. H., Luo, Z., Hogstrand, C., Wu, K., & Ling, S. C. (2018). SREBP1, PPARG and AMPK pathways mediated the Cu-induced change in intestinal lipogenesis and lipid transport of yellow

catfish Pelteobagrus fulvidraco. *Food Chemistry*, 269, 595-602.

Chen, Y., Sun, Z., Liang, Z., Xie, Y., Su, J., Luo, Q., & Wang, A. (2020). Effects of dietary fish oil replacement by soybean oil and L-carnitine supplementation on growth performance, fatty acid composition, lipid metabolism and liver health of juvenile largemouth bass, Micropterus salmoides. *Aquaculture*, 516, 734596.

Cheng, C., Geng, F., Li, Z., Zhong, Y., Wang, H., Cheng, X., Zhao, Y., Mo, X., Horbinski, C., & Duan, W. (2022). Ammonia stimulates SCAP/Insig dissociation and SREBP-1 activation to promote lipogenesis and tumour growth. *Nature Metabolism*, 4, 575–588.

Chuan, D., Jin, T., Fan, R., Zhou, L., & Guo, G. (2019). Chitosan for gene delivery: Methods for improvement and applications. *Advances in Colloid and Interface Science*, 268, 25-38.

Dai, W., Panserat, S., Terrier, F., Seiliez, I., & Skiba-Cassy, S. (2014) Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (Oncorhynchus mykiss). *American Journal of Physiology-Regulatory and Integrative Comparative Physiology*, 307, R1231-R1238.

de Oliveira Santana, K. N., Lelis, D. F., Mendes, K. L., Lula, J. F., Paraíso, A. F., Andrade, J. M. O., Feltenberger, J. D., Cota, J., da Costa, D. V., de Paula, A. M. B., Guimarães, A. L. S., & Santos, S. H. S. (2016) Metformin reduces lipogenesis markers in obese mice fed a low-carbohydrate and high-fat diet. *Lipids*, 51, 1375–1384.

DeBose-Boyd, R. A., & Ye, J. (2018). SREBPs in lipid metabolism, insulin signaling, and beyond. *Trends in Biochemical Sciences*, 43(5), 358-368.

Droubogiannis, S., & Katharios, P. (2022). Genomic and biological profile of a novel bacteriophage, Vibrio phage Virtus, which improves survival of Sparus aurata larvae challenged with Vibrio harveyi. *Pathogens*, 11(6), 630.

Eberlé, D., Hegarty, B., Bossard, P., Ferré, P., & Foufelle, F. (2004). SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie*, 86(11), 839-848.

Edwards, P. A., Tabor, D., Kast, H. R., & Venkateswaran, A. (2000). Regulation of gene expression by SREBP and SCAP. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1529(1-3), 103-113.

Egea, M., Metón, I., Córdoba, M., Fernández, F., & Baanante, I. V. (2008). Role of Sp1 and SREBP-1a in the insulin-mediated regulation of glucokinase transcription in the liver of gilthead sea bream (Sparus aurata). *General and Comparative Endocrinology*, 155(2), 359-367.

Elizalde-Velázquez, G. A., Herrera-Vázquez, S. E., Gómez-Oliván, L. M., & García-Medina, S. (2023). Health impact assessment after Danio rerio long-term exposure to environmentally relevant concentrations of metformin and guanylurea. *Chemosphere*, 341, 140070.

El-Naggar, M., Salaah, S., El-Shabaka, H., Abd El-Rahman, F., Khalil, M., & Suloma, A. (2021). Efficacy of dietary chitosan and chitosan nanoparticles supplementation on health status of Nile tilapia, Oreochromis niloticus (L.). *Aquaculture Reports*, 19, 100628.

El-Nokrashy, A. M., El-Banna, R. A., Edrise, B. M., Abdel-Rahim, M. M., Jover-Cerdá, M., Tomás-Vidal, A., & Goda, A. S. (2021). Impact of nucleotide enriched diets on the production of gilthead seabream, Sparus aurata fingerlings by modulation of liver mitochondrial enzyme activitity, antioxidant status, immune gene expression, and gut microbial ecology. *Aquaculture*, 535, 736398.

Engle, C. R., Hanson, T., & Kumar, G. (2022). Economic history of US catfish farming: Lessons for growth and development of aquaculture. *Aquaculture Economics & Management*, 26(1), 1-35.

Fabrikov, D., Sánchez-Muros, M. J., Barroso, F. G., Tomás-Almenar, C., Melenchón, F., Hidalgo, M. C., & Montes-Lopez, J. (2020). Comparative study of growth performance and amino acid catabolism in Oncorhynchus mykiss, Tinca tinca and Sparus aurata and the catabolic changes in response to insect meal inclusion in the diet. *Aquaculture*, 529, 735731.

Feidantsis, K., Pörtner, H. O., Vlachonikola, E., Antonopoulou, E., & Michaelidis, B. (2018). Seasonal changes in metabolism and cellular stress phenomena in the gilthead sea bream (Sparus aurata). *Physiological and Biochemical Zoology*, 91(3), 878-895.

Fernández, F., Miquel, A. G., Córdoba, M., Varas, M., Metón, I., Caseras, A., & Baanante, I. V. (2007). Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (Sparus aurata, L.) fingerlings. *Journal of Experimental Marine Biology and Ecology*, 343(1), 1-10.

Ferré, P., Phan, F., & Foufelle, F. (2021). SREBP-1c and lipogenesis in the liver: an update1. *Biochemical Journal*, 478, 3723–3739.

Forbes, J., Kostyniuk, D., Mennigen, J., & Weber, J. (2019) Unexpected effect of insulin on glucose disposal explains glucose intolerance of rainbow trout. *American Journal of Physiology-Regulatory and Integrative Comparative Physiology*, *316*, R387–R394.

Foretz, M., Guichard, C., Ferré, P., & Foufelle, F. (1999). Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proceedings of the National Academy of Sciences of the United States of America*, 96(22), 12737-12742.

Gao, Y., & Wu, Y. (2022) Recent advances of chitosan-based nanoparticles for biomedical and biotechnological applications. *International Journal of Biological Macromolecules*, 203, 379–388.

Gaspar, C., Silva-Marrero, J. I., Fàbregas, A., Miñarro, M., Ticó, J. R., Baanante, I. V., & Metón, I. (2018). Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. *Journal of Biotechnology*, 286, 5-13.

Gong, Y., Xi, L., Liu, Y., Lu, Q., Zhang, Z., Liu, H., & Han, D. (2023). Sequential Activations of ChREBP and SREBP1 Signals Regulate the High-Carbohydrate Diet-Induced Hepatic Lipid Deposition in Gibel Carp (Carassius gibelio). *Aquaculture Nutrition*, 2023, 672985.

González, J. D., Caballero, A., Viegas, I., Metón, I., Jones, J. G., Barra, J., & Baanante, I. V. (2012). Effects of alanine aminotransferase inhibition on the intermediary metabolism in Sparus aurata through dietary amino-oxyacetate supplementation. *British Journal of Nutrition*, 107(12), 1747-1756.

González, J. D., Silva-Marrero, J. I., Metón, I., Caballero-Solares, A., Viegas, I., Fernández, F., & Baanante, I. V. (2016). Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. *Marine Biotechnology*, 18, 85-97.

Gosmain, Y., Dif, N., Berbe, V., Loizon, E., Rieusset, J., Vidal, H., & Lefai, E. (2005). Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and

refeeding in rat tissues. *Journal of Lipid Research*, 46(4), 697-705.

Gosmain, Y., Lefai, E., Ryser, S., Roques, M., & Vidal, H. (2004). Sterol regulatory element-binding protein-1 mediates the effect of insulin on hexokinase II gene expression in human muscle cells. *Diabetes*, 53(2), 321-329.

Gratacap, R. L., Regan, T., Dehler, C. E., Martin, S. A., Boudinot, P., Collet, B., & Houston, R. D. (2020). Efficient CRISPR/Cas9 genome editing in a salmonid fish cell line using a lentivirus delivery system. *BMC Biotechnology*, 20, 1-9.

Guo, J. L., Zhou, Y. L., Zhao, H., Chen, W. Y., Chen, Y. J., & Lin, S. M. (2019). Effect of dietary lipid level on growth, lipid metabolism and oxidative status of largemouth bass, Micropterus salmoides. *Aquaculture*, 506, 394-400.

Ha, A. W., Kang, N. E., & Kim, W. K. (2015). Ethanol Extract of Peanut Sprout Lowers Blood Triglyceride Levels, Possibly Through a Pathway Involving SREBP-1c in Rats Fed a High-Fat Diet. *Journal of Medicinal Food*, 18(8), 850–855.

Hagens, W. I., Oomen, A. G., & de Jong, W. H. (2007) What do we (need to) know about the kinetic properties of nanoparticles in the body? *Regulatory Toxicology Pharmacology*, 49, 217–229.

Harugade, A., Sherje, A. P., & Pethe, A. (2023). Chitosan: A review on properties, biological activities and recent progress in biomedical applications. *Reactive and Functional Polymers*, 191, 105634.

Hematyar, N., Rustad, T., Sampels, S., & Kastrup Dalsgaard, T. (2019). Relationship between lipid and protein oxidation in fish. *Aquaculture Research*, 50(5), 1393-1403.

Horton, J. D., Bashmakov, Y., Shimomura, I., & Shimano, H. (1998). Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), 5987-5992.

Horton, J. D., Goldstein, J. L., & Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation*, 109(9), 1125-1131.

Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W., Brown, M. S., & Goldstein, J. L. (2003). Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proceedings of the National Academy of Sciences of the United States of America*, 100(21), 12027-12032.

Iaconisi, V., Secci, G., Sabatino, G., Piccolo, G., Gasco, L., Papini, A. M., & Parisi, G. (2019). Effect of mealworm (Tenebrio molitor L.) larvae meal on amino acid composition of gilthead sea bream (Sparus aurata L.) and rainbow trout (Oncorhynchus mykiss W.) fillets. *Aquaculture*, 513, 734403.

Iber, B. T., & Kasan, N. A. (2021). Recent advances in Shrimp aquaculture wastewater management. *Heliyon*, 7(11), e08283.

Inoue, J., & Sato, R. (2013). New insights into the activation of sterol regulatory element-binding proteins by proteolytic processing. *BioMolecular Concepts*, 4(4), 417-423.

Jideonwo, V., Hou, Y., Ahn, M., Surendran, S., & Morral, N. (2018). lmpact of silencing hepatic SREBP-1 on insulin signaling. *PLoS One*, 13(5), e0196704.

Kandyliari, A., Karavoltsos, S., Sakellari, A., Anastasiadis, P., Asderis, M., Papandroulakis, N., &

Kapsofefalou, M. (2021). Trace metals in six fish by-products of two farmed fishes, the gilthead sea bream (Sparus aurata) and the meager (Argyrosomus regius): Interactions with the environment and feed. *Human and Ecological Risk Assessment: An International Journal*, 27(4), 1126-1146.

Karim, M., Leemans, K., Akester, M., & Phillips, M. (2020). Performance of emergent aquaculture technologies in Myanmar; challenges and opportunities. *Aquaculture*, 519, 734875.

Katz, M. G., Fargnoli, A. S., Gubara, S. M., Fish, K., Weber, T., Bridges, C. R., & Ishikawa, K. (2019). Targeted gene delivery through the respiratory system: rationale for intratracheal gene transfer. *Journal of Cardiovascular Development and Disease*, 6(1), 8.

Kaushik, S. J., & Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research*, 41, 322–332

Kaya Öztürk, D., Öztürk, R., Baki, B., Karayücel, İ., & Karayücel, S. (2020). Monthly variation of growth, biochemical composition, fatty and amino acids patterns of gilthead sea bream (Sparus aurata) in offshore cage systems under brackish water conditions in the Black Sea. *Aquaculture Research*, 51(12), 4961-4983.

Keibler, M. A., Wasylenko, T. M., Kelleher, J. K., Iliopoulos, O., Vander Heiden, M. G., & Stephanopoulos, G. (2016) Metabolic requirements for cancer cell proliferation. *Cancer & Metabolism*, 4, 16.

Kim, S. Y., Kim, H. I., Kim, T. H., Im, S. S., Park, S. K., Lee, I. K., & Ahn, Y. H. (2004). SREBP-1c mediates the insulin-dependent hepatic glucokinase expression. *Journal of Biological Chemistry*, 279(29), 30823-30829.

Kooij G, van Horssen J, de Vries E (2005) Tight junctions of the blood-brain barrier. In: de Vries E, Prat A (eds) The blood-brain barrier and its microenvironment: basic physiology to neurological disease. CRC Press, Boca Raton, pp 69–92

Krebs, N., Bock, C., Tebben, J., Mark, F. C., Lucassen, M., Lannig, G., & Pörtner, H. O. (2023). Evolutionary Adaptation of Protein Turnover in White Muscle of Stenothermal Antarctic Fish: Elevated Cold Compensation at Reduced Thermal Responsiveness. *Biomolecules*, 13(10), 1507.

Kumar, G., Engle, C., & Tucker, C. (2018). Factors driving aquaculture technology adoption. *Journal of the World Aquaculture Society*, 49(3), 447-476.

Kumari, R., Gupta, S., Singh, A. R., Ferosekhan, S., Kothari, D. C., Pal, A. K., & Jadhao, S. B. (2013). Chitosan nanoencapsulated exogenous trypsin biomimics zymogen-like enzyme in fish gastrointestinal tract. *PloS One*, 8(9), e74743.

Kurniawan, S. B., Ahmad, A., Rahim, N. F. M., Said, N. S. M., Alnawajha, M. M., Imron, M. F., & Hasan, H. A. (2021). Aquaculture in Malaysia: Water-related environmental challenges and opportunities for cleaner production. *Environmental Technology & Innovation*, 24, 101913.

Laghari, M. Y. (2018). Aquaculture in Pakistan: Challenges and opportunities. *International Journal of Fisheries and Aquatic Studies*, 6(2), 56-59.

Lai, W., Xu, D., Li, J., Wang, Z., Ding, Y., Wang, X., & Ai, Q. (2021). Dietary polystyrene nanoplastics exposure alters liver lipid metabolism and muscle nutritional quality in carnivorous marine fish large yellow croaker (Larimichthys crocea). *Journal of Hazardous Materials*, 419, 126454.

Lee, J. W., Shin, Y. J., Kim, H., Kim, H., Kim, J., Min, S. A., & Park, K. (2019). Metformin-induced

endocrine disruption and oxidative stress of Oryzias latipes on two-generational condition. *Journal of Hazardous Materials*, 367, 171-181.

Li, H., Xu, W., Jin, J., Zhu, X., Yang, Y., Han, D., & Xie, S. (2019). Effects of dietary carbohydrate and lipid concentrations on growth performance, feed utilization, glucose, and lipid metabolism in two strains of gibel carp. *Frontiers in Veterinary Science*, 6, 165.

Liu, Z., Zhou, Y., Liu, S., Zhong, H., Zhang, C., Kang, X., & Liu, Y. (2012). Characterization and dietary regulation of glutamate dehydrogenase in different ploidy fishes. *Amino Acids*, 43, 2339–2348.

Liu, Z.-Q., Song, X.-M., Chen, Q.-T., Liu, T., Teng, J.-T., Zhou, K., & Luo, D.-Q. (2016). Effect of metformin on global gene expression in liver of KKAy mice. *Pharmacological Reports*, 68, 1332–1338.

Liu, K., Liu, H., Chi, S., Dong, X., Yang, Q., & Tan, B. (2018). Effects of different dietary lipid sources on growth performance, body composition and lipid metabolism-related enzymes and genes of juvenile golden pompano, Trachinotus ovatus. *Aquaculture Research*, 49(2), 717-725.

Liu, Q. Q., Xia, R., Deng, X., Yang, H. J., Luo, L., Lin, S. M., & Chen, Y. J. (2023). Effects of dietary starch and metformin levels on the growth performance, body composition, hepatic glycolipid metabolism, and liver histology of juvenile largemouth bass Micropterus salmoides. *Aquaculture*, 573, 739582.

López-Pedrouso, M., Lorenzo, J. M., Cantalapiedra, J., Zapata, C., Franco, J. M., & Franco, D. (2020). Aquaculture and by-products: Challenges and opportunities in the use of alternative protein sources and bioactive compounds. *Advances in Food and Nutrition Research*, 92, 127-185.

Lorin, S., Tol, M. J., Bauvy, C., Strijland, A., Poüs, C., Verhoeven, A. J., Codogno, P., & Meijer, A. J. (2013). Glutamate dehydrogenase contributes to leucine sensing in the regulation of autophagy. *Autophagy*, 9, 850–860.

Lu, S., Wu, X., Gao, Y., Gatlin III, D. M., Wu, M., Yao, W., & Dong, Y. (2018). Effects of dietary carbohydrate sources on growth, digestive enzyme activity, gene expression of hepatic GLUTs and key enzymes involved in glycolysis-gluconeogenesis of giant grouper Epinephelus lanceolatus larvae. *Aquaculture*, 484, 343-350.

MacLaren, R. D., Wisniewski, K., & MacLaren, C. (2018). Environmental concentrations of metformin exposure affect aggressive behavior in the Siamese fighting fish, Betta splendens. *PLoS One*, 13(5), e0197259.

Madiraju, A. K., Qiu, Y., Perry, R. J., Rahimi, Y., Zhang, X. M., Zhang, D., & Shulman, G. I. (2018). Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo. *Nature Medicine*, 24(9), 1384-1394.

Mao, W., Wang, K., Xu, B., Zhang, H., Sun, S., Hu, Q., & Peng, B. (2021). ciRS-7 is a prognostic biomarker and potential gene therapy target for renal cell carcinoma. *Molecular Cancer*, 20(1), 1-7.

Matejkova, J., & Podhorec, P. (2019). Sustained drug delivery system in fish and the potential for use of PLGA microparticles: a review. *Veterinární Medicína*, 64(7), 287-293.

Maulu, S., Hasimuna, O. J., Haambiya, L. H., Monde, C., Musuka, C. G., Makorwa, T. H., & Nsekanabo, J. D. (2021). Climate change effects on aquaculture production: sustainability implications, mitigation, and adaptations. *Frontiers in Sustainable Food Systems*, 5, 609097.

Melnik, B.C., & Schmitz, G. (2014). Metformin: an inhibitor of mTORC1 signaling. *Journal of Endocrinology, Diabetes and Obesity*, 2, 1029.

Metón, I., Caseras, A., Cantó, E., Fernández, F., & Baanante, I. V. (2000a). Liver insulin-like growth factor-I mRNA is not affected by diet composition or ration size but shows diurnal variations in regularly-fed gilthead sea bream (Sparus aurata). *Journal of Nutrition*, 130(4), 757-760.

Metón, I., Caseras, A., Fernández, F., & Baanante, I. V. (2000b). 6-Phosphofructo-2-kinase/fructose-2, 6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, Sparus aurata. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1491(1-3), 220-228.

Metón, I., Caseras, A., Fernández, F., & Baanante, I. V. (2004). Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (Sparus aurata): response of its mRNA levels and glucokinase expression to refeeding and diet composition. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 138(2), 145-153.

Metón, I., Egea, M., Anemaet, I. G., Fernández, F., & Baanante, I. V. (2006). Sterol regulatory element binding protein-1a transactivates 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase gene promoter. *Endocrinology*, 147(7), 3446-3456.

Metón, I., Mediavilla, D., Caseras, A., Cantó, E., Fernández, F., & Baanante, I. V. (1999). Effect of diet composition and ration size on key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (Sparus aurata). *British Journal of Nutrition*, 82(3), 223-232.

Mhalhel, K., Levanti, M., Abbate, F., Laurà, R., Guerrera, M. C., Aragona, M., & Montalbano, G. (2023). Review on Gilthead Seabream (Sparus aurata) Aquaculture: Life Cycle, Growth, Aquaculture Practices and Challenges. *Journal of Marine Science and Engineering*, 11(10), 2008.

Monroig, O., Tocher, D. R., & Castro, L. F. C. (2018). Polyunsaturated fatty acid biosynthesis and metabolism in fish. In: Polyunsaturated fatty acid metabolism, AOCS Press, pp 31-60.

Moon T. W. (2001). Glucose intolerance in teleost fish: fact or fiction?. *Comparative Biochemistry and Physiology Part B, Biochemistry & Molecular Biology*, 129(2-3), 243–249.

Moraes, G., & de Almeida, L. C. (2020). Nutrition and functional aspects of digestion in fish. In Biology and physiology of freshwater neotropical fish, Academic Press, pp 251-271.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., & Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences of the United States of America*, 106(36), 15103-15110.

Niemuth, N. J., & Klaper, R. D. (2018). Low-dose metformin exposure causes changes in expression of endocrine disruption-associated genes. *Aquatic Toxicology*, 195, 33-40.

Nikapitiya, C., Dananjaya, S. H. S., De Silva, B. C. J., Heo, G. J., Oh, C., De Zoysa, M., & Lee, J. (2018). Chitosan nanoparticles: A positive immune response modulator as display in zebrafish larvae against Aeromonas hydrophila infection. *Fish & Shellfish Immunology*, 76, 240-246.

Ofori-Mensah, S., Yıldız, M., Arslan, M., & Eldem, V. (2020). Fish oil replacement with different vegetable oils in gilthead seabream, Sparus aurata diets: Effects on fatty acid metabolism based on whole-body fatty acid balance method and genes expression. *Aquaculture*, 529, 735609.

Okar, D.A., Wu, C., & Lange, A.J. (2004). Regulation of the regulatory enzyme, 6-phosphofructo-2-

kinase/fructose-2,6-bisphosphatase. *Advances in Enzyme Regulation*, 44, 123–154.

Osmond, A. T., & Colombo, S. M. (2019). The future of genetic engineering to provide essential dietary nutrients and improve growth performance in aquaculture: advantages and challenges. *Journal of the World Aquaculture Society*, 50(3), 490-509.

Ottinger, M., Clauss, K., & Kuenzer, C. (2018). Opportunities and challenges for the estimation of aquaculture production based on earth observation data. *Remote Sensing*, 10(7), 1076.

Pai, J. T., Guryev, O., Brown, M. S., & Goldstein, J. L. (1998). Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *Journal of Biological Chemistry*, 273(40), 26138-26148.

Pan, Y-X., Zhuo, M.-Q., Li, D.-D., Xu, Y.-H., Wu, K., & Luo, Z. (2019). SREBP-1 and LXRa pathways mediated Cu-induced hepatic lipid metabolism in zebrafish *Danio rerio*. *Chemosphere*, 215, 370e379.

Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C., Aguirre, P., Larroquet, L., Chavernac, G., Medale, F., Corraze, G., Kaushik, S., & Moon, T. W. (2009). Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 297, R707–R715.

Panserat, S., Capilla, E., Gutierrez, J., Frappart, P. O., Vachot, C., Plagnes-Juan, E., & Kaushik, A. S. (2001). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 128(2), 275-283.

Panserat, S., Médale, F., Breque, J., Plagnes-Juan, E., & Kaushik, S. (2000). Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (Oncorhynchus mykiss). *Journal of Nutritional Biochemistry*, 11(1), 22-29.

Pateiro, M., Munekata, P. E., Domínguez, R., Wang, M., Barba, F. J., Bermúdez, R., & Lorenzo, J. M. (2020). Nutritional profiling and the value of processing by-products from gilthead sea bream (Sparus aurata). *Marine Drugs*, 18(2), 101.

Paulino, R. R., Diógenes, A. F., de Oliveira, K. R. B., Orlando, T. M., Rosa, P. V., Peres, H., & Oliva-Teles, A. (2022). Short-term metabolic responses of gilthead seabream, Sparus aurata, fed diets with different protein and protein: Energy levels. *Aquaculture Research*, 53(4), 1205-1217.

Perera, E., Simó-Mirabet, P., Shin, H. S., Rosell-Moll, E., Naya-Catalá, F., de las Heras, V., & Pérez-Sánchez, J. (2019). Selection for growth is associated in gilthead sea bream (Sparus aurata) with diet flexibility, changes in growth patterns and higher intestine plasticity. *Aquaculture*, 507, 349-360.

Phan, L. T. T., Masagounder, K., Mas-Muñoz, J., & Schrama, J. W. (2021). Differences in energy utilization efficiency of digested protein, fat and carbohydrates in snakehead (Channa striata). *Aquaculture*, 532, 736066.

Picos-Corrales, L. A., Morales-Burgos, A. M., Ruelas-Leyva, J. P., Crini, G., García-Armenta, E., Jimenez-Lam, S. A., Ayón-Reyna, L. E., Rocha-Alonzo, F., Calderón-Zamora, L., Osuna-Martínez, U., Calderón-Castro, A., De-Paz-Arroyo, G., & Inzunza-Camacho, L. N. (2023). Chitosan as an outstanding polysaccharide improving health-commodities of humans and environmental protection. *Polymers*, 15(3), 526.

Polakof, S., Moon, T.W., Aguirre, P., Skiba-Cassy, S., & Panserat, S. (2011). Glucose homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin interact in a tissue-dependent manner. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 300, R166–R174.

Polakof, S., Skiba-Cassy, S., & Panserat, S. (2009). Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 297, R1769–R1776.

Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B*, 182, 1015-1045.

Pulido-Rodriguez, L. F., Cardinaletti, G., Secci, G., Randazzo, B., Bruni, L., Cerri, R., & Parisi, G. (2021). Appetite regulation, growth performances and fish quality are modulated by alternative dietary protein ingredients in gilthead sea bream (Sparus aurata) culture. *Animals*, 11(7), 1919.

Ramos-Pinto, L., Machado, M., Calduch-Giner, J., Pérez-Sánchez, J., Dias, J., Conceição, L. E., & Costas, B. (2021). Dietary histidine, threonine, or taurine supplementation affects gilthead seabream (Sparus aurata) immune status. *Animals*, 11(5), 1193.

Rao, B. M., Kole, S., Gireesh-Babu, P., Sharma, R., Tripathi, G., & Bedekar, M. K. (2019). Evaluation of persistence, bio-distribution and environmental transmission of chitosan/PLGA/pDNA vaccine complex against Edwardsiella tarda in Labeo rohita. *Aquaculture*, 500, 385-392.

Rashidpour, A., Silva-Marrero, J. I., Seguí, L., Baanante, I. V., & Metón, I. (2019). Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (Sparus aurata). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 316(3), R265-R273.

Rashidpour, A., Wu, Y., Almajano, M. P., Fàbregas, A., & Metón, I. (2023). Chitosan-Based Sustained Expression of Sterol Regulatory Element-Binding Protein 1a Stimulates Hepatic Glucose Oxidation and Growth in Sparus aurata. *Marine Drugs*, 21(11), 562.

Rito, J., Viegas, I., Pardal, M. A., Metón, I., Baanante, I. V., & Jones, J. G. (2018). Disposition of a glucose load into hepatic glycogen by direct and indirect pathways in juvenile seabass and seabream. *Scientific Reports*, 8(1), 464.

Ruiz, R., Jideonwo, V., Ahn, M., Surendran, S., Tagliabracci, V. S., Hou, Y., Gamble, A., Kerner, J., Irimia-Dominguez, J. M., Puchowicz, M. A., DePaoli-Roach, A., Hoppel, C., Roach, P., & Morral, N. (2014). Sterol Regulatory Element-binding Protein-1 (SREBP-1) Is Required to Regulate Glycogen Synthesis and Gluconeogenic Gene Expression in Mouse Liver. *Journal of Biological Chemistry*, 289(9), 5510–5517.

Sáez, M. I., Vizcaíno, A. J., Alarcón, F. J., & Martínez, T. F. (2018). Feed pellets containing chitosan nanoparticles as plasmid DNA oral delivery system for fish: In vivo assessment in gilthead sea bream (Sparus aurata) juveniles. *Fish & Shellfish Immunology*, 80, 458-466.

Sáez, M.I.; Vizcaíno, A.J.; Alarcón, F.J.; Martínez, T.F. (2017) Comparison of lacZ reporter gene expression in gilthead sea bream (Sparus aurata) following oral or intramuscular administration of plasmid DNA in chitosan nanoparticles. *Aquaculture*, 474, 1–10.

Sáez-Arteaga, A., Wu, Y., Silva-Marrero, J. I., Rashidpour, A., Almajano, M. P., Fernández, F., & Metón, I. (2022). Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (Sparus aurata). *Aquaculture*, 555, 738221.

Sajan, M. P., Lee, M. C., Foufelle, F., Sajan, J., Cleland, C., & Farese, R. V. (2018). Coordinated regulation of hepatic FoxO1, PGC-1α and SREBP-1c facilitates insulin action and resistance. *Cellular Signalling*, 43, 62–70.

Salgado, M. C., Metón, I., Anemaet, I. G., González, J. D., Fernández, F., & Baanante, I. V. (2012). Hepatocyte nuclear factor 4α transactivates the mitochondrial alanine aminotransferase gene in the kidney of Sparus aurata. *Marine Biotechnology*, 14, 46-62.

Salgado, M. C., Metón, I., Egea, M., & Baanante, I. V. (2004). Transcriptional regulation of glucose-6-phosphatase catalytic subunit promoter by insulin and glucose in the carnivorous fish, Sparus aurata. *Journal of Molecular Endocrinology*, 33(3), 783-795.

Salmerón, C. (2018). Adipogenesis in fish. *Journal of Experimental Biology*, 221(Pt Suppl 1), jeb161588.

Sánchez-Gurmaches, J., Cruz-Garcia, L., Ibarz, A., Fernández-Borrás, J., Blasco, J., Gutiérrez, J., & Navarro, I. (2013). Insulin, IGF-I, and muscle MAPK pathway responses after sustained exercise and their contribution to growth and lipid metabolism regulation in gilthead sea bream. *Domestic Animal Endocrinology*, 45(3), 145-153.

Sánchez-Nuño, S., Eroldogan, O. T., Sanahuja, I., Özşahniğlu, I., Blasco, J., Fernández-Borràs, J., & Ibarz, A. (2018). Cold-induced growth arrest in gilthead sea bream Sparus aurata: metabolic reorganisation and recovery. *Aquaculture Environment Interactions*, 10, 511-528.

Shah, B. R., & Mraz, J. (2020). Advances in nanotechnology for sustainable aquaculture and fisheries. *Reviews in Aquaculture*, 12(2), 925-942.

Shi, X. C., Jin, A., Sun, J., Tian, J. J., Ji, H., Chen, L. Q., & Du, Z. Y. (2018). The protein-sparing effect of α-lipoic acid in juvenile grass carp, Ctenopharyngodon idellus: effects on lipolysis, fatty acid β-oxidation and protein synthesis. *British Journal of Nutrition*, 120(9), 977-987.

Shimano, H., & Sato, R. (2017). SREBP-regulated lipid metabolism: convergent physiology—divergent pathophysiology. *Nature Reviews Endocrinology*, 13(12), 710-730.

Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., & Goldstein, J. L. (1997). Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *Journal of Clinical Investigation*, 99(5), 846-854.

Sibiya, A., Al-Ghanim, K. A., Govindarajan, M., Nicoletti, M., Sachivkina, N., & Vaseeharan, B. (2023). Biochemical Patterns and Genotoxicity of the Endocrine Disruptor Metformin in the Freshwater Fish Labeo rohita. *Fishes*, 8, 380.

Sievers, M., Korsøen, Ø., Warren-Myers, F., Oppedal, F., Macaulay, G., Folkedal, O., & Dempster, T. (2022). Submerged cage aquaculture of marine fish: A review of the biological challenges and opportunities. *Reviews in Aquaculture*, 14(1), 106-119.

Silva-Marrero, J. I., Villasante, J., Rashidpour, A., Palma, M., Fàbregas, A., Almajano, M. P., & Metón, I. (2019). The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of Sparus aurata. *Biomolecules*, 9(8), 297.

Smith, L. J., & Peterson, S. (2019). An introduction to aquaculture development. In Aquaculture Development In Less Developed Countries, Routledge, pp 1-10.

Soengas, J. L., Sangiao-Alvarellos, S., Laiz-Carrión, R., & Mancera, J. M. (2019). Energy metabolism and osmotic acclimation in teleost fish. In Fish osmoregulation, CRC Press, pp 277-307.

Song, X., Han, D., Liu, H., Yang, Y., Jin, J., Zhu, X., & Xie, S. (2019). Regulations on glucose metabolism affected by dietary carbohydrate in different strains of juvenile gibel carp (Carassius gibelio). *Aquaculture Research*, 50(4), 1075-1086.

Sun, M., Wang, T., Pang, J., Chen, X., & Liu, Y. (2020a). Hydroxybutyl chitosan centered biocomposites for potential curative applications: A critical review. *Biomacromolecules*, 21(4), 1351-1367.

Sun, Q., Yu, X., Peng, C., Liu, N., Chen, W., Xu, H., Wei, H., Fang, K., Dong, Z., & Fu, C. (2020b). Activation of SREBP-1c alters lipogenesis and promotes tumor growth and metastasis in gastric cancer. *Biomedicine & Pharmacotherapy*, 128, 110274.

Sun, S. X., Wu, J. L., Lv, H. B., Zhang, H. Y., Zhang, J., Limbu, S. M., & Du, Z. Y. (2020c). Environmental estrogen exposure converts lipid metabolism in male fish to a female pattern mediated by AMPK and mTOR signaling pathways. *Journal of Hazardous Materials*, 394, 122537.

Takase, K., & Kakuta, I. (2023). Oral administration of wild plant-derived minerals and red ginseng ameliorates insulin resistance in fish through different pathways. *Physiological Reports*, 11, e15667.

Talijančić, I. (2019). Morphological and ecophysiological adaptations of wild gilthead seabream Sparus aurata associated with tuna farms. *Aquaculture Environment Interactions*, 11, 97-110.

Tan, S. Y., Sethupathi, S., Leong, K. H., & Ahmad, T. (2023). Challenges and opportunities in sustaining aquaculture industry in Malaysia. *Aquaculture International*, 1-31.

Tavlo, M., Skakkebæk, N. E., Mathiesen, E. R., Kristensen, D. M., Kjær, K. H, Andersson, A.-M. & Lindahl-Jacobsen, R. (2022). Hypothesis: Metformin is a potential reproductive toxicant. *Frontiers in Endocrinology*, 13, 1000872.

Teles, A. O., Couto, A., Enes, P., & Peres, H. (2020). Dietary protein requirements of fish–a meta-analysis. *Reviews in Aquaculture*, 12(3), 1445-1477.

Teles, M., Reyes-López, F. E., Fierro-Castro, C., Tort, L., Soares, A. M. V. M., & Oliveira, M. (2018). Modulation of immune genes mRNA levels in mucosal tissues and DNA damage in red blood cells of Sparus aurata by gold nanoparticles. *Marine Pollution Bulletin*, 133, 428-435.

Teodósio, R., Aragão, C., Conceição, L. E., Dias, J., & Engrola, S. (2022). Amino acid metabolism in gilthead seabream is affected by the dietary protein to energy ratios. *Aquaculture Nutrition*, 2022, 8230704.

Thambiliyagodage, C., Jayanetti, M., Mendis, A., Ekanayake, G., Liyanaarachchi, H., & Vigneswaran, S. (2023). Recent advances in chitosan-based applications—a review. Materials, 16(5), 2073.

Thomas, M., Pasquet, A., Aubin, J., Nahon, S., & Lecocq, T. (2021). When more is more: taking advantage of species diversity to move towards sustainable aquaculture. *Biological Reviews*, 96(2), 767-784.

Tocher, D. R., Betancor, M. B., Sprague, M., Olsen, R. E., & Napier, J. A. (2019). Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: Bridging the gap between supply and demand. *Nutrients*, 11(1), 89.

Torno, C., Staats, S., Michl, S. C., de Pascual-Teresa, S., Izquierdo, M., Rimbach, G., & Schulz, C. (2018). Fatty acid composition and fatty acid associated gene-expression in gilthead sea bream (Sparus aurata) are affected by low-fish oil diets, dietary resveratrol, and holding temperature.

Marine Drugs, 16(10), 379.

Torres, M., Navarro, J. C., Varó, I., Agulleiro, M. J., Morais, S., Monroig, Ó., & Hontoria, F. (2020). Expression of genes related to long-chain (C18–22) and very long-chain (> C24) fatty acid biosynthesis in gilthead seabream (Sparus aurata) and Senegalese sole (Solea senegalensis) larvae: Investigating early ontogeny and nutritional regulation. *Aquaculture*, 520, 734949.

Vagner, M., & Santigosa, E. (2011). Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. *Aquaculture*, 315(1-2), 131-143.

Wang, J., Han, S. L., Li, L. Y., Lu, D. L., Limbu, S. M., Li, D. L., & Du, Z. Y. (2018). Lipophagy is essential for lipid metabolism in fish. *Science Bulletin*, 63, 879-882.

Wang, L., Sagada, G., Wang, C., Liu, R., Li, Q., Zhang, C., & Yan, Y. (2023). Exogenous bile acids regulate energy metabolism and improve the health condition of farmed fish. *Aquaculture*, 562, 738852.

Wang, X., Gong, Y., Li, W., Liu, N., Fang, Z., Zhang, N., & Li, S. (2024). The beneficial effects of metformin inclusion on growth performance, glucose utilization, antioxidant capacity and apoptosis of largemouth bass (Micropterus salmoides) fed with high dietary carbohydrates. *Aquaculture*, 588, 740957.

Wang, Y. V., Wan, A. H., Krogdahl, Å., Johnson, M., & Larsen, T. (2019). 13C values of glycolytic amino acids as indicators of carbohydrate utilization in carnivorous fish. *PeerJ*, 7, e7701.

Wang, Y., Hamid, N., Jia, P. P., & Pei, D. S. (2021). A comprehensive review on genetically modified fish: key techniques, applications and future prospects. *Reviews in Aquaculture*, 13(3), 1635-1660.

Wei, J., Qi, H., Liu, K., Zhao, C., Bian, Y., & Li, G. (2020). Effects of metformin on life span, cognitive ability, and inflammatory response in a short-lived fish. *The Journals of Gerontology: Series A*, 75(11), 2042-2050.

Wisdom, K. S., Bhat, I. A., Chanu, T. I., Kumar, P., Pathakota, G. B., Nayak, S. K., & Sharma, R. (2020). Chitosan grafting onto single-walled carbon nanotubes increased their stability and reduced the toxicity in vivo (catfish) model. *International Journal of Biological Macromolecules*, 155, 697-707.

Wu, Y., Rashidpour, A., Almajano, M. P., & Metón, I. (2020). Chitosan-based drug delivery system: Applications in fish biotechnology. *Polymers*, 12(5), 1177.

Wu, Y., Rashidpour, A., Fàbregas, A., Almajano, M. P., & Metón, I. (2024). Chitosan-based delivery of fish codon-optimised Caenorhabditis elegans FAT-1 and FAT-2 boosts EPA and DHA biosynthesis in Sparus aurata. *Reviews in Fish Biology and Fisheries*, Epub: 09 April 2024. https://doi.org/10.1007/s11160-024-09852-4.

Xia, I. F., Cheung, J. S., Wu, M., Wong, K. S., Kong, H. K., Zheng, X. T., & Kwok, K. W. (2019). Dietary chitosan-selenium nanoparticle (CTS-SeNP) enhance immunity and disease resistance in zebrafish. *Fish & Shellfish Immunology*, 87, 449-459.

Xie, D., Chen, C., Dong, Y., You, C., Wang, S., Monroig, O., & Li, Y. (2021). Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. *Progress in Lipid Research*, 82, 101095.

Xie, S., Yin, P., Tian, L., Liu, Y., & Niu, J. (2020). Lipid metabolism and plasma metabolomics of juvenile largemouth bass Micropterus salmoides were affected by dietary oxidized fish oil. *Aquaculture*, 522, 735158.

Xu, C., Liu, W. B., Zhang, D. D., Cao, X. F., Shi, H. J., & Li, X. F. (2018). Interactions between dietary carbohydrate and metformin: implications on energy sensing, insulin signaling pathway, glycolipid metabolism and glucose tolerance in blunt snout bream Megalobrama amblycephala. *Aquaculture*, 483, 183-195.

Yamamoto, T., Shimano, H., Nakagawa, Y., Ide, T., Yahagi, N., Matsuzaka, T., & Yamada, N. (2004). SREBP-1 interacts with hepatocyte nuclear factor-4α and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *Journal of Biological Chemistry*, 279(13), 12027-12035.

Yin, F., Feng, F., Wang, L., Wang, X., Li, Z., & Cao, Y. (2019a). SREBP-1 inhibitor Betulin enhances the antitumor effect of Sorafenib on hepatocellular carcinoma via restricting cellular glycolytic activity. *Cell Death and Disease*, 10, 672.

Yin, P., Xie, S., Huo, Y., Guo, T., Fang, H., Zhang, Y., & Niu, J. (2019b). Effects of dietary oxidized fish oil on growth performance, antioxidant defense system, apoptosis and mitochondrial function of juvenile largemouth bass (Micropterus salmoides). *Aquaculture*, 500, 347-358.

Younus, N., Zuberi, A., Rashidpour, A., & Metón, I. (2020). Dietary cobalt supplementation improves growth and body composition and induces the expression of growth and stress response genes in Tor putitora. *Fish Physiology and Biochemistry*, 46, 371-381.

Zhang, D., Yan, Y., Tian, H., Jiang, G., Li, X., & Liu, W. (2018). Resveratrol supplementation improves lipid and glucose metabolism in high-fat diet-fed blunt snout bream. *Fish Physiology and Biochemistry*, 44, 163-173.

Zhang, Q., Xie, Y., Zhang, Y., Huang, E., Meng, L., Liu, Y., & Tong, T. (2024). Effects of Dietary Supplementation with Chitosan on the Muscle Composition, Digestion, Lipid Metabolism, and Stress Resistance of Juvenile Tilapia (Oreochromis niloticus) Exposed to Cadmium-Induced Stress. *Animals*, 14(4), 541.

Zhang, Y., Lu, R., Qin, C., & Nie, G. (2020). Precision nutritional regulation and aquaculture. *Aquaculture Reports*, 18, 100496.

Zhou, R., Ding, R.-C., Yu, Q., Qiu, C.-Z., Zhang, H.-Y., Yin, Z.-J., & Ren, D.-L. (2024). Metformin Attenuates Neutrophil Recruitment through the H3K18 Lactylation/Reactive Oxygen Species Pathway in Zebrafish. *Antioxidants*, 13, 176.

Zhou, W. H., Wu, C. C., Limbu, S. M., Li, R. X., Chen, L. Q., Qiao, F., & Du, Z. Y. (2022). More simple more worse: simple carbohydrate diets cause alterations in glucose and lipid metabolism in Nile tilapia (Oreochromis niloticus). *Aquaculture*, 550, 737857.

Zhou, W., Rahimnejad, S., Tocher, D. R., Lu, K., Zhang, C., & Sun, Y. (2019). Metformin attenuates lipid accumulation in hepatocytes of blunt snout bream (Megalobrama amblycephala) via activation of AMP-activated protein kinase. *Aquaculture*, 499, 90-100.

Appendix

Other publications of Ania Rashidpour

- Silva-Marrero JI; Villasante J; **Rashidpour A**; Palma M; Fàbregas A; Almajano MP; Viegas I; Jones JG; Miñarro M; Ticó JR et al. (2019) The Administration of Chitosan-Tripolyphosphate-DNA Nanoparticles to Express Exogenous SREBP1a Enhances Conversion of Dietary Carbohydrates into Lipids in the Liver of Sparus aurata. *Biomolecules*, 9:297.

- Younus N; Zuberi A; **Rashidpour A**; Metón I. (2020) Dietary cobalt supplementation improves growth and body composition and induces the expression of growth and stress response genes in Tor putitora. *Fish Physiology and Biochemistry*, 46:371-381.

- Sáez-Arteaga A; Wu Y; Silva-Marrero JI; **Rashidpour A**; Almajano MP; Fernandez F; Baanante IV; Metón I. (2022) Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (Sparus aurata). *Aquaculture*, 555: 738221.

- Wu Y; **Rashidpour A**; Fàbregas A; Almajano MP; Metón I. (2024) Chitosan-based delivery of fish codon-optimised Caenorhabditis elegans FAT-1 and FAT-2 boosts EPA and DHA biosynthesis in Sparus aurata. *Reviews in Fish Biology and Fisheries*, electronic publication 2024 April 09. https://doi.org/10.1007/s11160-024-09852-4.

biomolecules

MDPI

Article

The Administration of Chitosan-Tripolyphosphate-DNA Nanoparticles to Express Exogenous SREBP1a Enhances Conversion of Dietary Carbohydrates into Lipids in the Liver of *Sparus aurata*

Jonás I. Silva-Marrero [1], Juliana Villasante [1,2], Ania Rashidpour [1], Mariana Palma [3], Anna Fàbregas [4], María Pilar Almajano [2], Ivan Viegas [3,5], John G. Jones [5], Montserrat Miñarro [4], Josep R. Ticó [4], Isabel V. Baanante [1] and Isidoro Metón [1,*]

1 Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27–31, 08028 Barcelona, Spain
2 Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain
3 Center for Functional Ecology (CFE), Department Life Sciences, University of Coimbra, Calçada Martins de Freitas, 3000-456 Coimbra, Portugal
4 Departament de Farmàcia i Tecnologia Farmacèutica, i Fisicoquímica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain
5 Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal
* Correspondence: imeton@ub.edu; Tel.: +34-93-4024521

Received: 30 June 2019; Accepted: 19 July 2019; Published: 24 July 2019

Abstract: In addition to being essential for the transcription of genes involved in cellular lipogenesis, increasing evidence associates sterol regulatory element binding proteins (SREBPs) with the transcriptional control of carbohydrate metabolism. The aim of this study was to assess the effect of overexpression SREBP1a, a potent activator of all SREBP-responsive genes, on the intermediary metabolism of *Sparus aurata*, a glucose-intolerant carnivorous fish. Administration of chitosan-tripolyphosphate nanoparticles complexed with a plasmid driving expression of the N-terminal transactivation domain of SREBP1a significantly increased SREBP1a mRNA and protein in the liver of *S. aurata*. Overexpression of SREBP1a enhanced the hepatic expression of key genes in glycolysis-gluconeogenesis (glucokinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), fatty acid synthesis (acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2), elongation (elongation of very long chain fatty acids protein 5) and desaturation (fatty acid desaturase 2) as well as reduced nicotinamide adenine dinucleotide phosphate production (glucose-6-phosphate 1-dehydrogenase) and cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A reductase), leading to increased blood triglycerides and cholesterol levels. Beyond reporting the first study addressing in vivo effects of exogenous SREBP1a in a glucose-intolerant model, our findings support that SREBP1a overexpression caused multigenic effects that favoured hepatic glycolysis and lipogenesis and thus enabled protein sparing by improving dietary carbohydrate conversion into fatty acids and cholesterol.

Keywords: SREBP1; chitosan; nanoparticles; gene delivery; metabolism; *Sparus aurata*

1. Introduction

The sterol regulatory element binding protein (SREBP) family of transcription factors has a major role in regulating the expression of genes that control cellular lipid biosynthesis [1,2]. SREBPs are synthesized as membrane-bound precursors that remain anchored to the endoplasmic

reticulum membrane. The C-terminal domain of SREBP precursors associates with a sterol sensor, the SREBP-cleavage activating protein (Scap), which in turn interacts with insulin-induced gene (Insig) protein [3,4]. Sterol deprivation drives the SREBP/Scap complex to the Golgi apparatus, where a two-step cleavage process that involves site-1 and site-2 proteases (S1P and S2P, respectively) releases the N-terminal domain of SREBPs, which are transcriptionally active basic-helix-loop-helix leucine zipper (bHLH-Zip) factors [5]. After dimerization, mature SREBPs translocate to the nucleus and transactivate target genes by binding to sterol regulatory element (SRE) sequences [6].

The activity of alternate promoters in the *srebf1* gene generates two isoforms of SREBP1 in mammals (SREBP1a and SREBP1c), while a second gene, *srebf2*, encodes for the SREBP2 protein [7]. Alternatively spliced variants have been reported for both genes [8–10]. SREBP1c primarily transactivates genes required for fatty acid and triglyceride synthesis, while SREBP2 regulates the transcription of genes associated with cholesterol metabolism [7,11]. SREBP1a exhibits stronger transcriptional activity than SREBP1c due to the presence of a longer N-terminal transactivation domain, and it is a potent activator of all SREBP-responsive genes [12–14]. Recent advances highlight the role of insulin-dependent activation of SREBPs through the PI3K-Akt-mTOR pathway in pathophysiological processes associated with impaired lipid metabolism, such as diabetes mellitus, non-alcoholic fatty liver disease, neurogenerative diseases, innate immunity and cancer [1,7].

In addition to the well-known effect of SREBP1 isoforms on lipid metabolism [1,2,7], evidence supports the involvement of SREBP1 in glucose metabolism. In the mammalian liver, SREBP1c mediates insulin-dependent upregulation of hexokinase II and glucokinase (GK) transcription by transactivating the promoters of both genes [15–18]. Furthermore, overexpression of SREBP1a in transgenic mice decreases the hepatic gluconeogenic flux due to the suppression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G6Pase) transcriptional activation mediated by hepatocyte nuclear factor-4α [19]. In a carnivorous fish, gilthead sea bream (*Sparus aurata*), we previously reported that SREBP1 upregulates the transcription of GK and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1) by binding to SRE boxes located in their respective gene promoters [20,21].

Carnivorous fish make efficient use of dietary protein to provide amino acids that serve as building blocks for the synthesis of new proteins and catabolic substrates to produce energy. On the contrary, glucose resulting from dietary carbohydrate digestion and free sugars are metabolised markedly slower than in mammals, and give rise to prolonged hyperglycemia [22]. Dysregulation of rate-limiting enzymes in glycolysis-gluconeogenesis that control the hepatic flux through the glucose/glucose-6-phosphate substrate cycle, GK and G6Pase, seems to play a critical role in glucose intolerance of carnivorous fish. *S. aurata* GK exhibits lower affinity for glucose than rat GK and delayed postprandial stimulation of GK mRNA levels in the liver [23]. On the other hand, the content of dietary carbohydrate fails to regulate the hepatic expression of G6Pase, which in turn is poorly repressed by insulin [24–26].

Despite *S. aurata* being a carnivorous fish, we previously showed that it tolerates partial substitution of dietary protein by carbohydrates through a metabolic adaptation that involves increased glucose oxidation, decreased gluconeogenesis and weaker transamination capacity in the liver [26–30]. Given that SREBP1 can promote a dual effect on the expression of lipogenic genes and genes encoding key enzymes in the regulation of glycolysis-gluconeogenesis, this transcription factor may be a unique target to facilitate conversion of dietary carbohydrates into lipids. In this study, with the aim to increase de novo lipogenesis from dietary carbohydrates in carnivorous fish, *S. aurata* juveniles were administered intraperitoneally chitosan-tripolyphosphate (TPP) nanoparticles complexed with a plasmid expressing the N-terminal transactivation domain of SREBP1a. The metabolic effect of SREBP1a overexpression was addressed in fish fed diets differing in macronutrient composition.

2. Materials and Methods

2.1. Animals

S. aurata juveniles obtained from Piscimar (Burriana, Castellón, Spain) with an average weight of 13.03 g ± 0.28 (mean ± SEM, n = 45) were maintained at 20 °C in 260-L aquaria supplied with running seawater as described [31]. Two groups of fish received isoenergetic diets differing in protein to carbohydrate ratio (HLL: high protein, low lipid, low carbohydrate; and LLH: low protein, low lipid, high carbohydrate). The composition of diets HLL and LLH is shown in Table 1. The diets were supplied at a ration of 40 g/kg body weight (BW) once a day (10:00) for 15 days. To study the effects of the expression of exogenous SREBP1a on *S. aurata* liver metabolism, fish fed diets HLL and LLH were intraperitoneally injected with chitosan-TPP nanoparticles complexed with a plasmid expressing the N-terminal active domain of hamster SREBP1a (pSG5-SREBP1a; [20]), or empty vector (pSG5; Agilent Technologies, Palo Alto, CA, USA) as a negative control, at a concentration of 10 µg of plasmid per gram BW. Seventy-two hours following the treatment and 24 h after the last meal, fish were sacrificed by cervical section, blood was collected and the liver was dissected out, frozen in liquid N_2 and kept at −80 °C until use. To prevent stress, fish were anesthetised with tricaine methanesulfonate (MS-222; 78.4 mg/l) before handling. Experimental procedures involving fish were performed in accordance with the guidelines of the University of Barcelona's Animal Welfare Committee (proceeding #461/16), in compliance with local legislation and EU Directive 2010/63/EU for animal experiments.

Table 1. Composition of the diets supplied in this study to *S. aurata*.

	HLL	LLH
Formulation (%)		
Fish meal [1]	81.6	54.3
Fish oil [2]	0.8	6.0
Starch [3]	15.0	37.1
Vitamin mixture [4]	0.2	0.2
Mineral mixture [5]	0.9	0.9
Carrageenan [6]	1.5	1.5
Proximate composition (%)		
Protein	58.0	38.6
Carbohydrates [7]	15.0	37.1
Fat	9.9	12.1
Ash	15.4	10.5
Gross energy (kJ/g) [8]	20.1	20.0

[1] Corpesca S.A. Super-Prime fish meal (Santiago de Chile, Chile). [2] Fish oil from A.F.A.M.S.A. (Vigo, Spain). [3] Pregelatinised corn starch from Brenntag Química S.A. (St. Andreu de la Barca, Barcelona, Spain). [4] Vitamin mixture provided (mg/kg): choline chloride, 1200; myo-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-rac-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; piridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-trans-retinol, 2; biotin, 0,7 cholecalciferol, 0.05; cyanocobalamin, 0.05. [5] Mineral mixture provided (mg/kg): $CaHPO_4.2H_2O$, 7340; MgO, 800; KCl, 750; $FeSO_4.7H_2O$, 60; ZnO, 30; MnO_2, 15; $CuSO_4.5H_2O$, 1.7; $CoCl_2.6H_2O$, 1.5; KI, 1.5; Na_2SeO_3, 0.3. [6] Iota carrageenan (Sigma-Aldrich). [7] Carbohydrates were calculated by difference. [8] Calculated from the gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17 kJ/g).

2.2. Chitosan-TPP-DNA Nanoparticles

Chitosan-TPP nanoparticles complexed with pSG5 or pSG5-SREBP1a were prepared following the ionic gelation method as previously described [32]. Low molecular weight chitosan (Sigma-Aldrich, St. Louis, MO, USA) was dissolved (2 mg/mL) in acetate buffer solution (pH 4.5) under magnetic stirring for 3 h and then filtered. Three-hundred microgram of pSG5 or pSG5-SREBP1a was dissolved in 1.2 mL of 0.84 mg/mL TPP (Sigma-Aldrich, St. Louis, MO, USA). TPP-DNA solutions were added dropwise to 3 mL of the chitosan-acetate buffer solution (1:0.4 chitosan:TPP ratio) under vigorous magnetic stirring. No DNA was used to obtain naked chitosan-TPP nanoparticles. Chitosan-TPP nanoparticles complexed in the presence and absence of DNA were sedimented by centrifugation at 19,000× g for 10

min at 15 °C, followed by washing twice with ultrapure water and resuspension in 2 mL of 2% *w/v* mannitol, which was used as a cryoprotector during lyophilisation. After a freeze-drying cycle at −47 °C, an additional drying step was carried out at 25 °C to remove residual water. Morphological characterisation of nanoparticles was performed by means of atomic force microscopy using peak force tapping mode (Multimode 8 AFM attached to a Nanoscope III Electronics, Bruker, USA). A Zetasizer Nano ZS fitted with a 633 nm laser (Malvern Instruments, Malvern, UK) was used to determine particle diameter by dynamic light scattering and *Z* potential of nanoparticles by laser Doppler electrophoresis. Chitosan-TPP-DNA nanoparticles were intraperitoneally administered to *S. aurata* after resuspension in 0.9 % NaCl.

2.3. Enzyme Activity Assays and Metabolites

Enzyme activity assays were performed in liver crude extracts obtained by homogenisation (1:5, *w/v*) of powdered frozen tissue in 50 mM Tris-HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 250 mM sucrose. Homogenisation was performed with a PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland) (position 3, 30 s). After centrifugation at 20,000× *g* for 30 min at 4 °C, the supernatant was collected and used to perform enzyme activity assays. GK, 6-phosphofructo-1-kinase (PFK1), fructose-1,6-bisphosphatase (FBPase1) and total protein were assayed as described [23,27]. One unit of enzyme activity was defined as the amount of enzyme necessary for transforming 1 µmol of substrate per min, except for PFK1 activity, which expressed the amount of enzyme needed to oxidise 2 µmol of NADH per min. Enzyme activities were expressed per mg of soluble protein (specific activity). Glucose, triglycerides and cholesterol were measured with commercial kits (Linear Chemicals, Montgat, Barcelona, Spain). Spectrophotometric determinations were performed at 30 °C in a Cobas Mira S analyser (Hoffman-La Roche, Basel, Switzerland).

2.4. Reverse Transcriptase-Coupled Quantitative Real Time PCR (RT-qPCR)

Total RNA from the liver of *S. aurata* was isolated by means of RNeasy Tissue Mini Kit (QIAGEN, Sussex, UK) and reverse-transcribed with Moloney murine leukaemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 °C in the presence of random hexamer primers. The mRNA levels of hamster SREBP1a and *S. aurata* GK, PFKFB1, acetyl-CoA carboxylase 1 (ACC1), acetyl-CoA carboxylase 2 (ACC2), fatty acid synthase (FASN), elongation of very long chain fatty acids protein 5 (ELOVL5), fatty acid desaturase 2 (FADS2), glucose-6-phosphate 1-dehydrogenase (G6PD) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) were determined in a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The 20-µL mixture contained 0.4 µM of each primer (Table 2), 10 µL of SYBR Green (Applied Biosystems, Foster City, CA, USA), and 1.6 µL of diluted cDNA. The amplification cycle protocol was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. Dissociation curves were applied after each experiment to confirm the amplification of single products. The size of amplicons was checked by agarose gel electrophoresis. Specificity of the reaction was assayed by sequencing amplicons at least once for each gene. Serial dilutions of control cDNA were used to generate standard curves in order to determine the efficiency of the amplification reaction for each gene. The mRNA levels for genes of interest in each sample were normalised using *S. aurata* ribosomal subunit 18s, β-actin and elongation factor 1 α (EF1α) as endogenous controls. The standard $\Delta\Delta C_T$ method was used to calculate variations in gene expression [33].

Table 2. Primer sequences used for reverse transcriptase-coupled quantitative real time PCR (RT-qPCR) in the present study.

Gene	Forward Sequences (5′ to 3′)	Reverse Sequences (5′ to 3′)	GenBank Accession
SREBP1a	CCTCCTGCCTCCGAGTTTCC	GAAGGAAGGCTAGAATACCCC	U09103
GK	TGTGTCAGCTCTCAACTCGACC	AGGATCTGCTCTACCATGTGGAT	AF169368
PFKFB1	TCGTGATGGTGGGACTGCCG	CTCGGCGTTGTCGGCTCTGAAG	U84724
ACC1	CCCAACTTCTTCTACCACAG	GAACTGGAACTCTACTACAC	JX073712
ACC2	TGACATGAGTCCTGTGCTGG	GCCTCAGTTCGTATGATGGT	JX073714
FASN	TGGGTCAGGGGGAGTTGG	TTGTGTGGAGAAGAACGTGCTGT	JQ277708
ELOVL5	GGGATGGCTACTGCTCGACA	CAGGAGAGTGAGGCCCAGAT	AY660879
FADS2	CACTATGCTGGAGAGGATGCC	TATTTCGGTCCTGGCTGGGC	AY055749
G6PD	TGATGATCCAACAGTTCCTA	GCTCGTTCCTGACACACTGA	JX073711
HMGCR	ACTGATGGCTGCTCTGGCTG	GGGACTGAGGGATGACGCAC	MN047456
18s	TTACGCCCATGTTGTCCTGAG	AGGATTCTGCATGATGGTCACC	AM490061
β-actin	CTGGCATCACACCTTCTACAACGAG	GCGGGGGTGTTGAAGGTCTC	X89920
EF1α	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	AF184170

2.5. Western Blot

Fifty microgram of protein extract from liver samples was loaded per lane and submitted to electrophoresis in a 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. When electrophoresis was completed, the gel was equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20 % methanol, pH 8.3), and electroelution proceeded at 4 °C onto NytranN nylon membranes (Whatman, Kent, UK) for 2 h at 60 V. A rabbit polyclonal antibody against rodent SREBP1 was used as the primary antibody (sc-8984, Santa Cruz Biotechnology, Dallas, TX, USA; 1:500). The Immun-Star Chemiluminescent Kit (Bio-Rad, Hercules, CA, USA) was used to immunodetect SREBP1 with an alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, Saint Louis, MO, USA; 1:3000).

2.6. Obtention of Aqueous and Organic Fractions from the Liver

Organic and aqueous fractions were extracted from powdered frozen livers by the Folch method [34], with modifications. Briefly, 0.75 mL of methanol:water solution 2:1 (v/v) was added to 50 mg of liver sample and vortexed 1 min. Following the addition of 0.5 mL of chloroform and 0.25 mL of water (vortexing 1 min after every addition), and centrifugation at 2000× *g* for 20 min at 4 °C, upper and lower fractions were collected separately. The upper phase (methanol/water) contained water-soluble metabolites and was dried in a vacuum concentrator and stored at −20 °C until subsequent analysis by nuclear magnetic resonance (NMR). The lower phase (chloroform), containing lipids, was transferred to amber glass vials and dried under a nitrogen stream. After the addition of 2 mL hexane, samples were vortexed for 30 s and left to rest for 5 min to ensure fat dilution before adding 200 µL 2 N potassium hydroxide in methanol solution and centrifuging for 10 min at 2000× *g*. The upper phase was collected and kept at −80 °C until gas chromatography was performed.

2.7. NMR Analysis and Liver Metabolite Identification

The aqueous fractions were resuspended in 200 µL of 99.8 % $^{2}H_2O$ and 40 µL phosphate buffer (50 mM; pD 7.41; with 5 mM 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt and sodium azide in $^{2}H_2O$). Samples were transferred into 3 mm NMR tubes. Proton (^{1}H) NMR spectroscopy was conducted on a Varian VNMRS 600 MHz (Agilent, Santa Clara, CA, USA) spectrometer, equipped with a 3 mm ID-PFG broadband probe, at 298 K. Spectra were collected using a ^{1}H-Presat pulse sequence (spectral width: 7200 Hz; relaxation delay: 4 s; saturation time: 3 s; acquisition time: 3 s). All spectra were processed with the ACD/NMR Processor Academic Edition from ACD\Labs 12.0 software (Advanced Chemistry Development, Inc.) applying: zero-filling to 65 k, line broadening of 0.2 Hz, phasing and baseline correction. The chemical shifts were referenced to TSP peak at 0 ppm. Metabolite identification and quantification was performed using ACD/NMR Processor Academic Edition from

ACD\Labs 12.0 software (Advanced Chemistry Development, Inc.), assisted by the Chenomx NMR Suite Library version 10 (Chenomx Inc., Edmonton, Canada) and the Bayesil software [35].

2.8. Fatty Acid Methyl Ester (FAME) Analysis

Fatty acid composition was analysed as described [36], using a GC-2025 with autosampler (Shimadzu, Kyoto, Japan) equipped with flame ionization detector and BPX70, 30 m × 0.25 mm × 0.25 µm, capillary column (Trajan Scientific and Medical, Ringwood, Australia). The oven temperature was held 1 min at 60 °C and then raised to 260 °C at the rate of 6 °C/min. Injector (AOC-20i, Shimadzu, Kyoto, Japan) and detector temperatures were set at 260 °C and 280 °C, respectively. One microlitre of sample was injected with helium as carrier gas and split ratio 1:20. Fatty acids were identified by comparing FAME retention times with the Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA).

2.9. Statistics

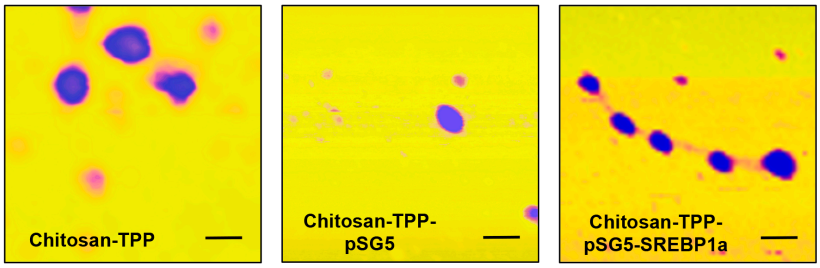
Analyses were performed with SPSS software Version 24 (IBM, Armonk, NY, USA). Data were submitted to two-way analysis of variance with diet (HLL vs. LLH) and treatment (control vs. SREBP1a-treated fish) as independent variables.

3. Results

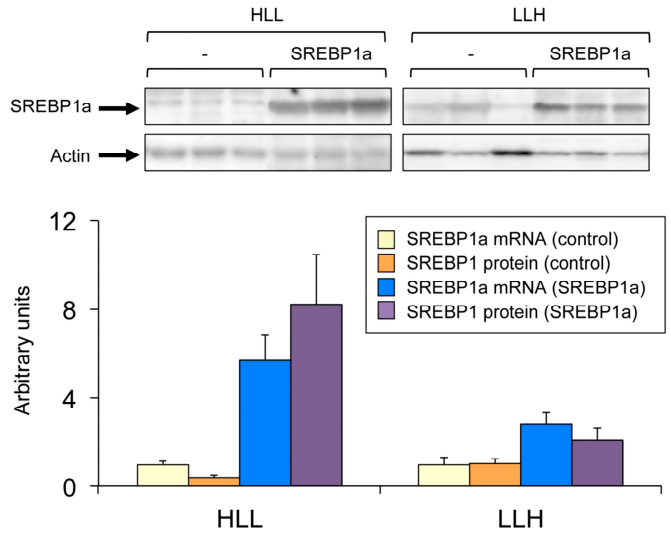
3.1. Intraperitoneal Administration of Chitosan-TPP-pSG5-SREBP1a Increases mRNA and Immunodetectable Levels of SREBP1a in the Liver

To study the metabolic effects resulting from overexpression of exogenous SREBP1a in the liver of *S. aurata*, ionic gelation was used to obtain chitosan-TPP nanoparticles complexed with an expression vector encoding the N-terminal nuclear fragment of hamster SREBP1a (pSG5-SREBP1a), which was shown to transactivate *S. aurata* GK and PFKFB1 genes in previous studies [20,21]. As a control, chitosan-TPP nanoparticles complexed with empty vector (pSG5) were also synthesised. Atomic force microscopy showed that chitosan-TPP nanoparticles presented rounded morphology, while dynamic light scattering indicated a mean diameter size of 230.9 nm ± 18.3 (mean ± standard error of the mean (SEM), $n = 3$). The mean Z potential was 31.2 mV ± 1.5 (mean ± SEM, $n = 3$). Incorporation of plasmid DNA to chitosan-TPP nanoparticles did not significantly affect morphology or diameter size, while decreased Z potential (Figure 1a). Mean diameter size and Z potential, were expressed as mean ± SEM, ($n = 3$), of chitosan-TPP-pSG5 were 233.6 nm ± 45.9 and 13.2 mV ± 0.4, respectively, while the values for chitosan-TPP-pSG5-SREBP1a were 272.3 nm ± 36.0 and 14.1 mV ± 2.3.

Twenty-four hours after the last meal, *S. aurata* fed HLL and LLH diets received an intraperitoneal injection of chitosan-TPP complexed with 10 µg/g BW of plasmid (pSG5-SREBP1a or empty vector, pSG5). To validate overexpression of SREBP1a in the liver of fish administered with chitosan-TPP-pSG5-SREBP1a, hepatic mRNA and immunodetectable levels of SREBP1a were determined 72 h post-treatment. As shown in Figure 1b, treatment with chitosan-TPP-pSG5-SREBP1a increased both mRNA abundance and the protein content of SREBP1a in the liver. A stronger effect was observed in fish fed HLL.



(**a**)



Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment
SREBP1a mRNA	NS	NS	**
SREBP1 protein	**	*	***

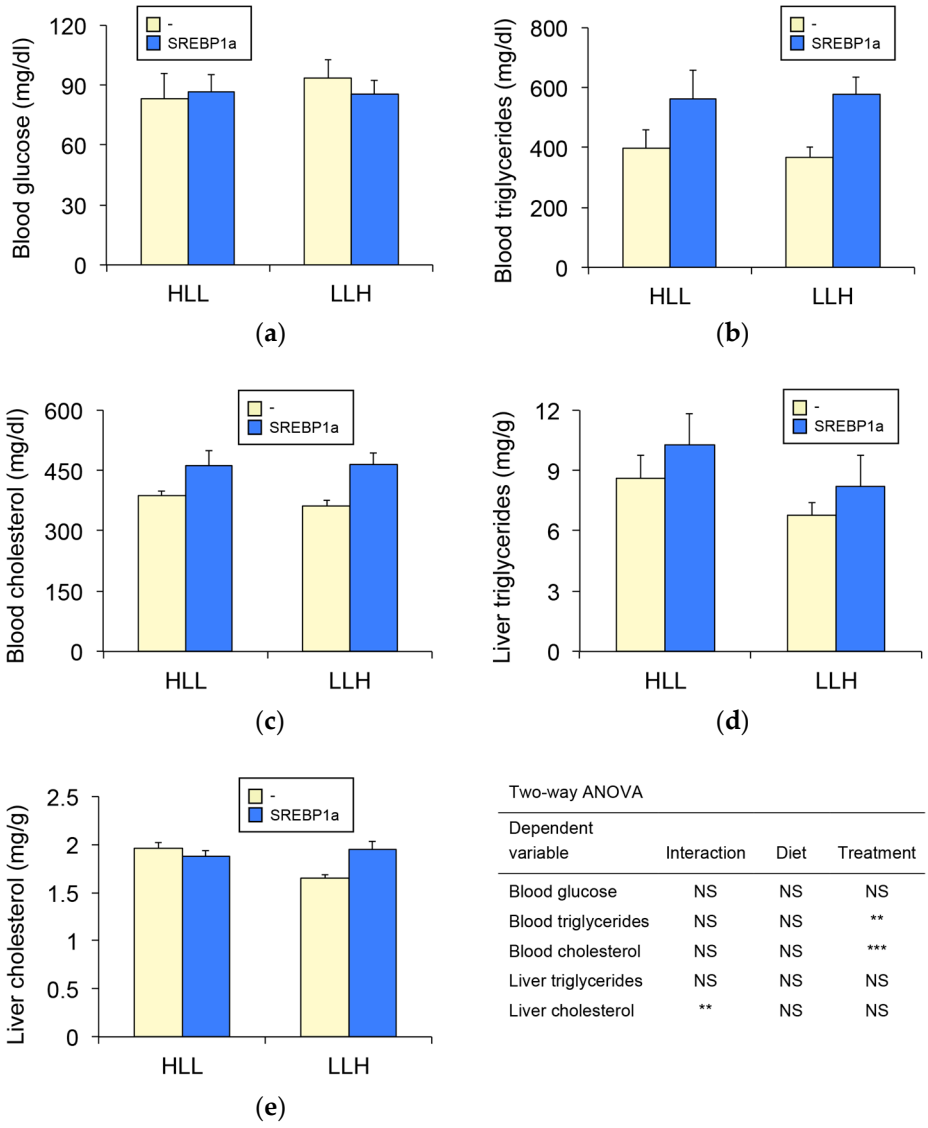
(**b**)

Figure 1. Effect of chitosan-TPP-pSG5-SREBP1a administration on SREBP1 mRNA and immunodetectable levels in the liver of *S. aurata*. (**a**) Representative images of chitosan-TPP, chitosan-TPP-pSG5 and chitosan-TPP-pSG5-SREBP1a nanoparticles obtained by atomic force microscopy. Black bars correspond to 200 nm. (**b**) The upper part of the panel shows a representative Western blot analysis of immunodetectable SREBP1 levels in liver extracts of of *S. aurata* fed diet HLL (high protein, low lipid, low carbohydrate) or LLH (low protein, low lipid, high carbohydrate) following 72 h of intraperitoneal administration with chitosan-TPP nanoparticles complexed with 10 μg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). Two independent experiments were performed. The lower part of the panel shows the effect of chitosan-TPP-pSG5 (control) and chitosan-TPP-pSG5-SREBP1a (SREBP1a) nanoparticle administration on SREBP1 mRNA and protein levels in the liver of *S. aurata* fed diet HLL or LLH. Analysis of SREBP1 mRNA levels relative to the geometric mean of ribosomal subunit 18s, β-actin and EF1α were performed by RT-qPCR in liver samples of *S. aurata* at 72 h post-treatment. The values are expressed as mean ± standard error of the mean (SEM) (n = 6–7). Statistical significance for independent variables (diet and treatment) is indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *NS* not significant.

3.2. *Effect of SREBP1a Overexpression on Serum and Aqueous Metabolites in the Liver*

Given that administration of chitosan-TPP-pSG5-SREBP1a significantly induced SREBP1a expression in the liver of *S. aurata*, we addressed the metabolic effects resulting from hepatic upregulation of SREBP1a. Concerning serum metabolites and regardless of the diet, SREBP1a overexpression significantly increased circulating triglycerides (1.2 to 1.3-fold) and cholesterol (1.4 to

1.6-fold), while did not affect blood glucose levels (Figure 2a–c). Although not significant, liver triglycerides also showed a trend to increase in SREBP1a-treated fish (Figure 2d), while only fish fed LLH presented higher cholesterol levels (Figure 2e).



Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment
Blood glucose	NS	NS	NS
Blood triglycerides	NS	NS	**
Blood cholesterol	NS	NS	***
Liver triglycerides	NS	NS	NS
Liver cholesterol	**	NS	NS

Figure 2. Effect of chitosan-TPP-pSG5-SREBP1a administration on serum and liver metabolite levels in *S. aurata*. Twenty-four h after the last meal, fish fed diet HLL or LLH were intraperitoneally administered with chitosan-TPP nanoparticles complexed with 10 µg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). Seventy-two hours following the treatment, the liver and blood were collected. The levels of blood glucose (**a**), blood triglycerides (**b**), blood cholesterol (**c**), liver triglycerides (**d**) and liver cholesterol (**e**) are presented as mean ± SEM (n = 6–9). Statistical significance for independent variables (diet and treatment) is indicated as follows: **$p < 0.01$; ***$p < 0.001$; *NS* not significant.

^{1}H-NMR studies allowed us to analyse the effect of SREBP1a overexpression in aqueous metabolites in the liver. Table 3 shows that 72 h post-treatment with chitosan-TPP-pSG5-SREBP1a nanoparticles significantly increased hepatic alanine (1.3 to 1.5-fold) and glycine (1.5 to 1.6-fold), while decreased formate (72 % to 80 % of the values observed in control animals, treated with empty vector). The SREBP1a-dependent effect on alanine levels was not affected by the diet. However, in addition to the effect of nanoparticle administration, the supply of the low protein, high carbohydrate diet (LLH)

significantly increased glycine (2.2-fold) and formate (1.3-fold). Acetate levels significantly increased (1.2-fold) in the liver of *S. aurata* fed diet LLH, while they were not affected by SREBP1a overexpression.

Table 3. Effect of SREBP1a overexpression on aqueous metabolites in the liver of *S. aurata*.

	HLL		LLH		Two-way ANOVA		
Metabolite (mM)	**-**	**SREBP1a**	**-**	**SREBP1a**	**Interaction**	**Diet**	**Treatment**
Lactate + Thr	0.96 ± 0.16	1.13 ± 0.19	0.78 ± 0.16	1.20 ± 0.35	NS	NS	NS
Alanine	3.21 ± 0.67	4.12 ± 0.72	2.87 ± 0.29	4.35 ± 0.31	NS	NS	*
Glucose	3.22 ± 0.44	4.44 ± 0.98	1.87 ± 0.31	3.27 ± 1.12	NS	NS	NS
Acetate	0.22 ± 0.02	0.21 ± 0.03	0.27 ± 0.02	0.28 ± 0.03	NS	*	NS
Isoleucine	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	NS	NS	NS
Valine	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.00	0.07 ± 0.01	NS	NS	NS
Creatinine	0.09 ± 0.02	0.09 ± 0.01	0.29 ± 0.05	0.23 ± 0.05	***	NS	NS
Choline compounds	0.44 ± 0.03	0.43 ± 0.08	0.36 ± 0.03	0.39 ± 0.03	NS	NS	NS
Succinate	0.37 ± 0.05	0.45 ± 0.09	0.24 ± 0.04	0.38 ± 0.09	NS	NS	NS
Sarcosine	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	NS	NS	NS
Taurine	6.46 ± 0.45	5.51 ± 0.75	5.77 ± 0.26	4.66 ± 0.40	NS	NS	NS
Glycine	0.86 ± 0.09	1.34 ± 0.20	1.90 ± 0.25	2.75 ± 0.38	NS	***	*
Formate	1.31 ± 0.11	0.94 ± 0.14	1.66 ± 0.18	1.32 ± 0.10	NS	*	*
Glycogen	1.28 ± 0.30	1.75 ± 0.16	1.84 ± 0.61	2.05 ± 0.63	NS	NS	NS
Lac/Ala	0.31 ± 0.03	0.28 ± 0.03	0.27 ± 0.03	0.26 ± 0.06	NS	NS	NS

Aqueous metabolites were assayed by ^{1}H-NMR in liver extracts of *S. aurata* fed diets HLL or LLH 72 h following administration of chitosan-TPP nanoparticles complexed with 10 µg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). The values are expressed as mean ± SEM ($n = 5$). Statistical significance for independent variables (diet and treatment) related to control fish (empty vector, pSG5) is indicated as follows: *$p < 0.05$; ***$p < 0.001$; *NS*, not significant.

3.3. *Effect of SREBP1a Overexpression on Hepatic Glycolysis-Gluconeogenesis*

We previously reported that the N-terminal nuclear fragment of SREBP1a transactivates GK and PFKFB1 gene expression in *S. aurata* by binding to sterol regulatory element (SRE) boxes located in the proximal promoter of both genes [20,21]. Therefore, the fact that chitosan-TPP-pSG5-SREBP1a administration stimulated the expression of SREBP1a in the liver prompted us to analyse the effect of SREBP1a overexpression on hepatic mRNA levels of GK, a rate-limiting enzyme in glycolysis, and PFKFB1, a bifunctional enzyme that exerts an essential contribution to the control of glycolysis and gluconeogenesis through the synthesis and degradation of fructose-2,6-bisphosphate (fru-2,6-P_2). In agreement with previous reports [26,28], a low protein, high carbohydrate diet (LLH) significantly increased GK and PFKFB1 mRNA levels in the liver of *S. aurata* by 2- and 1.6-fold, respectively (Figure 3a,c). In addition, SREBP1a overexpression upregulated the hepatic mRNA content of GK (2.8 to 3.9-fold) and PFKFB1 (1.3 to 1.4-fold). Consistently, GK activity significantly increased 1.5 to 2.1-fold as a result of SREBP1a overexpression (Figure 3b).

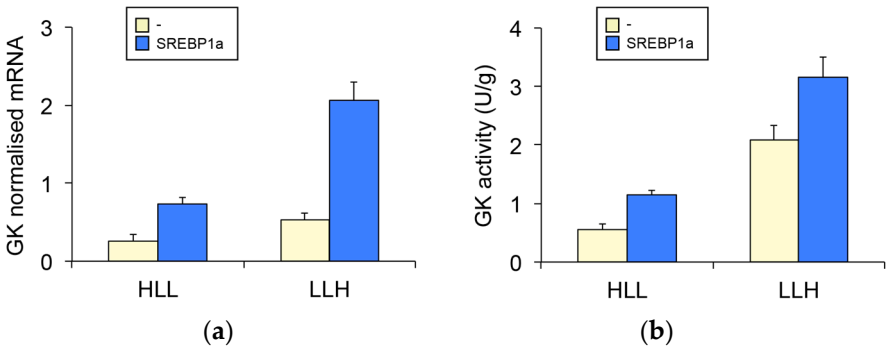
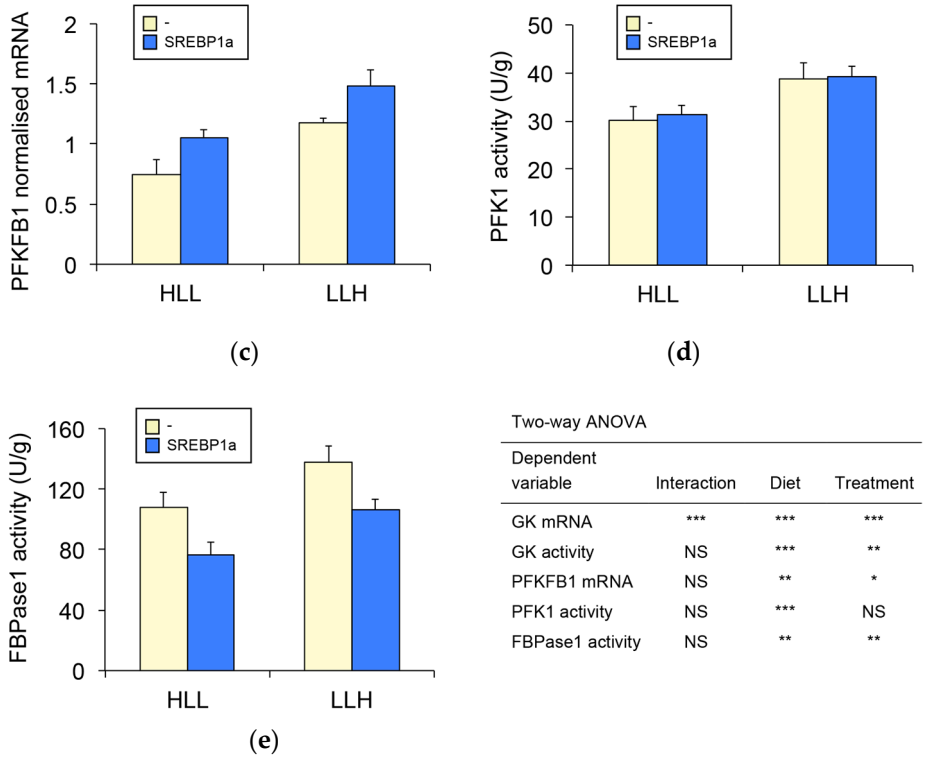


Figure 3. *Cont.*



Two-way ANOVA

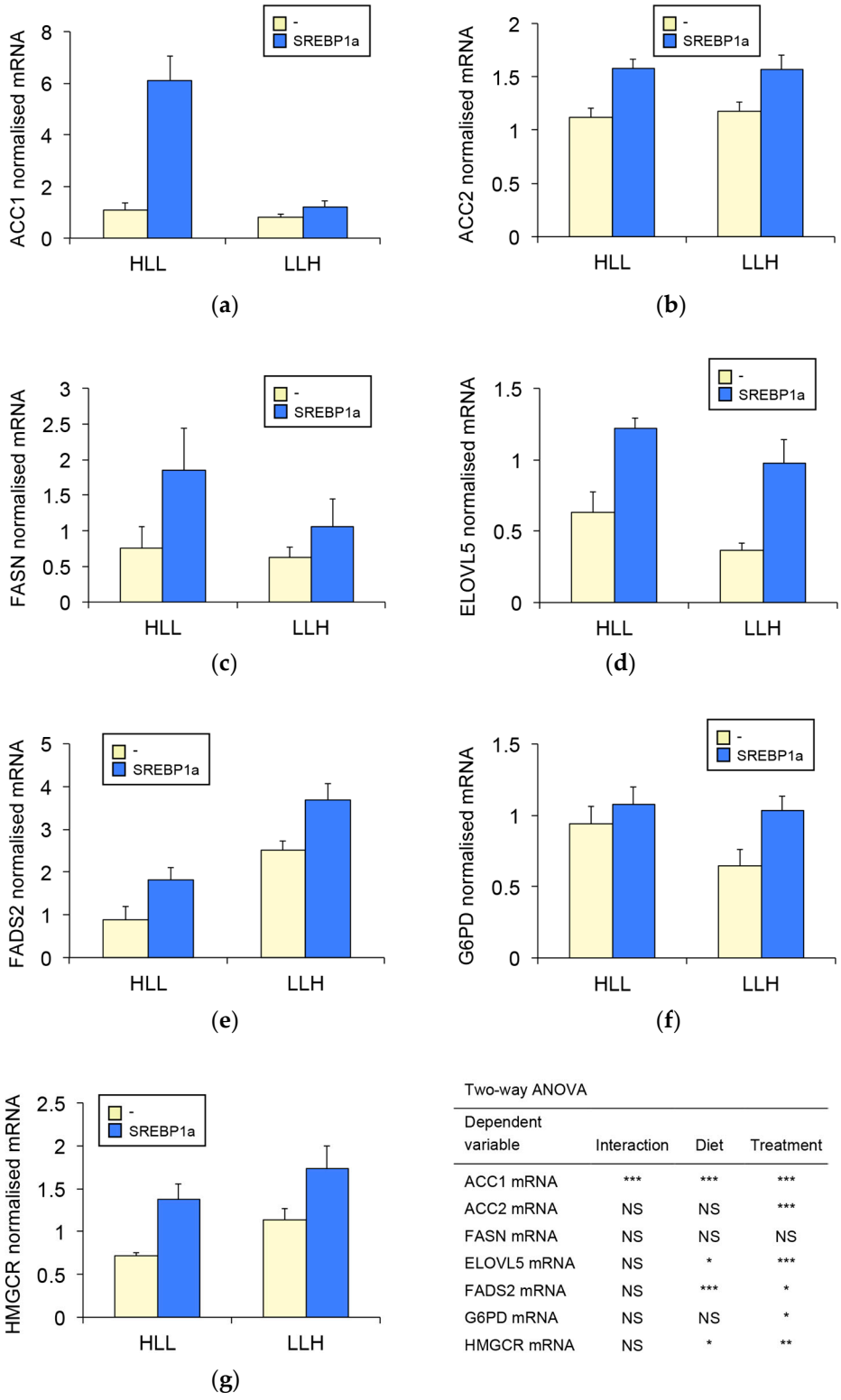
Dependent variable	Interaction	Diet	Treatment
GK mRNA	***	***	***
GK activity	NS	***	**
PFKFB1 mRNA	NS	**	*
PFK1 activity	NS	***	NS
FBPase1 activity	NS	**	**

Figure 3. Effect of chitosan-TPP-pSG5-SREBP1a administration on the expression of key enzymes in glycolysis-gluconeogenesis in the liver of *S. aurata*. Twenty-four hours after the last meal, fish fed diet HLL or LLH were intraperitoneally administered with chitosan-TPP nanoparticles complexed with 10 µg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). Seventy-two hours post-treatment, the liver was collected and RNA isolated. Hepatic mRNA levels and enzyme activity of GK (**a**, **b**), PFKFB1 (**c**), PFK1 (**d**) and FBPase1 (**e**) are presented as mean ± SEM (enzyme activity, $n = 8$; mRNA levels, $n = 6$). The mRNA levels for each gene were assayed by RT-qPCR and normalised with the geometric mean of ribosomal subunit 18s, β-actin and EF1α. Statistical significance for independent variables (diet and treatment) is indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *NS* not significant.

Given that SREBP1a-dependent upregulation of GK and PFKFB1 points to stimulation of the glycolytic flux in the liver, we also analysed the activity of PFK1 and FBPase1, which have a major role in the regulation of glycolysis-gluconeogenesis by catalysing the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle. Meanwhile, diet LLH had a similar stimulatory effect on both enzymes, SREBP1a overexpression did not affected PFK1 (Figure 3d), but significantly decreased FBPase1 (71 % to 77 % of the values observed in control fish; Figure 3e).

3.4. SREBP1a Overexpression Enhances Hepatic Biosynthesis of Fatty Acids and Cholesterol

Due to the well-known stimulatory effect of SREBP1a on the expression of genes involved in biosynthesis of fatty acids and cholesterol [1], we studied the effect of chitosan-TPP-pSG5-SREBP1a administration on gene expression levels of key enzymes involved in lipid biosynthesis in the liver of *S. aurata*. SREBP1a overexpression significantly upregulated mRNA levels of most genes analysed: rate-limiting enzymes in fatty acid synthesis (ACC1 and ACC2, Figure 4a–c), genes involved in fatty acid elongation (ELOVL5, Figure 4d) and desaturation (FADS2, Figure 4e) as well as key enzymes for the provision of NADPH (G6PD, Figure 4f) and cholesterol synthesis (HMGCR, Figure 4g). The highest stimulatory effect of SREBP1a was found for the expression levels of ACC1, which increased 5.6-fold in *S. aurata* fed diet HLL.



Two-way ANOVA

Dependent variable	Interaction	Diet	Treatment
ACC1 mRNA	***	***	***
ACC2 mRNA	NS	NS	***
FASN mRNA	NS	NS	NS
ELOVL5 mRNA	NS	*	***
FADS2 mRNA	NS	***	*
G6PD mRNA	NS	NS	*
HMGCR mRNA	NS	*	**

Figure 4. Effect of chitosan-TPP-pSG5-SREBP1a administration on the expression of key enzymes in the synthesis of fatty acid and cholesterol in the liver of *S. aurata*. Twenty-four hours after the last meal, fish fed diet HLL or LLH were intraperitoneally administered with chitosan-TPP nanoparticles complexed with 10 μg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). Seventy-two hours following the treatment, the liver was collected and RNA isolated. Hepatic mRNA levels of ACC1 (**a**), ACC2 (**b**), FASN (**c**), ELOVL5 (**d**), FADS2 (**e**), G6PD (**f**) and HMGCR (**g**) are presented as mean ± SEM (n = 6). Expression levels for each gene were assayed by RT-qPCR and normalised with the geometric mean of ribosomal subunit 18s, β-actin and EF1α. Statistical significance for independent variables (diet and treatment) are indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *NS* not significant.

Table 4 shows changes in the fatty acid profile in the liver of *S. aurata* treated with chitosan-TPP-pSG5-SREBP1a. All fatty acids that were significantly affected by the treatment (seven out of 29), increased their content in the liver of fish submitted to SREBP1a overexpression. Fatty acids affected by SREBP1a included pentadecanoic acid (15:0-n), heptadecanoic acid (17:0-n), myristoleic acid (14:1n-5), *cis*-10-pentadecenoic acid (15:1n-5) and long-chain polyunsaturated fatty acids (LC-PUFA), such as *cis*-11,14-eicosadienoic acid (20:2n-6), *cis*-11,14,17-eicosatrienoic acid (20:3n-3) and *cis*-13,16-docosadienoic acid (22:2n-6). Most remarkable SREBP1a-dependent increases were found for pentadecanoic acid (1.1 to 2.9-fold), *cis*-11,14-eicosadienoic acid (1.3 to 1.4-fold) and *cis*-11,14,17-eicosatrienoic acid (1.4-fold) as well as for myristoleic acid, *cis*-10-pentadecenoic acid and *cis*-13,16-docosadienoic acid, which presented barely detectable levels in non-treated *S. aurata* fed diet LLH.

Table 4. Effect of SREBP1a overexpression on fatty acid profile in the liver of *S. aurata*.

	HLL		LLH		Two-way ANOVA		
Fatty Acid	**-**	**SREBP1a**	**-**	**SREBP1a**	**Interaction**	**Diet**	**Treatment**
14:0	3.59 ± 0.31	3.10 ± 0.15	2.85 ± 0.12	2.76 ± 0.44	NS	NS	NS
15:0	0.28 ± 0.05	0.30 ± 0.02	0.11 ± 0.00	0.32 ± 0.01	*	*	**
16:0	22.26 ± 0.76	21.32 ± 0.54	22.09 ± 1.11	23.73 ± 0.96	NS	NS	NS
17:0	0.32 ± 0.03	0.41 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	NS	NS	*
18:0	7.14 ± 1.05	7.65 ± 0.37	8.69 ± 0.30	6.93 ± 0.37	NS	NS	NS
20:0	0.10 ± 0.02	0.12 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	NS	NS	NS
21:0	0.89 ± 0.14	1.21 ± 0.14	1.21 ± 0.47	1.27 ± 0.14	NS	NS	NS
22:0	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	0.16 ± 0.00	NS	NS	NS
23:0	0.01 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	NS	*	NS
24:0	0.13 ± 0.01	0.15 ± 0.02	0.18 ± 0.06	0.18 ± 0.02	NS	NS	NS
14:1n-5	0.09 ± 0.02	0.11 ± 0.01	0.00 ± 0.00	0.11 ± 0.04	NS	NS	*
15:1n-5	0.07 ± 0.02	0.09 ± 0.01	0.00 ± 0.00	0.08 ± 0.02	NS	*	*
16:1n-7	4.42 ± 0.26	4.53 ± 0.35	4.25 ± 0.14	3.96 ± 0.20	NS	NS	NS
17:1n-7	0.28 ± 0.02	0.32 ± 0.01	0.29 ± 0.05	0.33 ± 0.01	NS	NS	NS
18:1n-9c	26.48 ± 0.95	27.15 ± 1.83	26.10 ± 0.40	23.69 ± 1.53	NS	NS	NS
18:1n-9t	0.11 ± 0.01	0.12 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	NS	NS	NS
20:1n-9	1.29 ± 0.14	1.40 ± 0.01	1.70 ± 0.03	1.40 ± 0.08	NS	NS	NS
22:1n-9	0.74 ± 0.08	0.80 ± 0.13	0.96 ± 0.07	1.05 ± 0.08	NS	*	NS
24:1n-9	0.30 ± 0.04	0.39 ± 0.04	0.31 ± 0.06	0.24 ± 0.02	NS	NS	NS
18:2n-6c	2.12 ± 0.38	2.37 ± 0.59	2.09 ± 0.05	2.43 ± 0.07	NS	NS	NS
18:2n-6t	0.01 ± 0.01	0.02 ± 0.00	0.02 ± 0.02	0.03 ± 0.01	NS	NS	NS
18:3n-3	0.11 ± 0.03	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.01	NS	NS	NS
18:3n-6	0.36 ± 0.05	0.42 ± 0.09	0.32 ± 0.04	0.39 ± 0.03	NS	NS	NS
20:2n-6	0.13 ± 0.02	0.17 ± 0.01	0.14 ± 0.02	0.19 ± 0.01	NS	NS	*
20:3n-3	0.17 ± 0.02	0.24 ± 0.01	0.25 ± 0.04	0.36 ± 0.04	NS	*	*
20:4n-6	1.02 ± 0.07	0.96 ± 0.10	0.94 ± 0.02	1.38 ± 0.19	NS	NS	NS
20:5n-3	4.77 ± 0.14	4.27 ± 0.49	3.64 ± 0.22	4.98 ± 0.53	NS	NS	NS
22:2n-6	0.01 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	NS	NS	*
22:6n-3	14.46 ± 1.18	14.87 ± 1.84	12.46 ± 0.68	15.31 ± 1.16	NS	NS	NS
Saturated	35.48 ± 2.86	34.45 ± 0.85	35.76 ± 0.93	35.81 ± 0.74	NS	NS	NS
Monounsaturated	34.50 ± 0.52	34.91 ± 2.12	33.71 ± 0.54	30.95 ± 1.79	NS	NS	NS
PUFAs	23.01 ± 1.38	23.47 ± 3.11	19.99 ± 0.80	25.20 ± 1.83	NS	NS	NS
n-9	29.90 ± 0.52	29.86 ± 2.00	29.17 ± 0.46	26.47 ± 1.61	NS	NS	NS
n-6	3.49 ± 0.40	3.97 ± 0.79	3.53 ± 0.04	4.45 ± 0.18	NS	NS	NS
n-3	19.52 ± 1.23	19.49 ± 2.34	16.46 ± 0.82	20.75 ± 1.68	NS	NS	NS
n-6/n-3	0.18 ± 0.02	0.20 ± 0.02	0.22 ± 0.01	0.22 ± 0.01	NS	NS	NS

Fatty acid composition was assayed in liver extracts of *S. aurata* fed diets HLL or LLH 72 h following administration of chitosan-TPP nanoparticles complexed with 10 µg/g BW of pSG5 (-) or pSG5-SREBP1a (SREBP1a). Two replicate analyses per sample were performed and the results are expressed in % area as mean values ± SEM of five samples per condition. Statistical significance for independent variables (diet and treatment) related to control fish (empty vector, pSG5) is indicated as follows: $*p < 0.05$; $**p < 0.01$; *NS*, not significant.

4. Discussion

Chitosan is a cationic polymer derived from chitin that is composed of glucosamine and N-acetylglucosamine units linked by β-1,4-glycosidic bonds. Properties such as mucoadhesion, low toxicity, biodegradability, biocompatibility and protection of nucleic acids against nuclease degradation have made chitosan a valuable gene-carrier system to deliver nucleic acids in vivo [37,38]. In recent studies, we used the ionic gelation technique to obtain chitosan-TPP nanoparticles complexed with plasmids expressing short hairpin RNAs specifically designed to knockdown the expression of target genes in the liver of *S. aurata*. By using this methodology we were able to study the metabolic effect that results from silencing the expression of cytosolic alanine aminotransferase and glutamate dehydrogenase [32,39]. Herein, we obtained chitosan-TPP nanoparticles complexed with a plasmid that expresses the N-terminal activation domain of SREBP1a (pSG5-SREBP1a). The metabolic effect of SREBP1a overexpression was addressed in fish fed diets differing in macronutrient composition.

As a cationic polymer, it is considered that chitosan can absorb fatty acids and lower circulating triglycerides. In the present study, no significant differences were found for blood and liver triglycerides between non-treated *S. aurata* and fish treated with chitosan-TPP-pSG5 nanoparticles, which supports the notion that under our experimental conditions chitosan did not affect the hepatic content of fatty acids (Supplementary Table S1). The lack of effect due to chitosan may be attributed to the animal model and experimental design of the present study, where fish received a single dose of chitosan by intraperitoneal injection. This contrasts with reports that showed a chitosan-dependent lowering effect on circulating triglycerides, which have been essentially performed in mammals, mostly in rats submitted to an oral dose or fed with chitosan-containing diets for a period of time [40,41].

Intraperitoneal administration of chitosan-TPP-pSG5-SREBP1a significantly increased mRNA and immunodetectable levels of SREBP1a in the liver of *S. aurata*. The presence of SREBP1-stabilising factors in the liver of fish fed high protein, low carbohydrate diets may explain higher amounts of immunodetectable SREBP1 in fish fed HLL. Likewise, we previously showed that partial substitution of dietary protein by carbohydrates decreases body lipids and growth performance in *S. aurata* [29]. Consistent with previous findings that reported transactivation of *S. aurata* GK and PFKFB1 by SREBP1a [20,21], overexpression of SREBP1a upregulated the expression of both genes even in the group of fish fed the high carbohydrate/low protein diet, were in agreement with previous reports [26,28], dietary carbohydrates enhanced basal expression of glycolytic genes in the liver of *S. aurata*.

Enhancement of GK activity may be responsible for increasing hepatic glycogen, a trend that was found in the liver of fish treated with chitosan-TPP-pSG5-SREBP1a nanoparticles. Increased GK activity is needed to convert glucose into glucose-6-phosphate but may not be sufficient to increase the glycolytic flux. However, our findings support the notion that in addition to allocate part of the glucose-6-phosphate pool resulting from increased GK activity to synthesise glycogen, SREBP1a-dependent upregulation of PFKFB1 would be a key mechanism to favour glucose oxidation via glycolysis in the liver of fish that overexpressed SREBP1a. Consistently, glucose and insulin administration increases SREBP1 protein content and association with PFKFB1 promoter in the liver of *S. aurata*, leading to PFKFB1 upregulation [20]. PFKFB1 is a bifunctional enzyme that catalyses the synthesis and degradation of fru-2,6-P_2, which, in turn, is a major regulator of glycolysis–gluconeogenesis through allosteric activation of PFK1 and inhibition of FBPase1 [42,43]. Therefore, by controlling the activity of PFK1 and FBPase1, the cytosolic levels of fru-2,6-P_2 are essential to determine the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle.

In *S. aurata*, we previously reported that high carbohydrate diets upregulate the hepatic mRNA levels of PFKFB1 and the kinase activity of the bifunctional enzyme, leading to a huge increase of fru-2,6-P_2 [28]. In the present study, the effect of SREBP1a overexpression was also assessed in the activity of the two enzymes that regulate the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle: PFK1 and FBPase1. Consistent with increased expression of PFKFB1 and conceivably the fru-2,6-P_2 content, FBPase1 activity was significantly reduced in the liver of treated fish, which

suggests inhibition of the gluconeogenic pathway. Surprisingly, PFK1 activity was not affected by overexpression of SREBP1a. Similarly as in mammals, fru-2,6-P_2 behaves as allosteric activator of *S. aurata* PFK-1 in vitro [44]. However, the concomitant occurrence of other molecules that can act as allosteric inhibitors of PFK1, such as ATP and citrate, may counteract the stimulatory action of fru-2,6-P_2 in the liver of *S. aurata* overexpressing SREBP1a. In support of this hypothesis, it was shown that the liver isoform of PFK1 is highly susceptible to inhibition by ATP in *S. aurata* [44]. Nevertheless, considering the effect of SREBP1a overexpression on the PFK1/FBPase1 activity ratio, our findings suggest that by decreasing the gluconeogenic activity, SREBP1a favoured the glycolytic flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle. Increased levels of alanine and glycine in the liver of *S. aurata* support the enhancement of hepatic glycolysis from dietary carbohydrates as a result of SREBP1a overexpression. An increased glycolytic rate would favour further conversion of pyruvate into alanine and the use of glycolytic intermediates for biosynthetic purposes, such as 3-phosphoglycerate for subsequent biosynthesis of serine and glycine.

The results of this study suggest that SREBP1a-dependent increased glucose oxidation via glycolysis can assure a steady provision of carbons for de novo lipogenesis in the liver of *S. aurata*. Consistently, treatment with chitosan-TPP-pSG5-SREBP1a nanoparticles significantly upregulated the expression of key genes involved in biosynthesis of fatty acids and cholesterol, leading to increased circulating levels of triglycerides and cholesterol. In agreement with the role of SREBP1 in the transcription of lipogenic genes in mammals and fish [6,45–49], overexpression of SREBP1a in the liver of treated fish upregulated the expression of genes involved in synthesis (ACC1 and ACC2), elongation (ELOVL5) and desaturation (FADS2) of fatty acids, NADPH production (G6PD) and cholesterol biosynthesis (HMGCR).

By providing malonyl-CoA for the biosynthesis of fatty acids, ACC1 catalyses the rate-limiting step in fatty acid synthesis [50,51]. In the present study, the effect of SREBP1a overexpression on ACC1 was potentiated in fish fed the higher protein/lower carbohydrate diet. Albeit not significant, the same trend was observed for another important enzyme in fatty acid synthesis, FASN, which generates palmitate (16:0) from acetyl-CoA, malonyl-CoA and NADPH. Altogether, our findings suggest that dietary protein may either increase SREBP1a stability or have a synergistic effect with SREBP1a in the transcriptional activation of ACC1 and even FASN. In this regard, it is well known that in mammals insulin activates SREBP1 cleavage and production of the nuclear mature form of the protein through signalling pathways involving Akt and mTORC1 [7,52]. A similar behaviour may occur in carnivorous fish, where amino acids are considered the most potent insulin secretagogues [22]. Furthermore, consistent with our findings, it was proposed that dietary protein might enhance lipogenesis via mTOR signalling in rainbow trout [53–55].

SREBP1a-dependent activation of lipogenic genes caused significant changes in the hepatic fatty acid profile of treated fish. Upregulation of ACC1 and FASN combined with increased expression of genes involved in fatty acid elongation and desaturation significantly increased the relative concentration of a wide array of fatty acids, which is consistent with the fact that the activity of FADS2 and ELOVL5 can desaturate and condense, respectively, a broad range of fatty acids [56–58]. This seems to be also the case in fish species, where FADS2 enzymes exhibit predominantly Δ6 desaturase activity but display more varied substrate specificity than in mammals, while piscine ELOVL5 shows more versatility than its mammalian orthologues [59]. Affected fatty acids included saturated, monounsaturated and PUFAs. However, SREBP1a overexpression did not change the total content of saturated and unsaturated fatty acids as well as the n-6/n-3 ratio.

Even though SREBPs are conserved from yeast to humans, it remains uncertain to what extent the results of the present study can be generalised to mammals and other vertebrates. Physiological and metabolic features of fish, such as dependence on dietary amino acids to produce energy and adaptation to long-term food deprivation, and the fact that species-specific differences in SREBP targets and control pathways have been reported [7], make comparison between carnivorous fish and other vertebrates difficult.

5. Conclusions

In this study we show that treatment with chitosan-TPP-pSG5-SREBP1a nanoparticles led to SREBP1a overexpression in the liver, which in turn promoted a multigenic action that increased the glycolytic flux by stimulating GK and PFKFB1 expression as well as lipogenesis through upregulation of genes involved in biosynthesis of fatty acids and cholesterol. In addition to report for the first time the in vivo effects of exogenous SREBP1a in a glucose-intolerant model, our findings support that SREBP1a overexpression in the liver can trigger a protein sparing effect through conversion of dietary carbohydrates into lipids in *S. aurata*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/8/297/s1, Table S1: Triglyceride levels in serum and liver of non-treated *S. aurata* and *S. aurata* treated with chitosan-TPP-pSG5 and chitosan-TPP-pSG5-SREBP1a.

Author Contributions: Conceptualization, I.M. and I.V.B.; methodology, J.I.S.-M., J.V., A.R., M.P., A.F., I.V., J.G.J., M.M. and J.R.T.; validation, M.P.A., I.V., M.M., J.R.T., I.V.B. and I.M.; formal analysis, J.I.S.-M., J.V., A.R., M.P. and A.F.; investigation, J.I.S.-M., I.V., M.P.A., I.V.B. and I.M.; writing—original draft preparation, J.I.S.-M. and I.M.; writing—review and editing, J.I.S.-M., J.V., A.R., M.P., A.F., M.P.A., I.V., J.G.J., M.M., J.R.T., I.V.B. and I.M.; project administration, I.M.; funding acquisition, I.M.

Funding: This research was funded by Ministerio de Economía, Industria y Competitividad (Spain), grant number AGL2016-78124-R, co-funded by the European Regional Development Fund (EC).

Acknowledgments: The authors thank Piscimar (Burriana, Castellón, Spain) for providing *S. aurata* juveniles and Aquarium of Barcelona (Barcelona, Spain) for supplying filtered seawater.

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

References

1. DeBose-Boyd, R.A.; Ye, J. SREBPs in lipid metabolism, insulin signaling, and beyond. *Trends Biochem. Sci.* **2018**, *43*, 358–368. [CrossRef] [PubMed]
2. Bertolio, R.; Napoletano, F.; Mano, M.; Maurer-Stroh, S.; Fantuz, M.; Zannini, A.; Bicciato, S.; Sorrentino, G.; Del Sal, G. Sterol regulatory element binding protein 1 couples mechanical cues and lipid metabolism. *Nat. Commun.* **2019**, *10*, 1326. [CrossRef] [PubMed]
3. Edwards, P.A.; Tabor, D.; Kast, H.R.; Venkateswaran, A. Regulation of gene expression by SREBP and SCAP. *Biochim. et Biophys. Acta(BBA) - Mol. Cell Biol. Lipids* **2000**, *1529*, 103–113. [CrossRef]
4. Inoue, J.; Sato, R. New insights into the activation of sterol regulatory element-binding proteins by proteolytic processing. *Biomol. Concepts* **2013**, *4*, 417–423. [CrossRef] [PubMed]
5. Brown, M.S.; Goldstein, J.L. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **1997**, *89*, 331–340. [CrossRef]
6. Horton, J.D.; Shah, N.A.; Warrington, J.A.; Anderson, N.N.; Park, S.W.; Brown, M.S.; Goldstein, J.L. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12027–12032. [CrossRef]
7. Shimano, H.; Sato, R. SREBP-regulated lipid metabolism: Convergent physiology - divergent pathophysiology. *Nat. Rev. Endocrinol.* **2017**, *13*, 710–730. [CrossRef]
8. Harada, N.; Yonemoto, H.; Yoshida, M.; Yamamoto, H.; Yin, Y.; Miyamoto, A.; Hattori, A.; Wu, Q.; Nakagawa, T.; Nakano, M.; et al. Alternative splicing produces a constitutively active form of human SREBP-1. *Biochem. Biophys. Res. Commun.* **2008**, *368*, 820–826. [CrossRef]
9. Felder, T.K.; Klein, K.; Patsch, W.; Oberkofler, H. A novel SREBP-1 splice variant: Tissue abundance and transactivation potency. *Biochim. et Biophys. Acta(BBA) - Gene Struct. Expr.* **2005**, *1731*, 41–47. [CrossRef]
10. Wang, H.; Liu, F.; Millette, C.F.; Kilpatrick, D.L. Expression of a novel, sterol-insensitive form of sterol regulatory element binding protein 2 (SREBP2) in male germ cells suggests important cell- and stage-specific functions for SREBP targets during spermatogenesis. *Mol. Cell. Biol.* **2002**, *22*, 8478–8490. [CrossRef]
11. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Investig.* **2002**, *109*, 1125–1131. [CrossRef] [PubMed]

12. Pai, J.T.; Guryev, O.; Brown, M.S.; Goldstein, J.L. Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *J. Biol. Chem.* **1998**, *273*, 26138–26148. [CrossRef] [PubMed]
13. Shimano, H.; Horton, J.D.; Shimomura, I.; Hammer, R.E.; Brown, M.S.; Goldstein, J.L. Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J. Clin. Investig.* **1997**, *99*, 846–854. [CrossRef] [PubMed]
14. Horton, J.D.; Bashmakov, Y.; Shimomura, I.; Shimano, H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5987–5992. [CrossRef] [PubMed]
15. Foretz, M.; Guichard, C.; Ferre, P.; Foufelle, F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12737–12742. [CrossRef] [PubMed]
16. Gosmain, Y.; Lefai, E.; Ryser, S.; Roques, M.; Vidal, H. Sterol regulatory element-binding protein-1 mediates the effect of insulin on hexokinase II gene expression in human muscle cells. *Diabetes* **2004**, *53*, 321–329. [CrossRef] [PubMed]
17. Kim, S.Y.; Kim, H.I.; Kim, T.H.; Im, S.S.; Park, S.K.; Lee, I.K.; Kim, K.S.; Ahn, Y.H. SREBP-1c mediates the insulin-dependent hepatic glucokinase expression. *J. Biol. Chem.* **2004**, *279*, 30823–30829. [CrossRef]
18. Gosmain, Y.; Dif, N.; Berbe, V.; Loizon, E.; Rieusset, J.; Vidal, H.; Lefai, E. Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J. Lipid Res.* **2005**, *46*, 697–705. [CrossRef]
19. Yamamoto, T.; Shimano, H.; Nakagawa, Y.; Ide, T.; Yahagi, N.; Matsuzaka, T.; Nakakuki, M.; Takahashi, A.; Suzuki, H.; Sone, H.; et al. SREBP-1 interacts with hepatocyte nuclear factor-4 alpha and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *J. Biol. Chem.* **2004**, *279*, 12027–12035. [CrossRef]
20. Metón, I.; Egea, M.; Anemaet, I.G.; Fernández, F.; Baanante, I.V. Sterol regulatory element binding protein-1a transactivates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoter. *Endocrinology* **2006**, *147*, 3446–3456. [CrossRef]
21. Egea, M.; Metón, I.; Córdoba, M.; Fernández, F.; Baanante, I.V. Role of Sp1 and SREBP-1a in the insulin-mediated regulation of glucokinase transcription in the liver of gilthead sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol.* **2008**, *155*, 359–367. [CrossRef] [PubMed]
22. Polakof, S.; Panserat, S.; Soengas, J.L.; Moon, T.W. Glucose metabolism in fish: A review. *J. Comp. Physiol. B* **2012**, *182*, 1015–1045. [CrossRef] [PubMed]
23. Caseras, A.; Metón, I.; Fernández, F.; Baanante, I.V. Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). *Biochim. et Biophys. Acta(BBA) - Gene Struct. Expr.* **2000**, *1493*, 135–141. [CrossRef]
24. Panserat, S.; Médale, F.; Brèque, J.; Plagnes-Juan, E.; Kaushik, S. Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J. Nutr. Biochem.* **2000**, *11*, 22–29. [CrossRef]
25. Panserat, S.; Capilla, E.; Gutierrez, J.; Frappart, P.O.; Vachot, C.; Plagnes-Juan, E.; Aguirre, P.; Brèque, J.; Kaushik, S. Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. *Comp. Biochem. Physiol. Part B. Biochem. Mol. Biol.* **2001**, *128*, 275–283. [CrossRef]
26. Caseras, A.; Metón, I.; Vives, C.; Egea, M.; Fernández, F.; Baanante, I.V. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* **2002**, *88*, 607–614. [CrossRef] [PubMed]
27. Metón, I.; Mediavilla, D.; Caseras, A.; Cantó, E.; Fernández, F.; Baanante, I.V. Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* **1999**, *82*, 223–232. [CrossRef]
28. Metón, I.; Caseras, A.; Fernández, F.; Baanante, I.V. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, *Sparus aurata*. *Biochim. et Biophys. Acta(BBA) -Gene Struct. Expr.* **2000**, *1491*, 220–228. [CrossRef]

29. Fernández, F.; Miquel, A.G.; Cordoba, M.; Varas, M.; Metón, I.; Caseras, A.; Baanante, I.V. Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J. Exp. Mar. Biol. Ecol.* **2007**, *343*, 1–10. [CrossRef]
30. Rashidpour, A.; Silva-Marrero, J.I.; Seguí, L.; Baanante, I.V.; Metón, I. Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (*Sparus aurata*). *Am. J. Physiol. Integr. Comp. Physiol.* **2019**, *316*, R265–R273. [CrossRef]
31. Silva-Marrero, J.I.; Sáez, A.; Caballero-Solares, A.; Viegas, I.; Almajano, M.P.; Fernández, F.; Baanante, I.V.; Metón, I. A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). *BMC Genom.* **2017**, *18*, 768. [CrossRef] [PubMed]
32. González, J.D.; Silva-Marrero, J.I.; Metón, I.; Caballero-Solares, A.; Viegas, I.; Fernández, F.; Miñarro, M.; Fàbregas, A.; Ticó, J.R.; Jones, J.G.; et al. Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. *Mar. Biotechnol.* **2016**, *18*, 85–97. [CrossRef] [PubMed]
33. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]
34. Folch, J.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [PubMed]
35. Ravanbakhsh, S.; Liu, P.; Bjordahl, T.C.; Mandal, R.; Grant, J.R.; Wilson, M.; Eisner, R.; Sinelnikov, I.; Hu, X.; Luchinat, C.; et al. Accurate, fully-automated NMR spectral profiling for metabolomics. *PLoS ONE* **2015**, *10*, e0124219. [CrossRef] [PubMed]
36. Villasante, J.; Girbal, M.; Metón, I.; Almajano, M. Effects of pecan nut (*Carya illinoiensis*) and roselle flower (*Hibiscus sabdariffa*) as antioxidant and antimicrobial agents for sardines (*Sardina pilchardus*). *Molecules* **2019**, *24*, 85. [CrossRef]
37. Ragelle, H.; Riva, R.; Vandermeulen, G.; Naeye, B.; Pourcelle, V.; Le Duff, C.S.; D'Haese, C.; Nysten, B.; Braeckmans, K.; De Smedt, S.C.; et al. Chitosan nanoparticles for siRNA delivery: Optimizing formulation to increase stability and efficiency. *J. Control. Release* **2014**, *176*, 54–63. [CrossRef]
38. Sáez, M.; Vizcaíno, A.; Alarcón, F.; Martínez, T. Comparison of lacZ reporter gene expression in gilthead sea bream (*Sparus aurata*) following oral or intramuscular administration of plasmid DNA in chitosan nanoparticles. *Aquaculture* **2017**, *474*, 1–10. [CrossRef]
39. Gaspar, C.; Silva-Marrero, J.I.; Fàbregas, A.; Miñarro, M.; Ticó, J.R.; Baanante, I.V.; Metón, I. Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. *J. Biotechnol.* **2018**, *286*, 5–13. [CrossRef]
40. Wu, S.; Pan, H.; Tan, S.; Ding, C.; Huang, G.; Liu, G.; Guo, J.; Su, Z. In vitro inhibition of lipid accumulation induced by oleic acid and in vivo pharmacokinetics of chitosan microspheres (CTMS) and chitosan-capsaicin microspheres (CCMS). *Food Nutr. Res.* **2017**, *61*, 1331658. [CrossRef]
41. Liu, X.; Zhi, X.; Liu, Y.; Wu, B.; Sun, Z.; Shen, J. Effect of chitosan, O -carboxymethyl chitosan, and N -[(2-hydroxy-3- N, N -dimethylhexadecyl ammonium)propyl] chitosan chloride on overweight and insulin resistance in a murine diet-induced obesity. *J. Agric. Food Chem.* **2012**, *60*, 3471–3476. [CrossRef] [PubMed]
42. Houddane, A.; Bultot, L.; Novellasdemunt, L.; Johanns, M.; Gueuning, M.-A.; Vertommen, D.; Coulie, P.G.; Bartrons, R.; Hue, L.; Rider, M.H. Role of Akt/PKB and PFKFB isoenzymes in the control of glycolysis, cell proliferation and protein synthesis in mitogen-stimulated thymocytes. *Cell. Signal.* **2017**, *34*, 23–37. [CrossRef] [PubMed]
43. Okar, D.A.; Wu, C.; Lange, A.J. Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Adv. Enzyme Regul.* **2004**, *44*, 123–154. [CrossRef] [PubMed]
44. Mediavilla, D.; Metón, I.; Baanante, I.V. Purification and kinetic characterization of 6-phosphofructo-1-kinase from the liver of gilthead sea bream (*Sparus aurata*). *J. Biochem.* **2008**, *144*, 235–244. [CrossRef] [PubMed]
45. Rome, S.; Lecomte, V.; Meugnier, E.; Rieusset, J.; Debard, C.; Euthine, V.; Vidal, H.; Lefai, E. Microarray analyses of SREBP-1a and SREBP-1c target genes identify new regulatory pathways in muscle. *Physiol. Genom.* **2008**, *34*, 327–337. [CrossRef] [PubMed]

46. Lee, A.K.; Kyriakou, T.; Weston, A.J.; O'Dell, S.D. Functional single-nucleotide polymorphism in acetyl-CoA carboxylase ACACB gene promoter. *DNA Cell Biol.* **2010**, *29*, 703–712. [CrossRef] [PubMed]
47. Shikama, A.; Shinozaki, H.; Takeuchi, Y.; Matsuzaka, T.; Aita, Y.; Murayama, T.; Sawada, Y.; Piao, X.; Toya, N.; Oya, Y.; et al. Identification of human ELOVL5 enhancer regions controlled by SREBP. *Biochem. Biophys. Res. Commun.* **2015**, *465*, 857–863. [CrossRef]
48. Carmona-Antoñanzas, G.; Tocher, D.R.; Martinez-Rubio, L.; Leaver, M.J. Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene* **2014**, *534*, 1–9. [CrossRef]
49. Dong, X.; Tan, P.; Cai, Z.; Xu, H.; Li, J.; Ren, W.; Xu, H.; Zuo, R.; Zhou, J.; Mai, K.; et al. Regulation of FADS2 transcription by SREBP-1 and PPAR-α influences LC-PUFA biosynthesis in fish. *Sci. Rep.* **2017**, *7*, 40024. [CrossRef]
50. Du, X.; Cai, C.; Yao, J.; Zhou, Y.; Yu, H.; Shen, W. Histone modifications in FASN modulated by sterol regulatory element-binding protein 1c and carbohydrate responsive-element binding protein under insulin stimulation are related to NAFLD. *Biochem. Biophys. Res. Commun.* **2017**, *483*, 409–417. [CrossRef]
51. Kim, C.-W.; Addy, C.; Kusunoki, J.; Anderson, N.N.; Deja, S.; Fu, X.; Burgess, S.C.; Li, C.; Ruddy, M.; Chakravarthy, M.; et al. Acetyl CoA carboxylase inhibition reduces hepatic steatosis but elevates plasma triglycerides in mice and humans: A bedside to bench investigation. *Cell Metab.* **2017**, *26*, 394–406.e6. [CrossRef] [PubMed]
52. Wang, Y.; Viscarra, J.; Kim, S.-J.; Sul, H.S. Transcriptional regulation of hepatic lipogenesis. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 678–689. [CrossRef] [PubMed]
53. Lansard, M.; Panserat, S.; Plagnes-Juan, E.; Seiliez, I.; Skiba-Cassy, S. Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: Role of TOR. *Amino Acids* **2010**, *39*, 801–810. [CrossRef] [PubMed]
54. Skiba-Cassy, S.; Panserat, S.; Larquier, M.; Dias, K.; Surget, A.; Plagnes-Juan, E.; Kaushik, S.; Seiliez, I. Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*). *Br. J. Nutr.* **2013**, *109*, 1359–1372. [CrossRef] [PubMed]
55. Liang, H.; Mokrani, A.; Chisomo-Kasiya, H.; Ji, K.; Ge, X.; Ren, M.; Liu, B.; Xi, B.; Sun, A. Dietary leucine affects glucose metabolism and lipogenesis involved in TOR/PI3K/Akt signaling pathway for juvenile blunt snout bream *Megalobrama amblycephala*. *Fish Physiol. Biochem.* **2019**, *45*, 719–732. [CrossRef] [PubMed]
56. Leonard, A.E.; Bobik, E.G.; Dorado, J.; Kroeger, P.E.; Chuang, L.T.; Thurmond, J.M.; Parker-Barnes, J.M.; Das, T.; Huang, Y.S.; Mukerji, P. Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. *Biochem. J.* **2000**, *350*, 765–770. [CrossRef]
57. Geay, F.; Tinti, E.; Mellery, J.; Michaux, C.; Larondelle, Y.; Perpète, E.; Kestemont, P. Cloning and functional characterization of Δ6 fatty acid desaturase (FADS2) in Eurasian perch (*Perca fluviatilis*). *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **2016**, *191*, 112–125. [CrossRef]
58. Zhang, J.Y.; Kothapalli, K.S.D.; Brenna, J.T. Desaturase and elongase-limiting endogenous long-chain polyunsaturated fatty acid biosynthesis. *Curr. Opin. Clin. Nutr. Metab. Care* **2016**, *19*, 103–110. [CrossRef]
59. Castro, L.F.C.; Tocher, D.R.; Monroig, O. Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. *Prog. Lipid Res.* **2016**, *62*, 25–40. [CrossRef]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Fish Physiol Biochem (2020) 46:371–381
https://doi.org/10.1007/s10695-019-00723-5

Dietary cobalt supplementation improves growth and body composition and induces the expression of growth and stress response genes in *Tor putitora*

Naima Younus · Amina Zuberi · Ania Rashidpour · Isidoro Metón

Received: 23 May 2019 / Accepted: 22 October 2019 / Published online: 10 November 2019
© Springer Nature B.V. 2019

Abstract A 90-day randomized feeding experiment was performed to assess the effects of dietary cobalt (Co) supplementation on the growth performance, muscle composition, status of iron and manganese in the muscle as well as the expression of growth-related genes in the muscle (myoblast determination protein 1 homolog (MyoD) and myogenin) and the stress-related gene heat shock protein 70 KDa (Hsp-70) in the liver of mahseer (*Tor putitora*). Feeding trial was conducted in triplicate under controlled semi-static conditions, and graded levels of dietary cobalt (0.5–3 mg/kg) were fed to six groups of advanced fry of *T. putitora*. The results obtained indicated a curvilinear relationship of dietary Co levels with body crude protein content and weight gain (%). A positive correlation was observed with up to 2 mg Co/kg diet. However, a decreasing trend was found with values over 2 mg Co/kg diet. The expression of muscle growth biomarkers MyoD and myogenin showed a similar response, upregulation up to 2 mg Co/kg diet and decreased expression at 3 mg Co/kg diet. Indeed, the highest dietary Co supplementation increased the expression of Hsp-70, a key gene expressed in response to stress. Moreover, the muscle content of iron and manganese showed an inverse relationship with the dietary Co supplementation. Our findings suggest that 2 mg/kg Co dietary supplementation stimulates myogenesis and optimize muscle growth and body composition, while higher levels enhanced the expression of stress response genes and impaired growth of *T. putitora*.

Keywords Cobalt chloride · Myoblast determination protein 1 homolog · Myogenin · Heat shock protein 70 KDa · Mahseer

Introduction

Properly balanced feed formulation according to developmental stage and species is a prerequisite for optimal health status and growth performance in fish. Dietary protein and other essential nutrients such as lipids, vitamins, carbohydrates and minerals are required in optimum quantity for maintaining growth and improving health (Whitney and Rolfes 1993). Fortified feed with essential nutrient supplementation can prevent susceptibility to disease and nutrient-related deficiencies that may impair and disturb body functions.

Fish can absorb minerals and other compounds from surrounding water. Nevertheless, due to low concentration in water, freshwater fish generally require minerals

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10695-019-00723-5) contains supplementary material, which is available to authorized users.

N. Younus · A. Zuberi (✉)
Fisheries and Aquaculture program, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan
e-mail: amina.zuberi@qau.edu.pk

N. Younus · A. Rashidpour · I. Metón (✉)
Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain
e-mail: imeton@ub.edu

as dietary supplements to fulfill body requirements (Beveridge et al. 2013). Supplementation of any particular mineral in feedstuffs should be carefully screened to avoid metal accumulation at toxic levels (Malomo and Ihegwuagu 2017). The current knowledge about supplementation of metallic minerals in fish is mostly limited to iron, manganese, zinc and selenium, which are the main elements of body fluids and the basic components of non-enzymatic macromolecules and can also act as cofactors in enzymatic reactions (Robbins 1993). However, various spiteful symptoms associated with deficiencies of other minerals like chromium, copper, fluorine, iodine and molybdenum, are well known (Lall 2002; Terech-Majewska et al. 2016).

Cobalt is considered as an important essential micromineral in fish and other vertebrates (Perrault et al. 2014; Rahal and Shivay 2016). It regulates blood glucose levels, the activity of many enzymes and intestinal microbial synthesis of vitamin B_{12}, which is a cofactor of two important enzymes, i.e. methylmalonyl-CoA mutase (MCM) and methionine synthase (MS) (Speich et al. 2001; Siddiqui et al. 2014). MS is involved in the synthesis of methionine and nucleic acids (McDowell 1989), while MCM plays an important role in the Krebs cycle by contributing to the synthesis of succinyl-CoA (Kräutler 2005; Takahashi-Iñiguez et al. 2012; Moll and Davis 2017).

Worldwide Co abundance in the Earth's crust and in water is relatively low (Karadede-Akin and Ünlü 2007; Swanner et al. 2014). Therefore, it must be supplied with the feed (Robbins 1993; Mukherjee and Kaviraj 2009). Dietary Co requirement in fish varies with age, size, species and culture conditions (Wilson 1991). Although plant- and animal-based proteins are a source of Co for aquafeeds, some freshwater fish require additional Co in their diet for optimum growth (Wilson 1991). Though Co is ranked as an essential dietary mineral, knowledge about the optimum dietary requirement of cobalt in many fish species is limited.

Mahseer (*Tor putitora*) is a cyprinid fish, widely distributed throughout the Indian subcontinent. It is an important game fish and regarded as a valued fish due to its large size and high commercial value (Bhatt and Pandit 2016). It is classified as an endangered species due to the depletion of natural stocks. In Pakistan, it is cultured in captivity under controlled environment conditions. Contrary to natural bodies of water, culturing fish in captivity requires fortified nutrition and adequate feeding (Oliva-Teles 2012). Bearing in mind the importance of Co as an essential micromineral in feedstuffs for fish farming and the fact that limited literature has addressed the dietary requirement of Co, the present study was designed to determine optimal dietary supplementation levels of Co for *T. putitora*. To this end, we analyzed the effect of graded levels of dietary Co supplementation on growth rate, body composition, metal bioaccumulation and the expression of genes involved in growth and stress responses.

Material and methods

Experimental design

Mahseer (*T. putitora*) advanced fry were used for this study. Uniform-sized mahseer (average wet weight ± SEM, 1.35 ± 0.02 g) were selected and randomly distributed (stocking density = 1.5 g/L, i.e. 30 fish/tank) in 21 rectangular fish rearing fiberglass troughs (capacity = 45 L) equipped with heaters and an aeration system for maintaining water temperature (22.5°C) and dissolved oxygen (DO) level (6.0 mg/L). A basal diet containing 39.4% of crude protein was used as a control without Co supplementation (Table 1). Experimental diets A–F were designed on the basis of the basal diet but including increasing amounts of cobalt chloride hexahydrate (Sigma, USA), which was added to finely ground feed ingredients (A, 0.5 mg/kg diet; B, 1.0 mg/kg diet; C, 1.5 mg/kg diet; D, 2.0 mg/kg diet; E, 2.5 mg/kg diet; and F, 3.0 mg/kg diet). Feed pellets were prepared, dried and saved in Ziploc bags by following a standard method described previously (Amir et al. 2018). Fresh feed was prepared after every 15 days. Before providing feed to the respective groups, feed pellets were crushed by using a pestle motor and sieved through a fine mesh (150–250 μm), while feed particle size was adjusted after 1 month on the basis of the size of the fry. The experiment was conducted using three tanks per treatment. Feeding trial started after 1 week of acclimatization to corresponding tanks (control fish fed with the basal diet and fish fed with experimental diets A–F). At the beginning of the experiment, fish were fed three times a day (9:00, 13:00 and 16:00) at a daily rate of 8% body weight. After 1 month, daily rate was reduced to 4% body weight and supplied twice a day (9:00 and 18:00) until the end of the trial. Remaining feed was removed after 2 h of feeding while fish excreta were removed daily. Both were filtered and collected

separately. Every day, the total volume of water was maintained by addition of fresh water. Throughout the experiment, water quality parameters were checked by means of Multi-parameter Hanna HI 9829-01102. The feeding trial lasted for 90 days, during which temperature and DO levels fluctuated slightly, i.e. temperature ± 0.2°C and DO level ± 0.35 mg/L, while total ammonia remained < 0.5 mg/L. All experimental procedures were approved by the Quaid-i-Azam University's animal welfare committee in compliance with local legislation (BEC-FBS-67-QAU-2017).

Sampling and growth measurements

At the end of the trial (90 days), fish were starved for 24 h before sampling. Fish from each tank were captured separately, weighed and counted for evaluation of growth performance. Standard formulas reported previously (Zhou et al. 2013; Munir et al. 2016) were adopted for determining growth performance:

$$\text{Average weight gain} = \text{average } w_f - \text{average } w_i$$

$$\text{Weight gain } (\%) = w_f - w_i \div w_i \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed intake (g)} \div \text{Weight gain (g)}$$

$$\text{Specific growth rate } (\%) = (\ln w_f - \ln w_i) \div \text{experimental duration} \times 100$$

where w_i = initial body weight of fish, w_f = final body weight of fish.

About 6 fish per tank (18 fish/treatment) were anesthetized with buffered MS222 (0.1 mg/L) and dissected at low temperature (on an ice pad), and the muscle and liver were immediately collected and preserved in RNA later (Thermo Scientific CAT# AM7020) for molecular-based studies. To obtain sample in enough quantity for proximate analysis, 21 fry from each tank were caught and divided into three pools consisting of 7 fry each, thus representing three pools/tank (nine pools/treatment).

Body proximate composition

Standard protocols were adopted to determine mahseer body composition (AOAC 2000). All analyses were conducted at the ISO 17025 accredited laboratory facility of the Poultry Research Institute (PRI, Islamabad, Pakistan). The crude protein and fat contents were determined by using the Kjeldahl and Soxthlet extraction procedure, respectively, while ash content was determined by heating sample in a muffle furnace at 550°C.

Table 1 Formulation and composition of basal diet for *T. putitora*

Ingredients	Quantity (g/100 g)
White fish meal	45
Soybean meal	15
Sunflower meal	15
Gluten 60%	15
Rice polish	4
Wheat bran	4
CMC[a]	1
Vitamin premix[b]	1
Cobalt chloride[c]	0
Proximate composition (%)	
Crude protein	39.42
Crude fat	12.57
Ash	8.73

[a] Carboxy-methyl-cellulose

[b] Vitamin premix contains vitamins, amino acids and minerals premix/100g

[c] Cobalt chloride hexahydrate was added to experimental diets A (0.5 mg/kg diet), B (1.0 mg/kg diet), C (1.5 mg/kg diet), D (2.0 mg/kg diet), E (2.5 mg/kg diet) and F (3.0 mg/kg diet). The basal diet was not supplied with cobalt chloride

Metal bioaccumulation

Atomic absorption spectrometry was used to determine the muscle Co, iron and manganese contents. For this purpose, 1 g fish muscle was added to conical flasks containing 5 ml of HNO_3 and 1 ml $HClO_4$. The reaction mixture was digested on a hot plate at 200°C to 250°C until a clear and transparent solution was obtained. Samples were cooled at room temperature, filtered through a Whatman No. 42 filter paper, diluted by adding 50 ml of distilled water and analyzed for Co (λ = 240 nm), Fe (λ = 248 nm) and Mn (λ = 279.5 nm) by using Varian AA240FS (Palo Alto, CA, USA) for fast sequential atomic absorption spectroscopy. For each metal studied, standard calibration curves were performed to determine metal concentrations in samples.

Molecular cloning of *T. putitora* myoblast determination protein 1 homolog, myogenin and heat shock protein 70 KDa cDNA fragments

Total RNA from muscle and liver samples was isolated by using High Pure RNA tissue kit (Roche, Basel, Switzerland) and Illustra RNAspin Mini Isolation Kit (GE Healthcare, Chicago, IL, USA), respectively. Following quantification with NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA), 1 μg of total RNA was used to synthesize cDNA by incubating with M-MLV RT (Promega, Madison, WI, USA) for 1 h at 37°C in the presence of random hexamers. Reverse transcription polymerase chain reaction (RT-PCR) was performed with primers designed from conserved regions of myoblast determination protein 1 homolog (MyoD), myogenin and heat shock protein 70 KDa (Hsp-70) coding-domain sequences in closely related fish species (*Danio rerio*, *Ctenopharyngodon idella*, *Cyprinus carpio* and *Labeo rohita*) (Table 2). PCR products, MyoD and myogenin from the muscle, and Hsp-70 from the liver were purified and then ligated into pGEM-T Easy (Promega, Madison, WI, USA). The resulting constructs were used to transform competent *E. coli* cells. Positive colonies were grown overnight at 37°C in liquid LB media supplemented with ampicillin to isolate plasmid DNA by using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, San Luis, MI, USA). Plasmids with inserted fragments were totally sequenced on both strands. The cloning of amplified products and sequencing of three independent clones for each gene allowed us to obtain cDNA fragments of 421 bp for MyoD, 615 bp for myogenin and 353 bp for Hsp-70. The nucleotide sequences isolated for *T. putitora* MyoD, myogenin and Hsp-70 were submitted to the DDJB/EMBL/GenBank databases under accession numbers MH545701, MH545702 and MH545703, respectively. The inferred amino acid sequences of *T. putitora* MyoD, myogenin and Hsp-70 were aligned with GDH orthologues in other fish species and vertebrates to explore evolutionary relationships and generated phylogenetic trees by pair-wise alignments (Sievers et al. 2011).

Quantitative RT-PCR

Total RNA (1 μg) isolated from tissue samples was reverse transcribed for 1 h at 37°C with M-MLV RT (Promega, Madison, WI, USA). MyoD, myogenin and Hsp-70 mRNA levels were determined with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To this end, 0.4 μM of the corresponding primer pair (Table 3), 10 μl of SYBR Green (Applied Biosystems, Foster City, CA, USA) and 1.6 μl of diluted cDNA were mixed in a 20-μl reaction. The temperature cycle protocol for amplification was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 62°C for 1 min. A dissociation curve was run after each experiment to ensure amplification of single products. The efficiency of the PCR reaction was checked for each gene by generating standard curves with serial dilutions of a control cDNA sample. MyoD, myogenin and Hsp-70 mRNA levels were normalized to the expression of *T. putitora* β-actin (primers shown in Table 3). Variations in gene expression were calculated by the standard $\Delta\Delta C_T$ method (Pfaffl 2001).

Statistics

Statistical package program SPSS (version 20) was used to analyze data. Univariate generalized linear model (GLM) followed by LSD test was applied to determine significant differences ($P < 0.05$) among treatments for growth performance, proximate composition and metal accumulation in the muscle of *T. putitora*. One-way ANOVA was used to analyze gene expression data.

Results

Growth performance

The relative growth performance of *T. putitora* in response to different levels of dietary Co supplementation is shown in Table 4. At the end of the experiment, a positive relationship was observed between % weight gain and Co dietary dosage level up to 2 mg Co/kg diet ($R^2 = 0.889$). However, values over 2 mg Co/kg diet negatively affected weight gain ($R^2 = 0.98$). Among different treatments, the highest percentage of weight gain was observed in fish fed 2 mg/kg dietary Co ($P < 0.05$). However, in fish fed diets supplemented with 2.5 mg/kg (group E) and 3 mg/kg (group F), dietary Co reduced fish weight compared to the control by 8% and 11.3%, respectively.

Table 2 Putative primers used in the present study for isolation of MyoD, myogenin and Hsp-70 cDNA fragments

Gene	Primer type	Sequence 5′ to 3′	Amplicon size
MyoD	Forward	TTTCTACGACGACCCTTGCTTC	464 bp
	Reverse	TGCCATCAGAGCAGTTGGATC	
Myogenin	Forward	CCAGCGTTTTTACGAAGGCG	665 bp
	Reverse	ACGTCAGAGACCTCAGGTTGG	
Hsp-70	Forward	ATGGTCCTGGTGAAGATGAAG	396 bp
	Reverse	GATGTCCTTCTTGTGCTTCCTC	

Whole body composition

Whole body proximate composition in response to different levels of dietary Co supplied to mahseer is shown in Table 5. Crude protein content showed a positive correlation ($R^2 = 0.99$) with dietary Co supplementation up to 2 mg/kg diet (group D), having values significantly higher than those of the control fish ($P < 0.05$). However, the highest level of dietary Co (3 mg/kg, group F) showed a negative effect (deceased content) on muscle protein. In contrast to protein content, muscle fat and ash exhibited a positive correlation ($R^2 = 0.8587$ and $R^2 = 0.756$, respectively) with dietary Co even at the highest supplementation level.

Metal bioaccumulation

The effect of graded levels of dietary Co concentration on the accumulation of Fe, Mn and Co in muscle is shown in Table 6. The concentration of Mn and Fe in the muscle of *T. putitora* showed a negative correlation (Mn, $R^2 = 0.907$; Fe, $R^2 = 0.993$) while Co showed a positive correlation ($R^2 = 0.953$) with dietary Co supplementation (Fig. 1a–c).

Cloning of *T. putitora* MyoD, myogenin and Hsp-70 cDNA fragments

Among fish, *T. putitora* MyoD exhibited higher similarity with sequences reported for *C. idella*, *D. rerio* and *C. carpio* (100%, 99.3% and 97.9% of identity, respectively), while the identity with other fish species ranged from 87.1% to 92.4%. A lower similarity was observed when compared with mammalian orthologues: identity with *Homo sapiens* and *Mus musculus* was 82.1% and 82.9%, respectively (Supplementary Fig. S1). As seen in MyoD, *T. putitora* myogenin also exhibited higher similarity with *D. rerio* and *C. carpio* (90.2% and 94.1% of identity, respectively). However, the percent identity with other fish species ranged from 68.9% to 74.0%, while 59.3% and 58.7% similarity were found with *H. sapiens* and *M. musculus*, respectively (Supplementary Fig. S2). For *T. putitora* Hsp-70, we found 98.3% similarity with amino acid sequences reported for *D. rerio*, while for other fish species, similarity ranged from 88.9% to 93.2%. The similarity of *T. putitora* Hsp-70 with that of *H. sapiens* and *M. musculus* was 85.5% and 90.6%, respectively (Supplementary Fig. S3).

Table 3 Putative primers used in the present study for RT-qPCR

Gene	Accession no.(GenBank)	Primer type	Sequence 5′ to 3′	Amplicon size
β-actin	KU714644.1	Forward	GCTGTGCTGTCCCTGTATGC	99 bp
		Reverse	GGCGTAACCCTCGTAGATGG	
MyoD	MH545701	Forward	CGCTTTCGAGACCCTCAAGAG	107 bp
		Reverse	GCGCCTGCAGAGACTCAATG	
Myogenin	MH545702	Forward	GGACAAACCGTCTCCATCTTC	165 bp
		Reverse	CCTCCTCTTCTCCCTCAAAGTG	
Hsp-70	MH545703	Forward	GATTGCTGAAGCCTATTCTGG	83 bp
		Reverse	TTGCCTCTGGGAGTCATTG	

Table 4 Effect of graded levels of dietary cobalt supplement on the growth performance of *T. putitora*

Diet groups	Mean initial weight (g)	Mean final weight (g)	Average weight gain (g)	Weight gain (%)	Specific growth rate (%)	FCR
Control	1.36 ± 0.006^{a}	2.39 ± 0.051^{d}	1.03 ± 0.201^{e}	75.73 ± 0.413^{e}	0.63 ± 0.003^{bc}	5.61 ± 0.001^{c}
A	1.35 ± 0.005^{a}	2.41 ± 0.020^{cd}	1.06 ± 0.043^{d}	78.44 ± 0.132^{d}	0.64 ± 0.001^{bc}	5.52 ± 0.002^{d}
B	1.34 ± 0.001^{a}	2.46 ± 0.012^{c}	1.12 ± 0.054^{c}	83.61 ± 0.213^{c}	0.68 ± 0.005^{b}	5.13 ± 0.001^{e}
C	1.34 ± 0.003^{a}	2.73 ± 0.032^{b}	1.39 ± 0.023^{b}	103.98 ± 0.432^{b}	0.79 ± 0.009^{a}	4.06 ± 0.005^{f}
D	1.33 ± 0.005^{a}	2.98 ± 0.017^{a}	1.65 ± 0.053^{a}	123.47 ± 0.332^{a}	0.81 ± 0.006^{a}	3.32 ± 0.004^{g}
E	1.33 ± 0.003^{a}	2.24 ± 0.053^{e}	0.91 ± 0.033^{f}	67.77 ± 0.543^{f}	0.58 ± 0.002^{c}	6.36 ± 0.006^{b}
F	1.34 ± 0.002^{a}	2.20 ± 0.042^{e}	0.86 ± 0.013^{g}	64.46 ± 0.324^{g}	0.55 ± 0.031^{c}	6.19 ± 0.005^{a}

Data are expressed as mean ± SEM ($n = 3$). Different letters within the columns indicate significant differences between groups ($P < 0.05$)

A = 0.5 mg Co/kg diet, B = 1 mg Co/kg diet, C = 1.5 mg Co/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

Expression levels of MyoD, myogenin and Hsp-70

Availability of cDNA sequences for *T. putitora* MyoD, myogenin and Hsp-70 allowed us to design primers to determine the mRNA levels of the three genes by quantitative RT-PCR (RT-qPCR) in fish fed basal/control diet (devoid of Co supplementation) and diets supplemented with 2 and 3 mg /kg Co. Feeding dietary Co resulted in significant changes of mRNA levels for muscle MyoD and myogenin as well as hepatic Hsp-70 in *T. putitora*. In the muscle, the highest mRNA level for both MyoD and myogenin was found in fish fed 2 mg/kg dietary Co, while feeding 3 mg/kg dietary Co resulted in the lowest expression levels for both proteins, even when compared with control fish (Fig. 2a,b). Albeit MyoD and myogenin behaved similarly, the expression of myogenin showed better correspondence with weight gain and growth rate. In contrast to MyoD and myogenin expression in the muscle, the highest Hsp-70 mRNA levels in the liver were observed in fish fed 3 mg Co/kg diet (Fig. 2c).

Table 5 Effect of different levels of dietary Co chloride on muscle proximate composition of *T. putitora*

Diet groups	Protein (%)	Fats (%)	Ash (%)
Control	16.31 ± 0.042^{e}	1.53 ± 0.23^{f}	1.56 ± 0.035^{f}
A	16.90 ± 0.023^{d}	1.61 ± 0.45^{e}	1.63 ± 0.052^{e}
B	18.14 ± 0.032^{c}	1.63 ± 0.021^{e}	1.65 ± 0.075^{e}
C	18.98 ± 0.043^{b}	1.82 ± 0.065^{d}	1.71 ± 0.034^{d}
D	20.02 ± 0.023^{a}	2.13 ± 0.032^{c}	2.06 ± 0.065^{c}
E	17.01 ± 0.067^{d}	2.48 ± 0.012^{b}	2.26 ± 0.043^{b}
F	15.09 ± 0.054^{f}	3.14 ± 0.054^{a}	2.75 ± 0.032^{a}

Data are expressed as mean ± SEM ($n = 9$). Different letters within the columns indicate significant differences ($P < 0.05$)

A = 0.5 mg/kg Co, B = 1 mg/kg Co, C = 1.5 mg/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

Discussion

The results of the present study demonstrate beneficial effects of dietary Co supplementation on growth performance, body composition, muscle content of manganese and iron as well as expression of growth-regulating genes in *T. putitora*. Similarly to our results, many investigators observed beneficial effects of dietary Co supplement on growth, survival, protein synthesis, glucose metabolism, insulin effectiveness in utilizing glucose, and efficiency of protein in fish species such as rainbow trout (Blust 2011), *Labeo rohita* (Adhikari and Ayyappan 2002) and common carp (Satoh 1991).

In the present study, we observed a positive correlation between weight gain (%) and dietary Co supplementation ranging from 0 to 2 mg/kg. The highest percentage of weight gain was observed in fish fed 2 mg/kg dietary Co, while higher levels of Co supplementation had negative impact on weight gain. Diets supplemented with 2.5 mg/kg (group E) and 3 mg/kg (group F) dietary Co reduced fish weight compared to the control by 8% and 11.3%, respectively. According to our results, 2 mg/kg is the optimum level of dietary Co supplementation for early rearing of *T. putitora*. This level is somewhat higher than the 1 mg/kg diet, which was reported for gaining 40% increase in survival rates of *C. carpio* hatchlings (Mukherjee and Kaviraj 2009), and 0.05 mg/kg dietary Co as reported for channel catfish (Wilson 1991), while lower than the 2.5 and 5 mg/kg diet which are known to improve growth

Table 6 Effect of different levels of dietary Co chloride on metal accumulation in *T. putitora* muscle

Diet groups	Metal concentrations (μg/g of sample tissue)		
	Iron	Manganese	Cobalt
Control	0.0303 ± 0.0015^{a}	0.00422 ± 0.0054^{a}	0.01075 ± 0.0021^{d}
A	0.0281 ± 0.0023^{b}	0.00442 ± 0.0044^{a}	0.01082 ± 0.0043^{d}
B	0.0250 ± 0.0045^{c}	0.00415 ± 0.0032^{a}	0.01115 ± 0.0012^{c}
C	0.0211 ± 0.0023^{d}	0.0038 ± 0.0056^{b}	0.01135 ± 0.0065^{c}
D	0.0163 ± 0.0034^{e}	0.0034 ± 0.00432^{c}	0.01214 ± 0.0054^{b}
E	0.0133 ± 0.0014^{f}	0.003451 ± 0.002^{c}	0.01221 ± 0.0062^{b}
F	0.0096 ± 0.0052^{g}	0.00310 ± 0.002^{d}	0.01260 ± 0.0042^{a}

Data are expressed as mean ± SEM ($n = 3$). Different letters within the columns indicate significant differences ($P < 0.05$)

A = 0.5 mg/kg Co, B = 1 mg/kg Co, C = 1.5 mg/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

performance of sea bass (*Lates calcarifer*) and catfish (*Clarias batrachus*), respectively (Sapkale and Singh 2011). Reports indicating different optimum levels of Co supplement may reflect variations in metabolic and functional demand of micronutrients, which depend on species, age, size, sex, feed, feeding practices and farming conditions (Biesalski Hans and Jana 2018). The improved growth performance observed in the present study in response to dietary Co supplementation may result from the well-known involvement of Co in nitrogen assimilation, hemoglobin synthesis, manufacturing of muscular protein as well as in fish metabolism and biochemical processes (Silvers and Scott 2002).

Dietary Co supplements above optimum level (2 mg/kg diet) showed negative effects on the growth performance of *T. putitora*. Like our results, Mukherjee and Kaviraj (2011) also reported a decrease in the weight gain of catfish, *Heteropneustes fossilis* (Bloch), with dietary Co supplementation above 0.1%. According to Chanda et al. (2015), high dietary cobalt levels over 5 g/kg are toxic to rainbow trout. This might be due to toxicity resulting from increased dietary Co levels, leading to inhibition of key enzymes and biochemical pathways by displacing cations of metal-activated enzymes in their ion centers, and oxidative damage to DNA, lipids and protein structure due to the generation of reactive oxygen species (ROS). In fact, Co ions are considered cytotoxic at high concentrations (Simonsen et al. 2012), which may induce necrosis and inflammation (Abudayyak et al. 2017).

Exogenous factors such as environment and feed composition affect the proximate composition of cultured fish (Alemu et al. 2013). Dietary feed ingredients play significant roles in fish body composition. Body composition assessment allows us to study the efficiency of nutrient transfer from feed to fish (Whitney and Rolfes 1993). Fish is considered as a rich source of important minerals, vitamins and essential amino acids and fatty acids. The protein content of fish has a marked biological significance due to the presence of essential amino acids. Our findings indicate that dietary Co up to 2 mg/kg increased protein content in the muscle as compared to the control fish. Since dietary Co facilitates amino acid incorporation into fish, therefore, it can promote a protein sparing effect by improving glucose tolerance and reducing gluconeogenesis (Kawakami et al. 2012; Ghica et al. 2013). Consistent with our results, Tonye and Sikoki (2014) reported increased crude protein content in juvenile tilapia (*Oreochromis niloticus*), while a similar effect was observed in *C. carpio* fed dietary supplement of Co (Mukherjee and Kaviraj 2009). In contrast to protein content, crude fat in our experimental trial positively correlated with dietary Co concentration, even at levels above 2.5 mg/kg. Likewise with *T. putitora*, dietary Co chloride also increased crude fat content in *C. carpio* (Mukherjee and Kaviraj 2009). This might be due to the involvement of Co in lipid metabolism by decreasing circulating levels of LDL cholesterol and triglycerides and increasing HDL cholesterol (Kawakami et al. 2012).

Fish is also a source of essential minerals to consumers (Steffens 2006). However, inclusion of metals in aquafeeds needs to be cautiously screened to certify that the metal is not accumulated at levels that may elicit toxicological effects. We found no reports that addressed the standardization of recommended dietary

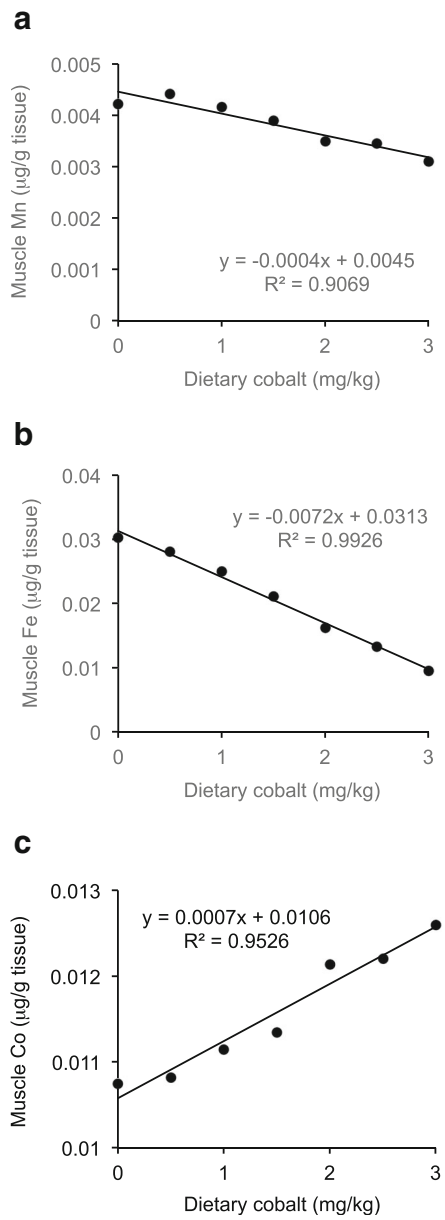


Fig. 1 Correlation between muscle accumulations of metals with graded level of dietary cobalt. Linear regression analysis for muscle accumulation of Mn (**a**), Fe (**b**) and Co (**c**) is shown. Each point represents the mean metal concentration in the muscle of three fishes

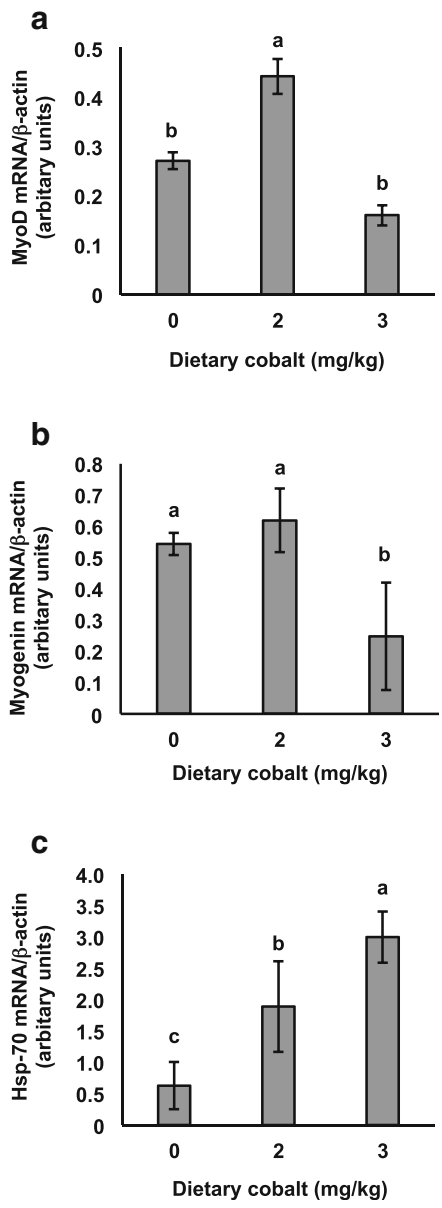


Fig. 2 MyoD, myogenin and Hsp-70 mRNA levels in the muscle and liver of *T. putitora*. Analysis of MyoD (**a**), myogenin (**b**) and Hsp-70 (**c**) mRNA levels relative to β-actin was performed by RT-qPCR in muscle and liver samples of *T. putitora* fed for 90 days with a diet supplemented with Co (2 mg/kg and 3 mg/kg) or without dietary Co (control). The values are expressed as mean ± SEM (n = 4). Statistical significance related to the control (without dietary Co supplementation) is indicated with different letters ($P < 0.05$)

allowance (RDA) of Co for fish. Indeed, micronutrient deficiency may result in increased accumulation of heavy metals from the environment (Golovanova 2008). In fish, the kidney, liver, gill, and gut are the main tissues where heavy metals are accumulated (Farkas et al. 2003; Ambreen et al. 2015). However, accumulation of dietary Co and its distribution in different tissues of fish are not precisely known. In our feeding trials, Co accumulation in the muscle positively correlated with dietary levels ($R^2 = 0.952$). Our findings are consistent with previous observations in other fish

species (Yildiz 2008). Although Co is an essential mineral that participates in biochemical processes, a high concentration may disrupt many enzymatic functions, causing toxicity (Javed 2013; Rai et al. 2015). In our experimental trial, iron accumulation shows a negative linear relationship with respect to increasing dietary Co supplementation. Most of the metals are competitively taken up in the intestinal tract of fish (Norwood et al. 2003). However, some intercellular transport systems are specific to one metal only, yet some are less selective, such as divalent metal transporter DMT1. Co is most commonly absorbed across the tissues through voltage-gated calcium channels and ligand-sensitive channels (Simonsen et al. 2012). Therefore, decreased levels of iron and manganese in the muscle resulting from increased dietary Co might be related to decreased absorption due to concentration-dependent interactive effects between metals (Kwong and Niyogi 2009).

For validating the results at the molecular level, the expression of growth and stress response genes through RT-qPCR was determined. Due to the unavailability of sequence information for *T. putitora* MyoD, myogenin and Hsp-70 messengers, genes of interest were first cloned and then sequenced. Isolation of cDNA fragments for these genes allowed us to assess changes in the expression levels of MyoD, myogenin and Hsp-70. *T. putitora* belongs to Cyprinidae, and as expected, alignment of the inferred peptide sequences of MyoD, myogenin and Hsp-70 with other cyprinids such as *D. rerio*, *C. carpio* and *C. idella* gave higher identities. Higher identity of amino acid sequences of *T. putitora* MyoD and Hsp-70 as compared to phylogenetically distant fish species and even with mammals suggest a high degree of structural conservation and conceivably functionality of MyoD and Hsp-70 during vertebrate evolution.

In contrast to MyoD and Hsp-70, *T. putitora* myogenin amino acid sequence displayed higher identity with species belonging to the same order (Cypriniformes), while the identity was markedly lower when comparing Cypriniformes to fish species belonging to Salmoniformes and Pleuronectiphormes as well as to mammals. Our findings argue for less conserved evolution of myogenin orthologues in vertebrates or specific evolution of myogenin in Cypriniformes.

MyoD and myogenin mRNA expression in the muscle of fish fed different dietary Co levels provided further insight about involvement of this mineral in the growth of *T. putitora.* The mRNA levels of MyoD and myogenin in the muscle showed a positive correlation with weight gain up to 2 mg/kg dietary level of Co. Cobalt chloride is a bioactive compound which induces the expression of a series of hypoxia response genes such as hypoxia-inducible factor (HIFα) by acting as a hypoxia-mimicking agent (Ji et al. 2012). HIFα is localized in the nucleus and cytoplasm of myotubes and myoblasts. Its increased expression in response to Co stimulates the myogenic differentiation and expression of myogenin and MyoD proteins (Wagatsuma et al. 2011). Co supplement-dependent increase in weight gain in our study might be due to the stimulation of myogenic regulating factors (MRFs) such as myogenic factor 5 (MyF5), MyoD, MRF4 and myogenin as well as increased skeletal muscle fiber formation (myogenesis) (Rescan 2001). These MRFs are highly conserved helix-loop-helix proteins in fish, and increased MyoD expression has a major role in regulating myogenesis and specification of newly formed skeletal muscles. MyoD is basically involved in the proliferation and activation of satellite cells towards the myogenic pathway while myogenin controls cell differentiation and myoblast fusions to form new myofibers. Additionally, Co is involved in the expression of several glycolytic enzymes and glucose transporters which enable anaerobic energy metabolism for regenerating myofibers (Wagatsuma et al. 2011). Since newly formed myofibers possess less capillary development to provide oxygen enough to regenerate skeletal muscle fibers, activation of glycolytic enzymes in response to Co may help newly formed myofibers.

Fish have well-developed networks of stress responses for adapting to environmental changes at the cellular level(Barton 2002). Such stress responses include the transcription of several stress proteins such as heat shock protein (Hsp) family members (Kregel 2002). Transcription of Hsp genes in response to different xenobiotics is considered a useful biomarker to assess the effect of metals in fish. We found a significant upregulation of Hsp-70 in the liver of *T. putitora* fed 3 mg/kg dietary Co compared to the control fish or fish fed with lower levels of dietary Co. This finding confirms the toxicity of Co when its level exceeded the optimum level. The liver plays a central role in metabolism, and it can be greatly affected by metal absorption in the gut (Soetan et al. 2010). Overexpression of Hsp-70 in response to higher dietary Co levels may indicate the stimulation of a cytoprotective mechanism, i.e. repair of metal-induced damaged proteins or bringing

them back to their normal conformation (Sener et al. 2003). Given that fish under stress conditions reduce feed intake (Lupatsch et al. 2010), retarded growth at higher dietary levels of Co could be the result of inadequate availability of nutrients required for proper growth performance (Yengkokpam et al. 2008).

In conclusion, our study revealed that 2 mg/kg of dietary Co supplementation is the optimum level where *T. putitora* showed improved growth performance by a mechanism involving increased protein content and enhanced expression of genes involved in muscle growth and differentiation. In contrast, higher levels of dietary Co increased the expression of the stress response gene Hsp-70 and had negative effects on growth.

Acknowledgements The work was supported by the Higher Education Commission of Pakistan under the International Research Support Initiative Program (IRSIP).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abudayyak M, Altincekic Gurkaynak T, Özhan G (2017) In vitro toxicological assessment of cobalt ferrite nanoparticles in several mammalian cell types. Biol Trace Elem Res 175: 458–465

Adhikari S, Ayyappan S (2002) Fertilization of freshwater fish ponds with cobalt and its adsorption and desorption in the pond sediment. Isr J Aquac 54:110–115

Alemu LA, Melese AY, Gulelat DH (2013) Effect of endogenous factors on proximate composition of nile tilapia (*Oreochromis niloticus* L.) fillet from Lake Zeway. Am J Res Commun 1:405–410

Ambreen F, Javed M, Batool U (2015) Tissue specific heavy metals uptake in economically important fish, *Cyprinus carpio* at acute exposure of metals mixtures. Pak J Zool 47: 399–407

Amir I, Zuberi A, Imran M, Ullah S (2018) Evaluation of yeast and bacterial based probiotics for early rearing of *Labeo rohita* (Hamilton, 1822). Aquac Res 49:3856–3863

AOAC (2000) Official methods of analysis. Association of Official Analytical Chemists. Washington, DC

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525

Beveridge MCM, Thilsted SH, Phillips MJ, Metian M, Troell M, Hall SJ (2013) Meeting the food and nutrition needs of the poor: the role of fish and the opportunities and challenges emerging from the rise of aquaculture. J Fish Biol 83:1067–1084

Bhatt JP, Pandit MK (2016) Endangered golden mahseer *Tor putitora* Hamilton: a review of natural history. Rev Fish Biol Fish 26:25–38

Biesalski Hans K, Jana T (2018) Micronutrients in the life cycle: requirements and sufficient supply. NFS J 11:1–11

Blust R (2011) Cobalt. In: Wood CM, Farrel AP, Brauner CJ (eds) Fish physiology, Homeostasis and toxicology of essential metals, vol 31A. Elsevier, Amsterdam, pp 291–326

Chanda S, Paul BN, Ghosh K, Giri SS (2015) Dietary essentiality of trace minerals in aquaculture—a review. Agric Rev 36: 100–112.

Farkas A, Salánki J, Specziár A (2003) Age- and size-specific patterns of heavy metals in the organs of freshwater fish *Abramis brama* L. populating a low-contaminated site. Water Res 37:959–964

Ghica ME, Carvalho RC, Amine A, Brett CMA (2013) Glucose oxidase enzyme inhibition sensors for heavy metals at carbon film electrodes modified with cobalt or copper hexacyanoferrate. Sensors Actuators B Chem 178:270–278

Golovanova I (2008) Effects of heavy metals on the physiological and biochemical status of fishes and aquatic invertebrates. Inland Water Biol 1:93–101

Javed M (2013) Chronic effects of nickel and cobalt on fish growth. Int J Agric Biol 15:575–579

Ji Q, Yang L, Zhou J et al (2012) Protective effects of paeoniflorin against cobalt chloride-induced apoptosis of endothelial cells via HIF-1α pathway. Toxicol in Vitro 26:455–461

Karadede-Akin H, Ünlü E (2007) Heavy metal concentrations in water, sediment, fish and some benthic organisms from Tigris River, Turkey. Environ Monit Assess 131:323–337

Kawakami T, Hanao N, Nishiyama K et al (2012) Differential effects of cobalt and mercury on lipid metabolism in the white adipose tissue of high-fat diet-induced obesity mice. Toxicol Appl Pharmacol 258:32–42

Kräutler B (2005) Vitamin B12: chemistry and biochemistry. Biochem Soc Trans 33:806–810

Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92:2177–2186

Kwong RWM, Niyogi S (2009) The interactions of iron with other divalent metals in the intestinal tract of a freshwater teleost, rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol Part C Toxicol Pharmacol 150:442–449

Lall SP (2002) The minerals. In: Halver JE, Hardy RW (eds) Fish nutrition, 3rd edn. Academic Press, San Diego, CA, pp 259–308

Lupatsch I, Santos GA, Schrama JW, Verreth JAJ (2010) Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. Aquaculture 298:245–250

Malomo GA, Ihegwuagu NE (2017) Some aspects of animal feed sampling and analysis. In: Stauffer MT (ed) Ideas and applications toward sample preparation for food and beverage analysis. IntechOpen, London, pp 77–93

McDowell LR (1989) Vitamins in animal nutrition: comparative aspects to human nutrition. Academic Press, San Diego, CA

Moll R, Davis B (2017) Iron, vitamin B_{12} and folate. Medicine (Baltimore) 45:198–203

Mukherjee S, Kaviraj A (2009) Evaluation of growth and bioaccumulation of cobalt in different tissues of common carp, *Cyprinus carpio* (Actinopterygii: Cypriniformes: Cyprinidae), fed cobalt-supplemented diets. Acta Ichthyol Piscat 39:87–93

Mukherjee S, Kaviraj A (2011) Bioaccumulation of cobalt, digestive enzyme activities, and growth of freshwater catfish, *Heteropneustes fossilis* (Bloch), fed cobalt-supplemented diets. Toxicol Environ Chem 93:575–584

Munir MB, Hashim R, Chai YH et al (2016) Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (*Channa striata*) fingerlings. Aquaculture 460: 59–68

Norwood WP, Borgmann U, Dixon DG, Wallace A (2003) Effects of metal mixtures on aquatic biota: a review of observations and methods. Hum Ecol Risk Assess An Int J 9:795–811

Oliva-Teles A (2012) Nutrition and health of aquaculture fish. J Fish Dis 35:83–108

Perrault JR, Buchweitz JP, Lehner AF (2014) Essential, trace and toxic element concentrations in the liver of the world's largest bony fish, the ocean sunfish (*Mola mola*). Mar Pollut Bull 79: 348–353

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Rahal A, Shivay YS (2016) Micronutrient deficiencies in humans and animals: strategies for their improvement. Biofortification Food Crop. Springer India, New Delhi, pp 217–228

Rai A, Ullah A, Haider J (2015) Determination of acute toxicity of copper and cobalt for *Tilapia nilotica*. J Bioresour Manag 2: 16–25

Rescan PY (2001) Regulation and functions of myogenic regulatory factors in lower vertebrates. Comp Biochem Physiol Part B Biochem Mol Biol 130:1–12

Robbins CT (1993) Wildlife feeding and nutrition. Academic Press, San Diego, CA

Sapkale P, Singh R (2011) Dietary zinc and cobalt requirements of fry of seabass (*Lates calcarifer*) and catfish (*Clarias batrachus*). Isr J Aquac 63:613

Satoh S (1991) Common carp, *Cyprinus carpio*. In: Wilson RP (ed) Handbook of nutrient requirements in finfish. CRC Press, Boca Raton, FL, pp 55–68

Sener G, Sehirli AO, Ayanoglu-Dulger G (2003) Melatonin protects against mercury(II)-induced oxidative tissue damage in rats. Pharmacol Toxicol 93:290–296

Siddiqui K, Bawazeer N, Scaria Joy S (2014) Variation in macro and trace elements in progression of type 2 diabetes. Sci World J 2014:1–9

Sievers F, Wilm A, Dineen D et al (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539

Silvers KM, Scott KM (2002) Fish consumption and self-reported physical and mental health status. Public Health Nutr 5:427–431

Simonsen LO, Harbak H, Bennekou P (2012) Cobalt metabolism and toxicology—a brief update. Sci Total Environ 432:210–215

Soetan KO, Olaiya CO, Oyewole OE (2010) The importance of mineral elements for humans, domestic animals and plants—a review. Afr J Food Sci 4:200–222

Speich M, Pineau A, Ballereau F (2001) Minerals, trace elements and related biological variables in athletes and during physical activity. Clin Chim Acta 312:1–11

Steffens W (2006) Freshwater fish—wholesome foodstuffs. Bulg J Agric Sci 12:320–328

Swanner ED, Planavsky NJ, Lalonde SV et al (2014) Cobalt and marine redox evolution. Earth Planet Sci Lett 390:253–263

Takahashi-Iñiguez T, García-Hernandez E, Arreguín-Espinosa R, Flores ME (2012) Role of vitamin B_{12} on methylmalonyl-CoA mutase activity. J Zhejiang Univ Sci B 13:423–437

Terech-Majewska E, Pajdak J, Siwicki AK (2016) Water as a source of macronutrients and micronutrients for fish, with special emphasis on the nutritional requirements of two fish species: the common carp (*Cyprinus carpio*) and the rainbow trout (*Oncorhynchus mykiss*). J Elem 21:947–961

Tonye IA, Sikoki FD (2014) Growth performance and proximate composition of *Oreochromis niloticus* (Trewavas) fed cobalt chloride incorporated diet. J Biol Agric Healthc 4:106–125

Wagatsuma A, Kotake N, Yamada S (2011) Spatial and temporal expression of hypoxia-inducible factor-1α during myogenesis in vivo and in vitro. Mol Cell Biochem 347: 145–155

Whitney EN, Rolfes SR (1993) Understanding nutrition. West Publishing Company, St. Paul, MN

Wilson RP (1991) Handbook of nutrient requirements of finfish. CRC Press, Boca Raton, FL

Yengkokpam S, Pal AK, Sahu NP et al (2008) Metabolic modulation in *Labeo rohita* fingerlings during starvation: Hsp70 expression and oxygen consumption. Aquaculture 285:234–237

Yildiz M (2008) Mineral composition in fillets of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*): a comparison of cultured and wild fish. J Appl Ichthyol 24: 589–594

Zhou Y, Yuan X, Liang X-F et al (2013) Enhancement of growth and intestinal flora in grass carp: the effect of exogenous cellulase. Aquaculture 416–417:1–7

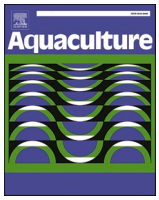
Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Aquaculture 555 (2022) 738221

Contents lists available at ScienceDirect

Aquaculture

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



Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (*Sparus aurata*)

Alberto Sáez-Arteaga [a], Yuanbing Wu [b], Jonás I. Silva-Marrero [b], Ania Rashidpour [b], María Pilar Almajano [c], Felipe Fernández [d], Isabel V. Baanante [b], Isidoro Metón [b,*]

[a] *Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Temuco, Chile*
[b] *Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain*
[c] *Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain*
[d] *Secció d'Ecologia, Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain*

ARTICLE INFO

Keywords:
Dietary macronutrients
Gene expression
Gene markers
Growth
Muscle
Sparus aurata

ABSTRACT

To increase our current knowledge on the nutritional regulation of growth and gene expression pattern in fish skeletal muscle, the effect of dietary macronutrient composition was assessed on digestibility, nutrient retention, growth performance, and the mRNA levels of key genes involved in functionality, growth and development of the skeletal muscle in gilthead sea bream (*Sparus aurata*). Long-term starvation decreased the expression of myogenic regulatory factors such as Myod2, Myf5, myogenin (Myog) and Myf6 in the skeletal muscle of *S. aurata*. The supply of high or medium protein, low carbohydrate diets enhanced growth parameters, feed efficiency ratio, feed conversion ratio and significantly upregulated *myod2*. However, the supply of low protein, high carbohydrate diets restricted growth and stimulated the mRNA levels of *myostatin*, while downregulated *follistatin* (*fst*), *igf1*, *mtor* and *rps6*. Microarray analysis revealed *igfals*, *tnni2*, and *gadd45a* as gene markers upregulated by diets enriched with protein, lipids and carbohydrates, respectively. The results of the present study show that in addition to *myod2*, *fst*, *igf1*, *mtor* and *rps6*, the expression levels of *igfals*, *tnni2* and remarkably *gadd45a* in the skeletal muscle can be used as markers to evaluate the effect of dietary macronutrient changes on fish growth and muscle development in *S. aurata*.

1. Introduction

The development of the skeletal muscle follows a well-ordered structure that is highly adaptable to changing conditions. The process of myogenesis has a marked plasticity, which constitutes a fundamental event for proliferation, differentiation, migration and fusion of new myoblasts (Johnston, 2006). This complex and dynamic process leads to fusion of myocytes with existing myofibers (hypertrophy) and increasing proliferation of myocytes (hyperplasia) (Zhu et al., 2014). Myogenesis is primarily controlled by myogenic regulatory factors (MRFs) which include Myod, Myf5, myogenin (Myog) and Myf6, among others (Braun and Gautel, 2011; Rescan, 2001; Alami-Durante et al., 2019). MRFs are highly conserved proteins from teleosts to mammals that belong to a larger group of transcription factors containing a basic DNA-binding motif and a helix-loop-helix dimerisation domain (Olson and Klein, 1994; Rossi and Messina, 2014). Myod and Myf5 are essential for initiation of the myogenic program, while Myog and Myf6 are expressed later, during myofibre differentiation (Holterman and Rudnicki, 2005; Rescan, 2001; Tan and Du, 2002). Additional factors, such as myostatin (Mstn) and follistatin (Fst), which belong to the transforming growth factor beta (Tgfb) superfamily, were also shown to have a major role in skeletal muscle growth and development. Mstn is a potent negative regulator of skeletal muscle growth (McPherron et al., 1997). Knockout mice for Mstn present 2 to 3-fold greater skeletal muscle mass than wild-type animals. Fst is an activin-binding protein essential for muscle fibre formation (Medeiros et al., 2009), and a potent antagonist of several members of the Tgfb superfamily, including Mstn (Amthor et al., 2004). Overexpression of Fst in mice and rainbow trout (*Oncorhynchus mykiss*) evidences the capacity of Fst to increase muscle mass (Lee and McPherron, 2001; Medeiros et al., 2009).

* Corresponding author.
E-mail address: imeton@ub.edu (I. Metón).

https://doi.org/10.1016/j.aquaculture.2022.738221
Received 11 November 2021; Received in revised form 22 February 2022; Accepted 1 April 2022
Available online 7 April 2022
0044-8486/© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Somatotropic compounds such as insulin-like growth factor 1 (Igf1) exert an important role in nutritional regulation of metabolism. Igf1 expression increases muscle mass and decreases muscle atrophy (Christoffolete et al., 2015; Glass, 2003a, 2003b; Vélez et al., 2014). Protein synthesis and glucose and amino acid uptake is stimulated by Igf1 in rainbow trout (*O. mykiss*) and gilthead sea bream (*S. aurata*) myocytes (Castillo et al., 2004; Montserrat et al., 2012). Igf1 and amino acids activate the phosphatidylinositol 3-kinase (Pi3k)-Akt pathway, which leads to phosphorylation of the serine/threonine protein kinase mechanistic target of rapamycin (Mtor) and activation of a critical pathway involved in cellular processes such as apoptosis, protein synthesis, gene transcription and cell proliferation (Vélez et al., 2016). Indeed, Mtor is an essential sensor of nutrient and amino acid availability through phosphorylation of 40S ribosomal protein S6 (Rps6) (Glass, 2005; Schiaffino and Mammucari, 2011).

Despite the fact that carnivorous fish are considered glucose intolerant (Panserat et al., 2019; Rashidpour et al., 2019), we previously showed that partial replacement of dietary protein by carbohydrates stimulates glucose oxidation via glycolysis and pentose phosphate pathway in the liver of *S. aurata* (Fernández et al., 2007; Metón et al., 1999). Indeed, the administration of chitosan-tripolyphosphate-DNA to overexpress srebp1a stimulates conversion of dietary carbohydrates into lipids in *S. aurata* through enhanced hepatic glycolysis, pentose phosphate pathway and lipogenesis (Silva-Marrero et al., 2019; Wu et al., 2020). Dietary carbohydrates upregulate the expression of glycolytic genes in the muscle of rainbow trout (Song et al., 2018), while it was claimed that glucose regulates protein synthesis and growth-related mechanisms in myogenic precursor cells from this species (Latimer et al., 2019). Although the fundamental events in muscle growth and development were well conserved during vertebrate evolution, fishes have unique features such as continuous growth and different proportion of white and red muscle fibres than mammals. Indeed, the molecular events that mediate the effect of dietary nutrients on signaling pathways that control growth of fish skeletal muscle remain unclear. With the aim to increase our current knowledge about the nutritional regulation of skeletal muscle growth and development in fish, we addressed the effect of dietary macronutrient composition on nutrient retention, growth performance and the gene expression pattern in the skeletal muscle of *S. aurata*, and identified novel gene markers for nutritional studies in cultured fish.

2. Materials and methods

2.1. Animals, feeding trial and sampling

S. aurata juveniles were obtained from Piscicultura Marina Mediterranea (Burriana, Castellón, Spain). A total of 330 fish (8.22 g ± 0.26 body weight) were transported to the laboratory, and distributed in 12 aquaria of 260 l supplied with running seawater at 21 °C in a closed system with active pump filter and UV lamps. The photoperiod was adjusted to a 12 h: 12 h dark-light cycle. Fish maintenance conditions were as previously described (Fernández et al., 2007). Three diets were formulated with gross energy at 20–22 kJ/g and macronutrient composition at levels above and below those in commercially available aquafeeds: HLL (high protein, low lipid, low carbohydrate), MHL (medium protein, high lipid, low carbohydrate) and LLH (low protein, low lipid, high carbohydrate) (Table 1). Following acclimation to our facilities, three groups of fish were fed twice a day (9:30 a.m. and 15:30 p.m.) with 25 g/kg body weight of the corresponding experimental diet for 23 days for microarray analysis and 37 days for evaluating growth, digestibility and gene expression (Fig. 1). Sampling points were selected according to previous studies showing that a period of 18–20 days is long enough for producing significant changes due to diet composition in the intermediary metabolism and gene expression profile of *S. aurata* juveniles (Metón et al., 1999; Silva-Marrero et al., 2017), while longer periods are necessary to obtain significant changes in growth and nutrient retention parameters (Fernández et al., 2007). A fourth group of fish was submitted to starvation during the same period of time. Each dietary condition was assayed in 3 aquaria. All fish were weighed at the beginning of the experiment and every 12 days during the experiment. To calculate apparent digestibility coefficient (ADC), 0.1% yttrium oxide (Y_2O_3) was included in each experimental diet and used as inert marker the last week of the experiment. To determine body composition, 30 fish from the initial stock and 5 fish per aquarium at the end of the experiment were sampled and stored at −20 °C for subsequent analysis. Prior to sampling, fish were starved for 24 h and sacrificed by anaesthesia overdose with 1:12,500 tricaine methanesulfonate (MS-222) diluted in seawater followed by cervical section. Samples of skeletal

Table 1
Composition of the diets supplied in this study to *S. aurata*.

	HLL	MHL	LLH
Formulation (%)			
Fish meal[a]	81.6	67.5	54.3
Fish oil[b]	0.8	13.1	6.0
Starch[c]	15.0	16.7	37.1
Carrageenan[d]	1.5	1.5	1.5
Mineral mixture[e]	0.9	0.9	0.9
Vitamin mixture[f]	0.2	0.2	0.2
Proximate Composition (%)			
Crude protein	59.5	50.1	40.6
Crude lipid	7.2	17.5	8.2
Carbohydrates[g]	18.4	20.9	40.9
Ash	14.9	11.5	10.3
Gross energy (kJ/g)[h]	20.2	22.4	19.9

[a] Corpesca S.A. Super-Prime fish meal (Santiago de Chile, Chile).
[b] Fish oil from A.F.A.M.S.A. (Vigo, Spain).
[c] Pregelatinised corn starch from Brenntag Química S.A. (St. Andreu de la Barca, Barcelona, Spain).
[d] Iota carrageenan (Sigma-Aldrich).
[e] Mineral mixture provided (mg/kg): $CaHPO_4 \cdot 2\ H_2O$, 7340; MgO, 800; KCl, 750; $FeSO_4 \cdot 7\ H_2O$, 60; ZnO, 30; MnO_2, 15; $CuSO_4 \cdot 5\ H_2O$, 1.7; $CoCl_2 \cdot 6\ H_2O$, 1.5; KI, 1.5; Na_2SeO_3, 0.3.
[f] Vitamin mixture provided (mg/Kg): choline chloride, 1200; myo-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-rac-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; piridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-trans retinol, 2; biotin, 0,7 cholecalciferol, 0.05; cyanocobalamin, 0.05.
[g] Carbohydrates were calculated by difference.
[h] Calculated from gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17 kJ/g).

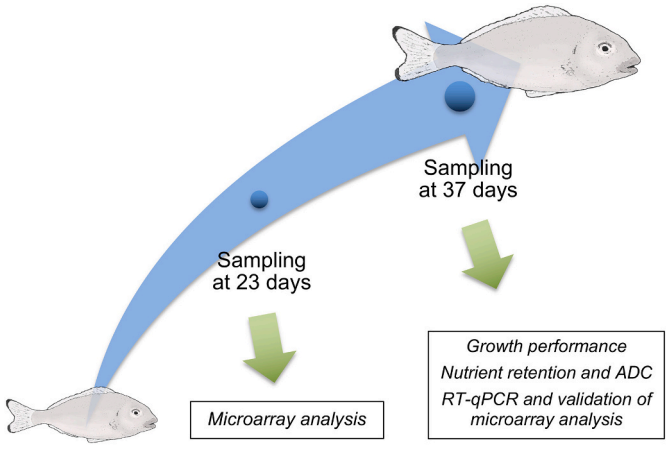


Fig. 1. Schematic workflow of sampling points and analysis carried out in the present study. ADC: apparent digestibility coefficient. RT-qPCR: reverse transcription coupled to quantitative real-time PCR.

muscle extracted from the middle/dorsal region were dissected out, immediately frozen in liquid nitrogen and kept at −80 °C until analysis. For fecal collection, the posterior intestine was dissected out and the intestinal content was obtained by stripping. Posterior intestine samples were pooled (pools of 5 fish per aquarium), dried at 70 °C and kept at −20 °C. Experimental procedures involving fish complied with the guidelines of the University of Barcelona's Animal Welfare Committee and EU Directive 2010/63/EU for animal experiments.

2.2. Diet and body composition

To determine moisture, samples were dried at 70 °C until constant weight was obtained (Busacker et al., 1990; Lucas, 1996). Samples were analysed for carbon (C) and nitrogen (N) with a Carlo Erba NA 2100 elemental analyser (CE Instruments, Wigan, UK). Lipid content was extracted with petroleum ether using a Foss Tecator Soxtec HT 1043 extraction system (Hillerød, Denmark). Ash content was determined after incineration of samples in muffle furnace at 450 °C for 12 h (Busacker et al., 1990; Lucas, 1996). Protein was calculated from N content, using a factor of 6.25. Gross energy was calculated on the basis of dietary protein (24 kJ/g), lipid (39 kJ/g) and carbohydrate (17 kJ/g) (Bradfield and Llewellyn, 1982). For assaying Ca, P and Y, samples were digested and analysed with an inductively coupled plasma spectrometer (Polyscan 61E, Thermo Jarrell Ash Corporation, Waltham, MA, USA).

2.3. Apparent digestibility and growth parameters

Specific growth rate (SGR) was calculated as $(\ln W_f - \ln W_i)*100/T$, where $\ln W_f$ and $\ln W_i$ are the natural logarithms of the final and initial mean fish weight in grams per aquarium, respectively, and T is time in days. Feed conversion ratio (FCR) was calculated as dry feed intake/wet weight gain. ADC of a given nutrient was calculated from the following equation (De Silva and Anderson, 1995):

$$ADC = 100–[100*(Nutrient_{Feces}/Nutrient_{Diet})*(Y_{Diet}/Y_{Feces})]$$

For dry matter, the equation became:

$$ADC = 100–[100*(Y_{Diet}/Y_{Feces})]$$

Other parameters calculated for each aquarium included: protein efficiency ratio (PER = g weight gain/g feed protein); protein retention (PR = g protein gain*100/g feed protein); lipid retention (LR = g lipid gain*100/g feed lipid) and hepatosomatic index (HSI = liver fresh weight*100/ fish body weight).

2.4. RNA isolation

Total RNA was extracted from the skeletal muscle using the RNeasy fibrous tissue Mini Kit (Qiagen, Sussex, UK) according to the manufacturer's recommendations. Concentration and purity were determined spectrophotometrically at 260/280 nm using Nanodrop ND-1000 (Thermo Fischer Scientific, Waltman, MA, USA). RNA integrity was determined with an Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA Integrity Number (RIN) > 9.2 were used for subsequent studies.

2.5. Quantitative real-time PCR

The mRNA levels of key genes involved in skeletal muscle growth and development, and differentially expressed genes selected from the microarray analysis were assayed by reverse transcription coupled to quantitative real-time PCR (RT-qPCR). One microgram of total RNA isolated from white skeletal muscle of *S. aurata* was reverse-transcribed to cDNA with Moloney murine leukemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 °C in the presence of random hexamer primers. The cDNA product was used for subsequent qPCR. The mRNA levels of *S. aurata* genes listed in Table 2 were determined in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 0.4 μM of each specific primer (Table 2), 10 μl of SYBR Green (Applied Biosystems Foster City, CA, USA), and 1.6 μl of the diluted cDNA product in a final volume of 18 μl. The temperature cycle protocol for amplification was: 1 cycle of initial activation at 95 °C for 10 min and 40 cycles of 15 s at 95 °C, 1 min at 62 °C. To validate the amplification efficiency of primers, standard curves with serial dilutions of a control cDNA were generated, and PCR amplicons were separated electrophoretically on 2% agarose gel for band size confirmation. 18S ribosomal RNA (18 s) and elongation factor 1 alpha (ef1α) were selected to normalise the amount of mRNA for the genes of interest in each sample. Variations in gene expression were calculated by the standard ΔΔCt method (Pfaffl, 2001). Results are presented as mean ± SD (n = 4–6 fish).

2.6. Microarray hybridisation and data analysis

A microarray analysis was performed to detect differentially expressed genes with potential interest for nutritional and diet formulation studies in *S. aurata*. To this end, labelling, hybridisation and scanning of an Agilent custom high-density oligonucleotide microarray (8 × 60 k; ID 079501; Agilent Technologies, Santa Clara, CA, USA), previously described to contain 2 60-mer probes for each of 25,392 assembled unique sequences of *S. aurata* transcriptome (Silva-Marrero et al., 2017), were performed with the *Two-Color Microarray-Based Gene Expression Analysis v. 6.5* kit (Agilent Technologies, Santa Clara, CA, USA). Total RNA isolated from the skeletal muscle of four fish was analysed for each condition (starved fish and fish fed diets HLL, MHL and LLH for 23 days). For each sample, 200 ng of total RNA was labelled with Cy3 or Cy5 using *Low Input Quick Amp Labeling Kit* (Agilent Technologies, Santa Clara, CA, USA), and microarray workflow for linearity, sensitivity and accuracy was monitored using *The Two-Color and RNA*

Table 2
Primers used to analyse gene expression by qPCR.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	GenBank accession nos.
18 s	TTACGCCCATGTTGTCCTGAG	AGGATTCTGCATGATGGTCACC	AM490061
ef1α	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	AF184170
fst	GGAAACGACGGGATCATCTATGC	CGACTTGGCCTTGATGCATTTTCC	AY544167
gadd45α	AGCGGGGTCGTTTTTTATTTCTTC	AGGAAGTGTGGTGTGTACCC	XM_030401611
igf1	ACTGCTGTGCGTCCTCACCCTGA	GTGCATTGGGGCCGTAGCCA	AY996779
igfals	TGTGGTGGAACGCCAGAGCTTTG	GAGGCCAGAGAATGTATGGGTTGTGAG	XM_030407223
mstn	GGATGCAGGAACACACACAC	AGACGACGAAGGACGAGAAA	AF258448.1
mtor	GGAGACTGTTTTGAGGTCGCC	ACCTCCATCACCGTGTGGCA	MH594580
myf5	CGACGGCATGGTTGACAGCA	TCCGGCTGTCTTATCGCCCA	JN034420
myf6	TCATCCCACAGCTTTAAAGGCA	AGTGAATCTTCGGCGTCCTCC	JN034421
myod2	CACTACAGCGGGGATTCAGAC	CCGTTTGCTTCTCCTGGACT	AF478569
myog	TTCCCTGACCAGCGCTCCTA	TCTGTTCCTGTCACCCCAAC	EF462191.1
rps6	CAGCAAGATCCGCAAGCTCT	CTTCTGGGTGCGCTGTCTCT	MN172174
tnni2	GCCCTGAAGAAAGTACGTATGTCTGC	CTCCCCTCCTCCTTGACCTCCTTC	XM_030426857

Spike-In Kit, Two-Color. The *RNeasy Mini Kit* (Qiagen, Hilden, Germany) was used to purify labelled cRNA. Microarray hybridisation was performed using 2.5 μg of each labelled sample at 65 °C for 17 h following the *Gene Expression Hybridization Kit* instructions. A double loop hybridisation with dye swap experimental design was followed (Kerr and Churchill, 2001), and included eight hybridisations to maximise discovery of significant changes among assayed conditions (starvation and feeding with diets HLL, MHL and LLH; $n = 4$ per condition). Scanning was performed using an Agilent Microarray Scanner G2565BA, and outlier spots and spot intensity for Cy3 and Cy5 channels were extracted with Agilent Feature Extraction software v. 10.7. Loess and Aquantile normalisation for within-and inter-array normalisation, respectively, was performed with R-Bioconductor package (Gentleman et al., 2004). Data analysis was only considered for unique sequences involved in growth and development with *E*-value <1e-10 and HSP/hit >30.

2.7. Statistics

Data concerning growth performance, nutrient retention, apparent digestibility and qPCR were analysed using SPSS Version 25 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by the Duncan post-hoc test was performed to identify significant differences between treatments. Statistical significance was considered when $P < 0.05$. For microarray data, a linear model analysis using Limma (Smyth, 2004) was conducted to determine differentially expressed genes with adjusted $P < 0.05$. Correlation coefficient values were calculated to assess relationships between growth, nutrient retention, ADC and gene expression variables, considering statistical significance when $P < 0.05$.

3. Results

3.1. Growth performance, nutrient retention and digestibility

The effect of dietary macronutrient composition on growth parameters and nutrient retention was studied in *S. aurata* juveniles fed 37 days with diets HLL (macronutrient composition similar to the diet of wild *S. aurata*), MHL (composition similar to commercial diets) and LLH (composition similar to HLL but with partial substitution of protein by carbohydrates). Fish fed the high protein, low lipid, low carbohydrate diet (HLL) and the medium protein, high lipid, low carbohydrate diet (MHL) presented significantly higher values of final body weight and SGR than fish fed the low protein, low lipid, high carbohydrate diet (LLH; Table 3). The highest FCR value was found in fish fed the LLH diet, while no significant difference was found between diets HLL and MHL. Fish fed the LLH diet also presented the highest LR value. The lowest PER levels were found for fish fed diet HLL, which were significantly different than in fish fed the MHL diet (Table 3). Diet composition did not significantly affect HSI or PR.

No significant differences were found in ADC values for calcium, carbon and dry matter in the posterior intestine of fish fed 37 days with different diets. However, fish fed LLH revealed a trend to present lower values than fish fed diets HLL and MHL (Table 4). ADC levels for protein were not affected by diet composition, while the highest values for phosphorus, calcium, carbon and dry matter were found in fish fed MHL. No significant correlation was found between ADC values and growth and nutrient retention parameters (Table 5).

Table 3
Growth performance and nutrient retention of *S. aurata* fed diets HLL, MHL and LLH for 37 days.

	HLL	MHL	LLH
Initial BW (g)	8.20 ± 0.04	8.52 ± 0.06	8.11 ± 0.21
Final BW (g)	17.69 ± 0.22^{b}	18.36 ± 0.18^{b}	14.76 ± 0.71^{a}
SGR	2.08 ± 0.04^{b}	2.08 ± 0.02^{b}	1.61 ± 0.10^{a}
FCR	1.58 ± 0.01^{a}	1.44 ± 0.04^{a}	1.96 ± 0.16^{b}
HSI	1.22 ± 0.08	1.11 ± 0.05	1.33 ± 0.11
PR (% intake)	17.92 ± 0.72	20.52 ± 1.05	20.34 ± 1.79
LR (% intake)	24.90 ± 4.14^{a}	33.21 ± 2.15^{ab}	41.95 ± 4.30^{b}
PER	1.06 ± 0.01^{a}	1.39 ± 0.04^{b}	1.27 ± 0.10^{ab}

Data are means ± SEM ($n = 3$ tanks). Different superscript letters indicate significant differences among dietary conditions ($P < 0.05$). BW: body weight.

3.2. Effect of diet composition and starvation on the expression of MRFs, *mstn* and *fst* in the skeletal muscle

Dietary macronutrient composition and food deprivation significantly affected the expression of MRFs in the white skeletal muscle of *S. aurata*. Starvation for 37 days significantly downregulated the mRNA levels of all MRFs assayed. Concerning fed animals, the supply of HLL and MHL significantly enhanced 1.7–1.9 fold the expression levels of *myod2* compared to fish fed with diet LLH (Fig. 2a). In a significant manner but to a lesser extent than *myod2*, feeding fish with the diet containing the lowest protein/carbohydrate ratio (LLH) decreased *myf5* and *myog* mRNA levels when compared to fish fed with medium protein, high lipid, low carbohydrate (MHL) and high protein, low lipid, low carbohydrate (HLL) diets, respectively (Fig. 2b-c). The only MRF assayed whose mRNA levels did not significantly differ as a result of diet composition was Myf6 (Fig. 2d). The mRNA levels of *myod2* and *myog* correlated positively with final body weight and SGR, and negatively with FCR (Table 5).

In regard of genes with a major role in regulation of skeletal muscle growth, we also analysed the expression of *mstn* and *fst*. Feeding with LLH significantly upregulated *mstn* 2.1–2.9 fold over the values observed in starved fish and *S. aurata* fed with HLL and MHL (Fig. 3a). A different expressional pattern was observed for *fst*. The expression levels of *fst* were 1.8–2.0-fold significantly higher in fish fed HLL and MHL than in fish fed LLH and submitted to starvation (Fig. 3b). The expression levels of *mstn* and *fst* exhibited significant but opposite correlation with final body weight, SGR, FCR, HSI and LR (Table 5).

3.3. Effect of diet composition and starvation on the expression of *igf1*, *mtor* and *rps6* in the skeletal muscle

The effect of diet composition and starvation on *igf1* mRNA levels in the skeletal muscle of *S. aurata* was also addressed. Starvation for 37 days significantly downregulated 2.9–6.0 fold *igf1* expression depending on the diet supplied. Among fed fish, significant upregulation of *igf1* (1.8–2.0 fold) was found in fish fed diets with improved growth parameters (MHL and HLL) (Fig. 4a).

Previous studies indicated that in addition to posttranslational regulation, the nutritional status regulates Mtor and downstream proteins at the mRNA level in fish (Lavajoo et al., 2020; Qin et al., 2019). We therefore investigated the effects of starvation and feeding diets differing in macronutrient composition on *mtor* and *rps6* expression in the skeletal muscle of *S. aurata*. Among fed fish, the mRNA levels of *mtor* and *rps6* showed a significant dependence on the protein/carbohydrate ratio in the diet. For *mtor*, diet HLL promoted the highest expression values, which were 1.7-fold and 2.4-fold greater than in fish fed diets MHL and LLH, respectively. Likewise, fish fed HLL presented 1.3-fold

Table 4
Apparent digestibility coefficient (ADC) values obtained for *S. aurata* fed HLL, MHL and LLH diets.

	HLL	MHL	LLH
Phosphorus	39.96 ± 1.11	50.97 ± 3.48	44.74 ± 6.27
Calcium	54.71 ± 1.98	59.25 ± 5.34	45.07 ± 6.72
Carbon	70.53 ± 6.78	79.94 ± 3.44	65.08 ± 8.17
Protein	79.24 ± 2.38	78.08 ± 3.45	77.46 ± 0.73
Dry matter	64.76 ± 1.54	67.21 ± 4.30	57.18 ± 5.24

Data are means ± SEM ($n = 3$ tanks).

Table 5
Correlation coefficient values for growth and nutrient retention parameters versus ADC and gene expression values.

	Final BW	SGR	FCR	HSI	PR	LR	PER
Phosphorus	0.151	0.091	−0.202	−0.342	0.520	0.254	0.590
Calcium	0.628	0.639	−0.634	−0.621	−0.091	−0.430	0.103
Carbon	0.490	0.639	−0.512	−0.552	0.119	−0.210	0.278
Protein	0.134	0.000	−0.120	−0.072	−0.190	−0.204	−0.156
Dry matter	0.600	0.548	−0.601	−0.577	−0.134	−0.442	0.048
myod2	0.744**	0.822**	−0.746**	−0.715**	−0.167	−0.548	0.059
myf5	0.494	0.513	−0.508	−0.524	0.027	−0.273	0.183
myog	0.613*	0.616*	−0.578*	−0.442	−0.569	−0.703*	−0.428
myf6	−0.308	−0.140	0.318	0.336	−0.034	0.162	−0.137
mstn	−0.858**	−0.768**	0.857**	0.815**	0.220	0.650*	−0.040
fst	0.785**	0.804**	−0.766**	−0.688**	−0.385	−0.756**	−0.108
igf1	0.767**	0.837**	−0.768**	−0.735**	−0.244	−0.591	−0.017
mtor	0.662**	0.841**	−0.615*	−0.449	−0.725**	−0.857**	−0.544*
rps6	0.612*	0.737**	−0.565*	−0.400	−0.667**	−0.800**	−0.503
tnni2	0.519	0.462	−0.565	−0.676*	0.384	−0.052	0.562
igfals	0.249	0.444	−0.249	−0.249	−0.440	−0.552	−0.440
gadd45a	−0.645*	−0.539*	0.623*	0.528	0.435	0.649*	0.259

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

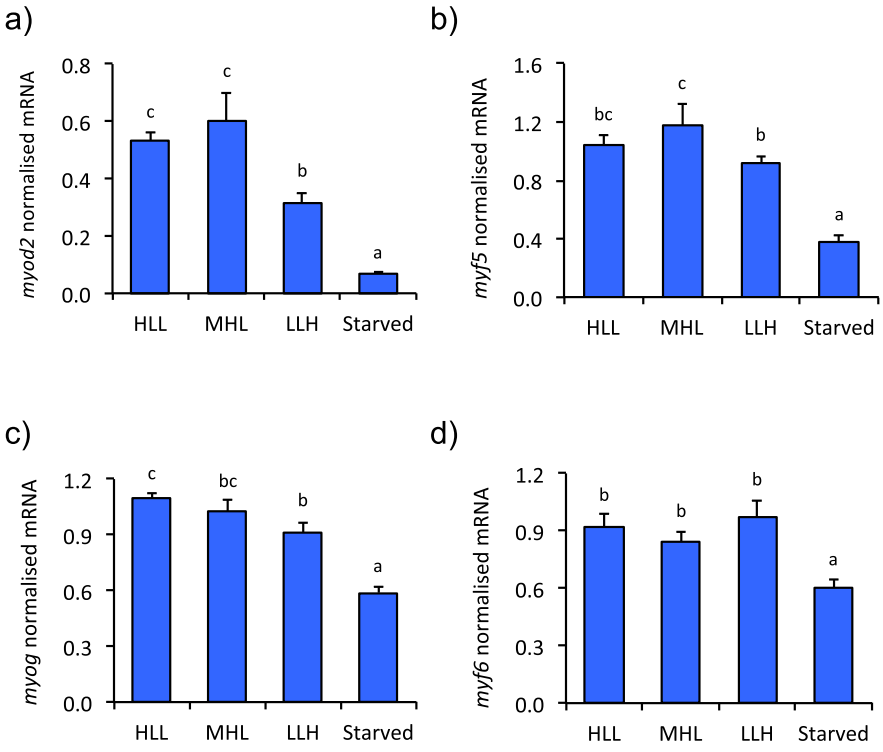


Fig. 2. Effect of diet composition and starvation on the expression of MRFs in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *myod2* (a), *myf5* (b), *myog* (c) and *myf6* (d) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata 18 s* and *ef1α*, which were used as housekeeping genes. Results are presented as mean ± SEM ($n = 4$–6 fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

and 1.6-fold higher *rps6* mRNA levels than fish supplied with MHL and LLH, respectively. The expression levels of *mtor* and *rps6* in starved fish were similar to those observed in the skeletal muscle of fish fed diet LLH (Fig. 4b-c). The mRNA levels of *igf1, mtor and rps6* positively correlated with final body weight and SGR, while negatively correlated with FCR (Table 5).

3.4. Microarray analysis

From a total of 247 genes involved in growth and development, the expression of 21 genes (included in the heat map hierarchical cluster shown in Fig. 5a) exhibited differential expression with an adjusted *P* value <0.05 and at least 2-fold difference in the normalised intensity ratio (Cy5/Cy3 or Cy3/Cy5) between 2 or more dietary conditions (starvation and feeding for 23 days with diets HLL, MHL and LLH) in the skeletal muscle of *S. aurata*. Starvation deeply affected the expression of most filtered genes. From the total of 21 genes selected, 3–6 genes (depending on the diet supplied) were significantly upregulated more than 2-fold in the skeletal muscle of starved fish, while food restriction downregulated 6–11 genes. Feeding upregulated genes associated with muscle contraction (*tnni2*, *tnnc2*, *stac3*, *ttn*, *actc1*, *calm*, *mpsf* and *cav3*), muscle development (*frg1*, *fgfr1* and *tagln*), as well as growth regulation and the growth hormone–Igf axis (*mstn*, *ghr1* and *igfals*), while downregulated genes were mostly involved in growth arrest and regulation (*gas6*, *ing3*, *gadd45a* and *naca*). Among upregulated genes by feeding with fold change >2, the greater values were found for *tnni2* (16.1, 39.1 and 29.0 fold change for fish fed HLL, MHL and LLH, respectively), followed by *stac3* (3.2, 3.5 and 2.8 fold change for fish supplied with HLL, MHL and LLH, respectively). The expression of *igfals* also increased in the skeletal muscle of fish fed diets HLL and MHL, but showed more

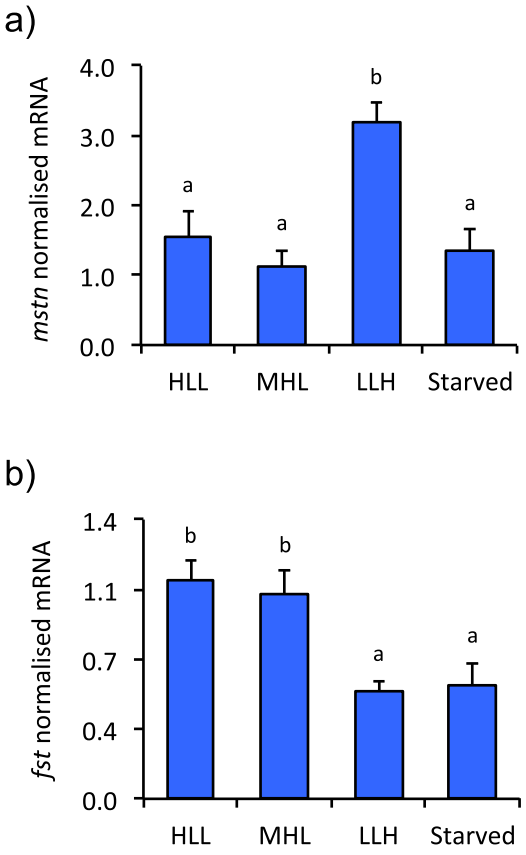


Fig. 3. Effect of diet composition and starvation on the expression of members of the Tgfb superfamily in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *mstn* (a) and *fst* (b) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata 18 s* and *ef1α*, which were used as housekeeping genes. Results are presented as mean ± SEM (n = 4–6 fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

dependence on diet composition (5.3, 3.1 and 1.0 fold change for fish supplied with diet HLL, MHL and LLH, respectively). Similar expression patterns were found for *fgfr1* and *igfals*. In regard of downregulated genes by feeding, the greater fold changes were observed for *ing3* (4.6–6.7 fold change, depending on the diet supplied) and *gas6* (4.0–4.2 fold change, depending on the diet supplied), while *gadd45a* exhibited a higher dependence on diet composition (2.6, 2.3 and 1.6 fold change for fish supplied with HLL, MHL and LLH, respectively).

With the aim to identify gene markers regulated by dietary macronutrient composition, the mRNA levels of *gadd45a*, *igfals* and *tnni2* were assayed by RT-qPCR in the skeletal muscle of fish starved or fed diets HLL, MHL and LLH for 37 days (Fig. 5b). Starvation significantly upregulated *gadd45a* over the expression values observed in fish fed diets HLL, MHL and LLH (2.4, 1.7 and 1.4 fold, respectively). The mRNA levels of *gadd45a* negatively correlated with final body weight and SGR, while positively correlated with FCR and LR (Table 5). Similarly as *igf1*, the lowest *igfals* mRNA levels were promoted by starvation, while feeding with HLL significantly upregulated *igfals* 1.9 fold. In regard of *tnni2*, the lowest mRNA abundance was found in food-deprived animals. Among fed fish the highest *tnni2* expression was observed in the skeletal muscle of *S. aurata* fed diet MHL (2.0-, 4.2- and 45.3-fold increased levels than in fish fed diets HLL, LLH and under starvation, respectively). Significant negative correlation was found between *tnni2* expression and HSI (Table 5).

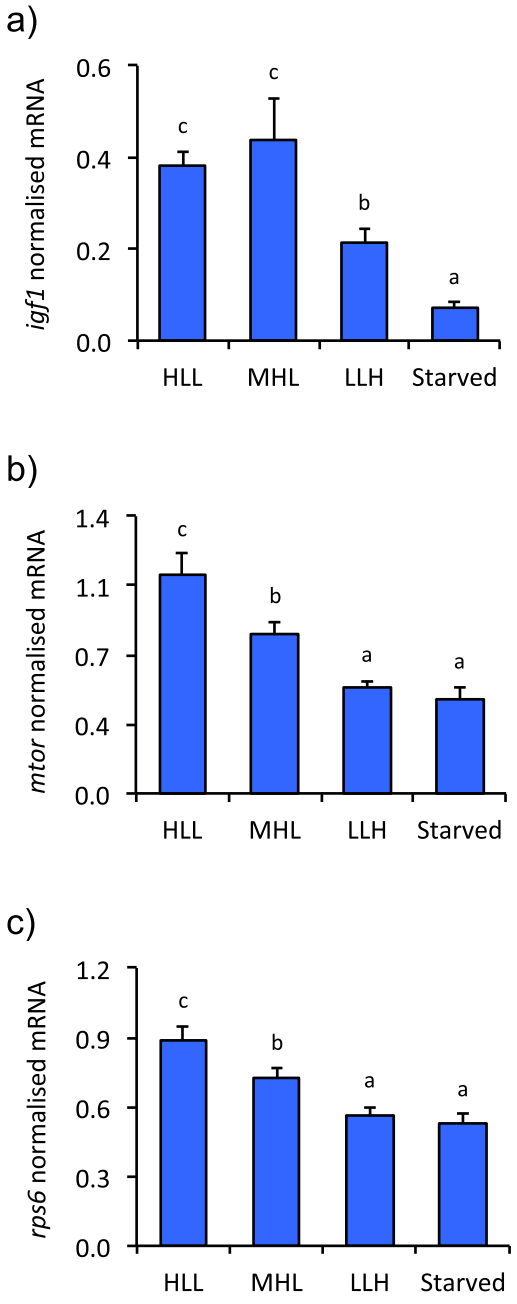


Fig. 4. Effect of diet composition and starvation on the expression of *igf1*, *mtor* and *rps6* in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *igf1* (a), *mtor* (b) and *rps6* (c) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata 18 s* and *ef1α*, which were used as housekeeping genes. Results are presented as mean ± SEM (n = 4–6 fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

4. Discussion

Substitution of dietary protein by cheaper and sustainable nutrients is a challenging question in fish nutrition (Panserat et al., 2019). The results of this study showed that partial substitution of fish meal by carbohydrates had a strong negative impact on growth performance of *S. aurata* and nutrient retention parameters such as body weight, SGR and FCR. On the contrary, the supply of medium protein, high lipid, low carbohydrate diets (MHL) improved growth performance, although at

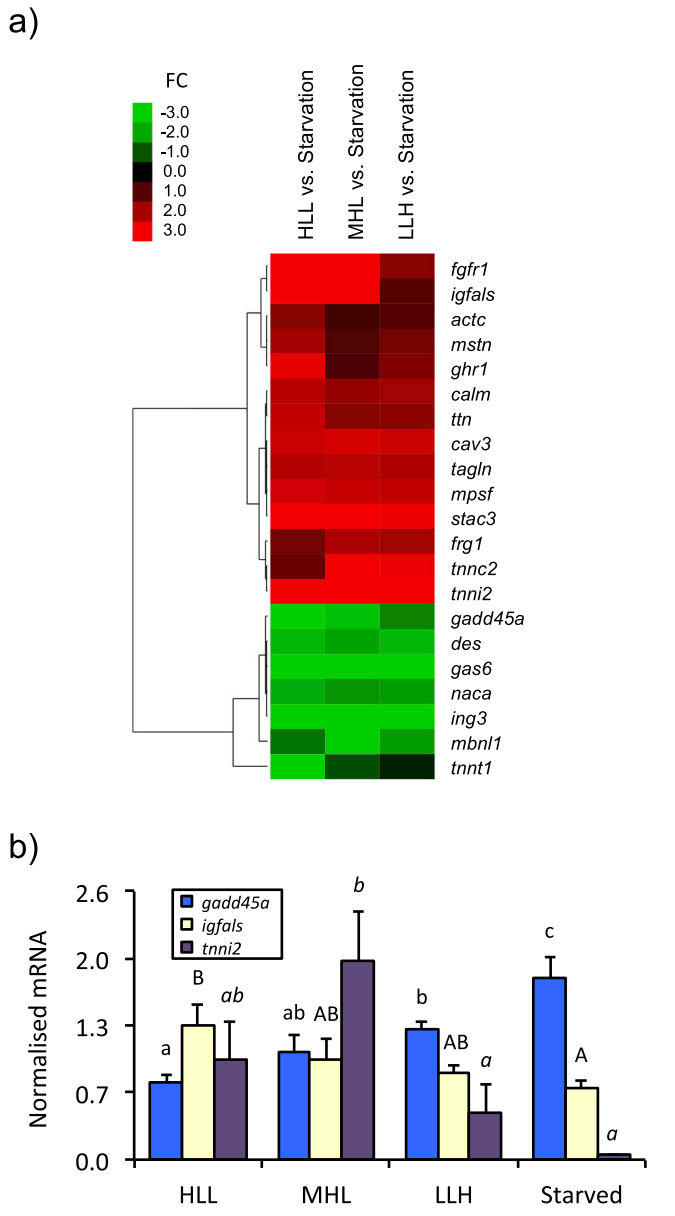


Fig. 5. Effect of diet composition and starvation on the expressional pattern of genes involved in skeletal muscle function, differentiation and growth, and validation of the expression of *gadd45a*, *igfals* and *tnni2*. (a) Heat map image of differentially transcribed genes involved in skeletal muscle function, differentiation and growth regulation. Three groups of fish were fed 23 days at a daily ration of 25 g/kg body weight with diets HLL, MHL and LLH, respectively. A fourth group of animals was submitted to starvation for the same period. Hierarchical clustering of differentially expressed genes in the skeletal muscle is represented from microarray data obtained from *S. aurata* fed with diets HLL, MHL and LLH versus starved fish, with an adjusted *P* value <0.05 and a difference of at least 2-fold in the normalised intensity ratio (Cy5/Cy3 or Cy3/Cy5) between two or more conditions. Results are presented as fold change (FC) mean value (n = 4 fish). Green color denotes downregulated genes and red color upregulated genes in fed animals. (b) RT-qPCR analysis of *gadd45a*, *igfals* and *tnni2* mRNA levels in the skeletal muscle of *S. aurata* following 37 days of starvation or feeding with diets HLL, MHL and LLH at a daily ration of 25 g/kg body weight. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata 18 s* and *ef1α*, which were used as housekeeping genes. Results are presented as mean ± SEM (n = 4–5 fish). Different letters above deviation bars indicate significant differences (P < 0.05) among conditions for a given gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels not far from those observed in fish fed high protein, low lipid, low carbohydrate diets (HLL). Increased PER values in fish fed MHL are in agreement with the increased weight gain exhibited by this group of fish. Consistent with our findings, diets with 54% of protein and 18% of carbohydrate, and therefore with macronutrient composition similar to MHL, are considered optimal to improve growth and nutritional parameters such as body weight and SGR in *S. aurata* (Fernández et al., 2007). A similar behaviour was observed in other fish species such as *Pagrus pagrus* (Schuchardt et al., 2008) and *O. mykiss* (Kamalam et al., 2012; Alami-Durante et al., 2019). A trend to present lower values for Ca, C and dry matter was observed in the group of fish fed LLH. Indeed, ADC levels for Ca, C and dry matter in turn showed a trend to correlate positively with final weight and negatively with FCR. Low tolerance of carnivorous fish to glucose (Polakof et al., 2012; Rashidpour et al., 2019), may determine the poor growth performance observed in fish fed the LLH diet.

Our observations also agree with the fact that long-term starvation and the supply of low protein, high carbohydrate diets in *S. aurata* affect similarly the expression of appetite-regulating peptides, leading to opposite effects than high protein, low carbohydrate diets on the expression of orexigenic and anorexigenic peptides (Babaei et al., 2017). Indeed, transcriptomic analysis revealed that both food deprivation and the supply of low protein, high carbohydrate diets also promoted similar effects on the expression levels of genes involved in mitochondrial oxidative phosphorylation in the skeletal muscle of *S. aurata* (Silva-Marrero et al., 2017).

In teleost fish, myoblast proliferation and hyperplasia occur mainly during the swim-up fry stage and through juvenile growth with a large increase in the number of white muscle fibres (Rowlerson and Veggetti, 2001). Herein, MRFs were measured to analyse myoblast proliferation and differentiation in *S. aurata* juveniles. Starvation downregulated *myod2*, *myf5*, *myog* and *myf6* in the skeletal muscle of *S. aurata*, which confirms previous observations in this species (García De La Serrana et al., 2014; Lavajoo et al., 2020). The strong reduction observed in the expression of *myod2* and *myf5* suggests that long-term starvation may promote muscular atrophy. Among fish fed different diets, the mRNA levels of *myod2* and to a lesser extent *myog* were more sensitive to the dietary protein to carbohydrate ratio than *myf5* and *myf6*. Therefore, *myod2* and *myog* mRNA levels significantly increased in fish that exhibited better growth performance (fish fed high or medium protein, low carbohydrate diets, MHL and HLL). However, low *myod2* mRNA levels in the skeletal muscle of fish fed LLH indicate that *myod2* can be used as sensitive marker of growth performance in *S. aurata* juveniles. In this regard, Igf-dependent expression of MRFs seems to play an important role in muscle differentiation and proliferation (Vélez et al., 2016). Indeed, in the present study, the expression of *igf1* positively correlated with growth rate and was highly dependent on nutritional status and diet composition. Consistent with our findings and the key role exerted in muscle growth, *igf1* was shown to stimulate growth and proliferation of *S. aurata* cultured myocytes (Vélez et al., 2014), while starvation markedly decreases *igf1* expression and Igf1 circulating levels in this species (Lavajoo et al., 2020; Metón et al., 2000; Pérez-Sánchez et al., 1995).

Upregulation of *mstn* expression inhibits cell growth and differentiation in the skeletal muscle of rainbow trout (Seiliez et al., 2012). Furthermore, high glucose supplementation inhibits protein synthesis in primary cultured muscle cells of olive flounder (*Paralichthys olivaceus*) by a mechanism involving downregulation of MRFs such as *myod* and *myog*, inhibition of Mtor signaling pathway and upregulation of *mstn1* (Liu et al., 2021). Similarly, *mstn* mRNA levels among fed fish were herein markedly higher in the skeletal muscle of *S. aurata* fed LLH, which was the diet that promoted the lowest growth performance. On the contrary, the lowest *mstn* expression was found in fish fed diet MHL, which in turn promoted better growth parameters. Therefore, downregulation of *mstn* in fish with enhanced growth performance may prevent *mstn*-dependent negative regulation of growth and induction of

muscle tissue hypertrophy. Consistent with this hypothesis, down-regulation of *mstn* strongly enhances hyperplasia, hypertrophy and body size in zebrafish (*Danio rerio*) (Fuentes et al., 2013; Rossi and Messina, 2014). The *mstn* mRNA levels in *S. aurata* submitted to long-term starvation were even significantly lower than those presented by fish fed LLH. In this regard, *mstn* expression in food-deprived fish seems to depend on the species and the starvation period. For instance, 30 days of starvation upregulates *mstn* in the muscle of *S. aurata* (García De La Serrana et al., 2014), while 28 days and 10 weeks of starvation did not significantly affect *mstn* expression in the muscle of adult tilapia and rainbow trout, respectively (Chauvigné et al., 2003; Rodgers et al., 2003). Specific adaptations and dietary conditions previous to starvation may explain variations in *mstn* expression. Fst is a potent antagonist of Mstn (Amthor et al., 2004). Overexpression of *fst* increases muscle mass in rainbow trout through enhanced muscle hypertrophy and hyperplasia (Medeiros et al., 2009). Our findings support that *fst* may have a major role in regulating muscle growth in *S. aurata*. Accordingly, *fst* expression showed strong dependence on dietary macronutrient composition and was significantly downregulated by supplying low protein, high carbohydrate diets (LLH).

In agreement with previous results in *S. aurata* (Lavajoo et al., 2020), muscle mRNA levels of *mtor* and *rps6* decreased upon starvation. The results of the present study indicate that the Mtor signaling pathway may be activated in fish fed high or medium protein, low carbohydrate diets. Our findings are supported by the fact that Mtor activation triggers biological responses such as cell proliferation and contribution to muscle hypertrophy (Glass, 2003a, 2003b). In agreement with this hypothesis, fish fed MHL and HLL exhibited better growth performance. Given that Akt phosphorylation is induced by growth factors, such as Igf1 (Glass, 2005; Vélez et al., 2016), upregulation of *igf1* in fish fed MHL and HLL would facilitate Pi3k phosphorylation and induction of the Igf1/Pi3k/Akt signaling cascade, whose components are considered key mediators in muscle mass increase (Stitt et al., 2004). Consistently, the combined action of Igf1 and amino acids stimulated Mtor and Akt activity in *S. aurata* myocytes (Vélez et al., 2014), and low protein diets diminished growth performance and Mtor signaling pathway in yellow catfish (*Pelteobagrus fulvidraco*) by downregulating the mRNA levels of key genes such as *igf1*, *mtor* and *akt* (Qin et al., 2019).

A microarray analysis performed on RNA isolated from skeletal muscle of *S. aurata* allowed us to address the effect of nutritional status and diet composition on muscle expression pattern and identify novel gene markers of interest for nutritional studies and diet formulation. In general, feeding stimulated the expression of genes involved in growth, development and muscle function, while starvation upregulated genes implicated in growth regulation and arrest. Some of the differentially expressed genes were also dependent on dietary macronutrient composition. Remarkably, dietary carbohydrates, protein and lipids specifically upregulated the mRNA levels of *gadd45a*, *igfals* and *tnni2*, respectively, in the skeletal muscle of *S. aurata*, suggesting that their expression level can be used as marker for monitoring the effect of changes in dietary macronutrient composition and feeding regime in cultured fish. Indeed, the expression of *gadd45a* negatively correlated with final body weight and SGR, which suggests that *gadd45a* can be also used as sensitive marker of growth performance.

Gadd45a is a ubiquitously expressed protein belonging to the growth arrest and DNA damage 45 stress sensor gene family and is involved in cell response to DNA-damaging agents and growth cessation signals, such as starvation, by mediating cell cycle arrest and inhibition of cell entry into S phase (Salvador et al., 2013). Downregulation of *gadd45a* in the skeletal muscle of fed fish, particularly those supplied with high or medium protein diets (HLL and MHL) agrees with reports indicating increased *gadd45a* mRNA levels in human and rat muscle under starvation (Bongers et al., 2013; Ibrahim et al., 2020; Wijngaarden et al., 2014). Regulation of *gadd45a* expression by dietary macronutrients in fish remains largely unknown. However, a number of evidences point to *gadd45a* as sensitive stress sensor gene also in fish. Exposition to cyanobacterial extracts, cadmium and DNA damage by acute exposure to hydrogen peroxide upregulate *gadd45* in zebrafish (Chen et al., 2014; Falfushynska et al., 2021; Reinardy et al., 2013). Moreover, the expression of *gadd45* increases in response to low temperature in rainbow trout, kelp grouper (*Epinephelus moara*) and large yellow croaker (*Larimichthys crocea*) (Borchel et al., 2017; Chen et al., 2020; Qian et al., 2020). Furthermore, the pattern of expression of *gadd45a* in tilapia (*Oreochromis* spp.) after infection with *Streptococcus agalactiae* and in grass carp (*Ctenopharyngodon idella*) following challenge with *Aeromonas hydrophila* suggests involvement of *gadd45a* in fish immunity against bacterial infection (Fang et al., 2018; Shen et al., 2016). In the present study, restricted-protein feeding (LLH diet) upregulated *gadd45a* to values not far from those observed in starved fish. Since *gadd45a* is critical mediator of muscle atrophy (Bongers et al., 2013; Ebert et al., 2012), the results of the present study suggest that protein restriction may shrink muscle growth in *S. aurata*.

Igfals encodes the insulin-like growth factor-binding protein complex acid labile subunit, which stabilises blood levels of Igf1, Igf2 and Igfbp proteins by binding binary complexes formed by Igf1 or Igf2 with Igfbp-3 or Igfbp-5 (Domené and Domené, 2020; Hwa et al., 2021). In the present study, changes in nutritional status and diet composition caused similar effects on *igfals* and *igf1* mRNA levels. Starvation downregulated *igfals*, while among fed fish the highest *igfals* expression was observed in *S. aurata* fed HLL. Our findings are consistent with reports in mammals showing that food deprivation reduces *igfals* expression and serum levels (Frystyk et al., 1999; Kong et al., 2002), while the supply of high protein diets and overfeeding increases Igfals circulating level (Khan et al., 2014; Rubio-Aliaga et al., 2011). Given that Igfals is well-established for its role in binding to Igf-Igfbp complexes and prolonge their half-life in serum (Boisclair et al., 2001), downregulation of *igfals* in fish fed low protein, high carbohydrate diets (LLH) would promote insulin insensitivity and growth impairment. Hence, Igfals deficiency decreases circulating levels of Igf and Igfbp proteins, leading to insulin insensitivity, growth impairment and puberty delay in humans (Domené and Domené, 2020; Hwa et al., 2021).

Tnni2 encodes the fast skeletal isoform of troponin I, which acts as the inhibitory subunit of the troponin complex during muscle contraction (Fu et al., 2009; Sheng and Jin, 2016). Knowledge of the effect of nutritional status and diet composition on *tnni2* expression in fish is scarce. However, our findings are consistent with previous observations that indicate downregulation of troponin in skeletal muscle of mandarin fish (*Siniperca chuatsi*) submitted to starvation (Liu et al., 2020). Accordingly, Lu et al. reported downregulation of troponin I in muscle of slow-growing grass carp (*C. idella*) (Lu et al., 2020). Conceivably, given that *S. aurata* is a carnivorous fish, starvation would promote the use of muscle protein as the major source of energy, while impairing muscle synthesis of proteins and contraction. However, the supply of dietary protein would avoid muscle breakdown, cessation of muscle contraction and facilitate muscle growth. Moreover, the fact that the highest *tnni2* expression was found in fish fed MHL suggests that dietary lipids could contribute to *tnni2* upregulation. In this regard, maternal dietary linoleic acid supplementation promotes muscle fibre transformation to type I fibre in a process that involves troponin I upregulation in the muscle of suckling piglets (Lu et al., 2017). Further studies are required to explore the hypothesis that the fatty acid profile of the diet can affect *tnni2* expression in fish.

5. Conclusions

The results of the present study show that the expression of MRFs and key genes in muscle growth and differentiation was markedly affected by nutritional status and dietary macronutrient composition in the skeletal muscle of *S. aurata*. In addition to the skeletal muscle mRNA levels of *myod2*, *fst*, *igf1*, *mtor* and *rps6*, our findings let us to report for the first time *gadd45a* and *igfals* as useful markers to study the effect of changes in feeding regime and diet composition on growth performance in fish.

Author contributions

Conception and design: Isidoro Metón, Isabel V. Baanante and Felipe Fernández. Material preparation, data collection and analysis were performed by Alberto Sáez-Arteaga, Yuanbing Wu, Jonás I. Silva-Marrero, Ania Rashidpour and María Pilar Almajano. Writing and original draft preparation: Isidoro Metón and Alberto Sáez-Arteaga. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Alberto Sáez-Arteaga: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Yuanbing Wu:** Investigation, Formal analysis, Writing – review & editing. **Jonás I. Silva-Marrero:** Investigation, Formal analysis, Writing – review & editing. **Ania Rashidpour:** Investigation, Formal analysis, Writing – review & editing. **María Pilar Almajano:** Investigation, Formal analysis, Writing – review & editing. **Felipe Fernández:** Conceptualization, Methodology, Writing – review & editing. **Isabel V. Baanante:** Conceptualization, Methodology, Writing – review & editing. **Isidoro Metón:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

This work was supported by the Ministerio de Economía, Industria y Competitividad, Spain (grant no. AGL2016-78124-R; cofunded by the European Regional Development Fund, European Commission) and the Agencia Nacional de Investigación y Desarrollo, Chile (Becas Chile/2011–72111506). The authors thank Piscicultura Marina Mediterranea (Burriana, Castellón, Spain) for providing *S. aurata* juveniles.

References

Alami-Durante, H., Cluzeaud, M., Bazin, D., Schrama, J., Saravanan, S., Geurden, I., 2019. Muscle growth mechanisms in response to isoenergetic changes in dietary non-protein energy source at low and high protein levels in juvenile rainbow trout. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 230, 91–99.

Amthor, H., Nicholas, G., McKinnell, I., Kemp, C.F., Sharma, M., Kambadur, R., Patel, K., 2004. Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. Dev. Biol. 270, 19–30.

Babaei, S., Sáez, A., Caballero-Solares, A., Fernández, F., Baanante, I.V., Metón, I., 2017. Effect of dietary macronutrients on the expression of cholecystokinin, leptin, ghrelin and neuropeptide Y in gilthead sea bream (*Sparus aurata*). Gen. Comp. Endocrinol. 240, 121–128.

Boisclair, Y., Rhoads, R., Ueki, I., Wang, J., Ooi, G., 2001. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J. Endocrinol. 170, 63–70.

Bongers, K., Fox, D., Ebert, S., Kunkel, S., Dyle, M., Bullard, S., Adams, C., 2013. Skeletal muscle denervation causes skeletal muscle atrophy through a pathway that involves both Gadd45a and HDAC4. Am. J. Physiol. Endocrinol. Metab. 305, E907–E915.

Borchel, A., Verleih, M., Rebl, A., Goldammer, T., 2017. Identification of genes involved in cold-shock response in rainbow trout (*Oncorhynchus mykiss*). J. Genet. 96, 701–706.

Bradfield, A.E., Llewellyn, M.J., 1982. Animal Energetics. Blackie and Son, Glasgow.

Braun, T., Gautel, M., 2011. Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. Nat. Rev. Mol. Cell Biol. 12, 349–361.

Busacker, G.P., Adelman, I.R., Goolish, E.M., 1990. Growth. In: Schreck, C.B., Moyle, P.B. (Eds.), Methods for Fish Biology. American Fisheries Society, Bethesda, pp. 363–387.

Castillo, J., Codina, M., Martínez, M.L., Navarro, I., Gutiérrez, J., 2004. Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R935–R941.

Chauvigné, F., Gabillard, J.C., Weil, C., Rescan, P.Y., 2003. Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. Gen. Comp. Endocrinol. 132, 209–215.

Chen, Y.Y., Zhu, J.Y., Chan, K.M., 2014. Effects of cadmium on cell proliferation, apoptosis, and proto-oncogene expression in zebrafish liver cells. Aquat. Toxicol. 157, 196–206.

Chen, Z., Tian, Y., Ma, W., Zhai, J., 2020. Gene expression changes in response to low temperatures in embryos of the kelp grouper, *Epinephelus moara*. Cryobiology 97, 159–167.

Christoffolete, M.A., Silva, W.J., Ramos, G.V., Bento, M.R., Costa, M.O., Ribeiro, M.O., Moriscot, A.S., 2015. Muscle IGF-1-induced skeletal muscle hypertrophy evokes higher insulin sensitivity and carbohydrate use as preferential energy substrate. Biomed. Res. Int. 2015, 282984.

De Silva, S., Anderson, T.A., 1995. Fish Nutrition in Aquaculture. Chapman and Hall, London.

Domené, S., Domené, H., 2020. The role of acid-labile subunit (ALS) in the modulation of GH-IGF-I action. Mol. Cell. Endocrinol. 518, 111006.

Ebert, S., Dyle, M., Kunkel, S., Bullard, S., Bongers, K., Fox, D., Adams, C., 2012. Stress-induced skeletal muscle Gadd45a expression reprograms myonuclei and causes muscle atrophy. J. Biol. Chem. 287, 27290–27301.

Falfushynska, H., Horyn, O., Osypenko, I., Rzymski, P., Wejnerowski, Ł., Dziuba, M., Sokolova, I., 2021. Multibiomarker-based assessment of toxicity of central European strains of filamentous cyanobacteria *Aphanizomenon gracile* and *Raphidiopsis raciborskii* to zebrafish *Danio rerio*. Water Res. 194, 116923.

Fang, Y., Xu, X., Shen, Y., Li, J., 2018. Molecular cloning and functional analysis of growth arrest and DNA damage-inducible 45 aa and ab (Gadd45aa and Gadd45 ab) in *Ctenopharyngodon idella*. Fish Shellfish Immunol. 77, 187–193.

Fernández, F., Miquel, A.G., Cordoba, M., Varas, M., Metón, I., Caseras, A., Baanante, I. V., 2007. Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. J. Exp. Mar. Bio. Ecol. 343, 1–10.

Frystyk, J., Delhanty, P., Skjaerbaek, C., Baxter, R., 1999. Changes in the circulating IGF system during short-term fasting and refeeding in rats. Am. J. Phys. 277, E245–E252.

Fu, C., Lee, H., Tsai, H., 2009. The molecular structures and expression patterns of zebrafish troponin I genes. Gene Expr. Patterns 9, 348–356.

Fuentes, E.N., Pino, K., Navarro, C., Delgado, I., Valdés, J.A., Molina, A., 2013. Transient inactivation of myostatin induces muscle hypertrophy and overcompensatory growth in zebrafish via inactivation of the SMAD signaling pathway. J. Biotechnol. 168, 295–302.

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

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Glass, D., 2003a. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nat. Cell Biol. 5, 87–90.

Glass, D.J., 2003b. Molecular mechanisms modulating muscle mass. Trends Mol. Med. 9, 344–350.

Glass, D.J., 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. Int. J. Biochem. Cell Biol. 37, 1974–1984.

Holterman, C.E., Rudnicki, M.A., 2005. Molecular regulation of satellite cell function. Semin. Cell Dev. Biol. 16, 575–584.

Hwa, V., Fujimoto, M., Zhu, G., Gao, W., Foley, C., Kumbaji, M., Rosenfeld, R., 2021. Genetic causes of growth hormone insensitivity beyond GHR. Rev. Endocr. Metab. Disord. 22, 43–58.

Ibrahim, M., Wasselin, T., Challet, E., Van Dorsselaer, A., Le Maho, Y., Raclot, T., Bertile, F., 2020. Transcriptional changes involved in atrophying muscles during prolonged fasting in rats. Int. J. Mol. Sci. 21, 1–28.

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

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J. Exp. Biol. 215, 2567–2578.

Kerr, M.K., Churchill, G.A., 2001. Experimental design for gene expression microarrays. Biostatistics 2, 183–201.

Khan, M.J., Jacometo, C.B., Graugnard, D.E., Corrêa, M.N., Schmitt, E., Cardoso, F., Loor, J.J., 2014. Overfeeding dairy cattle during late-pregnancy alters hepatic PPARα-regulated pathways including hepatokines: impact on metabolism and peripheral insulin sensitivity. Gene Regul. Syst. Bio. 8, 97.

Kong, S., Baxter, R., Delhanty, P., 2002. Age-dependent regulation of the acid-labile subunit in response to fasting-refeeding in rats. Endocrinology 143, 4505–4512.

Latimer, M.N., Reid, R.M., Biga, P.R., Cleveland, B.M., 2019. Glucose regulates protein turnover and growth-related mechanisms in rainbow trout myogenic precursor cells. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 232, 91–97.

Lavajoo, F., Perelló-Amorós, M., Vélez, E., Sánchez-Moya, A., Balbuena-Pecino, S., Riera-Heredia, N., Gutiérrez, J., 2020. Regulatory mechanisms involved in muscle and bone remodeling during refeeding in gilthead sea bream. Sci. Rep. 10, 184.

Lee, S.J., McPherron, A.C., 2001. Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. U. S. A. 98, 9306–9311.

Liu, X., Zeng, S., Liu, S., Wang, G., Lai, H., Zhao, X., Li, G., 2020. Identifying the related genes of muscle growth and exploring the functions by compensatory growth in mandarin fish (*Siniperca chuatsi*). Front. Physiol. 11, 553563.

Liu, J., Pan, M., Huang, D., Wu, J., Liu, Y., Guo, Y., Mai, K., 2021. High glucose induces apoptosis, glycogen accumulation and suppresses protein synthesis in muscle cells of olive flounder *Paralichthys olivaceus*. Br. J. Nutr. https://doi.org/10.1017/S0007114521002634.

Lu, Z., Ren, Y., Zhou, X., Yu, X., Huang, J., Yu, D., Wang, Y., 2017. Maternal dietary linoleic acid supplementation promotes muscle fibre type transformation in suckling piglets. J. Anim. Physiol. Anim. Nutr. (Berl). 101, 1130–1136.
Lu, X., Chen, H., Qian, X., Gui, J., 2020. Transcriptome analysis of grass carp (*Ctenopharyngodon idella*) between fast- and slow-growing fish. Comp. Biochem. Physiol. Part D. Genomics Proteomics 35, 100688.
Lucas, A., 1996. Bioenergetics of organisms: Methods. In: Priede, I.G. (Ed.), Bioenergetics of Aquatic Animals. Taylor & Francis, London, pp. 65–81.
McPherron, A., Lawler, A., Lee, S., 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387, 83–90.
Medeiros, E.F., Phelps, M.P., Fuentes, F.D., Bradley, T.M., 2009. Overexpression of follistatin in trout stimulates increased muscling. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R235–R242.
Metón, I., Mediavilla, D., Caseras, A., Cantó, E., Fernández, F., Baanante, I.V., 1999. Effect of diet composition and ration size on key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). Br. J. Nutr. 82, 223–232.
Metón, I., Caseras, A., Cantó, E., Fernández, F., Baanante, I.V., 2000. Liver insulin-like growth factor-I mRNA is not affected by diet composition or ration size but shows diurnal variations in regularly-fed gilthead sea bream (*Sparus aurata*). J. Nutr. 130, 757–760.
Montserrat, N., Capilla, E., Navarro, I., Gutiérrez, J., 2012. Metabolic effects of insulin and IGFs on gilthead sea bream (*Sparus aurata*) muscle cells. Front. Endocrinol. (Lausanne) 3, 55.
Olson, E., Klein, W., 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. 8, 1–8.
Panserat, S., Marandel, L., Seiliez, I., Skiba-Cassy, S., 2019. New insights on intermediary metabolism for a better understanding of nutrition in teleosts. Annu. Rev. Anim. Biosci. 7, 195–220.
Pérez-Sánchez, J., Martí-Palanca, H., Kaushik, S.J., 1995. Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (*Sparus aurata*). J. Nutr. 125, 546–552.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J. Comp. Physiol. B. 182, 1015–1045.
Qian, B., Qi, X., Bai, Y., Wu, Y., 2020. The p53 signaling pathway of the large yellow croaker (*Larimichthys crocea*) responds to acute cold stress: evidence via spatiotemporal expression analysis of p53, p21, MDM2, IGF-1, Gadd45, Fas, and Akt. PeerJ 8, e10532.
Qin, Q., Cao, X., Dai, Y., Wang, L., Zhang, D., Jiang, G., Liu, W., 2019. Effects of dietary protein level on growth performance, digestive enzyme activity, and gene expressions of the TOR signaling pathway in fingerling *Pelteobagrus fulvidraco*. Fish Physiol. Biochem. 45, 1747–1757.
Rashidpour, A., Silva-Marrero, J.I., Seguí, L., Baanante, I.V., Metón, I., 2019. Metformin counteracts glucose-dependent lipogenesis and impairs transdeamination in the liver of gilthead sea bream (*Sparus aurata*). Am. J. Physiol. Integr. Comp. Physiol. 316, R265–R273.
Reinardy, H., Dharamshi, J., Jha, A., Henry, T., 2013. Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish *Danio rerio*. Mutagenesis 28, 601–608.
Rescan, P.Y., 2001. Regulation and functions of myogenic regulatory factors in lower vertebrates. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 130, 1–12.
Rodgers, B.D., Weber, G.M., Kelley, K.M., Levine, M.A., 2003. Prolonged fasting and cortisol reduce myostatin mRNA levels in tilapia larvae ; short-term fasting elevates. Am. J. Physiol. Integr. Comp. Physiol. 284, R1277–R1286.
Rossi, G., Messina, G., 2014. Comparative myogenesis in teleosts and mammals. Cell. Mol. Life Sci. 71, 3081–3099.
Rowlerson, A.M., Veggetti, A., 2001. Cellular mechanisms of post-embryonic muscle growth in aquaculture species. Fish Physiol. 18, 103–140.
Rubio-Aliaga, I., Marvin-Guy, L., Wang, P., Wagniere, S., Mansourian, R., Fuerholz, A., Kussmann, M., 2011. Mechanisms of weight maintenance under high- and low-protein, low-glycaemic index diets. Mol. Nutr. Food Res. 55, 1603–1612.
Salvador, J.M., Brown-Clay, J.D., Fornace, A.J., 2013. Gadd45 in stress signaling, cell cycle control, and apoptosis. In: Liebermann, D., Hoffman, B. (Eds.), Gadd45 Stress Sensor Genes. Springer, New York LLC, New York, pp. 1–19.
Schiaffino, S., Mammucari, C., 2011. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skelet. Muscle 1, 4.
Schuchardt, D., Vergara, J.M., Fernández-Palacios, H., Kalinowski, C.T., Hernández-Cruz, C.M., Izquierdo, M.S., Robaina, L., 2008. Effects of different dietary protein and lipid levels on growth, feed utilization and body composition of red porgy (*Pagrus pagrus*) fingerlings. Aquac. Nutr. 14, 1–9.
Seiliez, I., Sabin, N., Gabillard, J.-C., 2012. Myostatin inhibits proliferation but not differentiation of trout myoblasts. Mol. Cell. Endocrinol. 351, 220–226.
Shen, Y., Ma, K., Liu, F., Yue, G., 2016. Characterization of two novel gadd45a genes in hybrid tilapia and their responses to the infection of *Streptococcus agalactiae*. Fish Shellfish Immunol. 54, 276–281.
Sheng, J., Jin, J., 2016. TNNI1, TNNI2 and TNNI3: evolution, regulation, and protein structure-function relationships. Gene 576, 385–394.
Silva-Marrero, J.I., Sáez, A., Caballero-Solares, A., Viegas, I., Almajano, M.P., Fernández, F., Metón, I., 2017. A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). BMC Genomics 18, 768.
Silva-Marrero, J.I., Villasante, J., Rashidpour, A., Palma, M., Fàbregas, A., Almajano, M. P., Metón, I., 2019. The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of Sparus aurata. Biomolecules 9, 297.
Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, 1–25.
Song, X., Marandel, L., Skiba-Cassy, S., Corraze, G., Dupont-Nivet, M., Quillet, E., Panserat, S., 2018. Regulation by dietary carbohydrates of intermediary metabolism in liver and muscle of two isogenic lines of rainbow trout. Front. Physiol. 9, 1579.
Stitt, T.N., Drujan, D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O., Glass, D.J., 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol. Cell 14, 395–403.
Tan, X., Du, S.J., 2002. Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (*Sparus aurata*). Dev. Genes Evol. 212, 207–217.
Vélez, E.J., Lutfi, E., Jiménez-Amilburu, V., Riera-Codina, M., Capilla, E., Navarro, I., Gutiérrez, J., 2014. IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. Gen. Comp. Endocrinol. 205, 296–304.
Vélez, E.J., Lutfi, E., Azizi, S., Montserrat, N., Riera-Codina, M., Capilla, E., Gutiérrez, J., 2016. Contribution of in vitro myocytes studies to understanding fish muscle physiology. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 199, 67–73.
Wijngaarden, M., Bakker, L., van der Zon, G., t Hoen, P., van Dijk, K., Jazet, I., Guigas, B., 2014. Regulation of skeletal muscle energy/nutrient-sensing pathways during metabolic adaptation to fasting in healthy humans. Am. J. Physiol. Endocrinol. Metab. 307, E885–E895.
Wu, Y., Rashidpour, A., Almajano, M.P., Metón, I., 2020. Chitosan-based drug delivery system: applications in fish biotechnology. Polymers (Basel). 12, 1177.
Zhu, K., Wang, H., Wang, H., Gul, Y., Yang, M., Zeng, C., Wang, W., 2014. Characterization of muscle morphology and satellite cells, and expression of muscle-related genes in skeletal muscle of juvenile and adult *Megalobrama amblycephala*. Micron 64, 66–75.

Rev Fish Biol Fisheries
https://doi.org/10.1007/s11160-024-09852-4

ORIGINAL RESEARCH

Chitosan-based delivery of fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 boosts EPA and DHA biosynthesis in *Sparus aurata*

Yuanbing Wu · Ania Rashidpour · Anna Fàbregas · María Pilar Almajano · Isidoro Metón

Received: 24 January 2023 / Accepted: 19 March 2024
© The Author(s) 2024

Abstract Omega-3 long-chain polyunsaturated fatty acids (*n*-3 LC-PUFA) are essential fatty acids required in healthy balanced diets for humans. To induce sustained production of *n*-3 LC-PUFA in gilthead seabream (*Sparus aurata*), chitosan-tripolyphosphate (TPP) nanoparticles encapsulating plasmids expressing fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 were intraperitoneally administered every 4 weeks (3 doses in total, each of 10 µg plasmid per g of body weight). Growth performance and metabolic effects of chitosan-TPP complexed with pSG5 (empty plasmid), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 were assessed 70 days post-treatment. Tissue distribution analysis showed high expression levels of fish codon-optimised FAT-1 and FAT-2 in the liver (> 200-fold). Expression of *fat-1* and *fat-1* + *fat-2* increased weight gain. Fatty acid methyl esters assay revealed that co-expression of *fat-1* and *fat-2* increased liver production and muscle accumulation of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total *n*-3 LC-PUFA, while decreased the *n*-6/*n*-3 ratio. Co-expression of *fat-1* and *fat-2* downregulated *srebf1* and genes encoding rate-limiting enzymes for de novo lipogenesis in the liver, leading to decreased circulating triglycerides and cholesterol. In contrast, FAT-2 and FAT-1 + FAT-2 upregulated hepatic *hnf4a*, *nr1h3* and key enzymes in glycolysis and the pentose phosphate pathway. Our findings demonstrate for the first time efficient and sustained production of EPA and DHA in animals after long-term treatment with chitosan-TPP-DNA nanoparticles expressing FAT-1 and FAT-2, which enabled the production of functional fish rich in *n*-3 LC-PUFA for human consumption.

Keywords Chitosan · Functional food · Gene therapy · Omega 3 long-chain polyunsaturated fatty acids · *Sparus aurata*

Y. Wu · A. Rashidpour · I. Metón (✉)
Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain
e-mail: imeton@ub.edu

A. Fàbregas
Departament de Farmàcia i Tecnologia Farmacèutica, i Fisicoquímica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Joan XXIII 27-31, 08028 Barcelona, Spain

M. P. Almajano
Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Diagonal 647, 08028 Barcelona, Spain

Introduction

All organisms can synthesise saturated and monounsaturated fatty acids. However, the biosynthetic rate of long-chain polyunsaturated fatty acids (LC-PUFA) in vertebrates is markedly low and cannot cover physiological demands. Linoleic acid (18:2*n*-6,

Published online: 09 April 2024

LA) and α-linolenic acid (18:3*n*-3, ALA) are precursors for the synthesis of omega-6 (*n*-6) and omega-3 (*n*-3) LC-PUFA series, respectively, and essential fatty acids for vertebrates, which lack Δ12/*n*-6 and Δ15/*n*-3 desaturases required to synthesise LA from oleic acid (18:1*n*-9c, OA) and ALA from LA (Castro et al. 2016; Tocher et al. 2019). LC-PUFA are critical components for growth and development, acting as bioactive components of membrane phospholipids, precursors of signalling molecules and modulators of gene expression. Although the specific physiological roles of *n*-3 LC-PUFA remain unclear, eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) are thought to exert protective roles preventing atherosclerosis, stroke, obesity, type-2 diabetes, inflammation and autoimmune diseases, among others (Calder 2018; Djuricic and Calder 2021). In contrast, *n*-6 LC-PUFA, particularly arachidonic acid (20:4*n*-6, ARA), are precursors of local hormones promoting acute and chronic inflammation. ARA is a highly abundant fatty acid in the membranes of many cell types. Inflammatory stimuli releases ARA from cell membranes and let cyclooxygenase, lipoxygenase and cytochrome P450 pathways convert ARA to eicosanoids, a family of mediators and regulators of the inflammatory response that include prostaglandins, thromboxanes and leukotrienes. Therefore, many eicosanoids are linked to inflammatory diseases, although some ARA-derived metabolites also seem to be involved in the resolution of inflammation (Djuricic and Calder 2021). The role of ARA on inflammation has been widely studied in mammals. In contrast, knowledge of ARA function is still scarce in fish and cannot be directly inferred from mammalian physiology. Nevertheless, it is well-known the role of eicosanoids, mainly prostaglandins, in the regulation of fish immunity and inflammation (Xu et al. 2022). Apart from plants, fungi and some aquatic microorganisms, few other organisms such as the nematode *Caenorhabditis elegans* and some invertebrates can synthesise de novo *n*-3 and *n*-6 LC-PUFA in significant amounts. Vegetable oils are rich in LA and ALA, and often contain high levels of *n*-6 LC-PUFA, but are devoid of significant amounts of *n*-3 LC-PUFA, particularly EPA and DHA unless obtained from transgenic crops. Therefore, trophic transfer from microalgae and plankton to marine fish and seafood are major sources of LC-PUFA, notably *n*-3 LC-PUFA, in the human diet (Tocher et al. 2019; Osmond and Colombo 2019).

Shortage of *n*-3 LC-PUFA and increased *n*-6/*n*-3 ratio in fish fillets due to substitution of fish oil (rich in *n*-3 LC-PUFA) by vegetable oils (poor in *n*-3 LC-PUFA, but frequently rich in *n*-6 LC-PUFA) in aquafeeds is nowadays a major challenge for the aquaculture sector. To face this problem and increasing demands of functional food with high nutritional value, intense research is being conducted in order to improve the *n*-3 LC-PUFA content in farmed fish, including dietary incorporation of microalgae, genetically modified organisms (GMOs) such as yeast and algae, and plant GMO-derived oils, such as oil from false flax expressing microalgal genes (Betancor et al. 2016; Tocher et al. 2019; Osmond and Colombo 2019; Sales et al. 2021; Carvalho et al. 2022). Production in large-scale fermenters and the supply of balanced amounts of EPA and DHA constrains the use of microalgae biomass in aquafeeds (Tocher et al. 2019). Transgenesis of fish fatty acid desaturases and elongases aiming to increase EPA and DHA levels was assayed in zebrafish (Alimuddin et al. 2007, 2008; Cheng et al. 2015). However, fish desaturases and elongases act on both *n*-3 and *n*-6 fatty acid series and generally do not substantially change the *n*-6/*n*-3 ratio (Pang et al. 2014). Efficient conversion of *n*-6 PUFA into *n*-3 PUFA in transgenic mice expressing *Caenorhabditis elegans n*-3 fatty acid desaturase *fat-1* (FAT-1), an *n*-3 fatty acid desaturase absent in vertebrates (Kang et al. 2004), led to use synthetically humanised and fish codon-optimised *C. elegans* FAT-1 to generate transgenic zebrafish (Pang et al. 2014), common carp (Zhang et al. 2019), channel catfish (Xing et al. 2023), and other vertebrates, including mice, cattle, pigs and sheep (Lai et al. 2006; Ji et al. 2009; Chen et al. 2013; Liu et al. 2016, 2017; Li et al. 2018; Tang et al. 2019; Luo et al. 2020; Sun et al. 2020; You et al. 2021). Transgenesis of *C. elegans fat-1* in zebrafish, common carp, channel catfish, pigs and sheep efficiently increases EPA and DHA, while decreases the *n*-6/*n*-3 ratio (Lai et al. 2006; Pang et al. 2014; Li et al. 2018; Zhang et al. 2019; Luo et al. 2020; Xing et al. 2023). The effect is potentiated in zebrafish and pigs by double transgenesis with codon-optimised *C. elegans* Δ12 fatty acid desaturase *fat-2* (FAT-2), a Δ12 desaturase that converts OA into LA and which is also absent in vertebrates (Pang et al. 2014; Tang et al. 2019).

Given that there are major concerns on environmental risk, sustainability, fish welfare, food safety as well as consumer perception and acceptance of GMOs (Tocher et al. 2019; Osmond and Colombo 2019), in recent years we developed an alternative methodology to GMO generation based on the production of chitosan-tripolyphosphate (TPP)-DNA nanoparticles for transient modification of the expression of target genes in the liver of gilthead seabream (*Sparus aurata*) (González et al. 2016; Gaspar et al. 2018; Silva-Marrero et al. 2019). Chitosan is a cationic polymer of glucosamine and N-acetylglucosamine derived from chitin by deacetylation. Chitosan is increasingly used as carrier for delivering nucleic acids in vivo due to its well-known mucoadhesion, low toxicity, biodegradability and biocompatibility (Wu et al. 2020).

With the aim to promote sustained production of *n*-3 LC-PUFA in *S. aurata*, in the present study chitosan-TPP nanoparticles encapsulating plasmids expressing fish codon-optimised *Caenorhabditis elegans* FAT-1 and FAT-2 were intraperitoneally administered every 4 weeks to *S. aurata* (3 doses in total). Seventy days post-treatment, the effect of chitosan-TPP-DNA nanoparticles was assessed on growth parameters, intermediary metabolism and fatty acid content in the liver and skeletal muscle of *S. aurata*.

Materials and methods

Animals

S. aurata juveniles (7.7 g ± 0.2, mean weight ± SEM) were obtained from Piscicultura Marina Mediterranea (AVRAMAR Group, Burriana, Spain) and maintained at 20 °C in 250-L aquaria supplied with running seawater in the aquatic animals facility of the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB) as described (Silva-Marrero et al. 2017). Fish were fed with commercial diet (Dibaq Microbaq 165, Dibaq, Segovia Spain), containing 52% protein, 18% lipids, 12% carbohydrates, 10% ash, 8% moisture and 21.3 kJ/g gross energy. For the acclimation regime, fish were fed twice daily (9:00 and 17:00) at a ration of 5% body weight (BW). Two weeks before experimental treatments, the ration was adjusted and kept to 3% BW until the end of the experiment. Fish were weighted every 2 weeks to readjust the feed amount. To study the long-term effect of fish codon-optimised *C. elegans fat-1* and *fat-2* expression, 4 groups of fish were intraperitoneally injected up to 3 times (once every 4 weeks) with chitosan-TPP nanoparticles complexed with pSG5 (empty plasmid, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2. Every single administration consisted of 10 µg plasmid per gram BW. Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed by cervical section, blood was collected and the liver, intestine, skeletal muscle and brain were dissected out, frozen in liquid nitrogen and kept at − 80 °C until use. To prevent stress, fish were anesthetised by tricaine methanesulfonate (MS-222; 1:12,500) before handling.

Preparation and characterisation of chitosan-TPP-DNA nanoparticles

Fish codon-optimised FAT-1 and FAT-2 cDNA sequences (GenBank accession nos. ON374024 and ON374025, respectively) were synthesised based on *C. elegans* FAT-1 and FAT-2 using GeneArt Instant Designer (Thermo Fisher Scientific, Waltham, MA, USA) and ligated into pSG5 (Agilent Technologies, Palo Alto, CA, USA). The resulting constructs (pSG5-FAT-1 and pSG5-FAT-2) were verified by cycle sequencing on both sides. Chitosan-TPP nanoparticles encapsulating pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 were prepared by ionic gelation (González et al. 2016). For each experimental condition, 1 mg of plasmid was mixed with 4 mL of 0.84 mg/mL TPP (Sigma-Aldrich, St. Louis, MO, USA). Chitosan-TPP-DNA nanoparticles were formed upon dropwise addition of the TPP-DNA solution into 10 mL of 2 mg/mL low molecular weight chitosan (Sigma-Aldrich, St. Louis, MO, USA)-acetate buffer (pH 4.4) solution. Nanoparticles were sedimented by centrifugation at 36,000 g for 20 min at 15 °C and resuspended in 2 mL of 2% w/v mannitol, which acted as cryoprotector during lyophilisation. Nanoparticles were subjected to a freeze–drying process at − 47 °C. Particle size and *Z* potential were determined by dynamic light scattering and laser Doppler electrophoresis, respectively, using Zetasizer Nano ZS fitted with a 633 nm laser (Malvern Instruments, Malvern, UK). Chitosan-TPP-DNA

nanoparticles were resuspended in 0.9% NaCl before intraperitoneal administration to *S. aurata*.

Body composition

For moisture determination, fish were dried at 85 °C until constant weight was reached (Busacker et al. 1990; Lucas 1996). Moisture was calculated as [wet weight (g) − dry weight (g)]*100/wet weight (g). Dried samples were further used for assaying nitrogen (N), lipid and ash. N content was determined with FlashEA 1112 analyser (Thermo Fisher Scientific, Waltham, MA, USA) and was subsequently used to estimate crude protein by multiplying N content by a factor of 6.25. Crude lipid was extracted with petroleum ether using a Soxhlet extractor. For ash determination, samples were incinerated in a Hobersal 12PR/300 muffle furnace (Hobersal, Caldes de Montbui, Spain) at 550 °C for 12 h (Busacker et al. 1990; Lucas 1996). Crude protein, lipid and ash are expressed as percentage of dry weight.

Growth parameters

Specific growth rate (SGR), feed conversion ratio (FCR), hepatosomatic index (HSI), protein retention (PR), lipid retention (LR) and protein efficiency ratio (PER) were calculated according to the following equations:

$$SGR = \left(\ln W_f - \ln W_i\right) * 100/T;$$

where W_f and W_i are mean final and initial body fresh weight (g) and T is time (days)

FCR = dry feed intake (g)/wet weight gain (g)

HSI = liver fresh weight (g) ∗ 100/fish body weight (g)

PR = body protein gain (g) ∗ 100/protein intake (g)

LR = body lipid gain (g) ∗ 100/lipid intake (g)

PER = weight gain (g)/feed protein provided (g)

Enzyme activity assays and metabolites

Enzyme activity assays and metabolites were spectrophotometrically determined at 30 °C in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Liver crude extracts were obtained by homogenisation of powdered frozen tissue (1:5, *w/v*) in 50 mM Tris-HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 250 mM sucrose, 30 s at 4 °C using PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland). Following centrifugation at 10,000 g for 30 min at 4 °C, the supernatant was collected for enzyme activity assays. Reaction mixtures for 6-phosphofructo-1-kinase (Pfkl), fructose-1,6-bisphosphatase (Fbp1) and total protein were as previously described (Metón et al. 1999b). Enzyme activities were expressed as specific activity (U/g protein). One unit of Pfkl activity was considered the amount of enzyme needed to oxidise 2 µmol of NADH per min. One unit of Fbp1 activity of was defined as the amount of enzyme necessary for transforming 1 µmol of substrate per min. Serum glucose, triglycerides and cholesterol were measured with commercial kits (Linear Chemicals, Montgat, Spain).

Reverse transcription coupled to quantitative real-time PCR (RT-qPCR)

Total RNA from *S. aurata* tissues was isolated using HigherPurity Tissue Total RNA Purification Kit (Canvax, Cordoba, Spain) and reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The mRNA expression levels of genes listed in Table 1 were determined using QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixture contained 0.4 µM of each primer (Table 1), 5 µL of SYBR Green (Thermo Fisher Scientific, Foster City, CA, USA), 0.8 µL of diluted cDNA and sterilized milli-Q water to final volume of 10 µL. The amplification cycle was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. For each gene, standard curves for determining efficiency of the amplification reaction were generated with serial dilutions of control cDNA. Amplification of single products was confirmed by checking dissociation curves after each experiment. Amplicon size was checked by agarose gel electrophoresis. *S. aurata* ribosomal subunit 18S (*18s*), β-actin (*actb*) and elongation factor 1 alpha (*eef1a*) were used as endogenous controls to normalise the mRNA levels for genes of interest in liver samples. For tissue distribution, normalisation

Table 1 Primer sequences used for RT-qPCR in the present study

Gene	Forward sequences (5′–3′)	Reverse sequences (5′–3′)	GenBank Accession
acaca	CCCAACTTCTTCTACCACAG	GAACTGGAACTCTACTACAC	JX073712
acacb	TGACATGAGTCCTGTGCTGG	GCCTCAGTTCGTATGATGGT	JX073714
actb	CTGGCATCACACCTTCTACAACGAG	GCGGGGGTGTTGAAGGTCTC	X89920
cpt1a	GAAGGGCAGATAAAGAGGGGC	GCATCGATCGCTGCATTCAGC	JQ308822
eef1a	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	AF184170
elovl4a	AAGAACAGAGAGCCCTTCCAG	TGCCACCCTGACTTCATTG	MK610320
elovl4b	TCTACACAGGCTGCCCATTC	CGAAGAGGATGATGAAGGTGAC	MK610321
elovl5	GGGATGGCTACTGCTCGACA	CAGGAGAGTGAGGCCCAGAT	AY660879
fads2	CACTATGCTGGAGAGGATGCC	TATTTCGGTCCTGGCTGGGC	AY055749
fasn	GTAGAGGACACGCCCATCGAT	TGCGTATGACCTCTTGGTGTGCT	JQ277708
fat-1	TTCAACCCCATTCCTTTCAGCG	TAGGCGCACACGCAGCAGCA	ON374024
fat-2	AAGAGGACTACAACAACAGAACCGCCA	CGAACAGTCTGCTCCAAGGCCAA	ON374025
fbp1	CAGATGGTGAGCCGTGTGAGAAGGATG	GCCGTACAGAGCGTAACCAGCTGCC	AF427867
gck	TGTGTCAGCTCTCAACTCGACC	AGGATCTGCTCTACCATGTGGAT	AF169368
g6pc1	GCGTATTGGTGGCTGAGGTCG	AAGGAGAGGGTGGTGTGGAAG	AF151718
g6pd	TGATGATCCAACAGTTCCTA	GCTCGTTCCTGACACACTGA	JX073711
hmgcr	ACTGATGGCTGCTCTGGCTG	GGGACTGAGGGATGACGCAC	MN047456
hnf4a	GTGGACAAAGACAAGCGAAATC	GCATTGATGGATGGTAAACTGC	FJ360721
nr1h3	GCATCTGGACGAGGCTGAATAC	ACTTAGTGTGCGAAGGCTCACC	FJ502320
pck1	CAGCGATGGAGGAGTGTGGTGGGA	GCCCATCCCAATTCCCGCTTCTGTGCT CCGGCTGGTCAGTGT	AF427868
pfkfb1	TGCTGATGGTGGGACTGCCG	CTCGGCGTTGTCGGCTCTGAAG	U84724
pfk1	TGCTGGGGACAAAACGAACTCTTCC	AAACCCTCCGACTACAAGCAGAGCT	KF857580
pklr	CAAAGTGGAAAGCCGGCAAGGG	GTCGCCCCTGGCAACCATAAC	KF857579
ppara	GTGAGTCTTGTGAGTGAGGGGTTG	AGTGGGGATGGTGGGCTG	AY590299
srebf1	CAGCAGCCCGAACACCTACA	TTGTGGTCAGCCCTTGGAGTTG	JQ277709
scd1a	TCCCTTCCGCATCTCCTTTG	TTGTGGTGAACCCTGTGGTCTC	JQ277703
18s	TTACGCCCATGTTGTCCTGAG	AGGATTCTGCATGATGGTCACC	AM490061

was performed against *S. aurata 18s* expression. The standard $\Delta\Delta C_T$ method was used to calculate variations in gene expression (Pfaffl 2001).

Fatty acid methyl ester (FAME) analysis

Fatty acid profiles of liver and muscle were analysed by gas chromatography with flame ionisation detection as previously described (Silva-Marrero et al. 2019), using GC-2025 (Shimadzu, Kyoto, Japan) with capillary column BPX70, 30 m × 0.25 mm × 0.25 µm (Trajan Scientific and Medical, Ringwood, Australia). Oven temperature started at 60 °C for 1 min and then it was raised to 260 °C (rate: 6 °C/min). Injector (AOC-20i, Shimadzu, Japan) and detector temperatures were 260 °C and 280 °C, respectively. Sample (1 µL) was injected with helium as carrier gas and split ratio 1:20. Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA) was used as reference for identifying fatty acids.

Statistics

To identify significant differences between treatments, the SPSS Version 25 software (IBM, Armonk, NY, USA) was used to submit experimental data to one-way analysis of variance followed by the Duncan post-hoc test (> 2 groups). Statistical significance was considered when $P < 0.05$.

Results

Delivery of chitosan-TPP complexed with pSG5-FAT-1 and pSG5- FAT-2 increases fish codon-optimised FAT-1 and FAT-2 mRNA levels in *S. aurata*

To assess the metabolic effects resulting from expression of *C. elegans fat-1* and *fat-2* in the liver of *S. aurata*, we designed fish codon-optimised *C. elegans* FAT-1 and FAT-2 cDNA sequences for further ligation into pSG5 and prepared chitosan-TPP nanoparticles complexed with empty pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 by ionic gelation. Particle size and *Z* potential of naked chitosan-TPP, expressed as mean ± SEM ($n = 3$), was 214.6 nm ± 20.2 and 37.5 mV ± 0.6, respectively. Incorporation of plasmid DNA to chitosan-TPP did not significantly modify particle size, which was 262.7 nm ± 74.0 (mean ± SEM, $n = 3$), but decreased *Z* potential to 12.0 mV ± 0.8 (mean ± SEM, $n = 3$).

For long-term sustained expression of fish codon-optimised *fat-1* and *fat-2* in the liver of *S. aurata*, each experimental group of fish received every 4 weeks up to 3 intraperitoneal injections of chitosan-TPP complexed with 10 µg/g BW of the corresponding plasmid (pSG5, pSG5-FAT-1, pSG5-FAT-2 or pSG5-FAT-1 + pSG5-FAT-2). The dosing schedule was based on preliminary studies showing that 28 days post-administration of chitosan-TPP-pSG5-FAT-1 and chitosan-TPP-pSG5-FAT-2 (10 µg/g BW of plasmid) to *S. aurata* increased the hepatic mRNA levels of fish codon-optimised *fat-1* and *fat-2* to levels even higher than those found at 72 h post-treatment. Expressed as mean ± SEM ($n = 3$), fold increase over control values at 72 h and 28 days post-treatment were 31.6 ± 4.1 and 74.8 ± 29.0, respectively, for *fat-1* mRNA levels, while for *fat-2* mRNA levels fold increase was 20.3 ± 2.8 and 70.2 ± 7.0, respectively. Seventy days after the beginning of the experiment (14 days following the last injection), the mRNA levels of fish codon-optimised *fat-1* and *fat-2* were determined by RT-qPCR in several tissues of treated fish, including the liver, intestine, skeletal muscle and brain (Fig. 1).

When compared with control fish, chitosan-TPP nanoparticles complexed with pSG5-FAT-1 and pSG5-FAT-2 significantly increased the mRNA levels of *fat-1* and *fat-2*, respectively, in the liver and intestine of *S. aurata*. Specifically, *fat-1* mRNA abundance in the liver of fish administered with pSG5-FAT-1 was 201.8-fold higher than in control fish, while treatment with pSG5-FAT-2 upregulated *fat-2* 297.4-fold. For the intestine, pSG5-FAT-1 and pSG5-FAT-2 upregulated 10.6-fold *fat-1* and 24.7-fold *fat-2*, respectively. Nanoparticle administration did not exert effects on the skeletal muscle and brain.

Effect of fish codon-optimised FAT-1 and FAT-2 expression on whole-body composition, growth performance and serum metabolites in *S. aurata*

Sustained expression of fish codon-optimised FAT-1 + FAT-2 in the liver of *S. aurata* caused a moderate but significant 7.6% decrease of whole-body crude protein values observed in control fish. No effect was observed in moisture, ash and crude lipid body composition (Table 2). Analysis of growth performance parameters showed significantly increased weight gain values in fish expressing *fat-1* (18% of increase) and *fat-1* + *fat-2* (26% of increase) compared to control fish. No significant difference was found between controls and treatment with FAT-2. Similarly, the highest SGR was found in fish treated with FAT-1 + FAT-2, followed by fish treated with FAT-1, controls and fish treated with FAT-2. Fish expressing *fat-2* also presented the lowest PER. HSI significantly decreased in fish treated with FAT-1 and FAT-1 + FAT-2 to 72% of control values. No significant differences were observed in PR and LR.

Serum glucose, triglycerides and cholesterol were also determined in 70-day treated *S. aurata*. Any of the treatments assayed affected blood glucose levels. However, co-expression of *fat-1* + *fat-2* significantly decreased 1.8-fold triglycerides and 1.5-fold cholesterol compared to control levels (Fig. 2).

Effect of fish codon-optimised FAT-1 and FAT-2 on the fatty acid profile in the liver and skeletal muscle

The effect of long-term expression of *fat-1* and *fat-2* was analysed on the fatty acid profile of the liver and skeletal muscle of *S. aurata*. Table 3 shows the fatty acid composition in the liver of *S. aurata* long-term treated with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2, and pSG5-FAT-1 + pSG5-FAT-2. Among

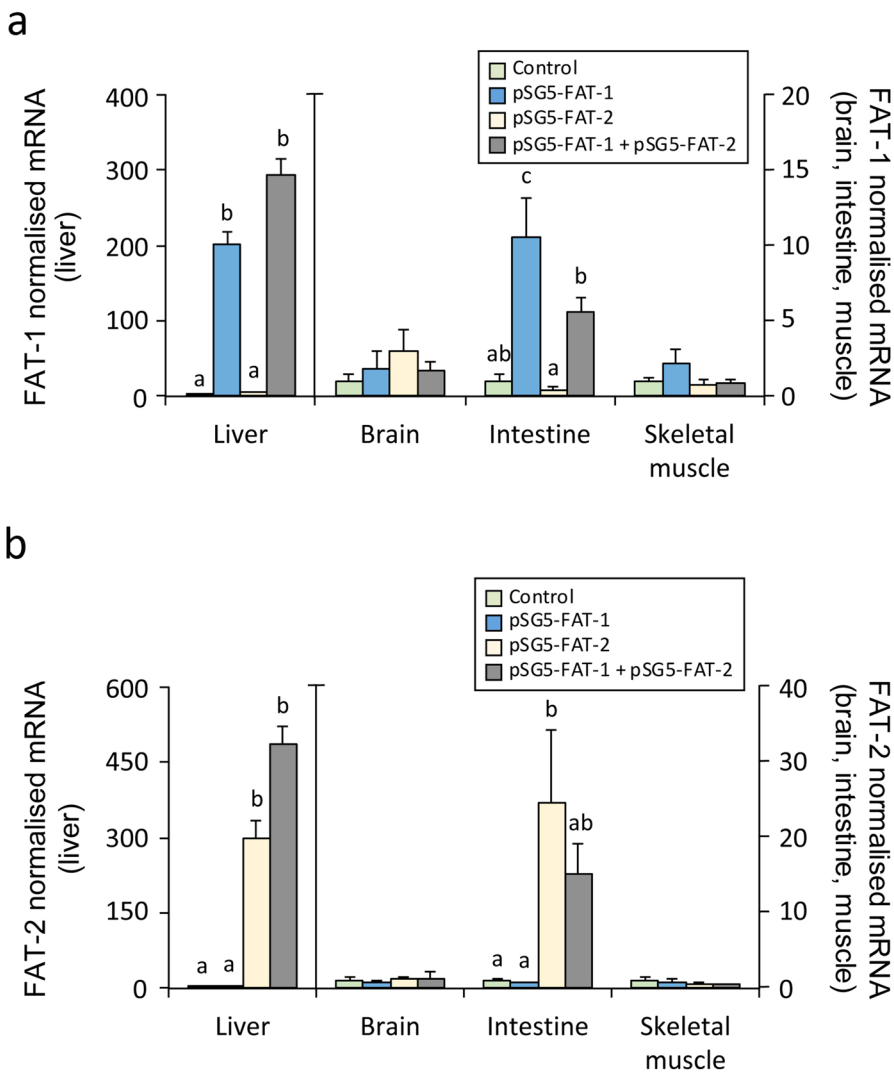


Fig. 1 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the mRNA levels of fish-codon optimised *C. elegans* FAT-1 and FAT-2 in *S. aurata* tissues (brain, skeletal muscle, liver and intestine). Fourteen days after the last injection and 24 h following the last meal, exogenous *fat-1* (**a**) and *fat-2* (**b**) expression was assayed by RT-qPCR, normalised to the *S. aurata 18 s* mRNA levels and represented as mean ± SEM ($n = 4$). For each tissue, homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

30 different fatty acids identified in this study, treatment with FAT-1 and FAT-1 + FAT-2 significantly increased EPA (1.5-fold and 1.6-fold, respectively), DHA (2.4-fold and 2.3-fold, respectively) and total *n*-3 fatty acids (1.7-fold in both cases). The *n*-6/*n*-3 ratio significantly decreased in fish expressing *fat-1* (to 60.3% of control values), *fat-2* (66.9%) and *fat-1* + *fat-2* (63.7%). A moderate 1.2-fold increase of *cis*-10-heptadecenoic acid (17:1*n*-7) was also observed in FAT-1 + FAT-2 treated fish, while expression of *fat-1* decreased palmitoleic acid (16:1*n*-7) to 58.4% of control levels.

The effect of long-term hepatic expression of *fat-1*, *fat-2* and *fat-1* + *fat-2* on the fatty acids profile in the skeletal muscle is shown in Table 4. Twenty-one out of 29 fatty acids identified in the skeletal muscle were significantly affected by co-expression of *fat-1* and *fat-2*. Total saturated fatty acids significantly decreased to 57.1% of control levels, mostly resulting from the low content in myristic acid (14:0; 34.4% of controls), palmitic acid (16:0; 56.3% of controls) and margaric acid (17:0; 50.0% of controls). In addition, treatment with FAT-1 and FAT-2 also decreased margaric acid to 50.0% of control values. In contrast, longer saturated fatty acids (with more than 17 carbons) presented increased values than in controls. Thus, stearic acid (18:0) increased 1.2-fold, while arachidic acid (20:0), behenic acid (22:0), tricosylic acid (23:0) and lignoceric acid (24:0) rised from non-detectable levels in control

Table 2 Growth performance, nutrient retention and body composition of *S. aurata* after intraperitoneal injection of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2

	Control	FAT-1	FAT-2	FAT-1 + FAT-2
Initial body weight (g)	9.97 ± 0.69	11.42 ± 0.90	9.74 ± 0.44	11.04 ± 0.55
Final body weight (g)	34.64 ± 1.03^{a}	40.51 ± 1.63^{b}	31.46 ± 1.57^{a}	42.15 ± 1.25^{b}
Weight gain (g)	24.67 ± 0.51^{a}	29.09 ± 1.06^{b}	21.73 ± 1.23^{a}	31.11 ± 0.73^{b}
SGR (%)	1.79 ± 0.05^{ab}	1.85 ± 0.08^{ab}	1.59 ± 0.04^{a}	2.01 ± 0.03^{b}
FCR	1.40 ± 0.03^{ab}	1.36 ± 0.04^{ab}	1.53 ± 0.08^{b}	1.29 ± 0.03^{a}
HSI (%)	1.55 ± 0.12^{b}	1.12 ± 0.08^{a}	1.49 ± 0.18^{ab}	1.12 ± 0.15^{a}
PR (%)	24.98 ± 3.24	21.96 ± 0.85	18.90 ± 2.45	21.81 ± 1.56
LR (%)	28.00 ± 7.50	31.47 ± 1.12	25.17 ± 2.03	26.24 ± 2.02
PER	1.40 ± 0.03^{ab}	1.45 ± 0.05^{ab}	1.31 ± 0.07^{a}	1.52 ± 0.04^{b}
Moisture (%)	71.34 ± 1.22	70.68 ± 0.29	72.03 ± 0.98	71.10 ± 1.28
Ash (%)	13.72 ± 1.47	13.93 ± 0.12	13.07 ± 0.73	12.94 ± 0.45
Protein (%)	62.20 ± 3.88^{b}	58.81 ± 1.80^{ab}	57.98 ± 1.04^{ab}	57.50 ± 1.24^{a}
Lipid (%)	27.28 ± 1.44	29.28 ± 0.55	26.09 ± 1.85	25.02 ± 2.3

All fish were fed twice a day at a total daily ration of 3% BW. Every 2 weeks, fish were individually weighted to readjust the feed amount. Data are expressed as mean ± SEM ($n = 3$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

SGR specific growth rate, *FCR* feed conversion ratio, *HSI* hepatosomatic index, *PR* protein retention, *LR* lipid retention, *PER* protein efficiency ratio

fish to low but detectable levels in fish treated with FAT-1 + FAT-2.

Monounsaturated, PUFA and total *n*-3, *n*-6 and *n*-9 fatty acids increased 1.3-fold, 1.3-fold, 2.2-fold, 1.1-fold and 1.5-fold, respectively, in the skeletal muscle of fish expressing *fat-1* + *fat-2*. As a result of greater effect on *n*-3 series than on *n*-6 fatty acids, the *n*-6/*n*-3 ratio significantly decreased to 52.0% of control levels. Considering unsaturated fatty acids with a content greater than 1% for any assayed treatment, expression of *fat-1* + *fat-2* significantly increased EPA (1.7-fold) and DHA (3.0-fold) from the *n*-3 series, LA (18:2*n*-6c; 1.1-fold) and OA (18:1*n*-9c; 1.5-fold), while decreased palmitoleic acid to 61.5% of control levels. For less abundant unsaturated fatty acids (content between 0.1 and 1%), treatment with FAT-1 + FAT-2 also resulted in significant increases of *cis*-10-heptadecenoic acid (1.5-fold), gondoic acid (20:1*n*-9; 3.2-fold), erucic acid (22:1*n*-9; 5.2-fold), eicosadienoic acid (20:2*n*-6; 5.5-fold) and ARA (20:4*n*-6; 1.4-fold).

Effect of fish codon-optimised FAT-1 and FAT-2 on the expression of key genes in de novo lipogenesis and fatty acid oxidation in the liver

The effect of chitosan-TPP-DNA nanoparticles expressing *fat-1* and *fat-2* was also assessed on the hepatic expression of genes involved in de novo lipogenesis and fatty acid oxidation. As shown in Fig. 3, treatment with FAT-1 significantly decreased the mRNA levels of elongation of very long chain fatty acids protein 4a (*elovl4a*; to 43.3% of control values), elongation of very long chain fatty acids protein 4b (*elovl4b*; to 45.4%), elongation of very long chain fatty acids protein 5 (*elovl5*; to 62.3%), sterol regulatory element-binding protein 1 (*srebf1*; to 41.3%) and peroxisome proliferator-activated receptor alpha (*ppara*; to 46.0%), while treatment with FAT-2 decreased *elovl5* (to 43.6%) and *ppara* (to 56.3%) mRNA levels (Fig. 3f–h, k, l). Co-expression of *fat-1* and *fat-2* also significantly down-regulated acetyl-CoA carboxylase 1 (*acaca*; to 31.4% of control values), acetyl-CoA carboxylase 2 (*acacb*; to 65.0%), acyl-CoA 6-desaturase (*fads2*; to 69.4%), *elovl4b* (to 59.1%), *elovl5* (to 41.8%), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*hmgcr*; to 64.2%) and *srebf1* (to 41.9%) (Fig. 3a, b, e, g, h, j, k). No significant differences were found for fatty acid

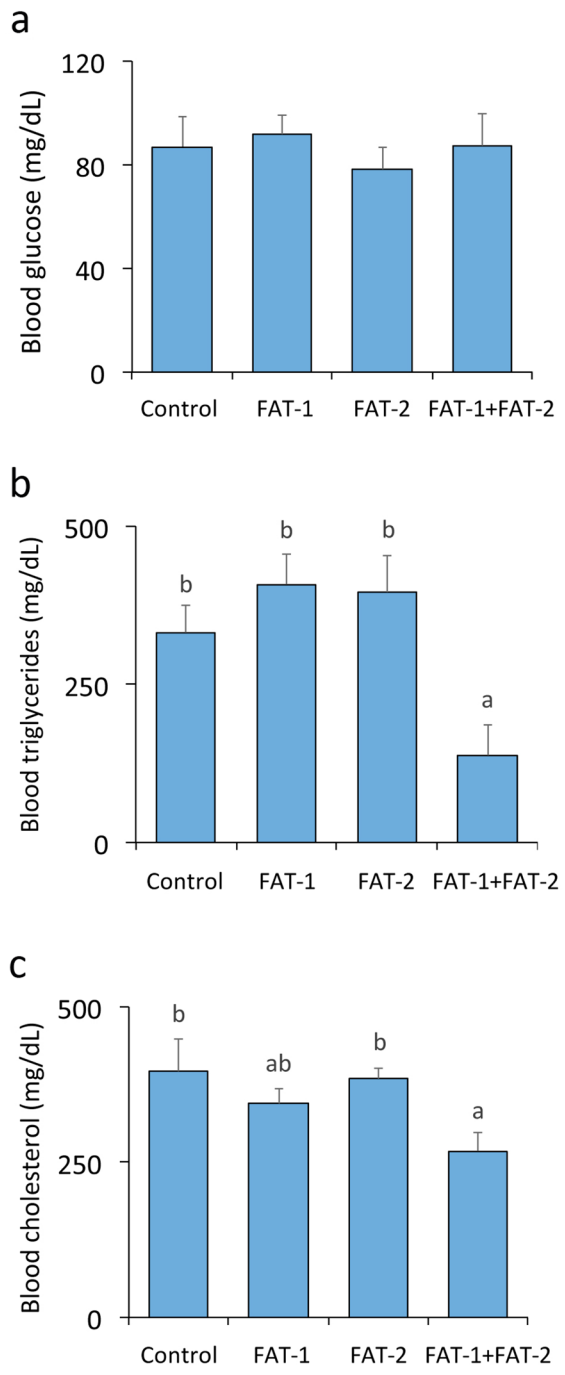


Fig. 2 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on serum glucose (**a**), triglycerides (**b**) and cholesterol (**c**) in *S. aurata*. Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the blood was collected. Values are represented as mean ± SEM ($n = 6$–7). Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

synthase (*fasn*), stearoyl-CoA desaturase-1a (*scd1a*) and carnitine O-palmitoyltransferase 1, liver isoform (*cpt1a*) (Fig. 3c, d, i).

Effect of fish codon-optimised FAT-1 and FAT-2 on glycolysis-gluconeogenesis, the pentose phosphate pathway, *hnf4a* and *nr1h3* in the liver

Figure 4a–h shows the effect of long-term expression of fish codon-optimised *fat-1* and *fat-2* on the hepatic expression of rate-limiting enzymes in glycolysis-gluconeogenesis. Due to the pivotal role of the enzymes that control the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate cycle in the regulation of glycolysis-gluconeogenesis, we measured both the mRNA levels and the enzyme activity of Pfkl and Fbp1. Gene expression of *pfkl* and *fbp1* was not significantly affected by the treatments (Fig. 4d, e). However, when considering the Pfkl/Fbp1 activity ratio, fish treated with FAT-1 + FAT-2 exhibited a significant increased glycolytic flux (29.9%) compared to control fish (Fig. 4f). Similarly, treatment with FAT-2 showed a trend to increase the Pfkl/Fbp1 activity ratio (22.7%).

In regard of other glycolytic-gluconeogenic enzymes, expression of *fat-2* and *fat-1* + *fat-2* significantly upregulated 1.5-fold and 1.8-fold, respectively, the mRNA levels of liver pyruvate kinase (*pklr*) and 1.4-fold those of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (*pfkfb1*) (Fig. 4c). Compared to controls, the expression levels for glucokinase (*gck*), glucose-6-phosphatase catalytic subunit (*g6pc1*) and phosphoenolpyruvate carboxykinase (*pck1*) were not affected (Fig. 4a, b, h).

In addition, expression of *fat-2* also significantly increased the hepatic mRNA levels (2.0-fold) of glucose-6-phosphate dehydrogenase (*g6pd*), the rate-limiting enzyme in the oxidative phase of the pentose phosphate pathway (Fig. 4i), while treatment with FAT-2 and FAT-1 + FAT-2 upregulated hepatocyte nuclear factor 4-alpha (*hnf4a*; 1.5-fold) and oxysterols receptor LXR-alpha (*nr1h3*; 1.6-fold) mRNA levels, respectively (Fig. 4j–k).

Discussion

With the aim to induce sustained production of *n*-3 LC PUFA in *S. aurata*, the ionotropic gelation technique was used to obtain chitosan-TPP nanoparticles

Table 3 Effect of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the fatty acid profile of *S. aurata* liver

Fatty acid	Control	FAT-1	FAT-2	FAT-1 + FAT-2
14:0	9.38 ± 0.46	7.31 ± 1.45	7.43 ± 0.60	7.40 ± 1.45
15:0	0.10 ± 0.10	0.32 ± 0.12	0.37 ± 0.13	0.32 ± 0.11
16:0	27.49 ± 0.66	27.09 ± 3.37	26.33 ± 1.42	25.29 ± 2.50
17:0	0.21 ± 0.03	0.11 ± 0.04	0.22 ± 0.11	0.11 ± 0.04
18:0	3.73 ± 0.39	3.88 ± 0.30	4.20 ± 0.38	4.03 ± 0.33
20:0	0.02 ± 0.02	0.20 ± 0.13	0.08 ± 0.03	0.08 ± 0.04
21:0	0.06 ± 0.06	0.16 ± 0.06	0.12 ± 0.07	0.11 ± 0.04
22:0	0.00 ± 0.00	0.06 ± 0.04	0.11 ± 0.04	0.05 ± 0.05
23:0	0.02 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
24:0	0.01 ± 0.01	0.03 ± 0.02	0.06 ± 0.02	0.04 ± 0.02
14:1*n*-5	0.06 ± 0.05	0.00 ± 0.00	0.14 ± 0.09	0.13 ± 0.04
15:1*n*-5	0.06 ± 0.02	0.02 ± 0.01	0.05 ± 0.03	0.05 ± 0.02
16:1*n*-7	6.93^{b} ± 0.27	4.05^{a} ± 1.23	5.58^{ab} ± 0.48	5.38^{ab} ± 0.42
17:1*n*-7	0.20 ± 0.02	0.22 ± 0.00	0.26 ± 0.02	0.23 ± 0.01
18:1*n*-9c	21.24 ± 1.24	22.04 ± 1.60	22.45 ± 1.15	21.84 ± 1.89
18:1*n*-9t	0.06 ± 0.03	0.04 ± 0.01	0.08 ± 0.02	0.04 ± 0.01
20:1*n*-9	0.58 ± 0.08	0.83 ± 0.02	0.71 ± 0.13	0.72 ± 0.14
22:1*n*-9	0.17 ± 0.03	0.30 ± 0.08	0.25 ± 0.05	0.31 ± 0.08
24:1*n*-9	0.00 ± 0.00	0.07 ± 0.05	0.07 ± 0.03	0.05 ± 0.03
18:2*n*-6c	23.23 ± 0.52	23.55 ± 1.4	22.42 ± 1.36	24.19 ± 1.29
18:2*n*-6t	0.26 ± 0.22	0.09 ± 0.03	0.09 ± 0.04	0.13 ± 0.05
20:2*n*-6	0.17 ± 0.05	0.25 ± 0.03	0.21 ± 0.05	0.19 ± 0.07
22:2*n*-6	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
18:3*n*-3	1.29 ± 0.10	1.26 ± 0.25	1.76 ± 0.45	1.16 ± 0.20
18:3*n*-6	0.76 ± 0.17	0.65 ± 0.14	0.81 ± 0.06	0.72 ± 0.14
20:3*n*-3	0.00 ± 0.00	0.06 ± 0.06	0.00 ± 0.00	0.07 ± 0.07
20:3*n*-6	0.23 ± 0.04	0.29 ± 0.06	0.21 ± 0.06	0.25 ± 0.02
20:4*n*-6	0.22 ± 0.02	0.29 ± 0.10	0.40 ± 0.14	0.30 ± 0.11
20:5*n*-3	1.79^{a} ± 0.09	2.76^{b} ± 0.29	2.43^{ab} ± 0.26	2.89^{b} ± 0.33
22:6*n*-3	1.70^{a} ± 0.15	4.02^{b} ± 0.65	3.15^{ab} ± 0.74	3.87^{b} ± 0.66
Saturated	41.02 ± 0.58	39.18 ± 4.49	38.92 ± 1.64	37.45 ± 3.54
Monounsaturated	29.3 ± 1.10	27.57 ± 2.60	29.59 ± 1.30	28.75 ± 1.79
PUFA	29.67 ± 0.60	33.25 ± 2.51	31.49 ± 1.39	33.80 ± 1.94
n-3	4.78^{a} ± 0.28	8.11^{b} ± 0.99	7.34^{ab} ± 0.99	8.00^{b} ± 0.93
n-6	24.89 ± 0.47	25.14 ± 1.63	24.15 ± 1.48	25.8 ± 1.54
n-9	22.05 ± 1.18	23.28 ± 1.74	23.56 ± 1.07	22.96 ± 2.06
n-6/*n*-3	5.26^{b} ± 0.28	3.17^{a} ± 0.26	3.52^{a} ± 0.65	3.35^{a} ± 0.42

Data are expressed as percentage of total fatty acids and represented as mean ± SEM ($n = 4$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

complexed with plasmids expressing fish codon-optimised *C. elegans fat-1* and *fat-2*. *C. elegans fat-1* and *fat-2* were chosen to improve *n*-3 LC PUFA biosynthesis in *S. aurata* on the basis of their functionality in transgenic vertebrates, including fish. In fact, methyl-end desaturases from nematodes, including *C. elegans fat-1* and *fat-2*, are considered one of the three main clades in the evolution of animal methyl-end desaturase genes, together with those from two distinct gene lineages of cnidarians and some lophotrochozoans and arthropods (Kabeya et al. 2018). Even though knowledge on pathways of PUFA biosynthesis in non-vertebrate animals is still limited, increasing availability of genomic data and functional characterisation of methyl-end desaturases from marine invertebrates will contribute to a better understanding

Table 4 Effect of chitosan-TPP complexed with empty vector (pSG5, control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the fatty acid profile of *S. aurata* skeletal muscle

Fatty acid	Control	FAT-1	FAT-2	FAT-1 + FAT-2
14:0	$8.83^{b\pm}0.37$	$7.86^{b\pm}1.39$	$7.44^{b\pm}1.22$	$3.04^{a\pm}0.10$
16:0	$29.58^{b} \pm 1.31$	$27.00^{b} \pm 3.05$	$26.93^{b} \pm 2.85$	$16.64^{a} \pm 0.34$
17:0	$0.18^{b} \pm 0.01$	$0.09^{a} \pm 0.00$	$0.09^{a} \pm 0.00$	$0.09^{a} \pm 0.00$
18:0	$2.77^{a} \pm 0.04$	$2.78^{a} \pm 0.15$	$2.96^{a} \pm 0.11$	$3.29^{b} \pm 0.08$
20:0	$0.00^{a} \pm 0.00$	$0.11^{a} \pm 0.07$	$0.09^{a} \pm 0.07$	$0.28^{b} \pm 0.03$
22:0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.10^{b} \pm 0.01$
23:0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.09^{b} \pm 0.03$
24:0	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.02^{a} \pm 0.02$	$0.08^{b} \pm 0.00$
14:1*n*-5	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
15:1*n*-5	0.02 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.05 ± 0.00
16:1*n*-7	$6.63^{b} \pm 0.16$	$6.42^{b} \pm 0.68$	$6.14^{b} \pm 0.56$	$4.08^{a} \pm 0.05$
17:1*n*-7	$0.16^{a} \pm 0.01$	$0.20^{ab} \pm 0.03$	$0.20^{ab} \pm 0.01$	$0.24^{b} \pm 0.00$
18:1*n*-9c	$19.6^{a} \pm 0.53$	$21.98^{a} \pm 2.33$	$21.65^{a} \pm 2.01$	$28.45^{b} \pm 0.30$
18:1*n*-9t	$0.11^{b} \pm 0.04$	$0.06^{ab} \pm 0.03$	$0.11^{b} \pm 0.04$	$0.00^{a} \pm 0.00$
20:1*n*-9	$0.26^{a} \pm 0.16$	$0.48^{ab} \pm 0.12$	$0.54^{ab} \pm 0.10$	$0.84^{b} \pm 0.01$
22:1*n*-9	$0.12^{a} \pm 0.01$	$0.20^{a} \pm 0.09$	$0.21^{a} \pm 0.09$	$0.62^{b} \pm 0.05$
24:1*n*-9	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.00^{a} \pm 0.00$	$0.08^{b} \pm 0.01$
18:2*n*-6c	$24.49^{a} \pm 0.62$	$24.57^{a} \pm 0.64$	$25.29^{a} \pm 0.67$	$27.98^{b} \pm 0.26$
18:2*n*-6t	0.40 ± 0.23	0.16 ± 0.16	0.17 ± 0.17	0.00 ± 0.00
20:2*n*-6	$0.06^{a} \pm 0.02$	$0.12^{a} \pm 0.05$	$0.13^{a} \pm 0.04$	$0.33^{b} \pm 0.02$
22:2*n*-6	$0.00^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.02^{a} \pm 0.02$	$0.10^{b} \pm 0.01$
18:3*n*-3	0.86 ± 0.26	0.89 ± 0.17	0.83 ± 0.16	1.11 ± 0.13
18:3*n*-6	0.97 ± 0.13	0.99 ± 0.06	0.90 ± 0.07	1.12 ± 0.04
20:3*n*-3	0.00 ± 0.00	0.04 ± 0.04	0.04 ± 0.04	0.09 ± 0.01
20:3*n*-6	0.13 ± 0.01	0.13 ± 0.03	0.13 ± 0.02	0.20 ± 0.02
20:4*n*-6	$0.23^{a} \pm 0.01$	$0.24^{a} \pm 0.03$	$0.28^{ab} \pm 0.03$	$0.32^{b} \pm 0.01$
20:5*n*-3	$2.36^{a} \pm 0.13$	$2.62^{a} \pm 0.44$	$2.79^{a} \pm 0.45$	$4.07^{b} \pm 0.03$
22:6*n*-3	$2.25^{a} \pm 0.31$	$2.97^{a} \pm 0.99$	$2.98^{a} \pm 0.98$	$6.69^{b} \pm 0.21$
Saturated	$41.36^{b} \pm 1.46$	$37.86^{b} \pm 4.21$	$37.53^{b} \pm 3.86$	$23.62^{a} \pm 0.38$
Monounsaturated	$26.9^{a} \pm 0.80$	$29.39^{a} \pm 1.95$	$28.92^{a} \pm 1.68$	$34.38^{b} \pm 0.34$
PUFA	$31.75^{a} \pm 0.67$	$32.75^{a} \pm 2.26$	$33.55^{a} \pm 2.19$	$42.00^{b} \pm 0.38$
n-3	$5.47^{a} \pm 0.19$	$6.52^{a} \pm 1.59$	$6.64^{a} \pm 1.62$	$11.96^{b} \pm 0.16$
n-6	$26.27^{a} \pm 0.82$	$26.23^{a} \pm 0.74$	$26.91^{a} \pm 0.76$	$30.04^{b} \pm 0.26$
n-9	$20.09^{a} \pm 0.65$	$22.73^{a} \pm 2.53$	$22.51^{a} \pm 2.16$	$29.99^{b} \pm 0.32$
n-6/*n*-3	$4.83^{b} \pm 0.30$	$4.52^{b} \pm 0.69$	$4.59^{b} \pm 0.76$	$2.51^{a} \pm 0.03$

Data are expressed as percentage of total fatty acids and represented as mean ± SEM ($n = 4$). Different superscript letters indicate significant differences between groups ($P < 0.05$)

of *n*-3 LC-PUFA biosynthesis in marine ecosystems (Monroig et al. 2022).

Monthly intraperitoneal administration of 3 doses of chitosan-TPP-DNA nanoparticles allowed long-standing high expressional levels of the exogenous proteins in the liver, mild expression in the intestine and barely detectable levels in the skeletal muscle and brain. Biodistribution of fish codon-optimised *fat-1* and *fat-2* expression shows that the particle size of chitosan-TPP-DNA complexes used in the present study favoured liver retention in *S. aurata*, which confirms previous reports where we analysed the acute effect of expressing exogenous SREBP1a and silencing of endogenous cytosolic alanine aminotransferase and glutamate dehydrogenase (González et al. 2016; Gaspar et al. 2018; Silva-Marrero et al. 2019). Possibly, discontinuous endothelia of the intestine enables chitosan-TPP-DNA nanoparticle absorption and transportation to the liver through portal circulation (Hagens et al. 2007), while the tight morphology of

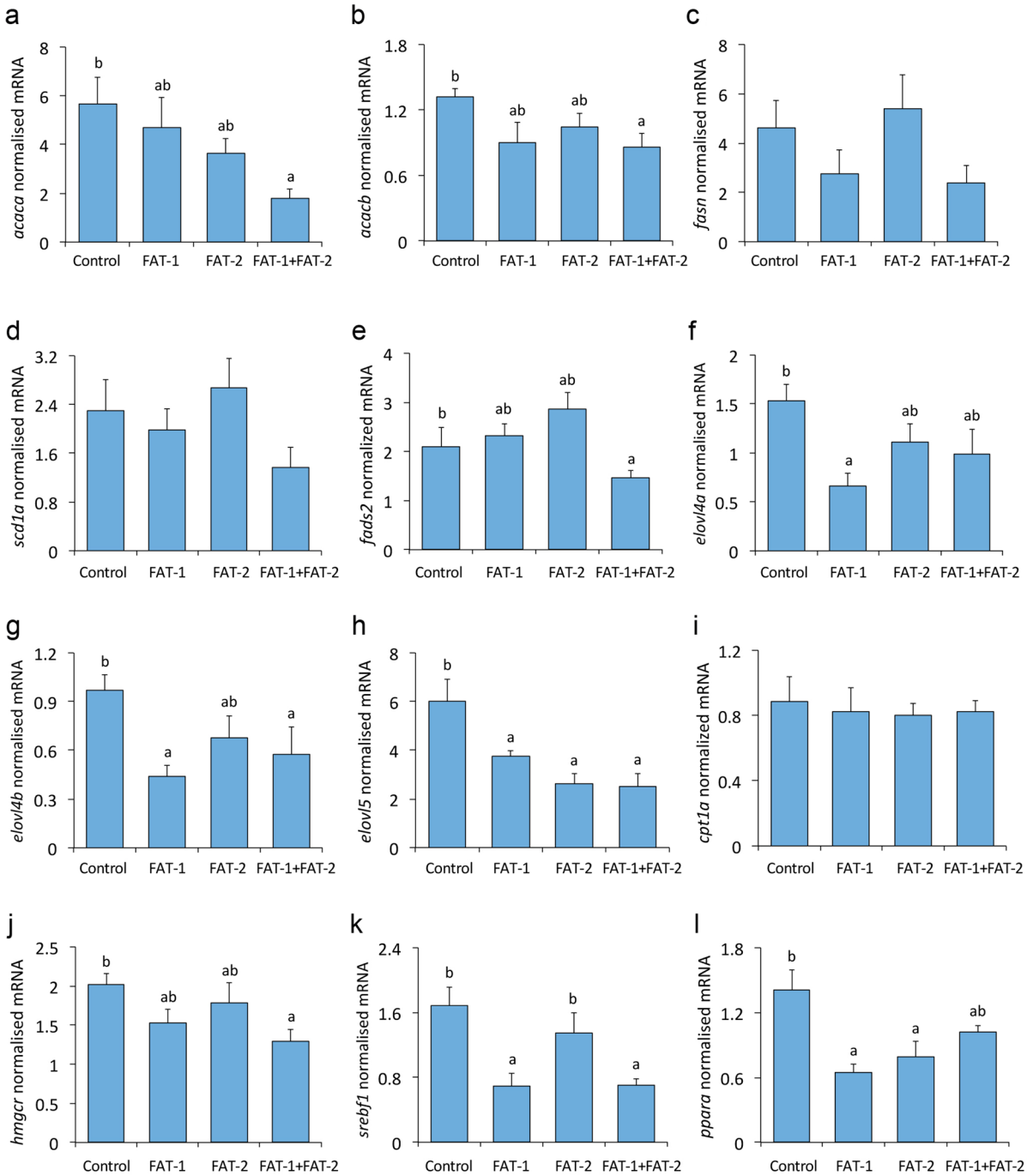


Fig. 3 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the expression of key genes in de novo lipogenesis and fatty acid oxidation in the liver of *S. aurata*. (a-l) Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the liver were collected. Data are means ± SEM ($n = 6$). Expression data were normalised by the geometric mean of *S. aurata 18 s*, *actb* and *eef1a* mRNA levels. Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

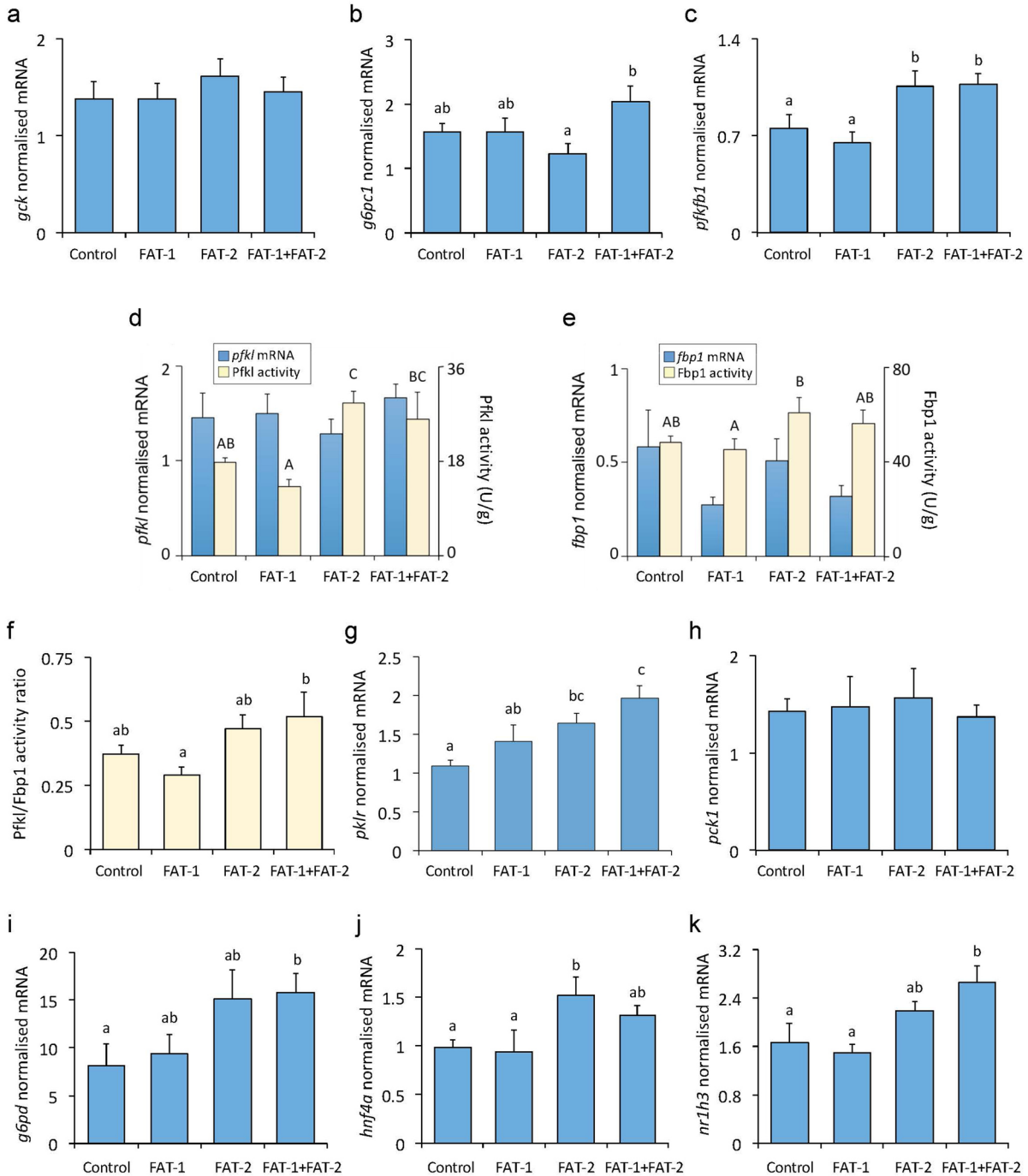


Fig. 4 Effect of long-term treatment with chitosan-TPP nanoparticles complexed with pSG5 (control), pSG5-FAT-1, pSG5-FAT-2 and pSG5-FAT-1 + pSG5-FAT-2 on the expression of key genes in glycolysis-gluconeogenesis and the pentose phosphate pathway, *hnf4a* and *nr1h3* in the liver of *S. aurata*. (**a**–**k**) Fourteen days after the last injection and 24 h following the last meal, fish were sacrificed and the liver were collected. Hepatic mRNA levels and enzyme activity of Pfkl and Fbp1 are presented as mean ± SEM ($n = 6$). Expression data were normalised by the geometric mean of *S. aurata 18 s*, *actb* and *eef1a* mRNA levels. Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$)

capillary endothelium in the muscle and brain may limit the transfer of nanoparticles and result in the scarce levels of transcript (Kooij et al. 2005). The feasibility of implementing chitosan-TPP-DNA administration in aquaculture relies on the fact that chitosan is recognised as safe by the U.S. Food and Drug Administration, on one hand, and that a procedure similar to that currently used to administer commercialised DNA vaccines for fish, could be applied to induce endogenous production of *n*-3 LC PUFA, on the other.

Transgenesis of *fat-1*, a gene that facilitates the conversion of *n*-6 to *n*-3 fatty acids, scarcely affected body growth in zebrafish, common carp, mice, pig and lamb (Bhattacharya et al. 2006; Ji et al. 2009; Liu et al. 2016; Zhang et al. 2018, 2019; Sun et al. 2020). However, in the present study long-term hepatic expression of *fat-1* and *fat-1*+*fat-2* significantly increased weight gain but not lipid content in *S. aurata*. A trend to increase SGR was observed in fish co-expressing *fat-1* and *fat*-2, while SGR values in control fish were in agreement with those reported for *Sparus aurata* under similar experimental conditions but non-treated with chitosan nanoparticles (Caballero-Solares et al. 2015; Sáez-Arteaga et al. 2022). Our findings suggest that increased levels of *n*-3 LC-PUFA and decreased *n*-6/*n*-3 fatty acid ratio resulting from expression of fish codon-optimised *fat-1* and *fat-2* may contribute to increased growth performance in *S. aurata*. In support of this hypothesis, substitution of fish oil (rich in *n*-3 LC-PUFA) by vegetable oil enhances the *n*-6/*n*-3 ratio and lowers *n*-3 LC-PUFA and weight gain in *S. aurata* (Houston et al. 2017), as well as in other marine fish such as cobia (Trushenski et al. 2012) and the anadromous Atlantic salmon (Qian et al. 2020). However, dietary fish oil does not significantly affect growth in other fish species such as zebrafish (Meguro and Hasumura 2018), common carp (Ljubojević et al. 2015), red hybrid tilapia (Al-Souti et al. 2012) and rainbow trout (Richard et al. 2006). Different adaptative responses to dietary *n*-3 LC-PUFA and the specific ability for converting *n*-3 and *n*-6 C_{18} PUFA into highly unsaturated long-chain fatty acids may result at least in part from functional diversification of Fads2 activity among teleosts, which in turn may have been influenced by a variety of factors such as phylogeny, trophic level, habitat (marine vs. freshwater) and trophic ecology (Castro et al. 2016; Garrido et al. 2019). Better growth performance of *S. aurata* submitted to sustained expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* may also result from improved health condition due to increased *n*-3 LC-PUFA and decreased *n*-6/*n*-3 ratio. Consistently, *fat-1* transgenesis prevents liver steatosis and lipid deposition in the abdominal cavity of zebrafish by a mechanism involving hepatic downregulation of lipogenic-related genes and upregulation of steatolysis-related genes (Sun et al. 2020). Moreover, *fat-1* transgenesis prevents glucose intolerance, insulin resistance, non-alcoholic fatty liver disease and allergic airway responses in mice (Bilal et al. 2011; Kim et al. 2012; Romanatto et al. 2014; Boyle et al. 2020), and exerts protective vascular effects on pigs and cattle by reducing inflammatory factors and improving the immune system (Liu et al. 2016, 2017). Accordingly, *S. aurata* treated with FAT-1 and FAT1 + FAT-2 showed decreased HSI levels, which therefore may essentially result from lower lipid deposition in the liver of fish expressing *fat-1*.

Body fatty acid composition is affected by multiple factors, including de novo fatty acid synthesis, physiological requirements and dietary fatty acid profile. Single-gene expression of either *fat-1* or *fat-2* enhanced fatty acid desaturation and, consequently, *n*-3 LC-PUFA synthesis in transgenic mice (Pai et al. 2014), pig (Tang et al. 2019), zebrafish (Pang et al. 2014), and common carp (Zhang et al. 2019). Similarly, *S. aurata* long-term treated with chitosan-TPP-DNA nanoparticles expressing either *fat-1* or *fat-2* showed a general trend to increase liver and muscle EPA, DHA, and total *n*-3 fatty acids and PUFA, while decreased the *n*-6/*n*-3 ratio and saturated fatty acids, conceivably by conversion into unsaturated fatty acids. Most of these effects were potentiated by hepatic co-expression of *fat-1* and *fat-2*. Combined activities of FAT-1 and FAT-2 decreased saturated fatty acids such as 14:0, 16:0 and 17:0, while increased unsaturated fatty acids, particularly *n*-3, and to a lesser extend *n*-9 and *n*-6. Thus, co-expression of *fat-1* and *fat-2* promoted a synergistic effect that favoured liver production of *n*-3 LC-PUFA and its accumulation in the muscle, particularly EPA and DHA. Given that *fat-1* and *fat-2* mRNA levels were scarcely detected in the skeletal muscle of *S. aurata*, changes in the muscle fatty acid profile between treatments may ascribe to hepatic fat exportation forming part of very low density lipoproteins (VLDL).

In contrast to most animals where *fat-1* transgenesis generally results in a significant decrease of the *n*-6 fatty acid series (Kang et al. 2004; Lai et al. 2006; Liu et al. 2016, 2017; Li et al. 2018; You et al. 2021), the effect of *fat-1* transgenesis in fishes seems species-dependent. Similarly as in *S. aurata*, transgenesis of *fat-1* did not affect total *n*-6 fatty acids in the muscle of channel catfish (Xing et al. 2023), while a slight decrease was observed in zebrafish muscle (Pang et al. 2014). In contrast, *fat-1* transgenesis largely decreased total *n*-6 PUFAs in common carp muscle (Zhang et al. 2019). The effect of *fat-2* transgenesis in the fatty acid profile also depended on the fish species. Thus, *fat-2* transgenesis in channel catfish decreased palmitic acid (16:0) and, as in *S. aurata*, led to a not significant trend to increase total *n*-6 and *n*-3 series in the muscle (Xing et al. 2023). However, *fat-2* increased total *n*-6 PUFAs while did not affect the *n*-3 series in zebrafish muscle (Pang et al. 2014). Divergent effects of *C. elegans fat-1* and *fat-2* among fish species may be attributed to the interaction between exogenous enzymes and the diversity of endogenous desaturases and elongases that are distributed within different teleost taxonomic groups, and which in turn are responsible for different specific abilities for converting *n*-3 and *n*-6 C_{18} PUFA into highly unsaturated LC-PUFAs in teleosts (Monroig et al. 2022).

Among biochemical parameters improved by *fat-1* transgenesis, reduced circulating levels for triglycerides and cholesterol were reported in mice (Romanatto et al. 2014), pigs (Liu et al. 2016), and cattle (Liu et al. 2017), while decreased hepatic triglycerides and cholesterol ester were found in zebrafish (Sun et al. 2020). Similarly, hepatic co-expression of *fat-1* and *fat-2* reduced serum triglycerides and cholesterol in *S. aurata*. In this regard, the present study showed that long-term co-expression of *fat-1* and *fat-2* promoted a general decrease of the expression of key enzymes for de novo lipogenesis in the liver of *S. aurata*. In addition, decreased serum triglycerides may also be attributed in part to the increase of *n*-3 LC-PUFA in the liver, where *n*-3 fatty acids are generally thought to reduce the production of VLDL and induce fatty acid β-oxidation (Shearer et al. 2012).

Treatment with FAT-1 + FAT-2 significantly downregulated *hmgcr*, which encodes the rate-limiting enzyme in cholesterol synthesis, and key genes in fatty acid synthesis, such as *acaca* and *acacb*, which catalyse conversion of acetyl-CoA into malonyl-CoA in the cytosol and mitochondrion, respectively, fatty acid elongases (*elovl4b* and *elovl5*) and *fads2* desaturase. Although no significant, the expression of *scd1a*, which catalyses the insertion of a *cis* double bond at the Δ9 position into saturated C_{16} and C_{18} fatty acyl-CoA (Wang et al. 2005), also showed a trend to decrease in fish treated with nanoparticles expressing *fat-1* + *fat-2*. Furthermore, the expression of the three fatty acid elongases herein analysed (*elovl4a*, *elovl4b* and *elovl5*) strongly decreased by sustained expression of *fat-1*. In transgenic animals, the effect of FAT-1 seems to depend on a variety of factors including the species, environmental conditions and dietary lipid content. Similarly as in *S. aurata*, a high-fat diet (13.4% of crude lipid *versus* 18.0% used in the present study) downregulated the hepatic expression of *acaca*, *fasn* and *scd1* in *fat-1* transgenic zebrafish. However, a low-fat diet (3.1% of crude lipid) caused the opposite effects, upregulating the expression of the three genes (Sun et al. 2020). In line with our findings, *fat-1* transgenesis in mice decreased the levels of phosphorylated ACACA and FASN (Romanatto et al. 2014). However, *fat-1* transgenic common carp showed upregulation of *fads2*, *elovl5* and *elovl2* in the liver, and transgenic pigs co-expressing *fat-1* and *fat-2* also presented increased expression levels of *elovl5* and *elovl2* in the muscle, skin and fat (Zhang et al. 2019; Tang et al. 2019).

Downregulation of *acacb* in *S. aurata* treated with FAT-1 + FAT-2 suggests a limited synthesis rate of mitochondrial malonyl-CoA. Any of the treatments affected the mRNA abundance of *cpt1a*, which is essential for the mitochondrial uptake of long-chain fatty acids and their subsequent β-oxidation in the mitochondrion. However, given that malonyl-CoA is a potent allosteric inhibitor of CPT1A (Saggerson 2008), our data suggest that in addition to decrease de novo lipogenesis, sustained co-expression of *fat-1* and *fat-2* may increase fatty acid oxidation in the liver of *S. aurata* fed medium- or high-fat diets. Similar results were reported for zebrafish fed a high-fat diet, where *fat-1* transgenesis stimulated lipolysis-related genes and mitochondrial energy metabolism-related genes while downregulated the hepatic expression of genes related with lipogenesis and lipid deposition (Sun et al. 2020). Accordingly, upregulation of hepatic fatty acid oxidation-related genes by *fat-1* transgenesis was also reported in common carp

(Zhang et al. 2019), mice (Romanatto et al. 2014; Boyle et al. 2020) and goat cells (Fan et al. 2016), as well as in *fat-1* and *fat-2* double transgenic zebrafish (Pang et al. 2014).

In mammals, alternate promoters in the *srebf1* gene generate SREBP1a and SREBP1c, which constitute transcription factors with a major role in de novo lipogenesis activation. SREBP1c primarily transactivates genes required for fatty acid and triglyceride synthesis while SREBP1a is a potent activator of all SREBP-responsive genes, including genes associated with cholesterol synthesis. Consistent with the role of *srebf1* in the transcription of lipogenic genes both in fish and mammals (Carmona-Antoñanzas et al. 2014; Silva-Marrero et al. 2019), downregulation of *srebf1* in the liver of *S. aurata* submitted to long-term expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* led to a trend to decrease the expression of genes involved in cholesterol synthesis (*hmgcr*) and fatty acid synthesis (*acaca*, *acacb* and *fasn*), desaturation (*scd1a* and *fads2*) and elongation (*elovl4a*, *elovl4b* and *elovl5*). In agreement with our findings, transgenic zebrafish expressing *fat-1* (when feeding a high-fat diet), *fat-2* and *fat-1*+*fat-2* and double transgenic pigs for *fat-1* and *fat-2* also showed downregulated expression levels of *srebf1* (Pang et al. 2014; Tang et al. 2019; Sun et al. 2020). Since DHA suppresses *srebf1* expression and enhances its protein degradation (Jump 2008), increased levels of DHA seem the main responsible for decreased expression of *srebf1* and de novo lipogenic genes in the liver of *S. aurata* expressing *fat-1* and *fat-1*+*fat-2*. Consistently, substitution of fish oil, rich in DHA, by vegetable oil leads to upregulation of *srebf1* and fatty acid synthesis-related genes in *S. aurata* (Ofori-Mensah et al. 2020).

In the present study, FAT-1 and FAT-2 downregulated *ppara* in the liver of *S. aurata*. PPARA is a nuclear receptor activated by a wide range of ligands including fatty acids and fatty acid metabolites, such as eicosanoids. In the mammalian liver, PPARA controls the expression of genes involved in fatty acid uptake, intracellular transport, acyl-CoA formation and fatty acid mitochondrial and peroxisomal oxidation, ketogenesis and lipoprotein metabolism (Bougarne et al. 2018). Supplementation of fish oil to rodents enhances *ppara* expression in the liver (Hein et al. 2010; Kamisako et al. 2012), possibly as a result of increased availability of *n*-3 LC-PUFA, particularly EPA. However, the effect of dietary fish oil on *ppara* expression in fish depends on the species. Similarly as in *S. aurata* expressing *fat-1* and *fat-2*, fish oil was shown to decrease the hepatic mRNA levels of *ppara* in *S. aurata* and juvenile turbot (Peng et al. 2014; Ofori-Mensah et al. 2020), while the opposite effect was reported in large yellow croaker and lean, but not fat, Atlantic salmon (Morais et al. 2011; Du et al. 2017). As pointed out by Peng et al. (2014), fatty acid-derived factors other than EPA-mediated activation may contribute to species-specific regulation of *ppara* expression in fishes.

In spite of limited knowledge of the effect of *C. elegans* FAT-1 and FAT-2 on glucose metabolism, *fat*-1 transgenesis was reported to improve glucose homeostasis by lowering hepatic gluconeogenesis and decreasing blood glucose and insulin in mice (Romanatto et al. 2014). Similarly, reduced plasma glucose was found in *fat*-1 transgenic cattle (Liu et al. 2017). In the present study, we found that long-term expression of fish codon-optimised *C. elegans* FAT-2 and FAT-1+FAT-2 stimulated glycolysis and the expression levels of *hnf4a* and *nr1h3* in the liver of *S. aurata*. By controlling the flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle, *pfkl* and *fbp1* exert critical roles in hepatic glycolysis-gluconeogenesis. Although the mRNA levels of *pfkl* and *fbp1* were not significantly affected by any of the treatments, expression of *fat-2* and *fat-1*+*fat-2* promoted higher levels of Pfkl/Fbp1 activity ratio, possibly as a result of *pfkfb1* upregulation. The bifunctional enzyme *pfkfb1* catalyses the synthesis and degradation of fructose-2,6-bisphosphate, which is a major regulator of glycolysis–gluconeogenesis through allosteric activation of Pfkl and inhibition of Fbp1 (Okar et al. 2004). We previously showed that refeeding and high carbohydrate diets upregulate *pfkfb1* and the kinase activity of the bifunctional enzyme in the liver of *S. aurata*, leading to a concomitant increase in fructose-2,6-bisphosphate levels (Metón et al. 1999a, 2000). As in mammals, fructose-2,6-bisphosphate is an allosteric activator of *S. aurata* PFKL (Mediavilla et al. 2008). Therefore, our results suggest that *pfkfb1* upregulation in the liver of fish expressing *fat-2* and *fat-1*+*fat-2* may be a key step favouring the glycolytic flux through the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle, which in turn will increase the hepatic content

of fructose-1,6-bisphosphate, an allosteric activator of Pklr.

The nuclear receptor HNF4A is a master regulator of liver metabolism through transcriptional regulation of target genes involved in glucose metabolism, lipid metabolism and hepatocyte differentiation (Meng et al. 2016). In mammals, HNF4A transactivates both glycolytic and gluconeogenic genes. Thus, HNF4A-binding to the gene promoter is required for insulin-stimulated upregulation of *gck* and *pklr* in the fed state, while a synergistic action of HNF4A and FOXO1 enhances the transcription of *g6pc1* and *pck1* during fasting (Hirota et al. 2008; Ganjam et al. 2009). Furthermore, HNF4A was previously shown to induce the expression of *nr1h3* (Theofilatos et al. 2016), which encodes LXR-alpha, a nuclear receptor stimulated by insulin that is also involved in glucose and lipid metabolism (Zhao et al. 2012). Indeed, LXR-alpha was shown to upregulate *pklr* mRNA levels in mice (Cha and Repa 2007), and behave as a key regulator of *pfkfb* expression in humans by binding and transactivating the gene promoter of the bifunctional enzyme (Zhao et al. 2012). Therefore, increased *hnf4a* and *nr1h3* mRNA abundance in *S. aurata* expressing *fat-2* and *fat-1*+*fat-2* may enhance hepatic upregulation of *pfkfb1* and *pklr* expression, and thus increase the glycolytic flux in the liver. Consistent with HNF4A-dependent enhancement of glycolysis in *S. aurata*, *hnf4a* expression was previously shown to increase in *S. aurata* under glycolytic conditions *versus* gluconeogenic conditions such as fasting and treatment with streptozotocin (Salgado et al. 2012). Increased levels of *n*-3 LC-PUFA may be a key factor leading to *hnf4a* and *nr1h3* upregulation in the *S. aurata* liver. In agreement, *fat-1* transgenic mice presented increased hepatic mRNA levels of *hnf4a* and to a lesser extend *nr1h3* (Kim et al. 2012). Similarly, dietary supplementation with dried marine algae, rich in *n*-3 LC-PUFA (particularly DHA), induced *hnf4a* expression in the pig liver (Meadus et al. 2011). Furthermore, fish oil upregulated *nr1h3* in *S. aurata* adipocytes (Cruz-Garcia et al. 2011), and in the liver of juvenile turbot, Nile tilapia and mice (Kamisako et al. 2012; Peng et al. 2014; Ayisi et al. 2018).

To our knowledge, the effect of *fat-1* and *fat-2* transgenesis on the pentose phosphate pathway was not previously addressed. In the present study, long-term expression of fish codon-optimised *fat-2* and *fat-1*+*fat-2* promoted higher expression levels of *g6pd*, which encodes the rate-limiting enzyme for the production of NADPH in the oxidative phase of the pentose phosphate pathway. Previous reports indicated that dietary carbohydrates are a key factor that enhances G6pd activity in the liver of *S. aurata* (Metón et al. 1999b). Nevertheless, our findings support that fatty acid composition, particularly the *n*-3/*n*-6 ratio, also seems to regulate the hepatic expression of *g6pd*. In agreement with *g6pd* upregulation by *n*-3 LC-PUFA, fish oil stimulated G6PD activity in the rat liver (Yilmaz et al. 2004), and dietary supplementation with *n*-3 PUFA increased *g6pd* mRNA levels in the pig muscle (Vitali et al. 2018). Furthermore, *n*-6 PUFA, particularly LA, decreased *g6pd* mRNA levels in rat hepatocytes (Kohan et al. 2011). Species-specific regulation of *g6pd* expression by fatty acid composition may occur in other fishes. In this regard, total replacement of fish oil by vegetable oil did not affect G6pd activity but increased the mRNA levels in the liver of Nile tilapia (Ayisi et al. 2018), while enhanced G6pd activity in the liver of Atlantic salmon (Menoyo et al. 2005). Bearing in mind a general trend to downregulate de novo hepatic lipogenesis in *S. aurata* co-expressing *fat-1* and *fat-2*, NADPH resulting from *g6pd* upregulation by *n*-3 LC-PUFA may reinforce cellular protection from oxidative stress.

Considered together, the metabolic effects of periodical administration of chitosan-TPP-DNA nanoparticles expressing fish codon-optimised *C. elegans fat-1* and *fat-2* included hepatic downregulation of de novo lipogenesis-related genes, leading to reduced circulating levels of triglycerides and cholesterol, stimulation of fatty acid oxidation and upregulation of glucose oxidation via glycolysis and pentose phosphate pathway without affecting glycemia. Although co-expression of *C. elegans fat-1* and *fat-2* in *S. aurata* reduced blood triglycerides, the action of *fat-1* and *fat-2* may allow protein sparing in the liver of a carnivorous teleost, such as *S. aurata*, by a mechanism involving increased glucose and fatty acid oxidation to obtain energy in fish fed medium- or high-fat diets.

Conclusion

The present study shows that long-term treatment with chitosan-TPP nanoparticles complexed with plasmids expressing fish codon-optimised *C. elegans fat-1* and *fat-2* allowed efficient expression of exogenous FAT-1 and FAT-2 desaturases in the liver of *S. aurata*, which in turn elevated the *n*-3 LC-PUFA content, particularly EPA and DHA, and decreased the *n*-6/*n*-3 ratio both in the liver and the skeletal muscle. Co-expression of fish codon-optimised *fat-1* and *fat-2* promoted the highest weight gain, *n*-3 LC-PUFA accumulation in the muscle, and had metabolic effects that included downregulation of lipid biosynthesis and stimulation of fatty acid and glucose oxidation in the liver. Expression of fish codon-optimised *fat-1* and *fat-1*+*fat-2* downregulated the hepatic expression of *srebf1* and as a consequence, the mRNA levels of key genes in de novo lipogenesis, while *fat-2* and *fat-1*+*fat-2* upregulated *hnf4a*, *nr1h3* and glucose oxidation through glycolysis and the pentose phosphate pathway. Our findings support that chitosan-TPP-DNA nanoparticles co-expressing fish codon-optimised *fat-1* and *fat-2* can alleviate the effect of fish oil replacement with vegetable oil currently occurring in aquafeeds and enable production of functional fish rich in EPA and DHA for human consumption. Future studies will help to determine dosage and the optimal developmental stage to administer *fat-1* and *fat-2* chitosan-TPP-DNA nanoparticles in cultured fish.

Availability of data

Data are available from the corresponding author upon reasonable request.

Acknowledgements The authors thank Piscicultura Marina Mediterranea (AVRAMAR Group, Burriana, Spain) for providing *S. aurata* juveniles and Eurocoyal S.L. (Sant Cugat del Vallès, Spain) for supplying dietary components.

Author contribution Conceptualization: IM; methodology: AF, MPA, IM; formal analysis and investigation: YW, AR, MPA, IM; writing—original draft preparation: YW, IM; writing—review and editing: YW, AR, AF, MPA, IM; funding acquisition: IM; supervision: IM.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This work was supported by grant AGL2016-78124-R funded by MICIU/AEI/10.13309/501100011033 and by ERDF A way of making Europe, and grant PID2021-125642OB-I00 funded by MICIU/AEI/10.13039/501100011033 and by ERDF, EU. YW and AR are the recipients of China Scholarship Council (P.R. China) and PREDOCS-UB (Universitat de Barcelona, Spain) predoctoral fellowships, respectively.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Experimental procedures involving fish were performed in accordance with the guidelines of the University of Barcelona's Animal Welfare Committee (proceeding #10811, Generalitat de Catalunya), in compliance with local legislation and EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Alimuddin YG, Kiron V et al (2007) Expression of masu salmon Δ5-desaturase-like gene elevated EPA and DHA biosynthesis in zebrafish. Mar Biotechnol 9:92–100. https://doi.org/10.1007/s10126-006-6003-y

Alimuddin KV, Satoh S et al (2008) Cloning and over-expression of a masu salmon (*Oncorhynchus masou*) fatty acid elongase-like gene in zebrafish. Aquaculture 282:13–18. https://doi.org/10.1016/j.aquaculture.2008.06.033

Al-Souti A, Al-Sabahi J, Soussi B, Goddard S (2012) The effects of fish oil-enriched diets on growth, feed conversion and fatty acid content of red hybrid tilapia, *Oreochromis* sp. Food Chem 133:723–727. https://doi.org/10.1016/j.foodchem.2012.01.080

Ayisi CL, Zhao JL, Hua XM, Apraku A (2018) Replacing fish oil with palm oil: effects on mRNA expression of fatty acid transport genes and signalling factors related to lipid metabolism in Nile tilapia (*Oreochromis niloticus*). Aquac Nutr 24:1822–1833. https://doi.org/10.1111/anu.12821

Betancor MB, Sprague M, Montero D et al (2016) Replacement of marine fish oil with de novo omega-3 oils from

transgenic *Camelina sativa* in feeds for gilthead sea bream (*Sparus aurata* L.). Lipids 51:1171–1191. https://doi.org/10.1007/s11745-016-4191-4

Bhattacharya A, Chandrasekar B, Rahman MM et al (2006) Inhibition of inflammatory response in transgenic fat-1 mice on a calorie-restricted diet. Biochem Biophys Res Commun 349:925–930. https://doi.org/10.1016/j.bbrc.2006.08.093

Bilal S, Haworth O, Wu L et al (2011) Fat-1 transgenic mice with elevated omega-3 fatty acids are protected from allergic airway responses. Biochim Biophys Acta Mol Basis Dis 1812:1164–1169. https://doi.org/10.1016/j.bbadis.2011.05.002

Bougarne N, Weyers B, Desmet SJ et al (2018) Molecular actions of PPARα in lipid metabolism and inflammation. Endocr Rev 39:760–802. https://doi.org/10.1210/er.2018-00064

Boyle KE, Magill-Collins MJ, Newsom SA et al (2020) Maternal fat-1 transgene protects offspring from excess weight gain, oxidative stress, and reduced fatty acid oxidation in response to high-fat diet. Nutrients 12:767. https://doi.org/10.3390/nu12030767

Busacker GP, Adelman IR, Goolish EM (1990) Growth. In: Schreck CB, Moyle PB (eds) Methods for fish biology. American Fisheries Society, Bethesda, pp 363–387

Caballero-Solares A, Viegas I, Salgado MC et al (2015) Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 444:79–87. https://doi.org/10.1016/j.aquaculture.2015.03.025

Calder PC (2018) Very long-chain n-3 fatty acids and human health: fact, fiction and the future. Proc Nutr Soc 77:52–72. https://doi.org/10.1017/S0029665117003950

Carmona-Antoñanzas G, Tocher DR, Martinez-Rubio L, Leaver MJ (2014) Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. Gene 534:1–9. https://doi.org/10.1016/j.gene.2013.10.040

Carvalho M, Marotta B, Xu H et al (2022) Complete replacement of fish oil by three microalgal products rich in n-3 long-chain polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (*Sparus aurata*). Aquaculture 558:738354. https://doi.org/10.1016/j.aquaculture.2022.738354

Castro LFC, Tocher DR, Monroig O (2016) Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of Fads and Elovl gene repertoire. Prog Lipid Res 62:25–40. https://doi.org/10.1016/j.plipres.2016.01.001

Cha JY, Repa JJ (2007) The liver X receptor (LXR) and hepatic lipogenesis. the carbohydrate-response element-binding protein is a target gene of LXR. J Biol Chem 282:743–751. https://doi.org/10.1074/jbc.m605023200

Chen Y, Mei M, Zhang P et al (2013) The generation of transgenic mice with fat1 and fad2 genes that have their own polyunsaturated fatty acid biosynthetic pathway. Cell Physiol Biochem 32:523–532. https://doi.org/10.1159/000354456

Cheng C-L, Huang S-J, Wu C-L et al (2015) Transgenic expression of omega-3 PUFA synthesis genes improves zebrafish survival during *Vibrio vulnificus* infection. J Biomed Sci 22:103. https://doi.org/10.1186/s12929-015-0208-1

Cruz-Garcia L, Sánchez-Gurmaches J, Bouraoui L et al (2011) Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.). Comp Biochem Physiol Part A Mol Integr Physiol 158:391–399. https://doi.org/10.1016/j.cbpa.2010.11.024

Djuricic I, Calder PC (2021) Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: an update for 2021. Nutrients 13:2421. https://doi.org/10.3390/nu13072421

Du J, Xu H, Li S et al (2017) Effects of dietary chenodeoxycholic acid on growth performance, body composition and related gene expression in large yellow croaker (*Larimichthys crocea*) fed diets with high replacement of fish oil with soybean oil. Aquaculture 479:584–590. https://doi.org/10.1016/j.aquaculture.2017.06.023

Fan Y, Ren C, Wang Z et al (2016) Transgenesis of humanized fat1 promotes n−3 polyunsaturated fatty acid synthesis and expression of genes involved in lipid metabolism in goat cells. Gene 576:249–255. https://doi.org/10.1016/j.gene.2015.10.013

Ganjam GK, Dimova EY, Unterman TG, Kietzmann T (2009) FoxO1 and HNF-4 are involved in regulation of hepatic glucokinase gene expression by resveratrol. J Biol Chem 284:30783–30797. https://doi.org/10.1074/jbc.m109.045260

Garrido D, Kabeya N, Betancor MB et al (2019) (2019) Functional diversification of teleost Fads2 fatty acyl desaturases occurs independently of the trophic level. Sci Rep 91(9):1–10. https://doi.org/10.1038/s41598-019-47709-0

Gaspar C, Silva-Marrero JI, Fàbregas A et al (2018) Administration of chitosan-tripolyphosphate-DNA nanoparticles to knockdown glutamate dehydrogenase expression impairs transdeamination and gluconeogenesis in the liver. J Biotechnol 286:5–13. https://doi.org/10.1016/j.jbiotec.2018.09.002

González JD, Silva-Marrero JI, Metón I et al (2016) Chitosan-mediated shRNA knockdown of cytosolic alanine aminotransferase improves hepatic carbohydrate metabolism. Mar Biotechnol 18:85–97. https://doi.org/10.1007/s10126-015-9670-8

Hagens WI, Oomen AG, de Jong WH et al (2007) What do we (need to) know about the kinetic properties of nanoparticles in the body? Regul Toxicol Pharmacol 49:217–229. https://doi.org/10.1016/j.yrtph.2007.07.006

Hein GJ, Bernasconi AM, Montanaro MA et al (2010) Nuclear receptors and hepatic lipidogenic enzyme response to a dyslipidemic sucrose-rich diet and its reversal by fish oil n-3 polyunsaturated fatty acids. Am J Physiol Endocrinol Metab 298:E429–E439. https://doi.org/10.1152/ajpendo.00513.2009

Hirota K, Sakamaki JI, Ishida J et al (2008) A combination of HNF-4 and Foxo1 is required for reciprocal transcriptional regulation of glucokinase and glucose-6-phosphatase genes in response to fasting and feeding. J Biol Chem 283:32432–32441. https://doi.org/10.1074/jbc.m806179200

Houston SJS, Karalazos V, Tinsley J et al (2017) The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. Br J Nutr 118:1010–1022. https://doi.org/10.1017/s0007114517002975

Ji S, Hardy RW, Wood PA (2009) Transgenic expression of n-3 fatty acid desaturase (fat-1) in C57/BL6 mice: effects on glucose homeostasis and body weight. J Cell Biochem 107:809–817. https://doi.org/10.1002/jcb.22179

Jump DB (2008) N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. Curr Opin Lipidol 19:242–247. https://doi.org/10.1097/mol.0b013e3282ffaf6a

Kabeya N, Fonseca MM, Ferrier DEK et al (2018) Genes for de novo biosynthesis of omega-3 polyunsaturated fatty acids are widespread in animals. Sci Adv. https://doi.org/10.1126/sciadv.aar6849

Kamisako T, Tanaka Y, Ikeda T et al (2012) Dietary fish oil regulates gene expression of cholesterol and bile acid transporters in mice. Hepatol Res 42:321–326. https://doi.org/10.1111/j.1872-034x.2011.00924.x

Kang JX, Wang J, Wu L, Kang ZB (2004) Fat-1 mice convert n-6 to n-3 fatty acids. Nature 427:504–504. https://doi.org/10.1038/427504a

Kim EH, Bae JS, Hahm KB, Cha JY (2012) Endogenously synthesized n-3 polyunsaturated fatty acids in fat-1 mice ameliorate high-fat diet-induced non-alcoholic fatty liver disease. Biochem Pharmacol 84:1359–1365. https://doi.org/10.1016/j.bcp.2012.08.029

Kohan AB, Qing Y, Cyphert HA et al (2011) Chylomicron remnants and nonesterified fatty acids differ in their ability to inhibit genes involved in lipogenesis in rats. J Nutr 141:171–176. https://doi.org/10.3945/jn.110.129106

Kooij G, van Horssen J, de Vries E (2005) Tight junctions of the blood-brain barrier. In: de Vries E, Prat A (eds) The blood-brain barrier and its microenvironment: basic physiology to neurological disease. CRC Press, Boca Raton, pp 69–92

Lai L, Kang JX, Li R et al (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436. https://doi.org/10.1038/nbt1198

Li M, Ouyang H, Yuan H et al (2018) Site-specific fat-1 knock-in enables significant decrease of n-6PUFAs/n-3PUFAs ratio in pigs. G3 8:1747–1754. https://doi.org/10.1534/g3.118.200114

Liu X, Pang D, Yuan T et al (2016) N-3 polyunsaturated fatty acids attenuates triglyceride and inflammatory factors level in hfat-1 transgenic pigs. Lipids Health Dis 15:89. https://doi.org/10.1186/s12944-016-0259-7

Liu X-F, Wei Z-Y, Bai C-L et al (2017) Insights into the function of n-3 PUFAs in fat-1 transgenic cattle. J Lipid Res 58:1524–1535. https://doi.org/10.1194/jlr.M072983

Ljubojević D, Radosavljević V, Puvača N et al (2015) Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.). J Food Compos Anal 37:44–50. https://doi.org/10.1016/j.jfca.2014.09.005

Lucas A (1996) Bioenergetics of organisms: methods. In: Priede IG (ed) Bioenergetics of aquatic animals. Taylor & Francis, London, pp 65–81

Luo R, Zheng Z, Yang C et al (2020) Comparative transcriptome analysis provides insights into the polyunsaturated fatty acid synthesis regulation of fat-1 transgenic sheep. Int J Mol Sci 21:1121. https://doi.org/10.3390/ijms21031121

Meadus WJ, Duff P, Rolland D et al (2011) Feeding docosahexaenoic acid to pigs reduces blood triglycerides and induces gene expression for fat oxidation. Can J Anim Sci 91:601–612. https://doi.org/10.4141/cjas2011-055

Mediavilla D, Metón I, Baanante IV (2008) Purification and kinetic characterization of 6-phosphofructo-1-kinase from the liver of gilthead sea bream (*Sparus aurata*). J Biochem 144:235–244. https://doi.org/10.1093/jb/mvn066

Meguro S, Hasumura T (2018) Fish oil suppresses body fat accumulation in zebrafish. Zebrafish 15:27–32. https://doi.org/10.1089/zeb.2017.1475

Meng J, Feng M, Dong W et al (2016) Identification of HNF-4α as a key transcription factor to promote ChREBP expression in response to glucose. Sci Rep 6:23944. https://doi.org/10.1038/srep23944

Menoyo D, López-Bote CJ, Obach A, Bautista JM (2005) Effect of dietary fish oil substitution with linseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. J Anim Sci 83:2853–2862. https://doi.org/10.2527/2005.83122853x

Metón I, Caseras A, Mediavilla D et al (1999a) Molecular cloning of a cDNA encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from liver of *Sparus aurata*: nutritional regulation of enzyme expression. Biochim Biophys Acta 1444:153–165. https://doi.org/10.1016/S0167-4781(98)00270-X

Metón I, Mediavilla D, Caseras A et al (1999b) Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). Br J Nutr 82:223–232. https://doi.org/10.1017/s0007114599001403

Metón I, Caseras A, Fernández F, Baanante IV (2000) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, *Sparus aurata*. Biochim Biophys Acta Gene Struct Expr 1491:220–228. https://doi.org/10.1016/s0167-4781(00)00040-3

Monroig S-C, Kabeya N et al (2022) Desaturases and elongases involved in long-chain polyunsaturated fatty acid biosynthesis in aquatic animals: from genes to functions. Prog Lipid Res 86:101157. https://doi.org/10.1016/j.plipres.2022.101157

Morais S, Pratoomyot J, Taggart JB et al (2011) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. BMC Genomics 12:1–17. https://doi.org/10.1186/1471-2164-12-255

Ofori-Mensah S, Yıldız M, Arslan M, Eldem V (2020) Fish oil replacement with different vegetable oils in gilthead seabream, *Sparus aurata* diets: effects on fatty acid metabolism based on whole-body fatty acid balance method and genes expression. Aquaculture 529:735609. https://doi.org/10.1016/j.aquaculture.2020.735609

Okar DA, Wu C, Lange AJ (2004) Regulation of the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Adv Enzyme Regul 44:123–154. https://doi.org/10.1016/j.advenzreg.2003.11.006

Osmond ATY, Colombo SM (2019) The future of genetic engineering to provide essential dietary nutrients and improve growth performance in aquaculture: advantages and challenges. J World Aquac Soc 50:490–509. https://doi.org/10.1111/jwas.12595

Pai VJ, Wang B, Li X et al (2014) Transgenic mice convert carbohydrates to essential fatty acids. PLoS ONE 9:e97637. https://doi.org/10.1371/journal.pone.0097637

Pang S-C, Wang H-P, Li K-Y et al (2014) Double transgenesis of humanized fat1 and fat2 genes promotes omega-3 polyunsaturated fatty acids synthesis in a zebrafish model. Mar Biotechnol 16:580–593. https://doi.org/10.1007/s10126-014-9577-9

Peng M, Xu W, Mai K et al (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. Aquaculture 433:442–449. https://doi.org/10.1016/j.aquaculture.2014.07.005

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45

Qian C, Hart B, Colombo SM (2020) Re-evaluating the dietary requirement of EPA and DHA for Atlantic salmon in freshwater. Aquaculture 518:734870. https://doi.org/10.1016/j.aquaculture.2019.734870

Richard N, Kaushik S, Larroquet L et al (2006) Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). Br J Nutr 96:299–309. https://doi.org/10.1079/bjn20061821

Romanatto T, Fiamoncini J, Wang B et al (2014) Elevated tissue omega-3 fatty acid status prevents age-related glucose intolerance in fat-1 transgenic mice. Biochim Biophys Acta 1842:186–191. https://doi.org/10.1016/j.bbadis.2013.10.017

Sáez-Arteaga A, Wu Y, Silva-Marrero JI et al (2022) Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (*Sparus aurata*). Aquaculture 555:738221. https://doi.org/10.1016/j.aquaculture.2022.738221

Saggerson D (2008) Malonyl-CoA, a key signaling molecule in mammalian cells. Annu Rev Nutr 28:253–272. https://doi.org/10.1146/annurev.nutr.28.061807.155434

Sales R, Galafat A, Vizcaíno AJ et al (2021) Effects of dietary use of two lipid extracts from the microalga *Nannochloropsis gaditana* (Lubián, 1982) alone and in combination on growth and muscle composition in juvenile gilthead seabream, *Sparus aurata*. Algal Res 53:102162. https://doi.org/10.1016/j.algal.2020.102162

Salgado MC, Metón I, Anemaet IG et al (2012) Hepatocyte nuclear factor 4alpha transactivates the mitochondrial alanine aminotransferase gene in the kidney of *Sparus aurata*. Mar Biotechnol 14:46–62. https://doi.org/10.1007/s10126-011-9386-3

Shearer GC, Savinova OV, Harris WS (2012) Fish oil—How does it reduce plasma triglycerides? Biochim Biophys Acta 1821:843–851. https://doi.org/10.1016/j.bbalip.2011.10.011

Silva-Marrero JI, Sáez A, Caballero-Solares A et al (2017) A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). BMC Genomics 18:768. https://doi.org/10.1186/s12864-017-4148-x

Silva-Marrero JI, Villasante J, Rashidpour A et al (2019) The administration of chitosan-tripolyphosphate-DNA nanoparticles to express exogenous SREBP1a enhances conversion of dietary carbohydrates into lipids in the liver of *Sparus aurata*. Biomolecules 9:297. https://doi.org/10.3390/biom9080297

Sun S, Castro F, Monroig Ó et al (2020) fat-1 transgenic zebrafish are protected from abnormal lipid deposition induced by high-vegetable oil feeding. Appl Microbiol Biotechnol 104:7355–7365. https://doi.org/10.1007/s00253-020-10774-x

Tang F, Yang X, Liu D et al (2019) Co-expression of fat1 and fat2 in transgenic pigs promotes synthesis of polyunsaturated fatty acids. Transgenic Res 28:369–379. https://doi.org/10.1007/s11248-019-00127-4

Theofilatos D, Anestis A, Hashimoto K, Kardassis D (2016) Transcriptional regulation of the human liver X receptor α gene by hepatocyte nuclear factor 4α. Biochem Biophys Res Commun 469:573–579. https://doi.org/10.1016/j.bbrc.2015.12.031

Tocher DR, Betancor MB, Sprague M et al (2019) Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: bridging the gap between supply and demand. Nutrients 11:89. https://doi.org/10.3390/nu11010089

Trushenski J, Schwarz M, Bergman A et al (2012) DHA is essential, EPA appears largely expendable, in meeting the n-3 long-chain polyunsaturated fatty acid requirements of juvenile cobia *Rachycentron canadum*. Aquaculture 326–329:81–89. https://doi.org/10.1016/j.aquaculture.2011.11.033

Vitali M, Dimauro C, Sirri R et al (2018) Effect of dietary polyunsaturated fatty acid and antioxidant supplementation on the transcriptional level of genes involved in lipid and energy metabolism in swine. PLoS ONE 13:e0204869. https://doi.org/10.1371/journal.pone.0204869

Wang J, Yu L, Schmidt RE et al (2005) Characterization of HSCD5, a novel human stearoyl-CoA desaturase unique to primates. Biochem Biophys Res Commun 332:735–742. https://doi.org/10.1016/j.bbrc.2005.05.013

Wu Y, Rashidpour A, Almajano MP, Metón I (2020) Chitosan-based drug delivery system: applications in fish biotechnology. Polymers (basel) 12:1177. https://doi.org/10.3390/polym12051177

Xing D, Shang M, Li S et al (2023) CRISPR/Cas9-mediated precision integration of fat-1 and fat-2 from *Caenorhabditis elegans* at long repeated sequence in channel catfish (*Ictalurus punctatus*) and the impact on n-3 fatty acid level. Aquaculture 567:739229. https://doi.org/10.1016/j.aquaculture.2023.739229

Xu H, Meng X, Wei Y et al (2022) Arachidonic acid matters. Rev Aquac 14:1912–1944. https://doi.org/10.1111/raq.12679

Yilmaz HR, Songur A, Özyurt B et al (2004) The effects of n-3 polyunsaturated fatty acids by gavage on some metabolic enzymes of rat liver. Prostaglandins Leukot Essent Fat Acids 71:131–135. https://doi.org/10.1016/j.plefa.2004.03.002

You W, Li M, Qi Y et al (2021) CRISPR/Cas9-mediated specific integration of *Fat-1* and IGF-1 at the *pRosa26* locus. Genes 12:1027. https://doi.org/10.3390/genes12071027

Zhang J, Cui ML, Nie YW et al (2018) CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. FEBS J 285:2828–2839. https://doi.org/10.1111/febs.14520

Zhang X, Pang S, Liu C et al (2019) A novel dietary source of EPA and DHA: metabolic engineering of an important freshwater species-common carp by fat1-transgenesis. Mar Biotechnol 21:171–185. https://doi.org/10.1007/s10126-018-9868-7

Zhao LF, Iwasaki Y, Nishiyama M et al (2012) Liver X receptor α is involved in the transcriptional regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene. Diabetes 61:1062–1071. https://doi.org/10.2337/db11-1255

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.