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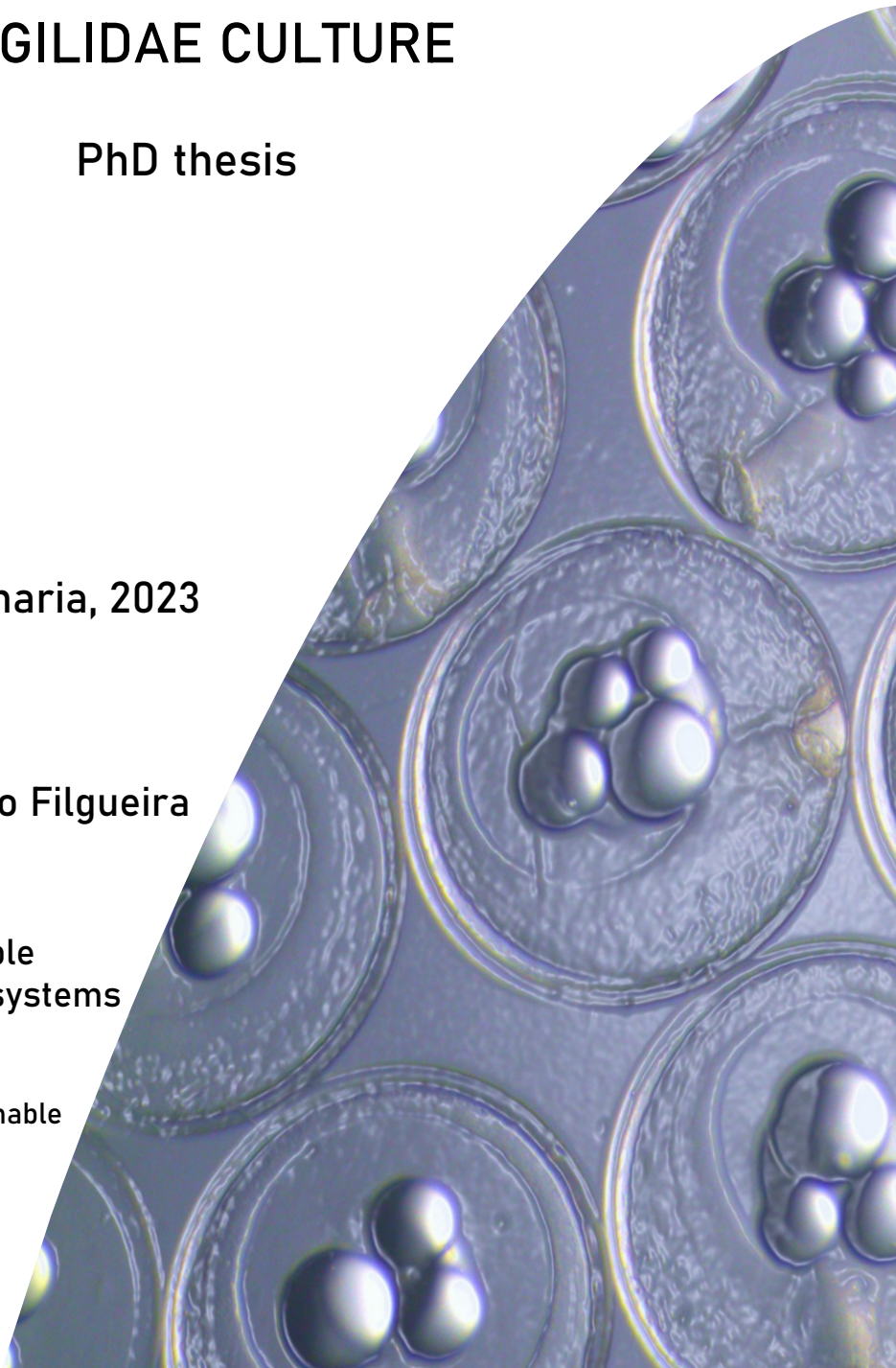
TOWARDS A SUSTAINABLE FISH PRODUCTION CONTRIBUTIONS TO THE OPTIMIZATION OF MUGILIDAE CULTURE

PhD thesis



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Las Palmas de Gran Canaria, 2023

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PhD programme in Sustainable Aquaculture and Marine Ecosystems

Institute of Aquaculture and Sustainable Marine Ecosystems (EcoAqua)

Aquaculture Research Group (GIA)

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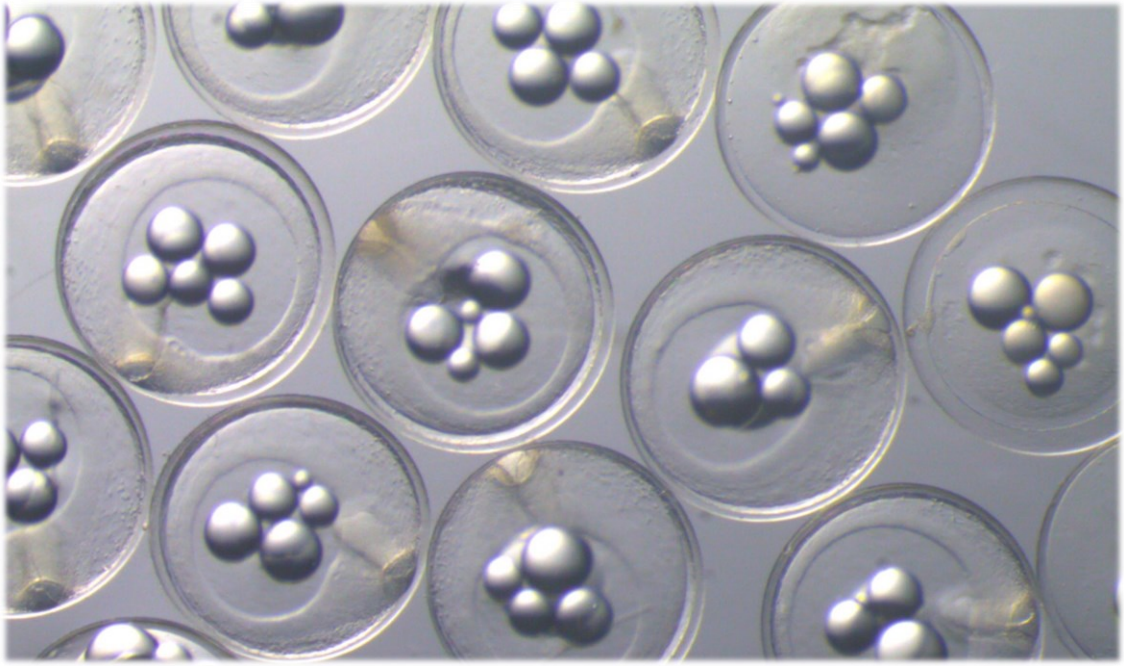

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PhD programme in Sustainable Aquaculture and Marine Ecosystems (ACUISEMAR)

Grupo de Investigación en Acuicultura (GIA), ECOAQUA Institute

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Las Palmas de Gran Canaria, June 2023

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INFORMA,

De que la Comisión Académica del Programa de Doctorado, en su sesión de fecha ..... tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Towards a sustainable fish production. Contributions to the optimization of Mugilidae culture" presentada por la doctoranda Dª Raquel Quirós Pozo y dirigida por la Doctora Lidia Esther Robaina Robaina y por el Doctor Francisco Javier Roo Filgueira.

Y para que así conste, y a efectos de lo previsto en el Art.º 11 del Reglamento de Estudios de Doctorado (BOULPGC 04/03/2019) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a.....de.....de dos mil veintitrés.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ESCUELA DE DOCTORADO

Programa de doctorado: **Acuicultura Sostenible y Ecosistemas Marinos**

Título de la Tesis: **Towards a sustainable fish production. Contributions to the optimization of Mugilidae culture.**

Tesis Doctoral presentada por Dª Raquel Quirós Pozo

Dirigida por la Dra. Dª Lidia Esther Robaina Robaina

Codirigida por el Dr. D Francisco Javier Roo Filgueira

Las Palmas de Gran Canaria, a 15 de Junio de 2023

La Directora,	El Codirector,	La Doctoranda,
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Ten en mente siempre a Ítaca. Llegar ahí es tu destino.

Más no apresures nunca el viaje.

La Odisea

A mis padres, que lo han hecho posible.

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List of Abbreviations

ARA: arachidonic acid

BHI: brain heart infusion

BHT: butylated hydroxytoluene

BW: brackish water

bw: body weight

°C: celsius degrees

cDNA: complementary deoxyribonucleic acid

CPE: carp pituitary extract

DHA: docosahexaenoic acid

DNA: deoxyribonucleic acid

DPA: docosapentaenoic acid

EPA: eicosapentaenoic acid

EVAc: ethylene-vinyl-acetate copolymer

LW: live weight

FAMEs: fatty acids methyl esters

FCR: feed conversion ratio

FI: feed intake

FM: fish meal

FSH: follicle stimulating hormone

FW: fresh water

FO: fish oil

FOM: final oocyte maturation

GnRH: gonadotropin releasing hormone

GnRHa: gonadotropin releasing hormone analogue

hCG: human chorionic gonadotropin

LC-HUFA: long chain highly unsaturated fatty acids

ICFP: industrial compound feed production

IU: international units

L: liter

LC-PUFA: long-chain polyunsaturated fatty acids

LH: luteinizing hormone

LH-RH: luteinizing hormone-releasing hormone

LH-RHa: luteinizing hormone-releasing hormone analogue

lnWi: initial weight neperian logarithm

lnWf: final weight neperian logarithm

M: molar

MT: 17α-methyltestosterone

MUFA: monounsaturated fatty acids

n3 LC-HUFA: omega 3 long chain highly unsaturated fatty acids

nm: nanometres

PG: prostaglandin

PPT: parts per thousand

PUFA: polyunsaturated fatty acids

rFSH: recombinant follicle stimulating hormone

rLH: recombinant luteinizing hormone

RNA: ribonucleic acid

rpm: revolutions per minute

RT-PCR: real time polymerase chain reaction

SFA: saturated fatty acids

SGR: specific growth ratio

SW: sea water

TBA: thiobarbituric acid

TCA: trichloroacetic acid

UN: United nations

USD: United States dolars

WG: weight gain

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Y la que parecía una de las páginas más sencillas de escribir de esta tesis doctoral se convierte ahora en una de las más complicadas, pues no es fácil expresar en unas líneas el agradecimiento a todas aquellas personas que, de una manera u otra, me han dado soporte en los últimos 5 años. Gracias a todos.

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1. Introduction

1.1. Sustainable aquaculture growth and 2030 Agenda

Human populations are predicted to reach ~10 billion by 2050 (UN, 2022a), turning climate change, resource depletion, food insecurity and biodiversity loss into the most urgent threats humanity must face.

On September 2015, the leaders of the UN (United Nations) adopted a set of global goals (sustainable development goals, SDGs) to eradicate poverty, protect the planet and ensure prosperity as part of a new sustainable development agenda. However, in 2020, between 720 million and 811 million persons globally were suffering from hunger, and above 30 per cent of the world's population was food-insecure, lacking regular access to suitable food. The COVID-19 crisis has driven those rising rates even higher and has aggravated all forms of malnutrition. In addition, the war in Ukraine is further disrupting global food supply chains and has given place to the most significant global food crisis since the Second World War (UN, 2022b).

Under that scenario, blue transformation is a strategy to meet the challenges of food security and environmental sustainability, focusing, among others, on sustainable aquaculture expansion worldwide. While the production by the fisheries declined a 4.4% in 2020, aquaculture grew continuously, with China accounting for 35% of the global aquatic animal production, and freshwater animal manufacture reaching 37% of the total. As a result, global aquaculture production achieved a historical record in 2020 with 122.6 million total tonnes and 87.5 million tonnes of aquatic animals produced, which reached a value of 264.8 billion USD (United States Dollars). By 2030, aquatic food production is predicted to increase by more than 15%, mainly by intensifying and expanding aquaculture production (FAO, 2022a). Additionally, fish and self-fish consumption play a crucial role in human nutrition and health, constituting the primary source of dietary animal protein in many low-income countries (Tacon and Metian, 2018). However, such growth must conserve aquatic ecosystem health, minimize pollution, and safeguard biodiversity and social fairness.

For those reasons, sustainable aquaculture development directly helps to accomplish almost 5 of the seventeen goals of the 2030 Agenda: zero hunger, good-health and well-being, decent work and economic growth, sustainable cities and communities, and sustainable use of the oceans, seas, and marine resources (UN, 2022b), although indirectly, it is considered to contribute to all the SDGs. In that way, aquaculture contributes importantly to human well-being; however, cohesive strategies between this sector and natural resources management, health and food sectors are crucial. Additionally, understanding and valorising the remarkable diversity of species and systems is imperative to achieve the SDGs in the short term (Troel *et al.*, 2023).

The success of sustainable aquaculture development depends on the sector's capability to adapt to serious challenges and uncertainties such as climate change, the effect of pathogens, pollution and price fluctuations (Ababouch *et al.*, 2023). Most animal aquaculture production is still represented by mollusc, herbivorous and omnivorous freshwater species that take advantage partially or entirely of the natural resources, presenting comparatively low environmental impacts and working even as environmental restorative systems. However, although most of the aquaculture

manufacture is carried out by small-scale producers from developing countries, 60-70% of the demand in value comes from Europe, North America, and Japan, primarily for fed finfish species such as salmon, shrimp or seabass (Ababouch *et al.*, 2023).

In this sense, 90% of the aquaculture supply chain's environmental impact is driven by aquafeeds (Little *et al.*, 2018). One of the reasons is that, although at low dietary levels, marine ingredients continue to be essential for the aquaculture industry. Fishmeal (FM) continues as one of the primary protein sources due to its good amino acid profile, which contributes to the digestibility and palatability of aquafeeds. Additionally, after much research supporting high substitution levels (Shepherd and Bachis, 2014; Shah *et al.*, 2018; Türkmen *et al.*, 2019; Hodar *et al.*, 2020), fish oil (FO) still also plays a crucial role mostly in marine aquaculture as it is the primary source used to provide essential n-3 long chain fatty acids, and therefore, is fundamental for fish health and for the quality of the final product for consumers (Kok *et al.*, 2020).

Global fishmeal trade reached 3.67 million tonnes in 2021, 12% more than in 2020, with China accounting for half of the global supply. The annual volume traded of fish oils also increased, although more modestly, from 2020 (972 000 tonnes) to 2021 (983 000 tonnes). Prices of fish meal and fish oil are rising steadily due to the high demand; for instance, fish oil reached a historical record of 3000 USD per tonne in 2021 (FAO, 2022b).

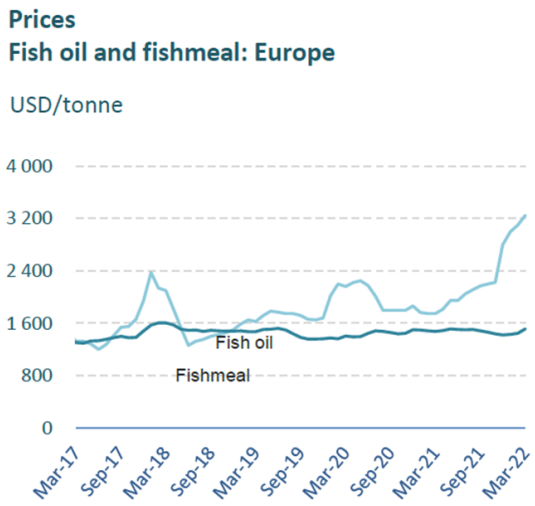


Figure 1.1. Fishmeal and fish oil prices in Europe (2017-2022) Source: FAO, 2022 (b).

In addition to escalating prizes, FM and FO use for aquafeeds is increasingly more controversial due to the expected collapse of the current fished stocks by the year 2050 if the current capture trends are maintained (Worm *et al.*, 2016). Although significant improvements have been achieved in the last decades, which have reduced the levels of dietary FM for carnivore species from 45% in 2000 to 23% in 2010 (Glenncross *et al.*, 2023), complete FM substitution for carnivore species is still uncommon due to alternative source's limitations, such as nutritional imbalances, reduced digestibility or the presence of antinutritional factors (Oliva-Teles *et al.*, 2015). For those reasons, the last decade's research has focused, among others, on finding suitable alternatives to dietary FM and FO. However, the substitution of FM based on traditional plant

ingredients, primarily soy and some wheat and corn gluten meals, produces a high carbon footprint, thus, increasing deforestation and freshwater consumption as it has increased its demand. For those reasons, the utilization of novel feedstuff such as yeasts, bacteria, microalgae or insects has been explored (Hauptman *et al.*, 2014; Zhao *et al.*, 2017; Delamare-Debouteville *et al.*, 2019; Rosas *et al.*, 2019; Mastoraki *et al.*, 2020; Zhang *et al.*, 2022). Additionally, substituting conventional plant ingredients with by-products is another vital tool to increase the sustainability of aquaculture (Figure 1.2). However, its use must necessarily expand to achieve the goals of sustainable growth and resource circularity, increasing the sector's resilience and promoting food safety (Glenncross *et al.*, 2023).

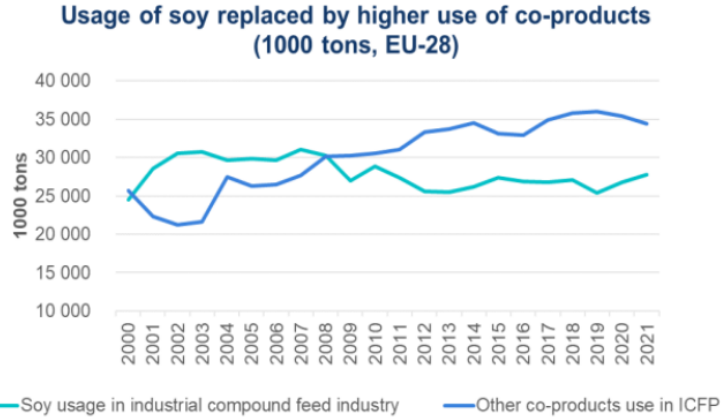


Figure 1.2. Replacement trend of soy usage by by-products. Source: FEFAC, 2023. Abbreviations: ICFP, industrial compound feed production.

As regards FO substitution, it has faced greater limitations than FM because of the reduced biosynthesis capacity of most marine species to convert 18C fatty acids into long-chain polyunsaturated fatty acids (LC-PUFA), which, together with its reduced availability, has placed FO as the most relevant potential bottleneck for the sustainable growth of marine aquaculture (Oliva-Teles *et al.*, 2015). Also, in this case, vegetable sources have been widely implemented until certain levels for FO substitution. Additionally, animal fats have also been broadly tested due to their competent price and availability; however, these lipids sources contain high levels of saturated (SFA) and monounsaturated fatty acids (MUFA), which are an excellent energy source but, on the other hand, constitute a meagre contribution of essential n3 LC-PUFA (Turchini *et al.*, 2009). For those reasons, marine lipids from microalgae or from by-products of seafood processing plants exhibit interesting perspectives for the aquaculture industry. Concerning to microalgae or derivates, the need for a stable commercial supply and the high production costs limits the further implementation of these sources. Oils from aquatic by-products are great alternatives due to their relatively low cost and sustainability; however, in many cases, these sources usually lack a stable composition and availability, and further improvements in the processing chain are needed even at a lower scale to increase the utilization of these ingredients.

Furthermore, dependency on FM and FO is almost inexistent in most herbivorous and omnivorous fish (Tacon and Metian; 2008; Oliva-Teles *et al.*, 2015; Glenncross *et al.*, 2023), therefore offering additional alternatives for the implementation of more sustainable aquafeeds.

1.2. Diversification, the role of the low trophic species

The vast expansion predicted for aquaculture has promoted an increasing interest in diversifying the number of fish species reared under controlled conditions, as diversification of the farmed species is a tool to reduce the spreading of diseases, expand the offer, reduce income fluctuations and market risks, and to the increase production efficiency, sustainability and food security (Abellán and Basurco, 1999; Kim *et al.*, 2022).

Global trends and advances in fish nutrition have shifted the diets of farmed species away from their wild counterparts, influencing the practical trophic level of many aquaculture species. Nonetheless, carnivorous species may suffer adverse effects on growth or health at high fish meal substitution levels (Chen *et al.*, 2019; Pham *et al.*, 2020; Liu *et al.*, 2021). In general, in non-obligate carnivores, lower or no FM/FO inputs align with natural dietary habits and are typically better tolerated due to physiological differences in their digestive tracts that allow more efficient use of low-grade plant material. However, effects on growth and health are also described for these species when inadequate levels of certain sources are provided (Amer *et al.*, 2019; Peng *et al.*, 2020). In that way, policies for sustainable aquaculture growth often promote the production of low trophic species (Allsopp., 2013; Cottrell *et al.*, 2021); nevertheless, not only the trophic level but also other physiological aspects as feeding efficiency are determinant factors to define the sustainability of a species (Cottrell *et al.*, 2021).

Aquaculture's sustainability and cost effectiveness depend on the species' ecological efficiency. In this way, each step in the trophic chain increase costs for the use of resources, waste production management and water quality maintenance. Herbivorous and detritivores species are usually farmed in extensive extractive (non-feed) aquaculture, with the potential for a low-cost sustainable culture which implies the use of natural food and even improves the water quality through self-waste treatment (Neobri and Nobre, 2012). Despite the high potential of extractive aquaculture with low trophic species for sustainable development, this type of production is confined to some environments with high nutrient availability (Krause *et al.*, 2022). However, in oligotrophic marine waters, a different approach must be made to meet the goals for sustainable food production.

Regarding feed aquaculture production, omnivorous freshwater species account for over half the outputs (Tacon, 2020); however, the production of marine fish placed low in the trophic chain is still uncommon. Few species of low trophic marine fish are cultured in significant quantities worldwide. For instance, the milkfish (*Chanos chanos*) is produced in warm waters of the Pacific and Indic oceans, reaching a total production of 1167.8 thousand tonnes in 2020 (FAO, 2022a). However, its production is limited due to the need for well-established seed production and its low market price in the international market (Mirera *et al.*, 2019).

Mullets are placed in the second position of the low trophic marine fish produced globally, with 291.2 thousand tonnes produced in 2020 (FAO, 2022a). Despite the high price of their processed products, such as the dry and salted roe, considered a delicacy in many regions worldwide, its culture in intensive systems still needs to be improved. Among the limiting factors for further intensification is the low market price of the whole fish and the absence of seed production at a commercial level, which causes their culture to be still supported by wild fry capture (Crosetti, 2015). However, its

omnivorous, euryhaline, and cosmopolitan nature, in addition to the outstripping of the supply of FM and FO for aquafeeds, have recently raised interest in this family, which had caused an increase in the production in the last years (FAO, 2022a).

1.3. Current state of mullet's aquaculture

1.3.1. Background and traditional mullet aquaculture

The first records of mullet captures were discovered in Ancient Egyptian tombs, dating back to 2340 B.C. Additionally, their crucial role as a fisheries resource date back centuries in other countries such as Mauritania or Australia. On the other hand, the first records of mullet aquaculture were reported by ancient Roman writers of the Italian Coast, which described how the fish were captured, taking advantage of their natural migrations from or towards the sea to coastal lagoons, to be later confined in restricted areas (Crosetti, 2015).

Figure 1.3. Tomb of princess Idut.

Vth Dynasty. Saqqara, Egypt.

Mullets are catadromous teleost that, at the juvenile stage, move from the open sea towards coastal areas, estuaries, bays, and coastal lagoons (Whitfield, 2015). Traditional mullet fry collection takes advantage of these migrations intercepting the juveniles and stocking them in confined areas to be cultured extensively or semi-intensively in most cases. For example, in Italy, a traditional culture practice named "Vallicultura" has been carried out for centuries until nowadays to culture different Mediterranean marine species, of which mullets represent a significant percentage (121.2 tonnes of Mullet sp. produced in Italy in brackish water in 2020) (FAO, 2022c). This method represents an integrated coastal management system with gates and channels to recruit the juveniles during their natural migration from the sea, stocking in confined areas ("valli") isolated from the lagoon by bars or soil banks, and wintering, to later capture the fish at the barriers in the autumn, when migrating back to sea. Therefore, this system takes advantage of the natural cycles and dynamics of the species and the environment (Figure 1.4), exploiting the ecosystem's natural resources with a multitrophic approach. However, natural recruitment is currently insufficient to meet the demand for fry and juveniles, so wild fry are commonly collected by fishermen from the coastal areas and transported to the culture valleys.

Figure 1.4. Systems for the capture of fish and juveniles stocking in *Valliculture*. Porto Pino lagoon, Sardinia, Italy.

The flathead grey mullet (*Mugil cephalus*) and the thicklipped grey mullet (*Chelon labrosus*) are usually the most expensive species, so are stocked in special areas free of predators ("peschiere"). One of the reasons for the high value of these species, (mostly for *Mugil cephalus* because of its higher gondado-somatic index), is the obtention of the egg roe to make the traditional "bottarga" (Figure 1.5), a dried and salted speciality that achieve high prices in the global market (~275 euros/Kg). Mullet dried and salted egg roe is not only highly appreciated in Italy but also in other countries like Spain ("hueva de mújol"), Greece ("avgotaracho"), Egypt ("batarekh"), Turkey ("haviar") or different sur-east Asian countries ("*karasumi*") (Crosetti, 2015).

Figure 1.5. Sardinian bottarga.

As bottarga production with local fish is not enough to meet the increasing demand, the ovaries are acquired from several distributors in different countries to be processed by the Sardinian companies according to tradition. This product is a natural source of n3 long chain highly unsaturated fatty acids (n3 LC-HUFA), especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), particularly in the form of wax esters (Rosa *et al.*, 2020), which are widely recognized by their beneficial effects for human nutrition and health (Beveridge *et al.*, 2013; Thilsted *et al.*, 2016).

Besides, mullet aquaculture has been carried out traditionally in other regions such as Hawaii (traditional ponds), China ("harbour culture"), Japan ("kava culture"), India ("bheris" of Gangetis estuaries) or Indonesia ("tambaks"). Until today, mullets are, in any case, a valuable food source in many regions worldwide (Crosetti, 2015).

1.3.2. Aquaculture of the different mullet species around the world

In 2020, the global aquaculture production of mullets was 291.2 thousand tonnes of live weight (Figure 1.6), representing 3.5% of the total marine and coastal finfish aquaculture and being comparable to productions of highly appreciated species such as seabream (282.1 thousand tonnes, 3.4% of the total) or seabass (243.9 thousand tonnes, 2.9% of the total) (FAO, 2022a). Compared to the total manufacture of Mugilidae in 2015, the production more than doubled in only 5 years (125.4% increase) (Figure 1.5), probably due to the recent advances in closing the live cycle in captivity (Besbes *et al.*, 2020; Vallainc *et al.*, 2021; Ramos-Júdez; 2022; Vallainc *et al.*, 2022) and to the increased interest in low trophic marine species for the sustainable development of aquaculture in the context of the expected blue growth in the short term (Tacon *et al.*, 2010; Neori and Nobre., 2012; Krause *et al.*, 2022).

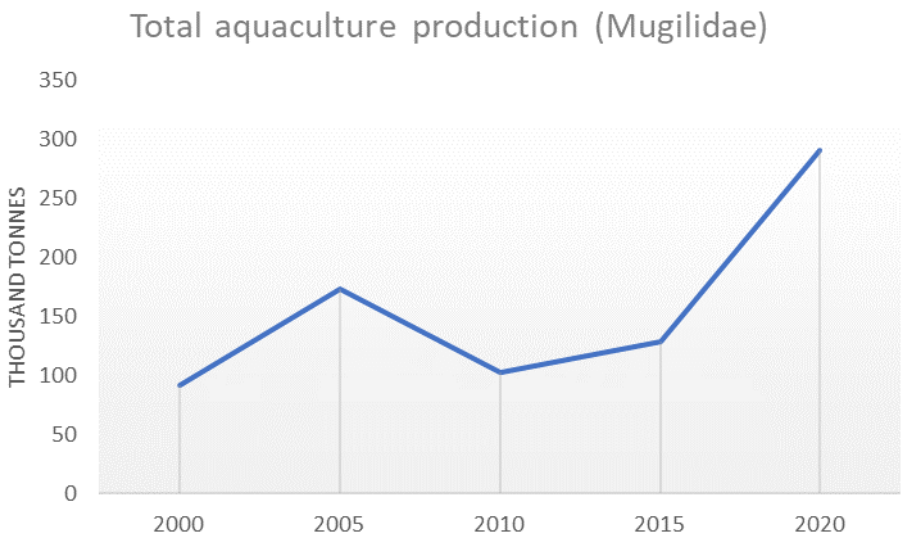


Figure 1.6. Total aquaculture production of mullets (2000-2020). Adapted from FAO, 2022a.

It must be considered that in ¾ of the countries, the data on mullet production are generic and do not identify the species, so it is difficult to know the actual production of a particular species (Crosetti, 2015). The total global production (fisheries and aquaculture) of the flathead, golden and thicklipped grey mullet (Figure 1.7) was 121 607 tonnes in 2019, of which the production of the flathead grey mullet accounted for 97.6%, while only 0.54% was from *Chelon labrosus* and interestingly a 2.9% from *Liza aurata* (FAO-FIGIS, 2022). For *Mugil cephalus*, it was observed a sharp increase from 1995 to its higher levels in 2004, the moment from which the production decreased up to 44.73% in 2007, to later recover to the levels of the 90s until 2019, when the production decreased to 118 056 tonnes. For *Liza aurata*, no production was recorded until 2002 (with only two tonnes), reaching a maximum level of 3183 tonnes in 2017. For *Chelon*

labrosus, no production data was recorded until 2009 (455 tonnes), reaching a peak in 2014 with 788 tonnes produced.

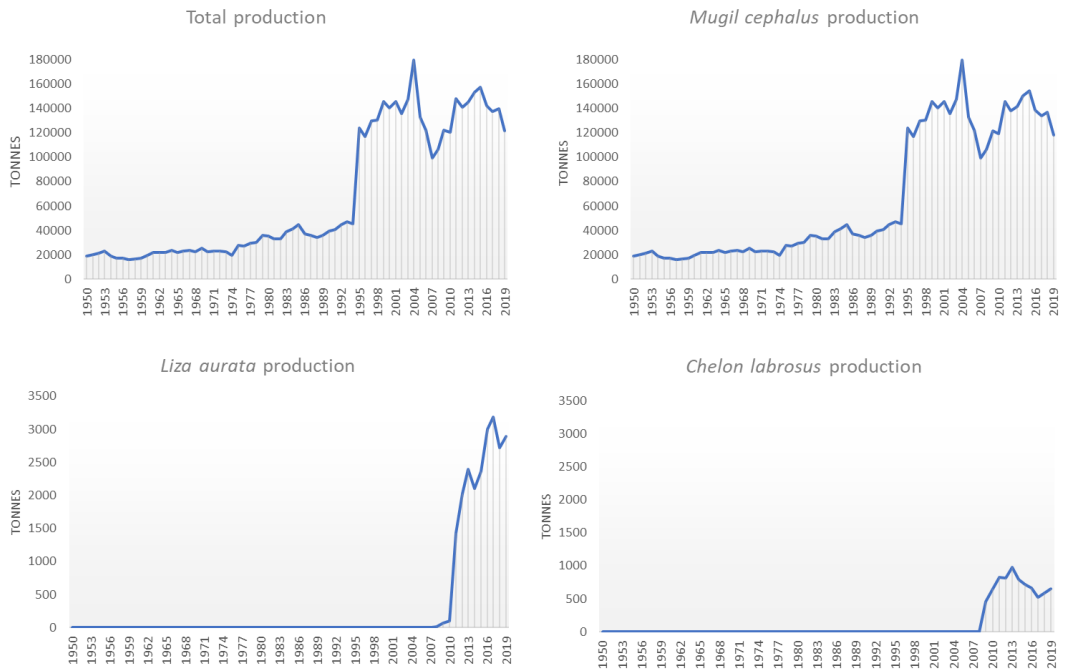


Figure 1.7. Total production (aquaculture and fisheries) of the flathead, thick-lipped and golden grey mullet, together and separately, from 1950 to 2019. Adapted from FAO-FIGIS, 2022.

In Europe, aquaculture production data is referred to Mugil spp. or Liza spp. being Italy the main producer with 153.2 tonnes of Mugil spp. and 376.1 of Liza produced in brackish water in 2021 (FAO, 2023). In Spain, the production records are lower with only 58.06 tonnes of Liza spp. manufactured in brackish water in 2021 (FAO, 2023).

Mullet aquaculture production has been recorded in almost 15 countries (Crosetti, 2015), being Egypt the first producer of farmed mullet with 250,000 tonnes in 2018 (El-Son *et al.*, 2021). The other leading countries contributing to mullet aquaculture are the Republic of Korea, Italy, Taiwan province of China and Israel. The aquaculture production of mullets still relies mainly on wild fry collection, and only in some countries like Israel, Hawaii and Italy the seed is produced under controlled conditions on a limited scale (Sukumaran *et al.*, 2022). Although wild fry capture is being regulated in many countries, fry collection and transport often result in high mortalities, up to 96%, and an important illegal market still contributes to the unsustainability of these practices (Crosetti, 2015).

1.4. Mullet's reproduction and larval rearing

1.4.1. Reproduction of Mugilidae

Mullets are oviparous bisexual teleost with no sexual dimorphism, with external fertilization and development. Both male and female mullet's gonads are internal paired organs which lay laterally to the gas bladder and join near the urogenital pore in the genital papilla.

The females are total spawners (Hunter, 1992) and have group synchronous ovaries (Wallace and Selman, 1981); therefore, usually only spawn once yearly. Although mullets are euryhaline fish, most species only spawn in saline water.

On the other hand, the high niche overlap and the usual co-existence of different mullet species in the same habitats have determined differential biological behaviours that help to avoid resource competition (Salvarina *et al.*, 2016). In that way, it is usual that mullet species with the same habitat distribution have different spawning seasons throughout the year. For instance, four of the most frequently farmed species in the Mediterranean region have been reported to have consecutive and differentiated spawning seasons, the flat-head grey mullet (July-September) (Besbes *et al.*, 2020), the golden grey mullet (September-November) (Hotos *et al.*, 2000; Ghaninejad *et al.*, 2010), the thin-lipped grey mullet (November-January) (Mousa *et al.*, 2018; Vallainc *et al.*, 2022) and the thick-lipped grey mullet (February-April) (Besbes *et al.*, 2020), which may also be a valuable fact by mullet seed's producers which can produce all the year along.

Oocyte development in mullets

As in another teleost, in mullet females, previously to the beginning of the reproductive cycle, the oogonia population in the ovary starts to proliferate through mitosis, to later enter the meiosis process, being arrested at prophase I and converting in primary oocytes (previtellogenic oocytes) (Ramos-Júdez, 2021). In this phase, known as the chromatin-nucleolus stage, the oocytes (50-200 µm in mullets) have scarce cytoplasm and a large central nucleus and are surrounded by few squamous cells (Figure 1.8 a). In the subsequent peri-nucleolus phase, the oocytes grow, and the nucleus increases in size, forming the germinal vesicle.

In the secondary growth (cortical-alveolus stage), the zona radiata develops around the oocyte, and the endogenous vitellogenesis starts through the synthesis of glycoproteins incorporated into the cortical alveolus. Later, during fertilization, the cortical alveolus will release its contents into the perivitelline space to prevent polyspermy and pathogen penetration (González-Castro and Minos, 2015).

In the next phase, vitellogenesis, extraovarian proteins begin to be stored in the oocytes, mainly vitellogenin from hepatic origin, which is the primary precursor of the yolk proteins (Figure 1.8 b). These proteins will accumulate in yolk globules which will fuse centripetally. Additionally, in this stage, the follicular cells multiply and differentiate into granulosa cells (internal layer) and theca cells (external layer), which in addition to the oocytes and the zona radiata, form the ovarian follicle (González-Castro and Minos, 2015).

Specifically, for the flathead grey mullet, Greeley *et al.* (1987) suggested a diameter of vitellogenic oocytes of 600 µm to achieve the capability to resume the meiotic process and to become a fertilizable egg.

The maturation stage, the last step of the oocyte development, is characterized by the resumption of meiosis, which implies the migration of the germinal vesicle through the periphery, the chromosomes condensation, the elimination of the first polar body, and the arresting of the remaining chromosomes on the second meiotic metaphase. Just before spawning, the oocytes hydrate, producing a considerable increase in the final egg size (900-1100 µm), which helps the final ovulation and expulsion of the oocytes to the ovarian cavity, and its consequent positive buoyancy in seawater (Cerdá, 2009). After this process, the remaining follicular cells will form the post-ovulatory follicles, which will be reabsorbed in a few days.

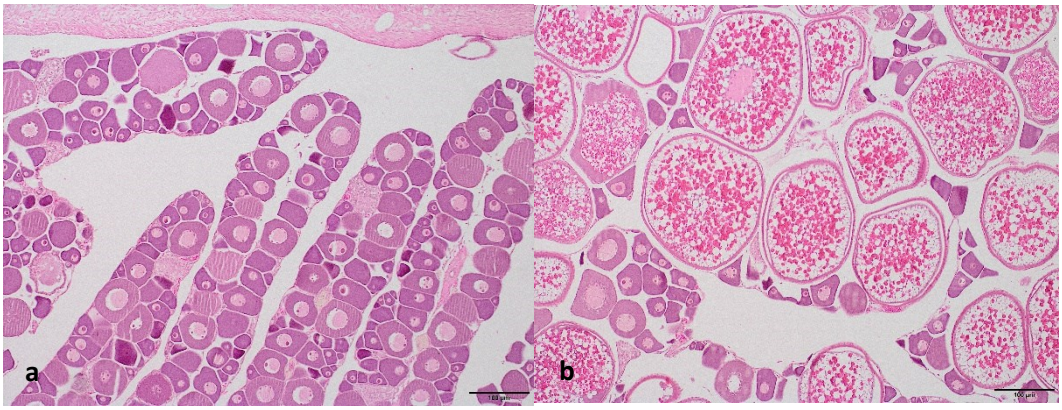


Figure 1.8. Sections of *Liza aurata*'s female inmature (a) and mature (b) gonads, stained with hematoxilin and eosin (H&E). Bars 100 µm.

Main reproductive dysfunctions of mullets in captivity

Reproductive dysfunctions in teleost are frequently associated with the stress response, which depends on a variety of factors as the fish species, the maturation stage, and the intensity and duration of the stressor. Confinement of wild fish in captivity, modification of the natural environmental parameters, handling and high densities are some of the main factors inducing corticosteroids-mediated suppression of the hypothalamus, pituitary and gonad functions (Corriero *et al.*, 2021).

Like many other marine teleosts, some mullet species as the flathead grey mullet (Aizen *et al.*, 2005; Ramos-Júdez *et al.*, 2022) or the thin-lipped grey mullet (Mousa *et al.*, 2018), usually fail during the final maturation stage and females do not complete the final oocyte maturation (FOM) in captivity, while males fail in the production of fluent milt. For that reason, some methods for mullet seed production are based on adult's collection during the spawning season and hormone induction treatments on highly ripened specimens (Vallainc *et al.*, 2021; Vallainc *et al.*, 2022), while other are based in maturation treatments followed by protocols to induce spawning (Aizen *et al*, 2005; Ramos-Júdez *et al.*, 2022). Nevertheless, other species as the thick-lipped grey mullet, do usually achieve the FOM under culture conditions (Sarasquete *et al.*, 2014; own data, GIA-Ecoaqua facilities); however, although controlled reproduction started more than

30 years ago (Cataudella *et al.*, 1988), in many mullet species is still common the need of high doses of inducing hormones to achieve the spawning of the mature females (Besbes *et al.*, 2020). This fact suggests the existence of inhibitory factors as the endogenous inhibitory dopamine impact on the hypothalamic-hypophyseal-gonadal axis (Aizen *et al.*, 2005), which may prevent the effects of GnRH treatments on the hypothalamic liberation of LH, and the consequent hormonal cascade that produces gametes liberation. For those reasons, it is usual that hormonal treatments to induce both maturation and spawning in grey mullets combine the effects of drugs that work as dopamine antagonists as the domperidone or the metoclopramide, with GnRH hormone analogous. Therefore, these treatments induce the endogenic liberation of LH and FSH hormones by the hypophysis and therefore, stimulate the endogenous hormonal cascade of the reproductive axis. A summary of the treatments used to induce maturation and spawning in the flathead, thicklipped and golden grey mullet are described in Table 1.

Table 1.1. Treatments to induce gonadal development and spawning in the flathead, thicklipped and golden grey mullet.

	Induction treatment	Temperature (°C)	Ratio Females/males	Time to spawning	Season	Country	Reference
Liza aurata	mullet PG 40 mg carp PG 70 mg	15-25				USSR	Apekin *et al.*, 1979
Chelon Labrosus							
	hCG (5000 UI/Kg), only females	15	3/4		April	Italy	Cataudella *et al.*,1988
	LH-RH (Zaporito, Cadiz), natural (INS els Alfacs, Tarragona)	14-16	19/13 and 11/9	48-72h	March	Spain	de las Heras *et al.*, 2012
	hCG at a priming dose of 10.000 IU, followed by a resolving dose of 10,000 IU hCG and 100 µg kg^{-1} LH-RH 24h apart.	15	1/2	72h		Tunisia	Besbes *et al.*, 2020
Mugil cephalus							
	Maturation treatment: Females: single injection of dopamine antagonist, domperidone. Males were treated with MT, administered via EVAc slow-release implants. Spawning treatment: Priming (GnRHa 10µg kg^{-1}; Met 15mg kg^{-1}) and resolving (GnRHa 20µg kg^{-1}; Met 15mg kg^{-1}) injections given 22.5h apart to fully mature females.	24-28	1/2	20h		Israel	Aizen *et al.*, 2005
	Maturation treatments: a) fish injected with 20 mg kg^{-1} carp pituitary extract (CPE) as a priming dose followed by 200 µg kg^{-1} GnRHa as resolving dose (24h apart), b) fish received 20 mg kg^{-1} CPE as a priming dose followed by injection of 200 µg kg^{-1} sGnRHa in Freund's incomplete adjuvant emulsion 24 h apart. All groups received 20 mg kg^{-1} BW metoclopramide.	18-22				Iran	Vazirzadeh *et al.*, 2015
	Spawning treatment: Slow-release LH-RHa preparation (leuprorelinacetate) 200 µg kg^{-1} BW for females 100 µg kg^{-1} BW for males (singular injection)		1/3 or 1/4	72 h		Italy	Vallainc *et al.*, 2017
	Maturation treatment: Weekly injections of recombinant follicle stimulating hormone (rFSH) and luteinizing hormone (rLH). Spawning treatment: priming dose of 30 µg kg^{-1} rLH and resolving dose of 40 mg kg^{-1} progesterone or priming and resolving doses of 30 µg kg^{-1} rLH (24h apart).	23	1/2 or 1/3		August-November	Spain	Ramos-Júdez *et al.*, 2022

Abbreviations: CPE, carp pituitary extract; hCG, human chorionic gonadotropin; EVAc: ethylene-vinyl-acetate; GnRHa, gonadotropin releasing hormone analogue; IU, international units; rLH, recombinant luteinizing hormone; LH-RHa, luteinizing hormone releasing hormone analogue; rFSH, recombinant follicle stimulant hormone; Met, metoclopramide; MT, 17α-methyltestosterone; PG, prostaglandin.

1.4.2. Embryonic development and larval rearing of Mugilidae

Embryonic and larval development knowledge varies significantly among the different mullet species. For instance, for *Liza aurata*, there are only a few studies which describe morphologically some larvae identified through plankton collections and date back more than 50 years ago (Vodyanitskii and Kazanova 1954, Demir 1971). On the other hand, several studies have been published for *Chelon labrosus* and *Mugil cephalus* ontogeny due to their important commercial value and the advances in their controlled reproduction in captivity (Boglione *et al.*, 1992; Sarasquete *et al.*, 2014; Besbes *et al.*, 2020; Loi *et al.*, 2020). For instance, several studies have targeted some aspects of *M. cephalus* and *C. labrosus* larval development as the ontogeny of the digestive, visual, and skeletal systems (Boglione *et al.*, 1992; Zouiten *et al.*, 2008; Sarasquete *et al.*, 2014; Loi *et al.*, 2020; Thieme *et al.*, 2021). Additionally, some aspects of larval rearing techniques and nutrition have been assessed in these two species, such as the beneficial effects of algal turbidity in the tanks or the effects of different protein levels of weaning diets in *Mugil cephalus* larval performance (Gisbert *et al.*, 2016; Koven *et al.*, 2019; Koven *et al.*, 2020), or the larval rearing and weaning conditions in mesocosm systems for *Chelon labrosus* (ben Khemis *et al.*, 2006). However, compared with well-established aquaculture species, there is still a vast gap of knowledge related to larval ontogeny, rearing practises, and nutrition requirements of grey mullets, information which may promote optimised rearing protocols and formulas to improve the efficiency and productivity of hatcheries.

<u>Embryonic development stages</u>

The fertilized eggs are generally pelagic, transparent, spherical, and buoyant. The egg diameter of *Chelon labrosus* is one of the highest among Mugilidae (1.3-1.4 mm) containing 1 to 13 oil globules (Boglione *et al.*, 1992; own data, GIA-Ecoaqua facilities). *Liza aurata* fertilized eggs diameter instead varies between 1.1-1.2 mm and have up to more than 30 oil globules (own data, GIA-Ecoaqua facilities, Figure 1.9), while *Mugil cephalus* fertilized eggs are smaller (0.88-0.99 mm) and usually contain a single oil globule having been described failures to complete de development in the eggs with more than four oil globules (Kuo *et al.*, 1973; Meseda and Samira., 2006; Berbes *et al.*, 2020). In the three species is usual that de oil globules merge in a single vacuole at latter stages of development, although sometimes the newly hatched larvae can be seen with more than one vacuole in the yolk sac.

Germinal disc stage and morula stage

After fertilization, the perivitelline space is formed and cell divisions starts forming the blastodisc (germinal disc) at the animal pole of the yolk, from the 2 cells to the 128 cells stage. Usually, the oil droplets progressively began to fuse. At the morula stage the blastodisc have multiple blastomeres forming a multicellular layer (Mousa *et al.*, 2010; González-Castro and Minos, 2015).

Blastula and gastrula stages

At the blastula stage, blastomeres division becomes less synchronous and it turns difficult to differentiate cells, which starts to encircle the yolk forming a germ ring with a thickened zone called the blastoderm and a central cavity (blastocoel). In the gastrula stage, cells start to proliferate and expand though the vegetal pole (Mousa *et al.*, 2010), forming the multi-layered embryo and the embryonic shield.

Organogenesis stage

Organogenesis began and the head and tail region start to be differentiable. The pigments start to cover the embryo and the oil globule in the form of star shaped melanophores (Boglione *et al.*, 1992; González-Castro and Minos, 2015). At the neurula stage the optic vesicles are recognizable and the tail developed. Internal organs can be seen and is possible to see the heart beating when the embryo round about halfway around the yolk. The embryo began to move and at hatching the larvae break the egg membrane though movements of the tail (González-Castro and Minos, 2015), which moment depends mostly on the species and the water temperature.

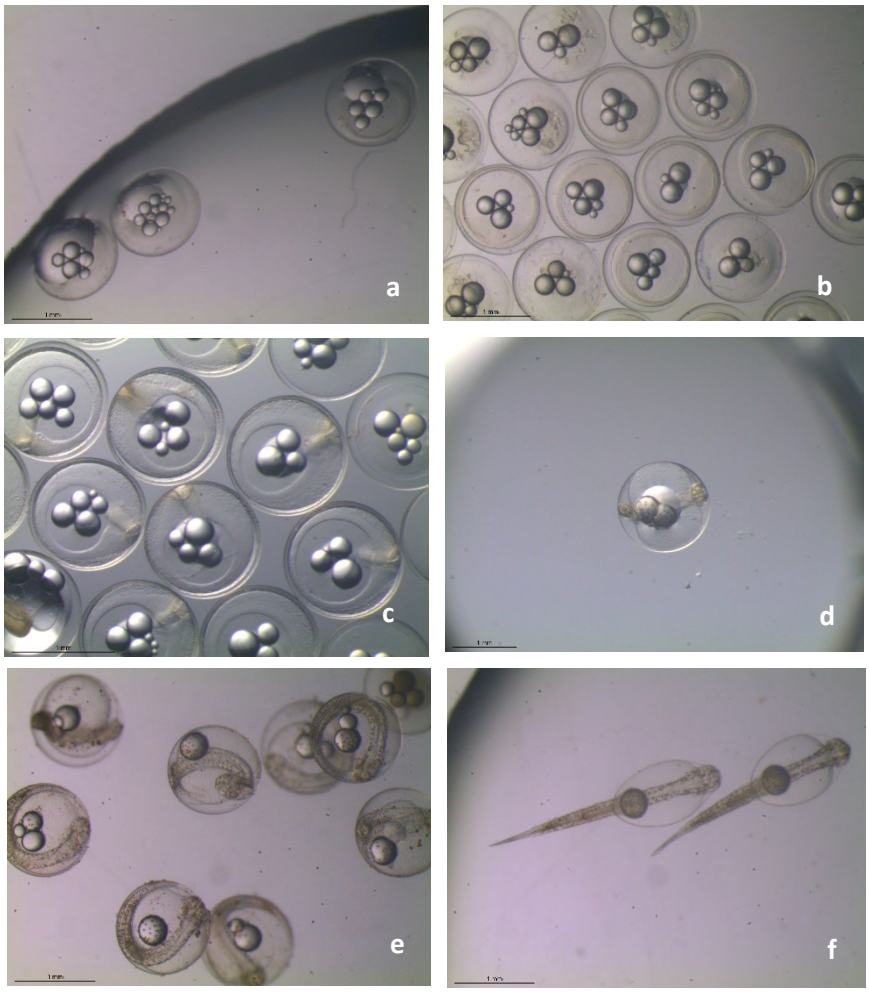


Figure 1.9. Embryonic development of *Liza aurata*; a) 2 h after spawning, b) 8 h after spawning (blastula stage), c) 12h after spawning (gastrula stage), d) 24 h embryo-stage, e) 32 h embryo-stage, f) hatching larvae (36h after spawning). Temperature 20-22°C. Bars 1 mm.

Like other marine fish species, Mugilidae larvae are born with undeveloped organs and systems which will mature during the larval phase to achieve full functionality. Size at hatching varies greatly among mullet species, affecting the type and size of the first feed that must be provided (Crosetti, 2015), for instance, from 4 mm at hatching in *Chelon labrosus* larvae, which allows the implementation of first feeding practises directly with *Artemia* sp. (Cataudella *et al.* 1988; de las Heras *et al.*, 2012), or 2.9 mm for

Liza aurata (own data, GIA-Ecoaqua facilities) to 1.97 mm for *Mugil cephalus* (Meseda and Samira, 2006) or 1.43 mm for larvae of *Mugil macrolepis* (Crosetti, 2015). Additionally, other physiological differences along the larval phase of the different mullet species as the yolk sac and oil globule size, reabsorption patterns, moment of mouth opening and mouth size, or visual and skeletal ontogeny, may all determine different rearing protocols and feed management to shift from the live to the inert feed and must be studied for each species to optimize the cost and effort investment by producers.

1.5. Mullet's ongrowing and culture management

Aquaculture is based on the growing of fish under controlled conditions which may determine the final profitability of the production. The number of parameters to be controlled is enormous and depends, among others, on the type of aquaculture system employed, the species used and the knowledge of its optimal growth conditions parameters. Some examples of parameters which may directly influence fish growth are water quality, temperature, photoperiod, stocking density, salinity, and feeding practices. As regards the environmental factors, water quality, temperature, and salinity depend mainly on the natural attributes of the water source when it comes to open flow aquaculture. However, these parameters must be controlled and can be easily modified when it comes to recirculation aquaculture systems (RAS). In this regard, salinity is a crucial factor to be considered for the culture of Mugilidae, as it has an important role in their natural physiology and behaviour which affects several factors of their productivity, from growth and reproduction to the final product quality.

Mullets are euryhaline fishes, and many species can live in a broad range of salinities, from freshwater to high salinity waters (rivers, lakes, coastal lagoons, seas, and oceans). Most species spawn in brackish or marine waters, and the fry migrates to near-shore areas or returns to coastal lagoons, estuaries, or even freshwaters (Nordlie *et al.*, 2015). However, there is great variability in the grade of euryhalinity depending on the species, and the salinity itself affects the species distribution along the same regions and, therefore, the community structure. The differential distribution in the wild in the function of salinity is usually promoted by the different osmoregulatory strategies among species and the optimum salinity level in which the growth is optimized, given the lower energy cost used for homeostasis (Cardona *et al.*, 2006). For instance, *Liza aurata* avoid freshwater environments and prefer polyhaline (15.1-30 ppt) and euhaline (30.1-40 ppt) waters. In contrast, *Mugil cephalus* usually select habitats with salinities under 15 ppt, while *Chelon labrosus* preferences varies greatly along the year. Nevertheless, the three species could be found in FW (freshwater) (Cardona *et al.*, 2006). In the case of *Mugil cephalus*, comparable growth is observed in both FW and SW (seawater), although it can be improved at intermediate salinities (Barman *et al.*, 2005; Olukolajo and Omolara, 2013; Loi *et al.*, 2022).

Thus, because of the natural migrations through the sea to freshwater environments and vice-versa, a huge salinity tolerance has been observed in many mullet species. For instance, *Mugil cephalus* and *Chelon labrosus* have been reported to support water salinities from FW to up to 100 ppt (Hotos *et al.*, 1998; Young and Potter, 2002). For *Liza aurata*, the minimum levels of salinity tolerance are controversial. Cardona *et al.* (2006)

described that although *Liza aurata* generally avoids freshwater areas, it was possible to find a few specimens in the island of Menorca, Spain. However, Mansour *et al.* (2013) described 100% of mortality for *Liza aurata* fingerlings placed in freshwater, even when the transition from SW was made gradually. For this species, the highest salinity tolerance reported is 76.16 ppt (Zismann and Ben-Tuvia, 1975), although a broader range is expected to be supported (Nordlie *et al.*, 2015).

Although these species are strongly euryhaline, abrupt changes in salinities of 20 ppt or more have been reported to cause mortality in the three species (Hotos *et al.*, 1998, Khodabandeh *et al.*, 2009; Shahriari Moghadam *et al.*, 2013); therefore, gradual acclimatisation is recommended.

Additionally, environmental salinity affects several other factors as the regulation of the enzymes involved in lipid metabolism. Hence, the elongase and Δ6 desaturase enzymes, which are involved in the synthesis of long-chain polyunsaturated fatty acids, are affected by the environmental salinity (Khériji *et al.*, 2003, Koven *et al.*, 2018, personal communication), thereby influencing the nutritional requirements of the animals and their final fatty acid profile for the consumers.

Among other management measures for improving the sustainability and rentability of Mugilidae culture, feeding management is one of the most meaningful economic and labour efforts in any aquaculture facility due to the rising prices of aquafeeds and the need for qualified staff. Therefore, optimising these practises will provide the optimum equilibrium between cost and benefit and will revert in the sustainability of the production, maximising the potentiality of these species.

Mullets have been defined as low trophic and continuous feeders which can feed from a wide range of materials such as macroalgae, plankton, detritus and microalgae growing in the sediment, exploiting a unique trophic niche (Lebreton *et al.*, 2011; Cardona, 2015). In addition to a well-sophisticated feeding apparatus, mullets have a muscular stomach to function as a mill for feed particles, and an extremely long intestine (González-Castro and Ghasemzadeh, 2015). These features have allowed the successful implementation of commensalism culture practises with other organisms in which the mullets decrease the organic matter in the water and sediments, thus improving the water quality and parasites incidence in polyculture (Hosseini Aghuzbeni *et al.*, 2016).

Hence, these aspects must also be considered under intensive culture systems to adapt feeding management and formulas to the biology and potential of the species. Although the Mugilidae family comprise very similar species, the co-existence of different mullet species in the same habitats has determined differential feeding behaviours to advise the competition for similar resources (Salvarina *et al.*, 2016). Thus, understanding the specific digestive physiology and feeding behaviour of the diverse mullet species may enhance the profitability of mullet aquaculture.

For instance, recent studies have addressed some aspects of feeding management, as the preferred pellet size and buoyancy feed type for *Mugil cephalus* (Ramos-Júdez and Duncan, 2022). This study recommends sinking and slow sinking pellets of 2-4 mm and 4 mm for juveniles (~366 g of live weight) and broodstock (~938 of live weight), respectively, given the preference of this species to feed in the water column or the bottom of the tank. Additionally, the optimal feeding frequency, which is closely related

to the digestion transit rate and the effectiveness of the digestion, has been studied in different mullet species as the flathead grey mullet (Solovyev and Gisbert, 2022) and the lebranche mullet (*Mugil liza*) (Calixto da Silva *et al.*, 2019), having been defined, in general, improved feed efficiency in the fish provided a more continuous regime, however, there is still a vast gap of knowledge respecting the differences on the digestive physiology between the diverse mullet species.

1.6. Mullet's feed and nutrition

In all species studied, mullet postlarvae are primarily zooplanktivores, and the shift to a benthic diet occurs from 20-30 mm of total length, although the exact size is species-specific. From that moment, the proportion of protein in the stomach contents decreases while the amount of detritus and plant material increases continuously, having the fish of 40-50 mm a similar diet to adults. After that shift, the wild juvenile's and adult's stomachs are found with a mixture of sand, detritus, green macroalgae, infauna, zooplankton, benthic fauna and microphytobenthos, this last representing a significant component of the diet of most mullet species. In that way, mullets take advantage of a unique trophic niche thanks to their sophisticated feeding behaviour on the sediment (Cardona, 2015). Additionally, detritus from vascular plants, cyanobacteria films, diatoms, polychaetes, and insects are part of the diet of Mugilidae.

Mullets usually take the sediment and associated food with the mouth. After working the material between the pharyngeal bones, they reject a part of the particles with the gills and mouth. The stomach possesses a species-specific number of pyloric caeca and a highly well-developed muscular layer to process the food. The gut is exceptionally long, although the relative length varies significantly among species and populations. Although the relative gut length has traditionally been considered a good indicator of the capacity to digest plant material, the enzymatic activity seems to be more relevant to define the different potentials of mullets to digest vegetable sources (Cardona, 2015; Pujante *et al.*, 2019). Carbohydrates usually represent half of the organic matter of the diet, although a relatively low assimilation efficiency (around 50%) has been suggested (Cardona *et al.*, 2001; Cardona *et al.*, 2015).

As respects the requirements of dietary protein, parameters of growth together with protein efficiency and hepatic health support the convenience of a 30% protein level in diets for *Mugil cephalus* fingerlings (~3 g bw) (Talukdar *et al.*, 2020). Also, for *Chelon labrosus* juveniles (~6 g bw), a 30% of dietary crude protein produced the best growth and feed conversion ratios (Altunok and Özden, 2017). In the case of *Liza aurata*, a study in higher juveniles (~ 50 g bw) revealed no statistical differences in fish growth when feeding from 25% to 45% of dietary crude protein. However, a trend of better growth and feed efficiency was founded with increased dietary protein levels of up to 45% (Karapanagiotidis *et al.*, 2014).

Regarding dietary lipid requirements, optimal levels are suggested to range from 5% to 9% in different mullet species (Rangaswamy *et al.*, 1998; Debasis *et al.*, 2012; Yang *et al.*, 2015). However, for both dietary protein and lipids, there is a great variation in the referenced levels utilized by different authors (25-45% for protein, 5-16% for lipids),

depending, between others, on the mullet species and size (Rangaswamy *et al.*, 1998; Wassef *et al.*, 2001; Karapanagiotidis *et al.*, 2014; Gisbert el al., 2016).

Mullets are opportunistic fishes with a great capacity to adapt their natural diet to the surrounding resources available (Whitfield *et al.*, 2015). In fact, there is a high controversy about the trophic level of mullet species, as the relevance of animal prey in mullet diets greatly varies between species and even geographically within the same species; having been suggested a trophic level between 2 and 3 for most studied species of Mugilidae (Cardona *et al.*, 2015; Salvarina *et al.*, 2016). In this regard, enzymatic studies are crucial to shed additional clues on this issue.

The escalating prizes and scarcity of fish meal and fish oil for animal nutrition have promoted research on alternative ingredients to fit the demand for aquafeed production. In this sense, mullets represent a unique opportunity to utilize different raw materials supported at low dietary levels in other marine species. For instance, the green algae *Ulva lactuca* at 20% of inclusion, yeast at up to 40% of inclusion (Wassef *et al.*, 2001), spirulina (*Arthrospira platensis*) at up to 22.5% (Rosas *et al.*, 2019), zooplankton biomass up to 30% (100% of FM substitution) (Abo-Taleb *et al.*, 2021a; Abo-Taleb *et al.*, 2021b), and fermented plant feedstuff up to 80% (75% of FM substitution) (De *et al.*, 2018) have been tested in mullets with successful results.

In addition to the suggested potential of mullet species to digest carbohydrates and proteins from vegetable sources in comparison with other marine aquaculture species, the Mugilidae offer great potential for the utilization of free fish oil diets due to the ability to transform 18 C fatty acids to LC-PUFA (Galindo *et al.*, 2021). Marine fish generally have a limited activity of the enzymes involved in the elongation (*Elovl* enzymes) and desaturation (*Fads2* enzymes) of 18 C fatty acids precursors, being the Δ4 and the Δ6 activities the latest responsible for the formation of LC-PUFA (Figure 1.10). For those reasons, LC-HUFA are considered essential in the diets of most marine fish, directly impacting fish welfare and health (Montero and Izquierdo, 2010; Oliva-Teles *et al.*, 2015). However, it has been described both Δ6 and Δ8 activities in *Fads2* from *Chelon labrosus*, appearing the species phylogeny a more relevant factor for the LC-PUFA biosynthesis capacity than the trophic level by itself (Garrido *et al.*, 2019). As well, studies in other mullet species provide some clues about the potential of this family to biosynthesize LC-PUFA (Mourente and Tocher 1993; Khériji *et al.*, 2003; Imen *et al.*, 2013; Rabeh *et al.*, 2015); although more research on this field is needed to fully understand the potential of Mugilidae as free-fish oil aquaculture species in the novel prospect of sustainable aquaculture.

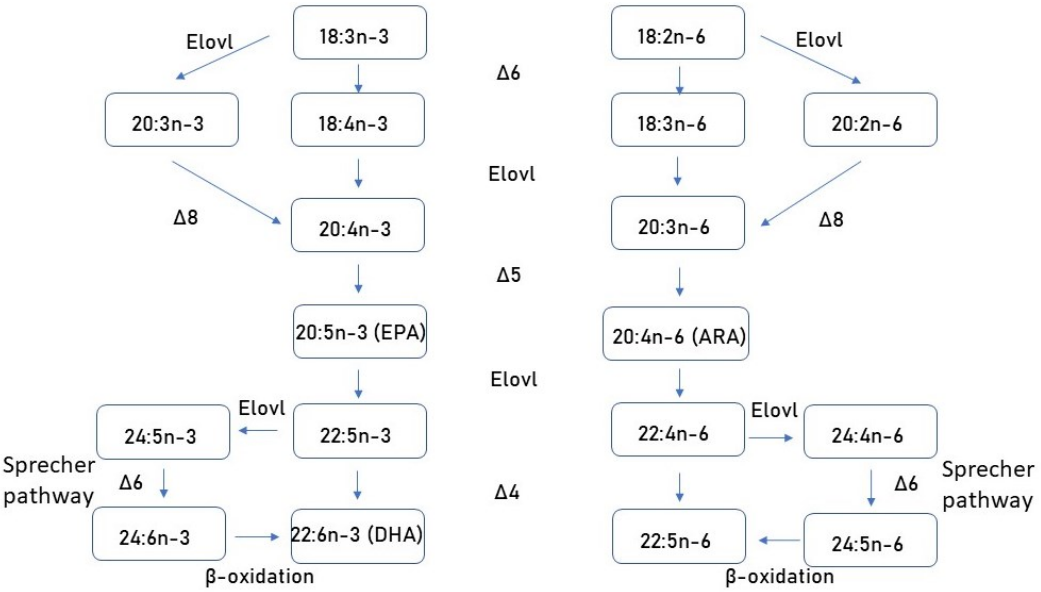


Figure 1.10. Metabolic routes of highly unsaturated fatty acid biosynthesis in teleost. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Δ: delta desaturases; Elovl, elongases. Adapted from Galindo *et al.* (2021).

1.7. Objetives

The main general objective of this thesis is to contribute to increasing the knowledge to optimize the culture of three important mullet species present in the Mediterranean and Atlantic coast, *Liza aurata*, *Chelon labrosus* and *Mugil cephalus*.

To achieve this general objective, 2 specific objectives were proposed:

1. Insight into the controlled reproduction and larval rearing of Mugilidae, which included fishing, acclimatization, the establishment of a suitable broodstock in captivity, a first approach to the reproductive lipid metabolism and final obtention of viable spawns (first chapter), and the improvement of the knowledge to optimize larval rearing practises (second chapter).

2. Increase the knowledge of the nutritional management under a sustainable approach, including a combined study assessing the feeding management of *Chelon labrosus* and *Liza aurata* reared under the same conditions (third chapter), the valorization of local *Aloe vera* by-product in diets for *Liza aurata* (fourth chapter) and the evaluation of the effects of diet and salinity on LC-HUFA biosynthesis potential of *Chelon labrosus* (fifth chapter).

2. General Methodology

2.1. General material and methods

To achieve this thesis's general and specific objectives, the PhD student participated in different zootechnical activities such as capture, transport, and acclimatization process to the captivity of wild specimens of *Chelon labrosus* and *Liza aurata*, both in the juvenile and adult phase, in possession of the government relevant permissions.

The experiments with animals, both with larvae and juveniles, were carried out in triplicates, considering the tank as the experimental unit. Both open seawater systems (chapters one, three and four) and recirculation aquaculture systems (RAS) (chapters two and five) were used. The manipulation and sacrifice of fish were rigorously conducted according to the European Union Directive (2010/63/EU) and Spanish legislation (RD 1201/2005) on animal welfare protection for scientific purposes, being the experimental protocols sent to the Bioethical Committee of the University of Las Palmas de Gran Canaria for approval.

The experimental part of chapter one was made in collaboration with the Centro Integrado de Formación Profesional (CIFP) Marítimo Zaporito, in San Fernando (Spain). The second chapter was done in collaboration with the International Marine Center (IMC) foundation in Oristano, Italy, as part of the stay in a foreign research centre contemplated by the PhD program of the ULPGC. The third, fourth and fifth chapters were carried out in the facilities of the Research Aquaculture Group (GIA) located at the ECOAQUA University Institute in the Marine Scientific Technological Park of Taliarte (PCTM) belonging to the University of Las Palmas de Gran Canaria (ULPGC).

Chapters one to four have been published in journals indexed in the first quartile score (Q1), while chapter 5 is currently being considered for publication.

2.2. Capture and acclimatization of mullets

The establishment of new species for aquaculture diversification relies on an initial phase of wild specimen's capture and their subsequent acclimatization to culture conditions. Fishing techniques depend on the capture site, the specie's behaviour, and the technical possibilities. Also, transport conditions and duration, and specificities of the acclimatization tanks are essential factors for the success of the adaptation to captivity.

There are reports from the ancient Romans which already established the mullet's susceptibility to handling, as it was reviewed by Raffaele del Rosso (1905), which states, "The muggine cannot be caught for stocking with a net, if its scales are touched it soon dies" (Crosetti, 2015).

During this PhD thesis, different artisanal techniques were applied to capture wild specimens of *Chelon labrosus* and *Liza aurata* depending on the capture site, the size of the objective animals, and the experience acquired. Since the referenced capture techniques for mullets depend greatly on the site, the capture techniques utilized in this PhD thesis were adapted following the recommendations of experimented local fishermen. The capture sites in two Sports piers of the island of Gran Canaria were chosen due to the recommendations of the local fishermen based on the high availability of specimens and the feasibility of the sites for capture and transport processes. However, in the first attempt, fish started with acute infection symptoms (fin and tale haemorrhage, desquamation, and debility) 24 h after the capture. For that reason, some specimens were sacrificed in iced cold water, and samples of the liver and spleen were evaluated for microbiological study. After isolating *Vibrio vulnificus* from the samples, preventive treatments were applied to the consequent fish acclimated to prevent the post-stress immunosuppression suffered by the animals. Additionally, a histological study of some wild specimens was done to evaluate the basal health status of the animals from the Sport Pier of Las Palmas de Gran canaria (samples of liver and intestine), and to compare it with some wild specimens of an open area (La puntilla, Las Palmas, Spain) in which it was possible to collect some specimens at a small scale. The study revealed an important presence of granulomas, necrosis and inflammatory infiltrate in the liver of the Sports Pier specimens (Figure 2.1) while in general, the animals from the open zone of "La Puntilla" presented a healthy structure of the tissues evaluated.

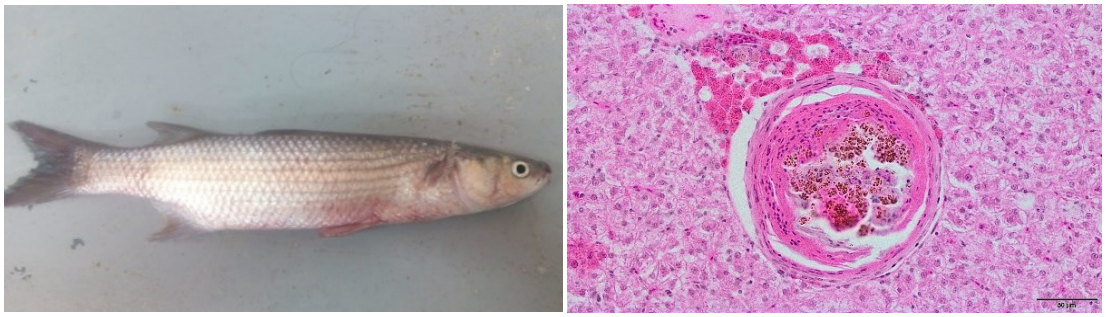


Figure 2.1. a) Post-capture mortality after acute septicaemia in an adult of *Chelon labrosus*. b) Hepatic granuloma surrounded by macrophages containing zymogen granules in a wild *Liza aurata* juvenile from the Sports Pier of Las Palmas de Gran Canaria. Bars 50 µm.

Table 2.1 summarize the protocols used for capture and acclimatization during this PhD thesis. As conclusions from experience acquired, extremely careful handling, preventive treatments at arrival, and relatively large acclimatization tanks are recommended to maximize survival. However, the success of this process depends on diverse factors which must be considered in each case. For instance, the initial health status of the animals had a significant impact, as can be observed in the last attempt of capture in San Fernando, Cádiz, in which the feasibility of capturing the animals in a site with high environmental quality and with the technical conditions to implement extremely smooth capture techniques, allowed to avoid the use of preventive treatments at arrival, with quite good survival results during the acclimatization process.

Table 2.1. Capture and acclimatization features for *Liza aurata* and *Chelon labrosus*.

Date	Place	Species	Fishing technique	Transport	Treatments	Acclimatization tanks	Symtoms	Mortality
October 2017	Las Palmas de Gran Canaria Sports Pier (Gran Canaria, Spain)	*Chelon labrosus* and *Liza aurata* (juveniles)	Fencing nets. Sunset	Tanks with oxygen	Oxytetracycline bath 20mg/L 5 consecutive days	500 L	Acute infection symtoms (desquamation, haemorrhages, lethargy)	>50%
June 2018	Castillo del Romeral (Gran Canaria, Spain)	*Liza aurata* (adults)	Fishing rod. Sunset	Tanks with aireation	Enrofloxacin bath 5 consecutive days (1mg/L)	500 L	Acute infection symtoms (desquamation, haemorrhages, lethargy)	>50%
June 2018	Pasito Blanco Sports Pier (Gran Canaria, Spain)	*Chelon labrosus* (adults)	Fishing rod. Sunset	Tanks with aireation	Enrofloxacin bath for 3 consecutive days (1mg/L) and intramuscularly injected at day 3 (20 mg/Kg). Formaldehyde baths from day 4 to 6	500 L	Acute infection symtoms (desquamation, haemorrhages, lethargy)	>50%
June 2018	Pasito Blanco Sports Pier (Gran Canaria, Spain)	*Chelon labrosus* (adults)	Fishing rod. Sunset	Tanks with oxygen	Two intramuscular enrofloxacin injection (20 mg/Kg) 48h apart. Formaldehyde baths from day 0 to 3	10,000 L	Sub-acute infection symtoms (fins and tale haemorrhages, lethargy)	<50%
September 2020	San Fernando (Cádiz, Spain)	*Liza aurata* adults (semi-wild population)	Fencing nets. Daylight	Plastic bags with oxygen	-	10,000 L	None	<10%

2.3. Species identification by molecular cloning

The Mugilidae family comprises many species with common morphological features such as medium-large size, subcylindrical body, muscular stomach, and long intestine. Due to their similar appearance, anatomical features such as the scale's structure, nostrils position, presence of an adipose eyelid or the number of pyloric caeca have been broadly used to discriminate between mullet species (Gonzalez-Castro and Ghasemzadeh, 2015). However, these procedures necessarily imply manipulation or sacrifice, so more recent techniques, such as molecular taxonomy, are gaining acceptance to differentiate mullet species.

For those reasons, one of the needs found during the PhD thesis was to efficiently corroborate the species of individuals recollected from the sea as future broodstock, with minimal animal damage. For that purpose, after acclimatization and a first classification by morphological parameters, the fish selected for broodstock purposes were anaesthetized (clove oil and ethanol 1:1 at 50 ppm), pit-tagged, and samples of the caudal fin were collected and stored in ethanol (70%) until molecular analyses were carried out (detailed in section 2.10.1).

2.4. Production of experimental diets

Diets for experiments three, four and five were produced in the Pilot Product and Processing Plant facilities at Ecoaqua-ULPGC Science and Technology Park in Taliarte, Telde, Spain. The ingredients were weighed, hand-mixed and pelletized to the desired pellet size for each trial (CL3, CPM, Spain). Pellets were dried in an air stove (38 °C) for 24 h before being stored at 11 °C.

2.5. Sampling protocols

For experiments with juveniles, samplings for measuring the total weight and length were done at the beginning and intermediate points (every 3 weeks), and the end of the trials after a fasting period of 24h, except for chapter 3, in which the final sampling was carried out in two steps, 15h and 40h after feeding to collect the gut samples at different moments along the digestion process. For proper manipulation, fish were anaesthetized with clove oil diluted with ethanol (1:1) at a concentration of 7-8 ml/100L of seawater. When needed, individuals were sacrificed by clove oil overdose (chapters one and four) or by immersion in iced cold water (chapter two, three and five). For the co-feeding trial with *Mugil cephalus* (chapter two), 25 larvae were randomly collected at initial, intermediate, and final points to measure the total length, wet and dry weight (15 larvae placed in cover glasses and weight prior and after being dehydrated in a laboratory oven (60°C, 48h). The specific samples taken from each experiment are defined in their correspondent chapters.

2.6. Biochemical analyses

Samples of whole fish and tissues for biochemical analyses were done in pools considering a minimum of three fish per tank (9 fish per treatment), which were properly homogenized and stored at -80°C until analyses could be conducted.

2.6.1. Moisture analysis

The moisture content was determined by dehydration of a known weight of the sample (around 0.5 g) in an oven at 110°C for 24h, and the weighing of the dehydrated sample in an analytical balance until constant weight (AOAC, 2000).

The percentage of moisture was calculated as follows:

% Moisture = (wet weight sample – dried weight sample) x 100

2.6.2. Ash content analysis

The ash content of the samples was determined by incineration in an oven at 600 °C for 24h (AOAC, 2000), so the organic matter is calcined, and the remaining material is composed just by the inorganic fraction.

The ash content was calculated as follows:

% Ash = (sample weight – ash weight) x 100

2.6.3. Crude protein content analysis

The protein content of the samples was calculated based on the total nitrogen content of the samples, following the Kjeldahl method (AOAC, 2000). For that, samples were digested with 10 ml sulfuric acid for 60 min at 400°C in the presence of a catalytic tablet with 1.5% $CuSO4 \cdot 5H2O$ and 2 % of selenium (Panreac, Barcelona, Spain). Subsequently, the digested material was distilled in 1% boric acid with 20 ml of distilled water and 50 ml of sodium hydroxide (40% w/v). Finally, the ammonia released was quantified through titration with hydrochloric acid (HCl) 0.1 M. Crude protein content was determined by the following formula:

% Protein = 100 x ((ml HCl samples–ml HCl blank) x 0.1 x 14.007 x 6.25) / sample weight

2.6.4. Total lipid content analysis

To determine the total lipidic content, around 0.2 g of the sample was weighed, and chloroform/methanol (2:1 v/v) with 0.01% butylated hydroxytoluene (BHT) was added. After 5 minutes of homogenization with an ultraturrax (T25 Digital Ultra-turrax, IKA®, Germany), the addition of 2 ml of 0.88% KCl, and the centrifugation of 5 minutes at 2000 rpm (revolutions per minute), the lower phase with the lipids was filtered and

evaporated to dryness under a nitrogen atmosphere (Folch *et al.*, 1957). Finally, the lipids were weighed, and their percentage in the sample was calculated following the formula:

% Lipids = (lipids weight / sample weight) x 100

2.6.5. Fatty acid profile

For fatty acid profile determination, the total lipids were trans-esterified by incubation with methanol: sulphuric (1%) in a nitrogen atmosphere and darkness, 16h at 50°C (Christie, 1989). Then, after cooling, ultrapure water and hexane:diethyl ether were added, and centrifugation was made at 2000 rpm (5 min) to separate the fames (fatty acid methyl esters) in the superior phase. Then, they were filtered, diluted in hexane, and evaporated to dryness under a nitrogen atmosphere to be weighed and diluted in hexane at a final concentration of 40 mg/ml. The identification and quantification of the different fatty acids were carried out through gas chromatography, following the protocol described by Izquierdo *et al.* (1992).

2.7. Thiobarbituric acid reactive substances (Tbars) determination

The malonaldehyde content was determined in a 10 mg/ml dilution of the total lipids of the sample, according to the modified protocol of Burk *et al.* (1980). First, the lipids were diluted with 2:1 chloroform-methanol with BHT. Then, 0.2% BHT in absolute ethanol, 0.02 M thiobarbituric acid (TBA), and 10% trichloroacetic acid (TCA) were added, and the mixture was incubated for 20 min on a heating mantle at 100°C.

After centrifugation for 5 minutes at 2000 rpm, the absorbance of the supernatant was detected by spectrophotometry at 532 nm, and the malonaldehyde was calculated following the formula:

Nmol malonaldehyde (MDA)/g lipid = (Abs λ 532/0.156) *(1/g lipids)

2.8. Histological analyses

After being fixed in 4% formalin a minimum of 24 h, the samples were placed in cassettes, washed with water to eliminate the remains of formaldehyde, and dehydrated by graded ethanol series in a sample processor (Histokinette 2000, Leica, Nussloch, Germanyand). After this, samples were embedded in paraffin blocks, and layers of 4 µm were made using a microtome (AUTOCUT JUNG 2055, LEICA, Lyon, France). Finally, the sections were placed in crystal slides and stained following the haematoxylin and eosin (H&E) technique (Martoja and Martoja-Pierson, 1970).

2.9. Serum parameters

For serum obtention, the latest techniques referred from GIA facilities by Serradell *et al*. (2020) were used, from which blood samples were obtained from the caudal vein of the anaesthetized animals and were stored at 4 °C for 24h. After this period, blood was centrifuged at 3000 rpm (5 min at 4°C), and the supernatant (serum fraction) was separated with a pipette and stored at -80°C until analyses.

2.9.1. Lysozyme activity

The determination of lysozyme activity was performed by turbidimetry according to the technique of Anderson and Siwicki (1994). The serum was faced with a suspension of *Mycrococcus luteus* (0.2 g/l) in a sodium phosphate solution (pH 6.3, 25°C). The absorbance was measured in a plate reader at 540 nm (Thermo Scientific Multiscan FC, type 357, Finland) every five minutes for one hour from the moment the bacterium solution was added.

To obtain a regression equation, a standard curve was also made facing the *Micrococcus* solution to different concentrations of standard egg lysozyme solution (40,000 units/mg protein). The results are expressed in units of lysozyme/ml of serum. A unit of lysozyme is defined as the amount of enzyme needed to produce a decrease in absorbance of 0.001/min.

2.9.2. Bactericidal activity

The bactericidal activity in serum was carried out to evaluate its effect on the growth curve of *Listonella anguillarum*, following the protocol described by Sunyer and Tort in 1995. For that, the bacterial culture was made in agar BHI (Brain Heart Infusion), from which one colony was inoculated in BHI solution and incubated for 16h at 25 °C in agitation. After this, a 1:10 dilution was made to be faced to the serum in the moment of exponential growth (0.5-0.8 of absorbance at 620 nm) in a 96 cells plate. Also, both positive control (only de bacteria solution) and a blank for each sample were made. Finally, the percentage of bactericidal activity was calculated as a function of the decrease in the absorbance at 620 nm of the bacterial solution because of the bactericidal activity of the serum after 24h incubation at chamber temperature, following the formula:

Serum bactericidal activity (%) = 100* (bacterial solution absorbance - sample absorbance)/ bacterial solution absorbance

2.10. Molecular analyses

2.10.1 DNA extraction and sequencing

All sample treatments and analyses were done at the GIAgen laboratories in Taliarte, Gran Canaria. The DNA of the fin samples was extracted using the kit DNAeasy of Quiagen. For that, the sample was first lysed by incubation overnight with proteinase K at 56 °C and agitation. Then, after adding the commercial buffer and ethanol, agitation and centrifugation, the mix was transferred to the column where the DNA was retained. Finally, after three washes and centrifugations with the corresponding commercial buffers, sterilized miliQ water was applied to the column to dissolve the DNA, which was recovered after the last centrifugation at 8000 rpm.

The quality of the DNA was then evaluated by electrophoresis in agarose gel (1%) and then quantified in a 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

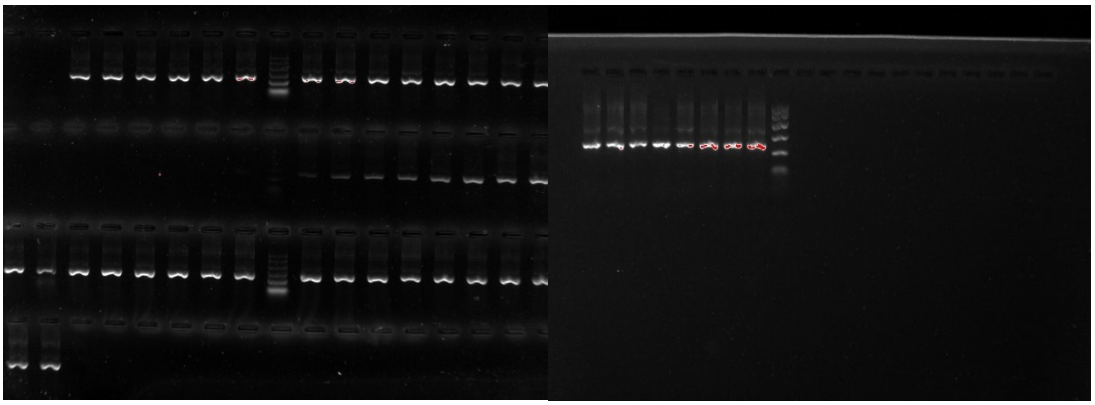


Figure 2.2. Agarose gel electrophoresis results of the DNA material obtained from mullet's fins. Each well correspond with an individual fish.

The genetic identification was carried out using the methodology described by Imsiridou *et al*. (2007) through polymerase chain reaction (PCR) amplification of the 5S rDNA gene, using the primers described by Pendas *et al*. (1994). The PCR products were sequenced by an external company (Macrogen CO., LTD) and the 5S rDNA sequences of each fish were aligned using Molecular Evolutionary Genetics Analysis Software (Mega X). The conserved segments were compared using the nucleotide databases of the National Center of Biotechnology Information website (NCBI), which allowed to corroborate the species membership.

2.10.2. RNA extraction and gene expression analyses

Gene expression analyses were carried out in the larvae of *Mugil cephalus* (second chapter) and the livers of *Chelon labrosus* juveniles maintained in different salinities and fed with a control diet and a diet without fish oil inclusion (fifth chapter). Samples were placed in RNA later solution, stored at 4°C for one week, and then frozen at -80°C until gene expression analyses.

The first step was to extract the RNA, for which the commercial kit RNAeasy® MiniKit from Quiagen was used. Next, 1 µg RNA of each sample conversed into cDNA through the iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California). For that, the RNA was denaturalized in a thermal cycler at 65 °C for 10 min. The denaturalized RNA was then combined with a mix containing the transcriptase enzyme (20 µl of final volume) and incubated for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The cDNA obtained was then diluted 1:10 with miliQ water.

Real-time PCRs were realized using an l-cycler with an optical module (Bio-Rad Hercules, Ca, USA) in a final volume of 15 µl containing 7.5 µl of iQTM-SYBER® Green Supermix (Bio-Rad Hercules, Ca, USA), 5 µl of cDNA, 0.75 µl of each primer (forward and reverse), and 1.0 µl of miliQ water. Three different housekeepers were evaluated for each study, being the 18S-rRNA gene those one with the best efficiency in both cases. All primer's efficiencies were evaluated with serial dilutions (1:5/1:10/1:100/1:1000) of a cDNA pool. All reactions were performed in duplicate for each sample, and blank control reactions were executed, replacing the cDNA for miliQ water. Relative gene expression was determined by the 2 −ΔΔ CT method (Livak & Schmittgen, 2001).

Primer sequences and conditions for the real-time reactions, adapted from referenced protocols to the conditions used in the laboratory, are resumed in a table for each experiment in chapters 2 and 5.

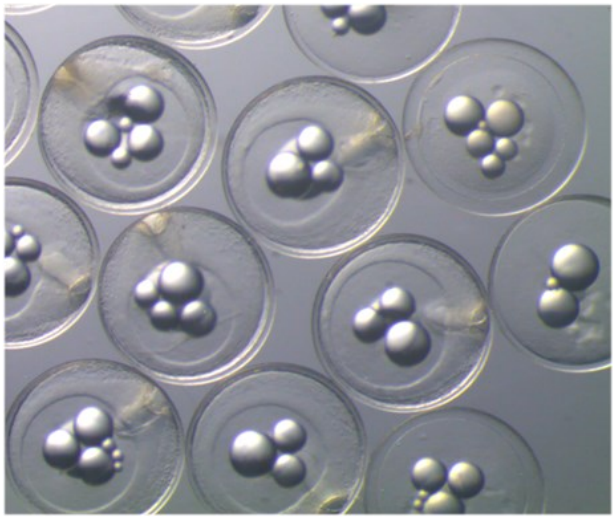
2.3. Statistical analyses

All experimental procedures with larvae and juveniles were performed in triplicates, where each tank worked as a replica. In general, data are expressed as means ± standard deviation. Chapters one, two, three and four were analysed with the program IBM® SPSS Statistic 20 (New York, USA). Data from chapter 5 was analysed with the program R Project for Statistical Computing. In all cases, it was confirmed that the data complied with the principles of normality and homoscedasticity (Shapiro-Wilk test and Levene's test, respectively) to compare the means with a one-way ANOVA (chapters one, two, and four) or a two-way ANOVA (chapter 3 and 5). In cases where data did not meet these requirements, medians were compared with a non-parametric test (Kruskal-Wallis). Additionally, in cases where less than three groups were compared, a T-student test was used instead of an ANOVA. For all cases, statistical differences were considered when $p<0.05$.

3. Results and discussion. Scientific publications

3.1. Reproductive management of the mugilid *Liza aurata* and characterization of proximate and fatty acid composition of broodstock tissues and spawnings



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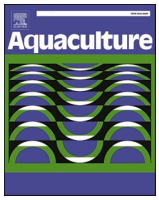

Reproductive management of the mugilid *Liza aurata* and characterization of proximate and fatty acid composition of broodstock tissues and spawnings

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ABSTRACT

The golden grey mullet (*Liza aurata*) is a promising species for aquaculture's sustainable expansion. However, the lack of sustainable juvenile provision, mainly related to the lack of reproductive control, is one of the most significant bottlenecks for further expanding the culture of this species. In many cases, mullet broodstock management needs the application of hormone treatments to induce gonadal maturation or spawning. However, no works are directly related to the broodstock collection, acclimation, and reproductive management of *Liza aurata*. On the other hand, the knowledge of essential fatty acids (EFA) requirements and mobilization patterns by the broodstock is a first step to designing appropriate feeding protocols and formulas, which are crucial for the success of reproduction and larval development.

For these reasons, this study aimed 1) to describe for the first time the reproductive management of *Liza aurata* broodstock under controlled conditions and 2) to offer a first approach to the reproductive lipid metabolism of the former species.

A selection of 22 *Liza aurata* broodstock from wild origin was acclimated in open seawater conditions. Additionally, the proximate and fatty acid composition of body tissues (gonads, liver, and muscle) of the initial wild population were evaluated, to be later compared with the profile of the eggs obtained after one year from the selected broodstock, for the first time described under cultured conditions.

The results highlighted the feasibility for the obtention of natural spawnings from broodstock with a mean weight of 787 g and 604 g (females and males, respectively), at a sex ratio of 2:1 (females/males), under natural photoperiod and marine water conditions with temperatures decreasing from 20.4 ± 0.3 °C to 18.8 ± 0.4 °C.

On the other hand, it was evidenced the crucial role of HUFA (highly unsaturated fatty acids) precursors for the gonadal development of *Liza aurata*, primarily for females. Additionally, the wild males' gonads presented a remarkable high content of HUFA, predominantly DHA (docosahexaenoic acid) (34% of the total fatty acids). In the eggs, significant variations appeared under captivity conditions, with lower levels of ARA (arachidonic acid) and EPA (eicosapentaenoic acid) and higher EPA/ARA and DHA/ARA ratios than the wild female gonad. Additionally, it was evidenced the significant role of the liver as a physiological reservoir of HUFA, which seem to be mobilized to the gonad during the maturation process.

Present results may help obtain a better insight to adjust broodstock management conditions and feeds, contributing to a more sustainable aquaculture growth.

1. Introduction

Aquaculture's diversification has been considered for the last decades as one of the major tools for a greater and more sustainable expansion of the sector (Abellán and Basurco, 1999). Among the different fish species considered for aquaculture diversification, mullets (Family Mugilidae) are great candidates due to their euryhaline, eurythermal, and opportunistic feeding nature, which have propitiated its extensive culture for centuries in different regions worldwide. However, its cultivation is supported traditionally, and still today, by wild fry capture, which

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produces high mortalities (from 70% to 96%), and, therefore, may be an unsustainable practice in the short term (Crosetti and Blaber, 2016). Despite fry collection being regulated in some of the countries involved, the lack of reproductive control is one of the most significant bottlenecks for further expanding the culture of these species.

Mullets generally have separate sex with no sexual dysmorphism, external fertilization and development. Females have group-synchronous ovaries (Wallace and Selman, 1981), being total or partial spawners, following the assignment of Hunter (1992). The golden grey mullet (*Liza aurata*) is one of the most abundant mullet species on the Atlantic coast and Mediterranean (Crosetti and Blaber, 2016). Despite having a smaller adult size than other mugilids, it has a great performance in the early stages (Hotos and Avramidou, 2020; Monzon et al., unpublished results), a slender body than other mullet species and a great fillet quality (Quirós-Pozo et al., 2021), making it an interesting choice for the development of its culture. However, the knowledge about reproductive characteristics is still very scarce, being the basic physiological and metabolic mechanisms unknown.

In this regard, the success in establishing new species is directly related to the controlled production of good quality eggs and larvae, which guarantee an adequate supply of juveniles (Izquierdo et al., 2001). Among the different parameters affecting egg quality, proximate composition, and especially, fatty acid profile are determinant factors affecting larval growth, survival, and early development of organs and tissues (Izquierdo and Koven, 2011, in Roo et al., 2015).

In this sense, broodstock nutrition not only affects the chemical composition of the eggs but also is one of the most significant factors affecting the success of the reproductive process (Fernández-Palacios et al., 2011). Particularly, dietary n3- HUFA affects ovarian steroidogenesis and vitellogenesis by altering FSH and LH levels in fish (Peng et al., 2015). Specifically, docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) play an essential role in maintaining the structure and function of cell membranes, being the DHA the main component of glycerophospholipids in marine fish eggs (Izquierdo, 1996). Furthermore, arachidonic acid (ARA, 20:4n-6) derivates also participate in several reproductive functions in fish, being involved in processes like pheromonal attraction (Stacey and Sorensen, 2006), steroidogenesis (Henrotte et al., 2011; Norberg et al., 2017), fecundity, hatching rate and yolk sac diameter (Røjbek et al., 2014; Asil et al., 2017; Stuart et al., 2018). Besides the optimal ratios between these nutrients are crucial in broodstock diets since DHA/EPA and EPA/ARA compete for the same enzymes, affecting egg and sperm quality (Fernández-Palacios et al., 2011; Estefanell et al., 2015). Fish eggs should contain all nutrients necessary for embryonic and early larval development, so their biochemical composition can be a great indicator to develop adequate feeds for broodstock in captivity conditions (Izquierdo et al., 2001). Besides, the body composition of wild fish in different reproductive stages may shed some clues on their nutritional requirements (Pérez et al., 2007).

For these reasons, this study aimed to describe the reproductive management which allowed the obtention of viable eggs of this species and to evaluate the biochemical composition and fatty acid profile of wild mature and immature *Liza aurata* broodstock tissues and the eggs obtained under culture conditions. Present results will help to define criteria for better management by producers and allow the formulation of more adequate diets for this species.

2. Material and methods

All the procedures with animals were rigorously conducted according to the European Union Directive (2010/63/EU) on animal welfare protection for scientific purposes and were carried out in coordination between the GIA-Ecoaqua institute (Canary Islands, Spain) and the aquaculture facilities of the CIFP Zaporito (San Fernando, Cádiz, Spain).

2.1. Broodstock collection and management

The experiment lasted for over one year, including fishing, sampling, broodstock acclimation and finally, the successful obtention of natural spawnings in captivity. In September 2019, 125 golden grey mullets (*Liza aurata*) were captured from a semi-natural estuary in San Fernando, Cádiz (Spain) (36° 27′ 56″ N; 6° 11′ 48″ W), where they were fed just from the natural food available.

After capture, fish were transferred to the aquaculture facilities of the CIFP Zaporito and sampled to be individually pit-tagged and biopsied, to determine the size, weight, sex ratio, and state of sexual maturation (Table 1). For the latter, fish were anesthetized with clove oil (40 ppm) and abdominal massage was applied to identify mature males. Negative animals for this test were gonadally biopsied with a 1.3-mm-internal-diameter catheter (Kruuse, Langeskov, Denmark). The sampled material was stored in Serra's fixative (6: 3: 1, 70% ethanol, 40% formaldehyde and 99.5% acetic acid) to later view under the microscope (148 LED Digital Zuzi®). The diameter of biggest oocytes (n = 30) was measured with the software TS View Zuzi®.

22 fish were selected as broodstock and acclimated in a separated tank of 10 m3 for one year, in an open seawater system under natural photoperiod (36° 27′ 56″ N; 6° 11′ 48″ W). Fish were fed once per day (0.5% of biomass day^{-1}) with a commercial extruded feed (R5 Europe, Skretting). To calculate the growth of the selected broodstock along the experimental period (Table 2), the following formulas were used:

$$\text{Weight gain (g)} = \text{final weight (g)} - \text{initial weight (g)}$$

$$\text{Weight gain (\%)} = (\text{final weight (g)–initial weight (g)}) \text{ x } 100/\text{initial weight}$$

$$\text{Specific growth ratio (SGR) (\%)} = (ln \text{ final weight}–ln \text{ initial weight})/\text{days of experiment x100}$$

2.2. Samplings of broodstock tissues and eggs

From the initial population (2019), four mature females, four mature males, and four immature mullets were sacrificed by clove oil overdose.

Table 1
Medium values of weight, length and somatic indexes in females, males and immature specimens of *Liza aurata* collected from the estuary.

	Mature females	Mature males	Immature
Weight (g)	475.00 ± 49.45^{a}	321.38 ± 66.74^{b}	280 ± 78.01^{b}
Lenght (cm)	38.80 ± 2.20^{a}	34.54 ± 3.02^{b}	30.98 ± 3.66^{c}
Population percentage (%)	8	10.4	81.6
HSI (%)	0.98 ± 0.19	0.94 ± 0.27	0.61 ± 0.07
GSI (%)	9.75 ± 1.40^{a}	2.53 ± 1.10^{b}	–

Data expressed as means ± SD (standard deviation). Values with different superscripts in the same row indicate the presence of significant differences ($p \leq 0.05$).

Table 2
Weight, length, and growth parameters of *Liza aurata* broodstock along the experimental period.

	Females	Males
Initial weight (g)	424.62 ± 74.54	352.75 ± 35.05
Initial lenght (cm)	37.23 ± 3.75	35.88 ± 1.36
Final weight (g)	787.40 ± 76.05	604.40 ± 48.64
Final lenght (cm)	38.55 ± 2.49	36.40 ± 2.72
Weight gain (g)	362.78	251.65
Weight gain (%)	85.43	71.34
SGR (%)	0.14	0.12

Data of weight and length expressed as means ± SD.

Table 3
Proximate composition in dry weight of different tissues of females, males and immature specimens of *Liza aurata* collected from the estuary.

	Gonad			Muscle			Liver
	Lipids	Protein	Ash	Lipids	Protein	Ash	Lipids
Females	32.88 ± 1.13^{a}	62.30 ± 2.30^{b}	3.31 ± 0.31^{b}	4.46 ± 1.88	90.88 ± 8.97	7.48 ± 0.58	20.67 ± 1.75^{b}
Males	24.49 ± 2.81^{b}	86.27 ± 4.68^{a}	7.67 ± 0.80^{a}	9.41 ± 1.39	90.77 ± 4.95	7.17 ± 0.42	22.73 ± 1.57^{ab}
Immature	-	–	–	6.23 ± 1.81	93.12 ± 4.33	7.69 ± 0.92	24.63 ± 1.42^{a}

Data expressed as means ± SD. Values with different superscripts in the same column indicate the presence of significant differences ($p \leq 0.05$).

Samples of muscle, liver, and gonads were collected for histological, biochemical, and gonadal development analyses. Gonado-somatic and hepato-somatic indexes (GSI and HSI) (Table 1) were calculated according to the following formulas:

$$\text{HSI}\ (\%) = \text{liver weight}\ (g)/\text{fish weight}\ (g) \times 100$$

$$\text{GSI}\ (\%) = \text{gonad weight}\ (g)/\text{fish weight}\ (g) \times 100$$

Samples of eggs from 5 different natural spawnings collected from October to November 2020 were also saved for biochemical analyses.

2.3. Biochemical and fatty acid analyses

Protein, moisture, and ash content of fish tissues (Table 3) and eggs (Table 5) were determined by the techniques described in AOAC (2000). Total lipids were quantified following the method described by Folch et al. (1957). For fatty acid determination (tissues, eggs, and commercial diet) (Tables 4 and 6), the total lipids were trans-esterified (Christie, 1989), and the fatty acids obtained were quantified by gas chromatography (Izquierdo et al., 1992).

2.4. Histological analysis

Gonadal samples from the initial population were fixed in 4% formalin and then dehydrated by graded ethanol series and placed in paraffin blocks. Thin layers (4 μm) were made using a microtome (AUTOCUT JUNG 2055, LEICA, Lyon, France), and the sections were placed in slides and stained following the haematoxylin and eosin (H&E) technique (Martoja and Martoja-Pierson, 1970). The histological evaluation was carried out by direct visualization to determine the maturation state of the fish according to the criteria described by Genten et al. (2009) and by González-Castro et al. (2011).

2.5. Statistical analyses

The statistical analyses were carried out using the program IBM®

Table 4
Fatty acid profile, expressed in % of the total fatty acids identified, of gonads, liver and muscle of wild females, males and immature specimens of *Liza aurata* collected from the estuary.

Gonads			Liver			Muscle		
	Females	Males	Females	Males	Immature	Females	Males	Immature
Saturated	15.36 ± 1.23	15.52 ± 1.68	34.29 ± 5.55	29.60 ± 2.27	29.03 ± 1.74	27.96 ± 2.39	22.62 ± 3.40	29.52 ± 5.03
Monoenoics	42.05 ± 1.76^{a}	17.57 ± 2.04^{b}	28.79 ± 3.62^{a}	28.01 ± 3.48^{a}	17.05 ± 3.55^{b}	38.23 ± 4.02^{ab}	40.86 ± 5.19^{a}	30.02 ± 3.48^{b}
n-3	24.16 ± 3.69^{b}	54.28 ± 3.81^{a}	22.11 ± 6.01^{b}	28.28 ± 2.20^{b}	39.57 ± 3.76^{a}	15.02 ± 1.38	18.44 ± 6.86	21.89 ± 6.38
n-6	16.24 ± 1.27^{a}	11.45 ± 1.38^{b}	13.70 ± 1.74	12.79 ± 1.03	12.96 ± 1.29	16.08 ± 2.11	15.76 ± 0.73	15.96 ± 1.02
n-9	27.00 ± 0.72^{a}	12.29 ± 1.57^{b}	17.55 ± 3.06^{a}	16.21 ± 2.34^{a}	9.20 ± 2.52^{b}	27.47 ± 1.13^{ab}	28.34 ± 0.42^{a}	21.02 ± 3.94^{b}
n3 PUFA	23.44.34 ± 3.75^{b}	53.34 ± 4.13^{a}	21.86 ± 6.01^{b}	28.03 ± 2.20^{b}	39.35 ± 3.75^{a}	13.95 ± 1.71	17.83 ± 6.81	21.06 ± 6.60
n6 PUFA	16.24 ± 1.27^{a}	11.43 ± 1.39^{b}	13.70 ± 1.74	12.79 ± 1.03	12.95 ± 1.30	16.06 ± 2.10	15.74 ± 0.73	15.94 ± 1.02
Total PUFA	41.16 ± 2.66^{b}	65.55 ± 2.93^{a}	36.13 ± 5.80^{b}	41.48 ± 2.46^{b}	52.89 ± 3.34^{a}	31.01 ± 1.35	34.56 ± 7.29	37.65 ± 7.41
14:0	0.76 ± 0.18a	0.30 ± 0.30b	0.88 ± 0.28	0.99 ± 0.64	0.98 ± 0.52	1.05 ± 0.63	1.39 ± 0.45	1.49 ± 0.68
16:0	10.65 ± 0.82	9.76 ± 1.86	21.80 ± 4.10	19.45 ± 3.56	17.26 ± 1.77	18.15 ± 1.66	15.06 ± 3.33	19.23 ± 3.15
16:1n-7	10.03 ± 1.81^{a}	1.39 ± 0.54^{b}	3.57 ± 0.32	4.13 ± 1.67	2.80 ± 0.34	4.65 ± 2.34	5.42 ± 1.22	4.55 ± 1.01
18:0	3.65 ± 0.30	4.99 ± 1.06	11.04 ± 1.36	9.83 ± 1.45	10.25 ± 0.46	8.16 ± 3.54	5.39 ± 0.62	7.96 ± 2.32
18:1n-9	25.80 ± 0.79^{a}	11.53 ± 1.38^{b}	16.93 ± 2.94^{a}	16.10 ± 2.76^{a}	8.72 ± 2.44^{b}	26.08 ± 1,17^{ab}	26.89 ± 3.84^{a}	19.98 ± 3,68^{b}
18:1n-7	4.68 ± 0.08^{a}	2.15 ± 0.28^{b}	5.14 ± 0.26^{a}	4.87 ± 0.46^{a}	3.29 ± 0.79 ^{b}	3.8 ± 0.32^{a}	4.03 ± 0.59^{a}	2.80 ± 0.21^{b}
18:2n-6	12.20 ± 1.12^{a}	3.66 ± 0.53^{b}	8.02 ± 1.10^{a}	6.99 ± 2.07^{ab}	4.25 ± 1.38^{b}	9.94 ± 0.46^{a}	10.30 ± 1.08^{a}	6.57 ± 1.60^{b}
18:3n-3	1.33 ± 0.24^{a}	0.56 ± 0.20^{b}	0.67 ± 0.22	0.89 ± 0.26	0.59 ± 0.11	0.97 ± 0.38	1.17 ± 0.25	0.76 ± 0.13
20:1n-9	0.07 ± 0.20	0.07 ± 0.02	0.13 ± 0.09	0.17 ± 0.04	0.06 ± 0.02	0.15 ± 0.06	0.21 ± 0.08	0.14 ± 0.04
ARA 20:4n-6	1.35 ± 0.17^{b}	3.87 ± 0.94^{a}	3.55 ± 0.66	3.34 ± 1.34	4.66 ± 0.80	3.43 ± 1.79^{ab}	2.38 ± 0.66^{b}	4.81 ± 0.60^{a}
EPA 20:5n-3	3.75 ± 0.26^{b}	10.28 ± 2.25^{a}	4.43 ± 0.74^{b}	4.47 ± 1.50^{b}	8.86 ± 2.13^{a}	3.48 ± 0.36	4.21 ± 1.68	6.82 ± 2.44
DHA 22:6n-3	12.90 ± 4.08^{b}	33.96 ± 5.53^{a}	13.86 ± 5.20^{b}	16.60 ± 6.36^{ab}	24.37 ± 3.27^{a}	6.65 ± 1.04	8.61 ± 3.80	9.19 ± 2.87
DPA 22:5n-6	0.71 ± 0.12^{b}	1.91 ± 0.40^{a}	0.65 ± 0.14^{b}	0.79 ± 0.38^{ab}	1.45 ± 0.52^{a}	0.88 ± 0.36	1.11 ± 0.56	2.17 ± 0.92
DHA/22:5n-6	18.03 ± 4.17	18.76 ± 5.91	21.64 ± 7.10	22.35 ± 8.86	19.02 ± 8.44	8.98 ± 5.17	7.99 ± 1.40	4.71 ± 2.06
EPA/ARA	2.79 ± 0.26	2.75 ± 0.58	1.26 ± 0.23	1.40 ± 0.44	1.91 ± 0.34	1.31 ± 0.83	1.77 ± 0.37	1.41 ± 0.45
DHA/EPA	3.44 ± 1.35	3.30 ± 0.89	3.13 ± 0.80	3.82 ± 0.58	2.86 ± 0.78	1.92 ± 0.32	2.03 ± 0.48	1.38 ± 0.31
DHA/ARA	9.54 ± 3.30	8.78 ± 3.17	3.90 ± 1.48	5.53 ± 1.91	5.42 ± 1.50	2.70 ± 2.18	3.59 ± 1.01	1.92 ± 0.63
Oleic/DHA	2.13 ± 0.53^{a}	0.35 ± 0.08^{b}	1.33 ± 0.49^{a}	1.22 ± 0.90^{ab}	0.37 ± 0.13^{b}	3.98 ± 0.56	3.75 ± 1.97	2.44 ± 1.17
Oleic/n3 PUFA	1.12 ± 0.18^{a}	0.22 ± 0.03^{b}	0.81 ± 0.25^{a}	0.58 ± 0.09^{a}	0.23 ± 0.08^{b}	1.89 ± 0.21	1.71 ± 0.73	1.06 ± 0.50
n-3/n-6	1.49 ± 0.36^{b}	4.74 ± 0.86^{a}	1.61 ± 0.55^{b}	2.22 ± 0.25^{ab}	3.08 ± 0.49^{a}	0.95 ± 0.19	1.16 ± 0.39	1.36 ± 0.34

Data expressed as means± SD. Values with different superscripts in the same row indicate significant differences ($p \leq 0.05$). Contains 14:1n-7, 14:1n-5, 15:0, 15:1n-5, 16:OISO, 16:1n-5, 16:2n-4, 16:3n-4, 16:3n-3, 16:3n-1, 16:4n-3, 17:0, 18:1n-5, 18:2n-9, 18:2n-4, 18:3n-6, 18:3n-4, 18:3n-1, 18:4n-3, 18:4n-1, 20:0, 20:1n-7, 20:1n-5, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 20:3n-3, 20:4n-3, 22:1n-11, 22:1n-9, 22:4n-6 and 22:5n-3.

SPSS Statistic 20 (New York, USA). Homogeneity of variances was performed using Levene's test ($p \geq 0.05$). To analyze the variance, a one-way ANOVA was used, being the means compared by Tukey post-hoc test ($p \leq 0.05$). In the cases where the data did not meet normality or homoscedasticity, the medians were compared using a non-parametric test (Kruskal Wallis) ($p \leq 0.05$).

3. Results

3.1. Broodstock collection and management

The average weight and length of the sampled population, as well as GSI and HSI from sacrificed animals are presented in Table 1. The percentage of mature females from the total identified (oocyte diameter ≥ 600 μm) was 39%, being their weight and length significantly higher than males and immature animals ($p \leq 0.05$). For the 22 fish selected as broodstock, both females and males almost double their weight in one year (Table 2), being the final sex ratio of 2:1 (females/males). Under these conditions, viable spawns were obtained from late September to late November of 2020 when temperature were decreasing from 20.4 ± 0.3 °C to 18.8 ± 0.4 °C.

3.2. Biochemical and fatty acid analyses

The proximate composition of the gonad from the initial population (Table 3) showed sex related differences. The lipid content in the ovaries was higher than in the testicles; on the other hand, protein and ash were lower in ovaries. In addition, females presented a lower percentage of hepatic lipids than immature animals.

The fatty acid composition of gonads, liver, and muscle (Table 4) also presented clear differences among groups. The gonad of initial mature females had higher levels of linolenic (LNA, 18:3n-3), linoleic (LA, 18:2n-6), monoenoic, n6, and n9 fatty acids than males. In comparison, initial male's gonads presented higher ARA, n3, and n3 PUFA (polyunsaturated fatty acids), particularly EPA and DHA, than female gonads, which caused an increase in the total PUFA and n3/n6 ratio.

In the liver, minor differences between sexes were found; however, in comparison with mature animals, immature ones presented lower levels of n9 fatty acids, oleic acid (18,1n-9), and oleic/n3 PUFA ratio, and higher levels of EPA, n3, n3 PUFA and total PUFA. In addition, levels of DHA and DPA (docosapentaenoic acid), were higher in immature animals than in females. Levels of n-6 were similar in all groups.

In the muscle, few differences between groups were found in the fatty acid composition. LA was higher in mature animals than in immature ones. ARA showed the highest levels in immature mullets, followed by females. EPA, DHA, DPA, total PUFA, n3, n6 fatty acids, and ratio n3/n6 were similar in all groups, the last one close to 1.

Regarding biochemical results of eggs obtained after one year in captivity (Table 5), they presented higher lipid content ($p \leq 0.05$) than the wild female gonad. The fatty acid composition of the eggs (Table 6), reflected in many cases the profile of the diet with some exceptions as the content of EPA, LNA, EPA/ARA and n3/n6 ratios, which were lower in the eggs, or the content of DHA and DHA/EPA ratio which were higher than those of the diet. The eggs fatty acid profile also showed many similarities with the wild female gonad, with some exceptions as the levels of ARA and EPA which were higher in the wild female gonad ($p \leq 0.05$). HUFA ratios also were quite different to those of the wild female gonad, with higher values of both EPA/ARA and DHA/ARA ratios for the eggs ($p \leq 0.05$).

Table 5
Proximate composition in dry weight of *Liza aurata* broodstock commercial diet and eggs.

Proximate composition (%)	Lipids	Protein	Ash
Diets	20	52	8.3
Eggs	39.15 ± 4.18	56.91 ± 9.24	4.23 ± 3.36

Data of the commercial diet obtained from the product label. Data of eggs is expressed as means ± SD.

Table 6
Fatty acid profile, expressed in % of the total fatty acids identified, of the commercial diet given to *Liza aurata* broodstock, and *Liza aurata* spawnings after one year in captivity.

	Diet	Eggs
Saturated	16.43	16.15 ± 0.67
Monoenoics	44.97	45.79 ± 0.99
n-3	22.52[a]	18.54 ± 0.93[b]
n-6	15.32	17.31 ± 0.90
n-9	29.12[b]	32.66 ± 0.94[a]
n3PUFA	22.15[a]	18.24 ± 0.77[b]
n6PUFA	15.05	17.07 ± 0.88
Total PUFA	43.59[a]	37.36 ± 1.00[b]
14:0	1.61	1.07 ± 0.32
16:0	10.67	11.31 ± 0.47
16:1n-7	2.90[b]	7.64 ± 1.09[a]
18:0	3.35	3.52 ± 0.26
18:1n-9	27.76	30.85 ± 1.08
18:1n-7	2.74	5.39 ± 0.96
18:2n-6	12.93	14.96 ± 0.75
18:3n-3	4.05[a]	2.46 ± 0.31[b]
20:1n-9	0.78[a]	0.10 ± 0.04[b]
ARA 20:4n-6	0.60	0.58 ± 0.09
EPA 20:5n-3	6.09[a]	2.33 ± 0.47[b]
DHA 22:6n-3	8.37[b]	9.83 ± 0.33[a]
DPA 22:5n-6	0.26	0.23 ± 0.02
DHA/22:5n-6	32.04	44.02 ± 4.27
EPA/ARA	10.09[a]	4.03 ± 0.47[b]
DHA/EPA	1.37[b]	4.35 ± 0.81[a]
DHA/ARA	13.87	17.33 ± 2.19
Oleic/DHA	3.32	3.14 ± 0.12
Oleic/n3 PUFA	1.25[b]	1.98 ± 0.12[a]
n-3/n-6	1.47[a]	1.07 ± 0.07[b]

Data expressed as means ± SD. Values with different superscripts in the same row indicate significant differences ($p \leq 0.05$). Contains 14:1n-7, 14:1n-5, 15:0, 15:1n-5, 16:OISO, 16:1n-5, 16:2n-4, 16:3n-4, 16:3n-3, 16:3n-1, 16:4n-3, 17:0, 18:1n-5, 18:2n-9, 18:2n-4, 18:3n-6, 18:3n-4, 18:3n-1, 18:4n-3, 18:4n-1, 20:0, 20:1n-7, 20:1n-5, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 20:3n-3, 20:4n-3, 22:1n-11, 22:1n-9, 22:4n-6 and 22:5n-3.

3.3. Histological analysis

Regarding the histological examination of the gonads (Fig. 1), the ovary of all females considered for the study showed a typical structure of fully mature spawners, with a synchronous group of larger oocytes full of vitellogenin next to a stock of previtellogenic oocytes. The male gonads were also fully mature, presenting testicular cysts full of spermatozoa.

4. Discussion

The obtained results of gonadal maturation and egg production identify the spawning season of *Liza aurata* in the north-eastern Atlantic region between September and November, which is in concordance with reported data from Hotos et al. (2000) for this species in the Mediterranean or by Ghaninejad et al. (2010) in the Caspian Sea, this last highlighting that the spawning is promoted by temperatures decreasing from 20 to 22 °C.

The average weight and length of mature females and males from the initial population sampled (475 g, 39 cm and 321 g, 35 cm, respectively) are quite similar to those reported by Fazli et al. (2008). The length reported in our study is higher than the length of first sexual maturity reported by Fehri Bedoui et al. (2002), who described an L50 (the length in which 50% of the animals were mature) of 21 cm for males, and 22 cm for females of *Liza aurata* in Tunisian waters, and also by Kesiktaş et al. (2020), who reported a length of first sexual maturity of 26 cm for males

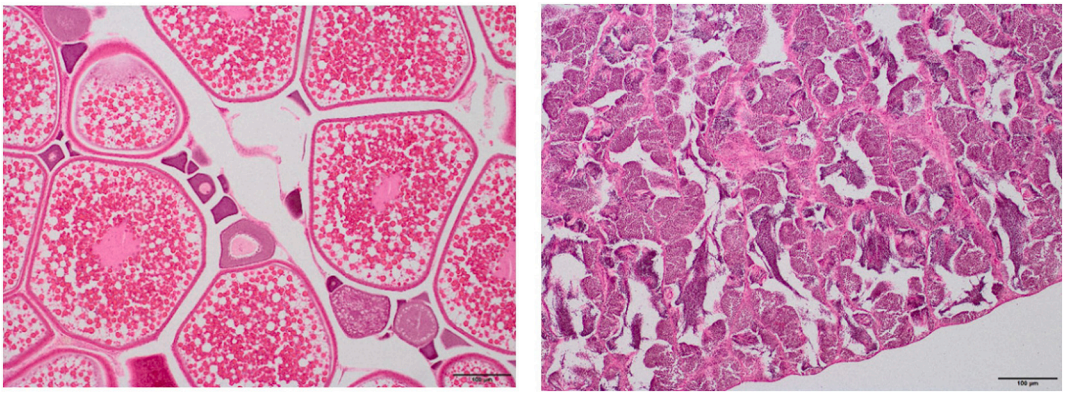

Fig. 1. Female (left) and male (right) gonads sections of mature *Liza aurata* specimens stained with H&E. Bars 100 μm.

and 24 cm for females. In this regard, present results suggest an adequate minimal weight for established wild collected *Liza aurata* broodstock above 300 g for males and 400 g for females.

Broodstock feeding with a commercial diet at 0.5% of biomass day^{-1}, resulted in fish almost doubling their weight in one year, with SGR values between 0.12 and 0.14 and successful natural spawns, indicating that this feeding dose seems to be adequate for broodstock maintenance. However, both dose and feeding frequency may be increased when the objective is the growth of the animals, as it has been described for mullet's juveniles (Calixto da Silva et al., 2020; Solovyev and Gisbert, 2022). Under the described conditions of salinity, temperature, and sex ratio, viable spawns have been obtained spontaneously, for first time described in captivity, which open new perspectives to increase the controlled culture of this species.

On the other hand, it has been widely described how the composition of the diet can be reflected in the fish tissues, especially in the gonads (Izquierdo et al., 2001; Jaya-Ram et al., 2008). As regards the evaluation of initial *Liza aurata* broodstock tissues, as the origin of the animals was the same, it can be supposed that the main differences in the lipids and fatty acids were primarily due to the specific physiological status of the animals. In general, for fish females, specific lipid mobilization patterns depend on whether the animals reduce their feed intake in the spawning season or not. In the first case, the body reserves supply the nutrients for ovary development (Aksnes et al., 1986; Lal and Singh, 1987). In species like the gilthead seabream (*Sparus aurata*), the Atlantic cod (*Gadus morhua*), or the smooth weakfish (*Cynoscion leiarchus*), which continue feeding during the spawning season, the lipids deposited in the ovary are both from the diet and the mobilization of the body reserves, mainly from the liver (Almansa et al., 2001; Dahle et al., 2003; do Carmo Silva et al., 2019). Based on the HSI and the condition factor (Kc), Fehri Bedoui et al. (2002) proposed that the golden grey mullet used both liver and muscle reserves to develop the gonads in both sexes. In the present study, the lipid content and fatty acid composition of liver was affected by the maturation state of the fish, being these differences less evident in the case of muscle. From the information listed above, it can be deduced that *Liza aurata* (primarily females) used both lipids from the diet and body reserves (including liver and muscle) to supply nutrients to the gonad during the maturation period.

In this study, the high levels of 18:1n-7, oleic acid and LA both in the wild female gonad and eggs reflected the importance of these fatty acids in the natural reproduction and embryonic processes of this species. Oleic acid has been described as a crucial fatty acid involved in gonadal maturation for both females and males of the white seabream (*Diplodus sargus*), increasing in the spawning season and decreasing after (Pérez et al., 2007). Also, in concordance with the previous study, in the present work the levels of oleic acid in the liver of both wild males and females were higher than those of immature animals, which confirm the role of the liver as a fatty acid reservoir to support the reproduction process along the spawning season. In the present study, also the amount of LA was high in both liver and muscle of mature specimens. This suggest that these fatty acids not only must be abundant in the natural diet of the fish under study but also that they have been selectively incorporated by mature animals to the tissues evaluated. LA levels were also markedly higher than those previously reported for other wild marine species (Pérez et al., 2007; Rodríguez-Barreto et al., 2012), however, they were like those reported for female gonads and eggs from fish maintained in captivity and fed with diets with certain contents of vegetable sources (Jaya-Ram et al., 2008; El-Husseiny et al., 2018; Ferosekhan et al., 2021).

Marine fish generally cannot transform 18C fatty acids into HUFA (Castro et al., 2012), so they must obtain these nutrients from the diet, however, it has been described how high availabilities of HUFA precursors can increase elongation and desaturation processes in some fish species (Ling et al., 2006; Jaya-Ram et al., 2008). In addition, it has been defined a certain capacity of HUFA biosynthesis for mullets (Garrido et al., 2019; Galindo et al., 2021), including fatty acyl desaturase 2 with Δ6 activity in *Liza aurata* (Mourente and Tocher, 1993). Also, it has been described for *Liza aurata* juveniles (Quirós-Pozo et al., 2021) higher levels of DHA and ARA in the whole body than those present in the diets, thus supporting the potential of this species to synthetize HUFA from its precursors. Moreover, the nutritional programming thought low FM and FO broodstock diets had been shown to influence HUFA biosynthesis metabolism in both parents and offspring, also affecting the posterior growth performance of the juveniles (Xu et al., 2019; Turkmen et al., 2019). Furthermore, increased dietary LNA/LA ratios produced higher plasma estradiol and gonadosomatic index in species with HUFA biosynthesis potential like the common carp (*Cyprinus carpio*) (Ma et al., 2020). This data supports the potential feasibility of Liza broodstock diets containing high levels of vegetable sources as an alternative to marine fish meal and oil ingredients, however, correct n3/n6 ratios may be taken under consideration.

On the other hand, male gonads showed a notary predominance of n-3 HUFA, primarily due to high levels of DHA, which is in concordance with its recognized influence on the reproductive process and fertility (Mansour et al., 2011). In different species such as the rainbow trout (*Oncorhynchus mykiss*) or the seabass (*Dicentrarchus labrax*), (Izquierdo et al., 2001), the sperm fatty acid composition depends on the essential fatty acid content of the broodstock diet. The levels of DHA in the sperm of *Liza aurata* males (34 ± 6%) were higher than those reported in other fish species like the Arctic char (*Salvelinus alpinus*) (26–24%) (Mansour et al., 2011), the sterlet (*Acipenser ruthenus*) (14%) (Engel et al., 2020), the wild white seabream (12.24%, calculated data) (Pérez et al., 2007), the gilthead seabream (*Sparus aurata*) (10.5%, calculated data) (Martín et al., 2009), or the rainbow trout (20%) (Vassallo-Agius et al., 2001), suggesting both the critical role of DHA content in *Liza aurata* sperm, the meaningful natural intake that the animals under study may have had in

the estuary, or even the probable biosynthesis of this fatty acid from precursors of the diet. Also, ARA is remarkably higher for male gonads than for females, in concordance with its key role for fish sperm motility and therefore for the ability to fertilize the eggs (Butts et al., 2015; Kowalski and Cejko, 2019).

The low percentage of EPA, DHA, n3, and n3 PUFA in the liver of mature animals in comparison with immature ones, may be explained by mobilization from the liver to the gonad in the first case, which highlights the role and selective mobilization of those fatty acids for the maturation process.

Selective retention of EFA, primarily DHA, has been described for different fish eggs (Izquierdo et al., 2001; Fernández-Palacios et al., 2011). This is in concordance with the present results, in which although the eggs reflected the fatty acid profile of the diet in many cases, the levels of EPA were quite lower in the eggs, while DHA levels were higher, showing a preferential conservancy of DHA compared to EPA. Additionally, the eggs presented lower levels of EPA, ARA, and a lower n3/n6 ratio than the wild female gonad, which may indicate a nutritional disbalance of the commercial diet for these animals. Therefore, until more studies could be done on the potential use of high HUFA precursors levels for this species' diets, ARA and EPA supplementation is recommended to achieve similar amounts in the eggs than in the wild female gonad.

As regards the histological evaluation of the mature gonads of *Liza aurata*, these animals presented similar features than those reported for other mullet species like de thin-lipped grey mullet (*Liza ramada*) or the grey mullet (*Mugil cephalus*) (Chang et al., 1995; Mousa et al., 2018). According to the recommended classification of Crosetti and Blaber (2016), the stage of ovarian maturity of the females under study was the IV stage (advanced maturity), in agreement with the categorization of González-Castro et al. (2011) for the "*tainha*" (*Mugil platanus*).

To summarize, *Liza aurata* seems to be a promising species for aquaculture diversification due to its feasible reproductive management in captivity, which may lead to viable egg production in only one year of acclimation under the present conditions. Furthermore, the present findings highlight that *Liza aurata* diets in nature may include important quantities of vegetable sources, opening the possibility of utilizing high levels of vegetable ingredients in *Liza aurata* broodstock diets as an alternative to marine fish meal and oil, although more studies are needed to evaluate this potential for reproductive and larval performance. Thus, the present findings contribute to more sustainable aquaculture while open also clear possibilities for the circular economy close to this species' production sites.

CRediT authorship contribution statement

Raquel Quirós-Pozo: Conceptualization, Investigation, Writing – original draft. **Lidia Robaina:** Conceptualization, Writing – review & editing, Funding acquisition. **Juan Antonio Calderón:** Investigation. **Javier Roo Filgueira:** Supervision, Conceptualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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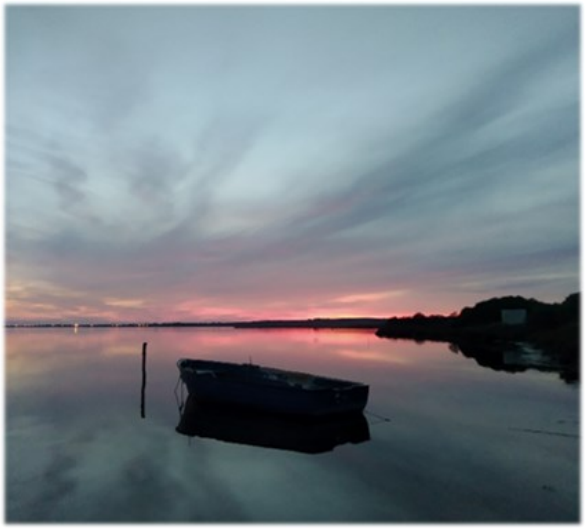
3.2. Effects of different co-feeding protocols on the early weaning of flathead grey mullet (*Mugil cephalus*) larvae.

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Effects of Different Co-Feeding Protocols on the Early Weaning of Flathead Grey Mullet (*Mugil cephalus*) Larvae

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Simple Summary: Sustainable aquaculture expansion will be crucial for the food security of a growing population expected to reach ~10 billion by 2050. Larval rearing is one of the most complex phases of marine aquaculture due to the need for additional facilities and labor to produce zooplankton to feed the larvae. Therefore, establishing adequate protocols for each species to shift from the live to the inert feed is crucial for more suitable management and profitability of the production. For those reasons, three different co-feeding protocols, A100 (2 initial *Artemia* sp. mL^{-1} day^{-1}), A50 (1 initial *Artemia* sp. mL^{-1} day^{-1}), and A0 (0 *Artemia* sp. mL^{-1} day^{-1}, only rotifers administered as live feed), were evaluated from 22 to 36 days post-hatching (dph) in the weaning of the flathead grey mullet, a promising species for sustainable aquaculture diversification. Survival, growth, biochemical composition, and gene expression of digestive enzymes and growth hormones were assessed. The A0 treatment performed better in survival, while the A100 treatment was the best for growth performance. The expression of the different genes evaluated did not show differences between treatments. In conclusion, rotifers should be maintained until 30–32 dph (total larval length of at least 10 mm) to maximize survival, while *Artemia* sp. supply is recommended from 26 to 29 dph (total larval length of 8 to 9 mm) to improve larval growth and minimize size dispersion.

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Abstract: The sustainable expansion of aquaculture relies on a sufficient supply of eggs and larvae, which are the first step of life cycle management. However, marine fish larval rearing generally depends on live feed production, which needs additional facilities and labor. The flathead grey mullet (*Mugil cephalus*), a promising species for aquaculture diversification, has a precocious digestive system development, supporting the feasibility of early weaning strategies. For these reasons, this study evaluated survival, growth, proximate and fatty acid composition, and gene expression of *Mugil cephalus* larvae reared under three different weaning protocols. Three co-feeding treatments, two with different *Artemia* sp. concentrations (A100 and A50, 2 and 1 *Artemia* sp. mL^{-1} day^{-1}, respectively) and one with only rotifers administered as live feed along the feeding trial (A0), were assessed from 22 to 36 days post-hatching (dph). The A0 treatment performed better in survival (64.79 $\pm$ 7.40%) than the A100 protocol (32.46 $\pm$ 12.82%). In contrast, the larvae of the A100 treatment presented significantly higher final length (15.51 $\pm$ 0.86 mm) than those of the A0 treatment (12.19 $\pm$ 1.45 mm) and higher final weight (41.28 $\pm$ 1.48 mg) than those of the A50 and A0 treatments (31.23 $\pm$ 3.65 mg and 24.03 $\pm$ 7.99 mg, respectively). On the other hand, the expression of digestive enzyme- and somatotropic factor-related genes did not show differences between treatments. The present results support the convenience of treatment A0 in maximizing survival, as rotifers should be maintained until 30–32 dph (until a total larval length of at least 10 mm). However, to improve growth and minimize size dispersion, *Artemia* sp. addition is recommended from day 26 to day 29 post-hatching (total larval length of 8 to 9 mm).

Keywords: Mugilidae; rotifers; *Artemia* sp.; diversification; gene expression

1. Introduction

Aquaculture has become the world's fastest-growing food production sector in the last few decades [1,2], driven by increased demand for aquatic products, overexploitation of wild stocks, and demographic growth. However, one of the most significant bottlenecks for further expansion of the sector is the supply of quality eggs and larvae that can guarantee an adequate volume of juveniles [3].

Larval rearing of marine fish is one of the most expensive and complex phases of the fish production cycle due to the need for auxiliary facilities to produce zooplankton (rotifers (*Brachionus plicatilis* mostly used) and *Artemia* sp.), which in general is critical for the success of the on-growing larval phase and consequently for the rest of the production cycle [4,5]. Many standard larval rearing protocols for Mediterranean species end between 30 and 40 dph [6]; however, the success of early weaning strategies is variable between species. For instance, for the Senegalese sole (*Solea senegalensis*), recent advances have allowed early weaning protocols starting as soon as 15 dph [7], while for the gilthead seabream (*Sparus aurata*), early weaning can begin around 20 dph [8,9]. However, early weaning protocols (16–20 dph) can lead to an increase in mortality or deformity incidence in species such as the European seabass (*Dicentrarchus labrax*) [6,10]. In this context, a suitable weaning protocol may optimize several parameters such as survival, larval growth, and size distribution, directly affecting the subsequent quality of the fingerlings [11]. Therefore, establishing adequate periods and protocols for larval weaning of different species is critical to promoting improved management procedures by hatcheries.

Another measure for the sustainable expansion of aquaculture is species diversification. Among the fish species suitable for this purpose, the Mugilidae family presents great potential due to its euryhaline, eurythermal, and low trophic nature [12]. Nevertheless, its cultivation is still mainly supported by wild fry capture, which results in high mortalities (70% to 96%), and hence appears to be an unsustainable practice [13]. However, the rising interest in mullets in recent years has promoted the investigation of reproductive techniques and physiology in different regions worldwide [14–17], offering a more sustainable alternative to increase the culture of these species. Specifically, the flathead grey mullet (*Mugil cephalus*) is a great candidate due to its fast growth, its large size, and the high market value of its salted and dried egg roe [18,19].

Recent studies have focused on some aspects of *M. cephalus* larval development under intensive culture conditions, particularly the ontogeny of the digestive, visual, and skeletal systems [19,20]. In addition, the beneficial effects of algal turbidity on larval performance [21] and the effects of different protein levels in weaning diets [22,23] have been recently assessed. Marine larvae such as the flathead grey mullet are born with undeveloped organs and systems that mature during the larval phase to achieve full functionality. For other mullet species such as the thick-lipped grey mullet (*Chelon labrosus*), premature intestinal maturation has been suggested, which may allow the use of early weaning strategies between 14 and 20 days post-hatching [24,25]. In the flathead grey mullet, mouth opening and first intestinal villi formation occur at 3 dph (88 degree days), while gastric glands and other main structures of the digestive tract are developed by 17 dph, which suggests the feasibility of shifting from live to inert feeds from this age onwards [19]. In this context, the expression of the digestive enzyme- and growth factor-related genes in the function of the employed weaning protocol would offer some clues to adapting feeding and rearing conditions [26].

For these reasons, the present study aimed to assess the effects of three different weaning protocols, two with different *Artemia* sp. concentrations (A100 and A50, 2 and 1 *Artemia* sp. mL^{-1} day^{-1}, respectively) and one with only rotifers administered as live feed along the feeding trial (A0). Survival, growth performance, proximate and fatty acid composition, and the expression of growth- and digestive enzyme-related genes of *M. cephalus* larvae were evaluated. The present results will provide essential information for tailored management protocols and hatchery cost optimization.

2. Materials and Methods

2.1. Larval Rearing

Two grey mullet mature females and two mature males were captured during the natural spawning season of the species (September 2020) at the Porto Pino lagoon (Sardinia, Italy), taking advantage of their seasonal migration towards the sea. The animals were transferred to a flow-through 3500 L tank of the San Giuseppe fishing cooperative (50 m from the site of capture) and injected with an LH-RH analog (ENANTONE, Takeda Italia S.p.a) as described by Vallainc et al. [16]. Fertilized eggs were spontaneously obtained 30 h post-injection and transferred to the International Marine Centre Foundation (IMC) facilities in Oristano (Italy). Eggs were incubated in a 2000 L tank in a seawater recirculating aquaculture system (RAS). Ammonia, nitrite, and nitrate levels were monitored and kept below harmful levels (0.5, 1, and 25 mg L^{-1}, respectively). After hatching, *M. cephalus* larvae were reared until 14 dph, following a standard protocol for marine larvae [27]. Enriched rotifers (*Brachionus* sp.) were adjusted twice daily to a concentration of 5 rotifers mL^{-1} to achieve a daily dose of 10 rotifers mL^{-1}. Two commercial products based on concentrated marine microalgae were used following manufacturer instructions to enrich the rotifers and produce the green water used for the larval rearing (Nanno Star, AlgaSpring, Almere, The Netherlands).

On the fourteenth day post-hatching, larvae were individually counted and manually distributed in nine 300 L tronco-conical tanks in a seawater RAS (1090 larvae per tank, three tanks per treatment), with an initial flow rate of 25% of the tank volume per hour.

Oxygen and temperature were monitored daily with a digital probe (Hach Lange HQ 40 d, Loveland, CO, USA), and the mean recorded values were 8.14 ± 0.10 mg L^{-1} and 23.0 ± 0.16 °C, respectively. Salinity, pH, ammonia, nitrite, and nitrates were measured every 48 h, and the median values were 38.22 ± 0.17 ppt, 7.80 ± 0.03, 0.09 ± 0.10 mg L^{-1}, 0.37 ± 0.37 mg L^{-1}, and 8.80 ± 5.69 mg L^{-1}, respectively. Salinity was measured with a refractometer (ATC Optika S.r.l. Ponteranica, Italy), pH was measured with a pH-meter (Halo HI11312, Hanna Instruments, Padua, Italy), and ammonia, nitrite, and nitrates were measured with a commercial kit (Prodac laboret test kit, Prodac S.L., Citadella, Italy). The bottom of each tank was siphoned daily to remove mortality and uneaten food. The water surface was also cleaned manually every day.

2.2. Feeding Protocols

Larvae were acclimated to experimental tanks for one week, receiving two daily doses of 5 rotifers mL^{-1} in green water culture (*Isocrysis galbana* and *Tetraselmis suecica*, 1:1 in volume ratio in the morning, and Nanno Star green in the afternoon). A 12 h light:12 h darkness artificial photoperiod was used during the whole experiment. Starting from 22 dph, 3 different weaning protocols named A100, A50, and A0 were applied, according to an initial dose of 2, 1, and 0 *Artemia* sp. mL^{-1} day^{-1}, respectively (Figure 1). For the control protocol (A100 treatment), initially, rotifers were provided twice daily at a concentration of 2.5 rotifers mL^{-1} (5 rotifers mL^{-1} day^{-1}) until 26 dph (end of rotifer administration), and *Artemia* sp. metanauplii were provided four times per day at an initial concentration of 0.5 metanauplii mL^{-1} (2 *Artemia* sp. mL^{-1} day^{-1}) with the dose being progressively reduced until 32 dph; thereafter, the larvae were fed only the inert feed until the end of the feeding trial (35 dph). In the A50 treatment, the larvae received the same amount of rotifers (5 rotifers mL^{-1} day^{-1}) and a half dose of *Artemia* sp. metanauplii compared to the A100 protocol (four times per day at an initial concentration of 0.25 metanauplii mL^{-1}, equal to 1 *Artemia* sp. mL^{-1} day^{-1}). In contrast, in the A0 treatment, larvae were fed only rotifers at an initial dose of 10 rotifers mL^{-1} day^{-1} (four daily doses of 2.5 rotifers mL^{-1}). In all treatments, the initial doses of live feed were progressively and proportionally decreased, until the end of the live prey feeding period (32 dph) (Figure 1). *Artemia* sp. metanauplii used in all treatments were enriched with the same commercial product used for rotifers, following the manufacturer's instructions. Furthermore, in all treatments, a commercial microdiet (Gemma micro 0.1 type, Skretting, Vervins, France) (D1) was also offered from the

start (22 dph) until the end of the experiment 15 times per day (8:00–18:00 h), and the fed amount was weighed daily. From 31 dph until the end of the feeding period (35 dph), this diet was mixed (1:1) with Gemma wean 0.2 (Skretting, Vervins, France) (D2). From 33 dph onwards, all larvae from the different treatments were fed only with the microdiet. The amount of microdiet was offered considering, on the one hand, the estimated consumption and growth of the larvae to ensure enough feed and, on the other, the visual inspection of the tanks, which allowed the adjustment of the dose given to avoid an excess of uneaten food. The average amount of feed given to each tank was 0.56 g day^{-1} from day 22 to day 29 post-hatching, and 0.88 g day^{-1} from 29 to 35 dph.

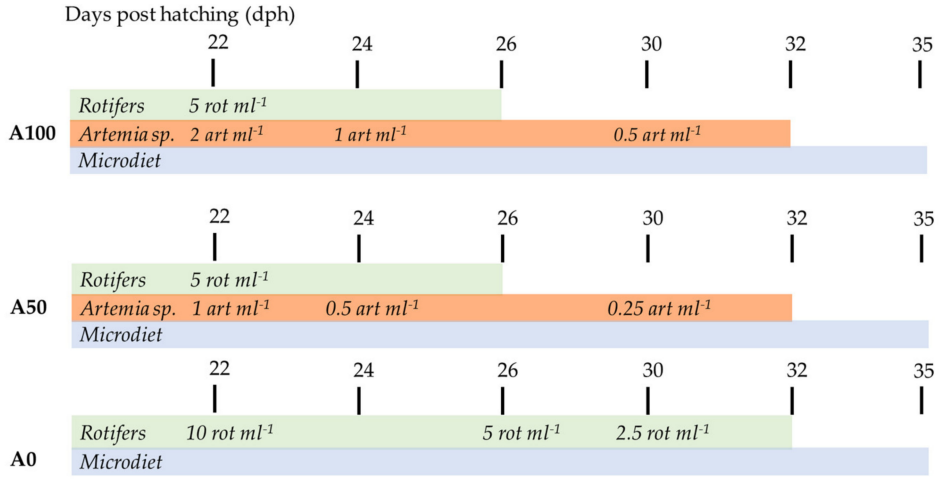

Figure 1. Protocols of daily doses of feed administration along the experiment in the treatments A100, A50, and A0 (2, 1, and 0 initial *Artemia* sp. mL^{-1} day^{-1}, respectively).

2.3. Sampling Protocols

The initial sampling was conducted after the acclimatization period (22 dph), prior to the beginning of the experiment. From each tank, 25 larvae were randomly collected and sacrificed with ice-cold water. A subsample of twenty larvae was then measured for total length under a microscope (Leica DMC2900, Wetzlar, Germany) using the LAS 4.5 software (Leica, Wetzlar, Germany). After measurements, a subsample of 15 larvae was placed in 3 groups of 5 larvae on cover glasses previously weighed to determine the wet weight. The glasses were then placed in a laboratory oven at 60 °C for 48 h to determine the dry weight. In addition, 5 larvae were also placed in RNAlater solution, stored at 4 °C for one week, and then frozen at −80 °C until gene expression analyses.

The same procedure was also used to sample larvae at 29 dph (intermediate sampling) and 36 dph (final sampling). At the latter sampling point, a pool of 17 larvae from each tank was also collected for biochemical analyses, frozen at −80 °C, and subsequently lyophilized until analyses could be conducted.

Furthermore, 3 different samples of enriched rotifers and *Artemia* sp. metanauplii were collected, frozen at −80 °C, and lyophilized until biochemical analyses. Samples of the inert diets were also stored at −80 °C until analyses. The biochemical and fatty acid composition of the diets is presented in Table 1.

Table 1. Proximate (% of dry weight) and fatty acid composition (% of total fatty acids (TFAs)) of experimental feeds.

	Artemia sp.	Rotifers	D1	D2
Proximate composition (% dry weight)				
Lipids	20.11 ± 2.32	16.07 ± 1.13	22.01 ± 1.57	16.20 ± 0.75
Protein	63.66 ± 2.80	62.35 ± 3.58	60.12 ± 0.22	67.97 ± 0.06
Ash	11.55 ± 3.06	12.43 ± 1.20	16.40 ± 0.18	9.08 ± 0.13

Table 1. *Cont.*

	Artemia sp.	Rotifers	D1	D2
Fatty acid composition (% TFAs)				
14:0	0.70 ± 0.22	1.29 ± 0.17	1.33	2.44
16:0	14.94 ± 4.67	14.09 ± 1.31	18.4	21.5
16:1n-7	2.81 ± 0.61	9.27 ± 0.36	1.98	3.86
18:0	7.59 ± 2.19	4.61 ± 0.37	4.88	5.48
18:1n-9	23.85 ± 4.35	13.08 ± 2.77	15.85	21.25
18:1n-7	8.33 ± 1.08	4.67 ± 0.21	2.36	2.99
18:2n-6	4.26 ± 1.15	10.71 ± 0.17	29.59	17.07
18:3n-3	21.58 ± 9.95	7.43 ± 0.05	2.67	2.31
20:1n-9	0.06 ± 0.02	0.55 ± 0.13	0.35	0.36
ARA (20:4n-6)	0.74 ± 0.27	3.89 ± 0.70	0.9	0.79
EPA (20:5n-3)	2.13 ± 0.94	5.72 ± 1.68	3.72	3.99
DHA (22:6n-3)	1.30 ± 0.58	3.01 ± 0.71	8.02	6.11
DPA (22:5n-6)	0.27 ± 0.12	1.47 ± 0.36	0.69	0.4
DHA/DPA	4.77 ± 0.03	2.05 ± 0.02	11.59	15.41
ARA/EPA	0.35 ± 0.03	0.69 ± 0.08	0.24	0.2
DHA/EPA	0.74 ± 0.60	0.53 ± 0.03	2.16	1.53
DHA/ARA	2.04 ± 1.53	0.77 ± 0.04	8.9	7.76
Oleic/DHA	6.09 ± 1.06	1.59 ± 0.50	1.98	3.48
Oleic/n-3 PUFAs	0.92 ± 0.53	0.63 ± 0.22	1.01	1.5
n-3/n-6	4.73 ± 0.92	1.09 ± 0.05	0.49	0.75
Total SFAs	24.00 ± 6.87	20.56 ± 1.93	25.29	30.38
Total MUFAs	38.17 ± 6.83	34.93 ± 2.94	26.3	35.09
Total n-3	29.60 ± 12.30	22.75 ± 2.86	15.94	14.51
Total n-6	6.12 ± 1.41	20.79 ± 1.67	32.58	19.32
Total n-9	24.39 ± 4.35	15.85 ± 1.94	16.68	22.18
Total n-3 PUFAs	29.51 ± 12.32	21.45 ± 3.23	15.76	14.17
Total n-6 PUFAs	1.28 ± 0.22	7.71 ± 1.48	2.08	1.67

Data expressed as means ± SD. Contains: 14:1n-7, 15:1n-5, 16:2n-6, 16:2n-4, 16:3n-4, 16:3n-3, 16:3n-1, 16:4n-3, 16:4n-1, 18:1n-5, 18:2n-4, 18:3n-6, 18:3n-4, 18:3n-1, 18:4n-1, 20:0, 20:1n-5, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 22:1n-11, 22:1n-9, and 22:4n-6. Abbreviations: ARA, arachidonic acid; D1, microdiet Gemma micro 0.1 type, Skretting; D2, microdiet Gemma wean 0.2 type, Skretting; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SD, standard deviation; SFAs, saturated fatty acids.

2.4. Survival and Growth

To determine survival, the dead larvae siphoned daily were counted. In addition, at the end of the experiment, the larvae in each tank were counted. Considering the number of larvae sampled during the experimental period, the percentage of survival was calculated using the following formula:

$$\text{Survival (\%)} = (\text{final living larvae}/(\text{initial larvae at the beginning of the treatments})) \times 100$$

Larval growth was measured using total length and wet and dry weight at the different sampling points. The coefficient of variation of total length (CV) and the specific growth ratio (SGR) were calculated using the following formulas:

$$\text{CV} = (\text{standard deviation of total final length}/\text{mean final length}) \times 100$$

$$\text{SGR (\%)} = (\ln Wf - \ln Wi)/\text{days of experiment} \times 100,$$

where Wf represents the final weight and Wi represents the initial weight.

2.5. Biochemical Analyses

Feed moisture, crude lipid, crude protein, and crude ash content (enriched rotifers, *Artemia* sp. metanauplii, and commercial diets) and larva moisture, crude lipid, and crude protein content were determined in the laboratories of the Instituto Eco-aqua, University of

Las Palmas de Gran Canaria (Canary Islands, Spain), following the techniques described by the AOAC [28]. Total crude protein content was determined by the Kjeldahl method [29], and total crude lipid content was determined as described by Folch et al. [30]. The total lipids extracted were trans-esterified [31], and the fatty acids were subsequently quantified by gas chromatography [32]. Analyses of diets were carried out in triplicate, while analyses of larvae were performed in duplicate for each tank (one pool per tank and three pools per treatment).

2.6. Gene Expression Analyses

Gene expression analyses were also carried out in the laboratories of the Instituto Eco-aqua, University of Las Palmas de Gran Canaria (Canary Islands, Spain). The present study aimed to assess the expression of genes related to growth (*growth hormone* (*gh*) and *insulin-like growth factor 1* (*igf1*)) and digestive enzymes (*pancreatic alpha amylase* (*amy2a*), *carboxyl ester lipase precursor* (*cel*), *chymotrypsinogen precursor* (*ctr*), *pancreatic phospholipase A2* (*pla2g1b*), and *trypsinogen 2 precursor* (*try2*)). The total RNA of 3 pooled larvae per tank was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, USA) and the extraction kit RNeasy Mini Kit from Qiagen. The reverse transcription (RT) of RNA to cDNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad Hercules, CA, USA) in 20 µL reaction volume according to the manufacturer's instructions to be later diluted 1/10 with Milli-Q water. Real-time PCRs were performed using an l-cycler with an optical module (Bio-Rad Hercules, CA, USA) in a final volume of 15.2 µL containing 7.5 µL of iQ-SYBER Green Supermix (Bio-Rad Hercules, CA, USA), 5 µL of cDNA, 0.6 µL of each primer (forward and reverse), and 1.5 µL of Milli-Q water. The conditions for the real-time reactions, which depended on each primer, were adapted from referenced protocols using a gradient of annealing temperatures to determine those with the best efficiency (Table 2). All reactions were performed in duplicate for each sample, and blank control reactions in which the cDNA was replaced with Milli-Q water were included. Relative gene expression was determined by the $2-\Delta\Delta CT$ method [33] using *18S-rRNA* as a housekeeping gene.

2.7. Statistical Analyses

All the statistical analyses were carried out using the program IBM SPSS Statistic 20 (New York, NY, USA). After the normality and homoscedasticity test ($p \geq 0.05$), the analysis of variance was performed using a one-way ANOVA, and the means were compared using the Tukey post hoc test ($p \leq 0.05$). When the data did not meet the assumption of normality or homoscedasticity, the medians were compared using a Kruskal–Wallis test ($p \leq 0.05$).

Table 2. Primer sequences and RT-PCR conditions of the different genes analyzed.

Gene	Access. Number		Primer Sequence 5′–3′	Initial Denaturation (°C) (Duration in min)	Denaturing Temperature (°C) (Duration in s)	Annealing Temperature (°C) (Duration in s)	Extension Temperature (°C) (Duration in s)	Number of Cycles	Reference
pla2g1b	MH350433	F	ACACCTGTTGATGACCTGGA	95 (3)	95 (15)	58.5 (30)	58.5 (30)	35	[26]
		R	GTCTTGGTGGCCTTGTCAC	95 (3)	95 (15)		58.5 (30)	35	
amy2a	KF684941	F	CCAAACTGGGAACTGTCATCAG	95 (3)	95 (15)	58.5 (30)	58.5 (30)	35	[26]
		R	TCTGGTTGTCGTGGTTGTCA	95 (3)	95 (15)		58.5 (30)	35	
cel	MH350432	F	CTGACCATGCTGATGACCTG	95 (3)	95 (15)	60 (30)	58.5 (30)	35	[26]
		R	GGCAATCATGTAACCGGAGA	95 (3)	95 (15)		58.5 (30)	35	
ctr	KC195969	F	CGTCCCTTCAGGATTATACCG	95 (3)	95 (15)	60 (30)	58.5 (30)	35	[26]
		R	AGTTGGAGGAACGGTCATGTT	95 (3)	95 (15)		58.5 (30)	35	
try2	KF684940	F	CTCCAGAACACAGCCATGAAG	95 (3)	95 (15)	60 (30)	58.5 (30)	35	[26]
		R	ACGTTCAGAGAGGCCTGGTAG						
igf1	AY427954.1	F	TCT TCA AGA GTG CGA TGT GC	95 (3)	95 (15)	53.8 (30)	72 (30)	35	[34]
		R	ACA GCT TTG GAA GCA GCA CT						
gh	AF134605.1	F	CATG CAC AAG GTG AGG AAG A	95 (3)	95 (15)	53.8 (30)	72 (30)	35	[34]
		R	AGG TCT CAA CCT GCA AAC ATC						
18S-rRNA		F	CACATCCAAGGAAGGCAGCA	94 (2)	94 (30)	60 (30)	68 (30)	30	[35]
		R	AAGATACGCTATTGGAGCTG						

Abbreviations: *gh, growth hormone; igf1, insulin-like growth factor 1; amy2a, pancreatic alpha amylase; cel, carboxyl ester lipase precursor; ctr, chymotrypsinogen precursor; pla2g1b, pancreatic phospholipase A2; RT-PCR, real-time polymerase chain reaction; try2, trypsinogen 2 precursor*.

3. Results

3.1. Survival and Growth

The survival of the larvae was significantly different between treatments (p = 0.02) (Figure 2). The A100 treatment presented the lowest survival, followed by the A50 treatment, while the A0 treatment resulted in the highest survival.

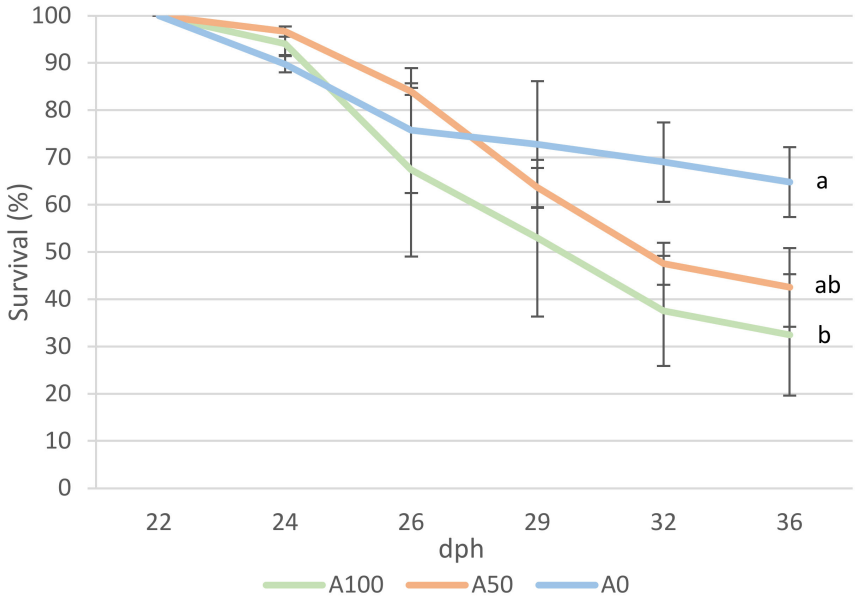

Figure 2. Survival (%) of *Mugil cephalus* larvae fed A100, A50, and A0 protocols (2, 1, and 0 of *Artemia* sp. mL^{-1} day^{-1}, respectively), during the experiment (means ± SD). Lines with different superscripts indicate the presence of significant differences (Tukey post hoc test $p \leq 0.05$). Abbreviations: dph, days post-hatching; SD, standard deviation.

Regarding growth performance, the final length showed differences between treatments; larvae reared under the A100 protocol were significantly longer than those of the A0 treatment at the end of the trial ($p < 0.01$) (Table 3). The CV showed the opposite trend, with the larvae reared under the A0 protocol presenting the highest value, even if not significantly (p= 0.27) (Figure 3).

Table 3. Wet weight (WW), dry weight (DW), and total length (TL) of *Mugil cephalus* larvae reared under the different protocols, during the experiment.

	Initial (22 dph)			Intermediate (29 dph)			Final (36 dph)		
	WW (mg)	**DW (mg)**	**TL (mm)**	**WW (mg)**	**DW (mg)**	**TL (mm)**	**WW (mg)**	**DW (mg)**	**TL (mm)**
A100	1.82 ± 0.80	0.46 ± 0.09	5.60 ± 0.16	6.31 ± 1.33 ^{a}	1.83 ± 0.27 ^{a}	8.79 ± 0.33	41.28 ± 1.48 ^{a}	9.84 ± 0.26 ^{a}	15.51 ± 0.86 ^{a}
A50	1.79 ± 0.64	0.42 ± 0.06	5.56 ± 0.08	3.78 ± 1.16 ^{b}	1.09 ± 0.21 ^{b}	8.03 ± 0.12	31.23 ± 3.65 ^{b}	7.23 ± 1.02 ^{b}	14.01 ± 1.24 ^{ab}
A0	1.74 ± 0.69	0.46 ± 0.08	5.73 ± 0.27	3.95 ± 1.61 ^{b}	1.15 ± 0.23 ^{b}	8.01 ± 0.75	24.03 ± 7.99 ^{b}	5.31 ± 2.02 ^{b}	12.19 ± 1.45 ^{b}

Data expressed as means ± SD. Values of the means in the same column with different superscripts indicate the presence of significant differences (Tukey post hoc test $p \leq 0.05$). Abbreviations: dph, days post-hatching; SD, standard deviation.

Larvae from the A100 treatment also presented a significantly higher wet and dry weight, both at the intermediate and final sampling points ($p < 0.01$) (Table 3). A similar trend was observed for SGR (Figure 4), although the observed differences were not statistically significant (p = 0.44).

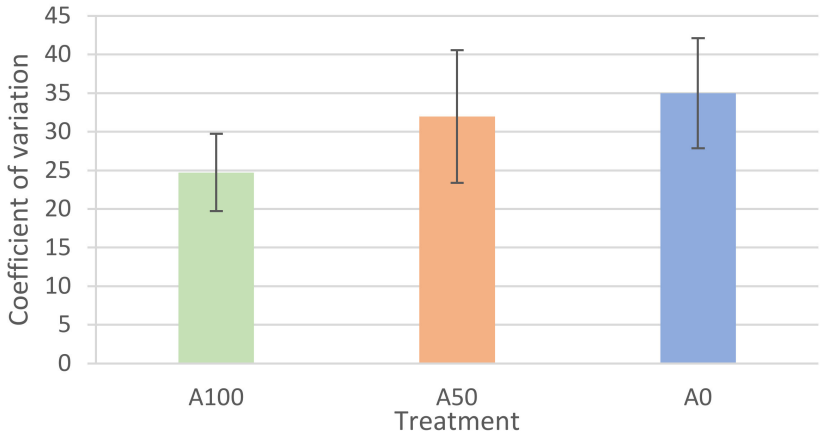

Figure 3. Coefficient of variation (CV) of total length at 36 dph of *Mugil cephalus* larvae fed A100, A50, and A0 protocols (2, 1, and 0 of *Artemia* sp. mL^{-1} day^{-1}, respectively) (means ± SD). Columns without superscripts indicate the absence of significant differences (Tukey post hoc test $p \geq 0.05$). Abbreviations: dph, days post-hatching; SD, standard deviation.

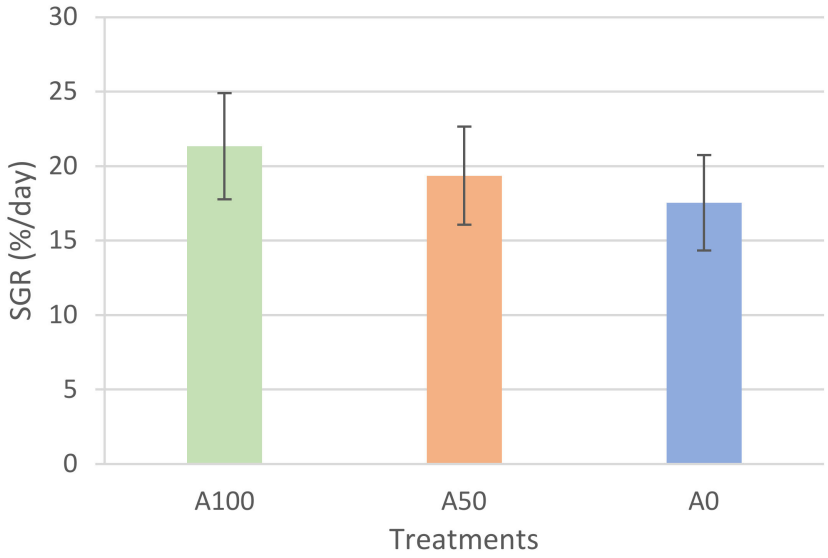

Figure 4. Specific growth rate (SGR (%/day)) at 36 dph of *Mugil cephalus* larvae fed A100, A50, and A0 protocols (2, 1, and 0 of *Artemia* sp. mL^{-1} day^{-1}, respectively) (means ± SD). Columns without superscripts indicate the absence of significant differences (Tukey post hoc test $p \geq 0.05$).

3.2. Biochemical Analyses

The proximate composition of larvae at the end of the experiment (Table 4) showed a similar trend to those observed for survival and final length, as evidenced by the total lipid contents found for the different treatments. The lipid content of the larvae from A100 was significantly higher ($p = 0.03$) than that of larvae under the A0 treatment, while larvae reared with the A50 protocol showed intermediate values. This trend of extreme values for A100 and A0 protocols and intermediate values for A50 was also observed in many cases for the larva fatty acid profile (Table 4). Specifically, the larvae from the A0 treatment presented the highest values of linoleic acid (18:2n-6) ($p = 0.02$), docosapentaenoic acid (DPA) ($p = 0.03$), and DHA/EPA (docosahexaenoic acid/eicosapentaenoic acid) ratio ($p = 0.02$), while the larvae from the A100 and A50 treatment showed the highest levels of oleic acid (18:1n-9) ($p = 0.01$), 18:1n-7 ($p = 0.01$), linolenic acid (18:3n-3) ($p < 0.01$), and n3/n6 ratio ($p < 0.01$).

Table 4. Proximate (% of dry weight) and fatty acid composition (% of total fatty acids (TFAs)) of the whole larvae from the different experimental groups.

	A100	A50	A0
Proximate composition (% dry weight)			
Lipids	25.49 ± 0.73 ^{a}	23.42 ± 0.86 ^{ab}	23.17 ± 1.01 ^{b}
Protein	66.82 ± 1.78	69.72 ± 2.28	69.46 ± 2.28
Fatty acid composition (% TFAs)			
14:0	1.43 ± 0.05	1.31 ± 0.15	1.43 ± 0.32
16:0	21.61 ± 1.69	20.52 ± 1.41	21.41 ± 2.66
16:1n-7	3.62 ± 0.16	3.32 ± 0.42	3.86 ± 0.46
18:0	7.76 ± 0.73	7.92 ± 0.20	7.98 ± 0.51
18:1n-9	22.42 ± 0.91 ^{a}	21.04 ± 1.02 ^{ab}	19.18 ± 0.80 ^{b}
18:1n-7	6.20 ± 0.46 ^{a}	5.54 ± 0.33 ^{a}	4.36 ± 0.10 ^{b}
18:2n-6	8.67 ± 1.09 ^{b}	10.00 ± 0.72 ^{ab}	11.50 ± 0.79 ^{a}
18:3n-3	6.32 ± 0.98 ^{a}	5.49 ± 0.27 ^{a}	2.82 ± 0.24 ^{b}
20:1n-9	0.30 ± 0.02 ^{b}	0.34 ± 0.03 ^{b}	0.42 ± 0.02 ^{a}
ARA (20:4n-6)	1.05 ± 0.08	1.21 ± 0.19	1.45 ± 0.21
EPA (20:5n-3)	2.51 ± 0.41	2.86 ± 0.41	2.99 ± 0.39
DHA (22:6n-3)	5.37 ± 1.10	7.38 ± 1.73	9.18 ± 1.94
DPA (22:5n-6)	0.37 ± 0.06 ^{b}	0.50 ± 0.11 ^{ab}	0.69 ± 0.15 ^{a}
DHA/DPA	14.46 ± 0.88 ^{b}	14.81 ± 0.28 ^{ab}	13.26 ± 0.55 ^{a}
ARA/EPA	0.42 ± 0.05	0.42 ± 0.01	0.49 ± 0.06
DHA/EPA	2.14 ± 0.11 ^{b}	2.58 ± 0.24 ^{ab}	3.07 ± 0.38 ^{a}
DHA/ARA	5.09 ± 0.72	6.07 ± 0.46	6.32 ± 0.69
Oleic/DHA	4.33 ± 1.13	2.97 ± 0.74	2.17 ± 0.60
Oleic/n-3 PUFAs	1.30 ± 0.30	1.11 ± 0.16	1.07 ± 0.22
n-3/n-6	1.56 ± 0.12 ^{a}	1.47 ± 0.06 ^{a}	1.18 ± 0.09 ^{b}
Total SFAs	31.50 ± 2.50	30.49 ± 1.52	31.57 ± 3.27
Total MUFAs	37.51 ± 1.71	35.48 ± 1.52	33.35 ± 0.97
Total n-3	17.88 ± 2.97	19.46 ± 2.12	18.49 ± 2.98
Total n-6	11.43 ± 1.29 ^{b}	13.23 ± 1.14 ^{ab}	15.65 ± 1.37 ^{a}
Total n-9	23.76 ± 0.87 ^{a}	22.31 ± 1.05 ^{ab}	20.58 ± 0.73 ^{b}
Total n-3 PUFAs	17.64 ± 2.96	19.20 ± 2.11	18.26 ± 2.98
Total n-6 PUFAs	11.06 ± 1.23 ^{b}	12.74 ± 1.04 ^{ab}	14.96 ± 1.23 ^{a}

Data expressed as means ± SD. Values of the means in the same row with different superscripts indicate the presence of significant differences (Tukey post hoc test $p \leq 0.05$). Contains: 14:1n-7, 15:1n-5, 16:2n-6, 16:2n-4, 16:3n-4, 16:3n-3, 16:3n-1, 16:4n-3, 16:4n-1, 18:1n-5, 18:2n-4, 18:3n-6, 18:3n-4, 18:3n-1, 18:4n-1, 20:0, 20:1n-5, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 22:1n-11, 22:1n-9, and 22:4n-6. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SD, standard deviation; SFAs, saturated fatty acids.

3.3. Gene Expression Analyses

No differences in the gene expression were found between treatments, neither for the digestive enzyme-related genes nor for the growth hormone-related genes at any of the sampling points (Figure 5). Additionally, the evaluation of the gene expression over time in the different treatments showed that the *carboxyl ester lipase precursor* (*cel*) increased significantly from the intermediate (29 dph) to the final sampling point (36 dph) ($p = 0.03$). In contrast, the *chymotrypsinogen precursor* (*ctr*) increased continuously during the trial ($p < 0.01$) (Figure 6).

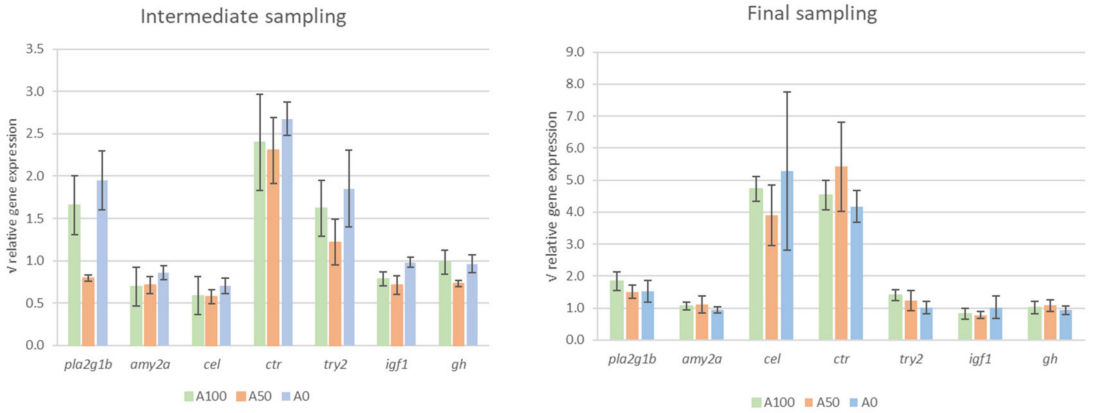

Figure 5. Relative gene expression of digestive enzymes and growth hormones for the different treatments in the intermediate (29 dph) and final sampling (36 dph). Data are normalized by the initial expression rates and are presented as square root of expression ratios ± standard error. Columns without superscripts indicate the absence of significant differences (Tukey post hoc test $p \geq 0.05$). Abbreviations: *gh*, *growth hormone*; *igf1*, *insulin-like growth factor 1*; *amy2a*, *pancreatic alpha amylase*; *cel*, *carboxyl ester lipase precursor*; *ctr*, *chymotrypsinogen precursor*; *pla2g1b*, *pancreatic phospholipase A2*; *try2*, *trypsinogen 2 precursor*.

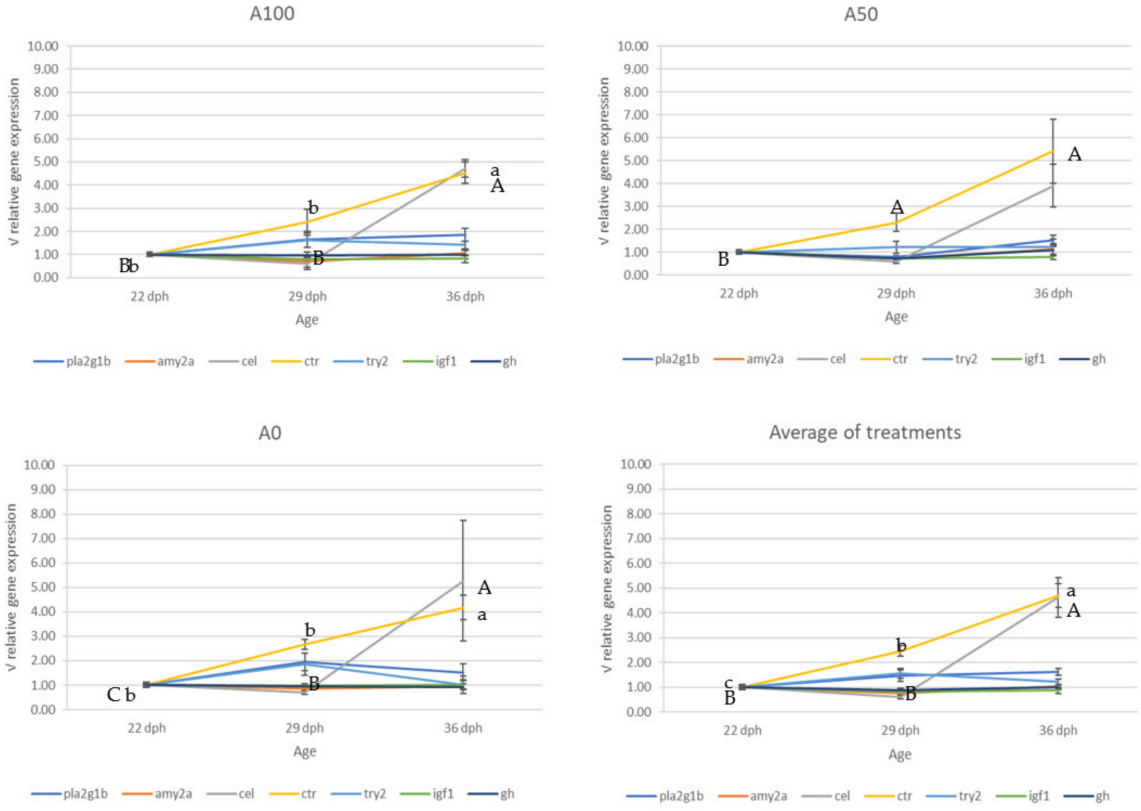

Figure 6. Relative gene expression of digestive enzymes and growth hormones for the different sampling moments. Data are normalized by the initial expression rates and are presented as the square root of normalized expression ratios ± standard error. Different lowercase letters indicate significant differences in *cel* gene expression, while different uppercase letters indicate significant differences in *ctr* gene expression among sampling points (Tukey post hoc test $p \leq 0.05$). Abbreviations: *gh*, *growth hormone*; *igf1*, *insulin-like growth factor 1*; *amy2a*, *pancreatic alpha amylase*; *cel*, *carboxyl ester lipase precursor*; *ctr*, *chymotrypsinogen precursor*; *pla2g1b*, *pancreatic phospholipase A2*; *try2*, *trypsinogen 2 precursor*.

4. Discussion

Weaning of the flathead grey mullet usually is carried out using a feeding sequence that includes both rotifer and *Artemia* sp. administration until the final shift to inert feeding [15,16,19], as described for other marine aquaculture species. In the present experiment, the live feeding period and the shift to the inert feed were carried out by comparing a standardized protocol (A100) [27] with two protocols, one with reduced *Artemia* sp. administration (A50) and another with no *Artemia* sp. provision (A0). The results evidenced the highest final weight but the lowest survival for the A100 treatment. In contrast, the highest survival and lowest final weight were registered in treatment A0. Survival during the experimental period (Figure 2) remained similar until day 26 post-hatching, the last day of rotifer administration in the A100 and A50 treatments. From that moment onward, mortality continued to increase in the A100 and A50 treatments but not in the A0. This could be related to the relatively small larval mouth size at the time of rotifer removal from the diet which may have prevented an efficient switch from the smaller rotifers to the larger *Artemia* sp. (720 µm average, own data). In this regard, Shirota et al. [36] reported a mouth size between 636 and 882 µm in 5.2 mm *Mugil cephalus* larvae, suggesting an optimum size of the live feed as less than half the size of the mouth gape [36,37]. In our study, at the intermediate sampling point (29 dph), small larvae of around 5.5 mm were still present. Oz et al. [38] also described an important size dispersion in cultured *M. cephalus* larvae and suggested that larval length is a better predictor than age for the larval developmental stage and, therefore, adapting rearing conditions. According to the previous data, this fact may suggest that the smaller larvae in A50 and A100 may have been unable to feed efficiently and cover their nutritional needs when rotifers were removed and *Artemia* sp. became the only live feed available. This may have resulted in higher mortality of the smaller individuals in these treatments and, consequently, larger larval sizes of the survivors.

Additionally, several other factors may have influenced larval survival, particularly when *Artemia* sp. was the primary live feed. For instance, Roo et al. [39] described larval mortality associated with malnutrition in 15–30 dph longfin yellowtail larvae (*Seriola rivoliana*) due to the low digestibility of the chitin exoskeleton of *Artemia* sp., reporting undigested and even alive *Artemia* sp. metanauplii in the feces after their transition thought the larval digestive tract. In addition, the elevated associated bacterial load in *Artemia* sp. (mainly *Vibrio* sp.) has been suggested to cause larval mortality when *Artemia* sp. is the primary food source [40]. Furthermore, present results are consistent with those obtained by Yanes-Roca et al. [41] for pikeperch larvae (*Sander lucioperca*), in which larvae fed only rotifers also presented lower weight and length but higher survival in comparison with those from the treatments where *Artemia* sp. and rotifers were provided. Hence, these authors suggested the higher rotifer concentration as a critical point that increased the chances of the smaller larvae to feed and avoid the point of no return.

The higher weight and length of the larvae in the A100 treatment may have been driven, as discussed above, by the probable higher mortality of the smallest larvae in this treatment and, on the other hand, by the higher lipid content of the *Artemia* sp. and the reduced feeding effort (greater energy intake per capture) which may have also promoted higher growth performance for these larvae. The growth results presented here are in line with previous observations in other species, such as the pikeperch, the Atlantic cod (*Gadus morhua*), and the cobia (*Rachycentron canadum*), in which better growth was obtained for larvae fed a combined protocol of rotifers and *Artemia* sp. in comparison with those fed with only rotifers [41–44]. Although the results for the CV were not statistically significant, a tendency of greater size dispersion in the larvae from the A0 protocol was observed; this tendency, together with the survival and growth data, supports the hypothesis that the smaller larvae from the A0 protocol were more able to cover their nutritional needs with rotifers and microdiets and therefore achieve a better survival in this treatment.

The higher lipid content of *Artemia* sp. metanauplii compared to rotifers was reflected in the proximate composition of the larvae reared under those protocols, particularly larvae under the A100 treatment. Moreover, the larvae of all treatments presented remarkably

higher lipid levels in comparison with those reported for other marine larvae, such as the red porgy (*Pagrus pagrus*) [45], the gilthead seabream [9,46], and the Senegalese sole [46], even when these were fed with equal or even higher lipid content diets compared to those used in the present trial.

The fatty acid profile of the live feed was reflected in the larvae of the different treatments, with higher levels of total n-9, 18:1n-9, 18:1n-7, and 18:3n-3 for the larvae reared under the *Artemia* sp. protocols (A100, A50) and higher levels of 18:2n-6, DPA, EPA and DHA for the larvae reared with rotifers in the A0 protocol, reflecting the fatty acid composition of the prey consumed. Higher DHA and linoleic levels have also been reported for pikeperch larvae reared only with rotifers as live feed compared with those reared with *Artemia* sp. or with a combined protocol [41]. Optimum DHA levels in marine larval feeds range from 0.5 to 2.5% [47], while for *M. cephalus* larvae, Koven et al. (2018, unpublished results) suggested that 1.7% of DHA in the total fatty acids is sufficient in the live feed when a source of 18:3n-3 is provided, which probably may indicate some potential to elongate and desaturate precursors of DHA. In the present study, there were differences in survival between the treatment fed with the higher content of *Artemia* sp. and the treatment fed only with rotifers (with higher highly unsaturated fatty acid (HUFA) content). However, the relationship between dietary DHA and survival seems to be highly species-dependent as it has been demonstrated to have effects on some marine larvae such as the red porgy [45] and the greater amberjack (*Seriola dumerili*) [48], while no effect of this fatty acid in larval survival has been described for other species such as the Senegalese sole [49] and the California halibut (*Paralichthys californicus*) [50]. Galindo et al. [51] concluded that the phylogeny of the fish species might be a relevant factor in the biosynthesis potential of long-chain polyunsaturated fatty acids (LC-PUFAs) from precursors, having demonstrated the ability of both the sand sole (*Pegusa lascaris*) and the thick-lipped grey mullet to synthesize 22:6n-3 from 18:3n-3. These data may suggest a lower compromise of DHA as an essential fatty acid for mullets.

Additionally, the optimal ratios of DHA/EPA for marine larvae have been described to be between 1.2 and 8 [47]. In the present study, the DHA/EPA ratio was lower than these values for rotifers and *Artemia* sp. metanauplii. However, in the larvae, the DHA/EPA ratio was between 2.14 (A100 treatment) and 3.07 (A0 treatment), being comparable to or even higher than those described for other marine larvae [9,45,52] and suggesting a sort of compensatory mechanism for conserving the physiological HUFA ratios, as has been described for other marine species as the Senegalese sole [53] and the gilthead seabream [54].

Regarding gene expression, the different protocols caused no appreciable differences in the digestive enzymes or the growth factors under study. The present findings contrast with those of Koven et al. [21], which suggested the modulation of lipases and alkaline proteases by the diet in *M. cephalus* larvae maintained under different water turbidity conditions. In addition, Koven et al. [23] recorded differential enzyme activities in pancreatic α-amylase, alkaline protease, and trypsin depending on the protein content of the weaning diet. In the present study, the inert weaning diets were the same in all the protocols assayed, which may have masked more subtle changes due to differences in the live feed and may explain the absence of differential expression of the digestive enzymes. Regarding the evaluation of the gene expression during the experiment and irrespectively of the treatment, as in the present study, Gilannejad et al. [26] also recorded a rising trend with time in the gene expression of *ctr* and *cel* in *C. labrosus* larvae. In contrast, they also registered a rising trend in the expression of *pepsinogen 2 precursor*, *pla2g1b*, *gh*, and *ifg1* genes during the same period, which may be explained by the normal ontogenetic variations between the two species or by the different rearing conditions.

The pronounced increase in *cel* and *ctr* observed in our study, mainly from 29 to 36 dph, is indicative of the improved ability of the *Mugil cephalus* larvae at that time to digest both proteins and lipids of the diet. Since in the present experiment, the proximate composition of the two microdiets was quite similar to those of *Artemia* sp. and rotifers, the rise in the

expression of both genes may be explained mainly by the intestinal maturation of the larvae during this period, which coincides with the end of the metamorphosis from larvae to the juvenile stage [15]. This hypothesis is also supported by the results of Koven et al. [23], which showed that chymotrypsin and lipase activities of *Mugil cephalus* fry during the weaning phase were independent of the diet provided.

Concerning the gene expression of the somatotropic factors in the different treatments, diverse studies have suggested that poor nutritional status in fish larvae can lead to changes in the expression of the *gh*/*igf* axis [55], indicating that smaller larvae with poorer nutritional status showed higher expression of the growth hormone. Thus, the lack of differences in the expression of the growth-related genes in the present study suggests that the treatments evaluated performed equally in terms of growth potential.

5. Conclusions

In conclusion, the present results confirm information on other marine larval weaning protocols and provide new data for a more efficient *Mugil cephalus* weaning. Flathead grey mullet larvae can be successfully weaned directly from rotifers to microdiet by 32 dph with good survival rates. Weaning protocols including artificial diets from 22 dph promoted higher final weight when *Artemia* sp. was provided, while larval survival was reduced. Therefore, to ensure better survival and a smoother transition from live prey to formulated feeds for the smaller larvae, rotifers should be maintained until 30–32 dph (total length of at least 10 mm). Additionally, to minimize size dispersion and to promote a smoother transition between live prey, newly hatched *Artemia* sp. may be provided prior to metanauplii for one to two days; however, *Artemia* sp. metanauplii administration is not recommended until 26–29 dph (total length of 8.0–8.8 mm). The present results highlight the feasibility of applying different weaning strategies for this species and provide hatcheries with new information to optimize the management effort regarding the final costs and benefits during the *Mugil cephalus* weaning phase; however, more studies are needed on this topic for a further understanding of the effects of different weaning protocols on the long-term performance.

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3.3. Evaluation of the effects of two different feeding frequencies on the digestive biochemistry of two mullet species (*Chelon labrosus* and *Liza aurata*)

animals

MDPI

Article

Evaluation of the Effects of Two Different Feeding Frequencies on the Digestive Biochemistry of Two Mullet Species (*Chelon labrosus and Liza aurata*)

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Simple Summary: Diversification of cultured fish species is one key measure to promote the sustainable development of marine aquaculture. Mullets (Mugilidae) present great potential due to their eurythermal, euryhaline, and low-trophic nature. However, there are many similar mullet species, and their physiological differences and optimal culture conditions are quite unknown, mainly when reared under intensive systems. In this sense, increasing the knowledge about changes in their digestive biochemistry related to feeding strategies is essential for a sustainable and cost-effective culture of these species. For these reasons, the present study aimed to characterize the digestive biochemistry of two mullet species (*Chelon labrosus* and *Liza aurata*) and to evaluate how it is affected when fish are fed using two different feeding frequencies: one or three times per day. In addition, changes in the potential bioavailability of nutrients determined by the two feeding patterns were assessed using digestive simulations performed in vitro. The results demonstrated the convenience of a feeding pattern of three meals per day instead of one. Moreover, the results evidenced that although *Chelon labrosus* and *Liza aurata* are species placed in a similar trophic level, their digestive response to feeding patterns, as well as body composition, present differences when fish are reared under the same conditions. This suggests that such interspecific variation must be considered when fish are reared under intensive conditions.

Abstract: Mullets (Mugilidae) present significant potential for sustainable aquaculture diversification due to their eurythermal, euryhaline, and low-trophic nature. However, the physiological differences and optimal cultured conditions among the diverse mullet species are quite unknown. For these reasons, the present study aimed to address two main objectives: (1) to characterize the differences in digestive biochemistry, somatic indexes, and body composition between two mullet species (*Liza aurata* and *Chelon labrosus*); and (2) to evaluate the interactions of two different feeding frequencies (one against three meals per day) on the above-mentioned parameters, and also on the potential bioavailability of nutrients determined using in vitro assays. The results evidenced higher protease and amylase activities for *Chelon labrosus* than for *Liza aurata*, while the latter species presented a higher percentage of eviscerated weight and muscle lipids. Furthermore, the results from in vitro assays supported the higher enzyme activity of *Chelon labrosus* by an observed increase in the release of amino acids and reducing sugars measured for this species. Regarding feeding patterns, the results of the in vitro assays simulating enzyme: substrate (E:S) ratios corresponding to one or three meals per day point to a clear increase of nutrient bioavailability when the daily ration is split into several meals. The present results improve the physiological knowledge of mullet species and define criteria to develop better management protocols by producers.

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1. Introduction

Global aquaculture production has more than tripled from 1997 to 2017 [1], reaching a historical record in 2020 with 122.6 million tons produced and a total value of USD 281,500 (United States Dollar), thus increasing the availability of aquatic foods to the population [2]. However, the diversity of products offered by aquaculture is still lower compared to those coming from fisheries due to different factors such as difficulties in the management of reproduction, production of suitable feeds, and other operational issues of the selected species. For this reason, diversification is one of the most critical challenges that aquaculture faces today. In the case of the EU (European Union), marine fish production is focused mainly on species belonging to high trophic levels, such as the seabass (*Dicentrarchus labrax*), or the turbot (*Scophthalmus maximus*). In contrast, the production of marine species placed at a low trophic level is still uncommon, despite their recognized importance for the sustainable development of aquaculture [3,4].

Nowadays, the EU's efforts to increase aquaculture diversification include species such as the grey mullet (*Mugil cephalus*). Mullets are catadromous teleosts living in temperate waters worldwide, which can feed on a wide range of materials, including plankton, macroalgae, and detritus [5]. There are up to 71 recognized species [6] but only some of them are currently interesting for aquaculture, including the flathead grey mullet (*Mugil cephalus*), the thick-lipped grey mullet (*Chelon labrosus*), the golden grey mullet (*Liza aurata*), the thin-lipped grey mullet (*Liza ramada*), and the feral leaping mullet (*Liza saliens*), the most frequently farmed species in the Mediterranean region [7]. In 2020, 291.2 thousand tonnes of mullets were produced globally from aquaculture [2]; their traditional cultivation being carried out in ponds and estuaries due to their greatly appreciated flesh and processed products in many regions worldwide [8]. However, despite being cultured extensively or semi-intensively in many territories, there is still not enough information about their digestive capacity, nutritional requirements, or body composition when reared under intensive culture conditions.

Additionally, the natural co-existence of different mullet species in the same habitats has determined different feeding behaviours that help to avoid competition for similar resources [9]. This suggests that despite the important similarities between species, differences in key features related to food selection and other aspects of their digestive biochemistry and physiology must be considered when adopting suitable species-specific rearing practices. Among these, feed intake and feeding frequency are closely linked to the digestion transit rate, and, therefore, to the effectiveness of digestion.

Considering the above-detailed points, the aim of the present study was to (1) characterize differences in the digestive biochemistry (activities of alkaline protease and amylase), body composition, and somatic indexes between two similar mullet species (*Chelon labrosus* and *Liza aurata*) when reared under the same intensive culture conditions and (2) to evaluate the effects and interactions of two feeding frequencies (one and three meals per day) on the parameters mentioned above for both species. Both aspects were additionally connected using an in vitro assay simulating the digestion of proteins and carbohydrates under the conditions used in the in vivo experiment. Comparative information obtained may help optimize feed formulation and nutritional management for a more sustainable intensive culture of these species.

2. Materials and Methods

2.1. Fish and Facilities

The fish used in the present experiment were captured close to the Sports Pier of Las Palmas de Gran Canaria and then transported and acclimated in the GIA-ECOAQUA facilities, where they were maintained in 500-litre tanks in open-flow system conditions. The two species were identified and classified according to morphological parameters [10]. During this period, the fish were fed until apparent satiation twice per day with a commercial marine feed (Skretting). For each species, thirty-six juveniles (39.25 ± 5.28 g and 15.69 ± 0.86 cm of total weight and length, respectively) were distributed in six cylindrical tanks of 250 L (six fish per tank, twelve total tanks). The trial was run under an open seawater system and controlled photoperiod (12 h of light and 12 h of darkness), close to that naturally present in the season and latitude. Temperature and oxygen concentration were measured daily, and the average values were 20.95 ± 0.39 °C and 6.19 ± 0.28 mg/L, respectively.

2.2. Experimental Design

Fish were fed a 3 mm diet produced in the pilot plant of products and processes at GIA-ECOAQUA facilities in Taliarte, Gran Canaria (Spain), following the formula described for *Liza aurata* juveniles by Quirós-Pozo et al. [11] (Table 1), which adapted referenced levels of dietary protein and lipids for mullets [12–14]. The fish were fed at two different patterns that were established following the approximation described by Busti et al. [15], considering the maximum amount of food accepted in a single meal as a reference for the fixed daily ration of feed given to the animals. This dose, previously determined to be around 1% of the body weight, was then provided to triplicate groups of six fish in either one meal (8:00 h) or equally divided into three meals per day (8:00, 11:00, and 14:00 h). The feeding was performed carefully to avoid the loss of food. The experiment lasted 6 weeks to allow the fish to adapt to the new systems and feed and, thus, to better determine the digestive biochemistry adaptation to the feeding patterns. The fish were sampled at a middle point to adjust the fixed ration of offered feed. As the daily ration of feed was the same, feeding frequencies and species were chosen as the two variables under study.

Table 1. Ingredients (g/100 g) and proximate composition of the diet used in the experiment.

Ingredients (%)	
Fish meal (Peruvian origin) [a]	20.0
Blood meal [b]	3.0
Ulva meal [c]	10.0
Rapeseed meal (0.0) [d]	8.0
Corn meal [e]	6.0
Wheat gluten [e]	6.0
SPC (soy protein concentrate) [f]	20.0
Wheat meal [e]	6.0
Fish oil [a]	8.5
Soy lecithin [g]	1.0
Vitamin mix [h]	2.0
Mineral mix [i]	2.0
$Ca(H_2PO_4)_2$ [j]	1.0
CMC [k]	0.5
Analytical composition (% dry weight)	
Protein	40.9 ± 0.4
Lipids	14.1 ± 0.6

Ash	11.4 ± 0.1
Moisture	8.1 ± 0.6

[a] Fish meal and fish oil from South America (supplied by Skretting, Spain); [b] Blood meal (supplied by Dibaq, Spain); [c] Ulva meal (supplied by Puerto Muiños S.L., Spain); [d] Rapeseed 0.0 (supplied by Dibaq, Spain); [e] Flours obtained from local producers; [f] Soy protein concentrate (Sopropeche, France); [g] Soy lecithin (92% fat) (supplied by Korott S.L., Spain); [h] Vitamin premix containing (mg kg^{-1} or IU/kg of dry feed): thiamine 40 mg, riboflavin 50 mg, pyridoxine 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, folic acid 10 mg, cyanocobalamin, 0.5 mg, choline chloride 2700 mg, myo-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg, cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU, vitamin E (DL-alpha-tocopherol acetate) 250 mg; [i] Mineral premix containing (g/kg of dry feed): calcium orthophosphate 1.60 g, calcium carbonate 4 g, ferrous sulphate 1.5 g, magnesium sulphate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminium sulphate 0.02 g, zinc sulphate 0.24 g, copper sulphate 0.20 g, manganese sulphate 0.08 g, potassium iodate 0.02 g; [j] Sigma-Aldrich, Munich, Germany; [k] carboxymethylcellulose (sodium salt, Sigma-Aldrich, Munich, Germany).

After 6 weeks of feeding, fish samples were collected to measure the length of the digestion process and the total production of alkaline protease and amylase, which was the information required to develop the in vitro digestive simulation assay. For this, after a fasting period of 48 h, fish were fed following the two established patterns (one or three meals/day) and sampled at two different moments (15 and 40 h post-feeding) to check potential differences in enzyme activities between a fully established and almost finished digestion process. The manipulation and sacrifice of fish were rigorously conducted according to the European Union Directive (2010/63/EU) on animal welfare protection for scientific purposes. The experimental protocol performed for this work was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (OEBA_ULPGC_09/2020).

Once sacrificed using immersion in ice-cold water, fish were measured and then dissected to separate the intestines. At that moment, body indexes were determined and samples from the liver and muscle were also conserved for biochemical analysis. The hepatosomatic index (HSI), percentage of eviscerated weight, and percentage of digestive tract weight were calculated with the following formulas:

Hepatosomatic index (HSI) (%) = liver weight (g)/fish weight (g) × 100.

Eviscerated weight (%) = eviscerated weight (g)/fish weight (g) × 100.

Digestive weight (%) = digestive tract weight (including perivisceral fat) (g)/fish weight (g) × 100.

2.3. Biochemical Assays

The muscle and liver samples (3 fish per tank) were pooled (9 fish and 3 pools per treatment) and stored at −80 °C until analysed. The analyses of the proximate composition of tissues and the experimental diet were performed following the techniques described in AOAC [16], with proteins being determined using the Kjeldahl technique [17], and the total lipids being determined following the protocol described by Folch et al. [18].

Crude enzyme extracts used for measuring enzyme activities and to develop the in vitro digestion assays were prepared with manual homogenization of the whole intestine and its contents in distilled water (1:10, weight/volume), followed by centrifugation (12.000 rpm, 3 °C, 15 min) (6 fish per tank and 3 pools in total per treatment). Total alkaline protease activity in the extracts was measured at pH 8.5 according to Kunitz's method [19] which was modified by Walter [20] using casein as substrate. One unit of enzyme activity (U) was defined as the amount of enzyme needed to catalyse 1 μg of tyrosine formation per minute. Total amylase activity was measured at pH 7.5, following the 3,5-di-nitrosalicylic acid (DNSA) method [21], using starch as substrate. One unit of amylase activity was defined as the amount of enzyme needed to catalyse the formation of 1 μg of maltose equivalent per minute.

2.4. In Vitro Digestion Assays

The in vitro digestion assays were performed using membrane bioreactors modified from those described by Morales and Moyano [22]. The device consists of two chambers separated by a semipermeable membrane of 3500 kDa MWCO (Kilodalton, molecular weight cut-off). Fish enzyme extracts and feed samples were placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. Hydrolysis products (either amino acids or reducing sugars) passing across the membrane into the lower chamber were recovered at different time intervals during the reaction time. The whole system was maintained at 25 °C within a temperature-controlled chamber. The release of amino acids was quantified using the orthophthaldehyde (OPA) method described by Church et al. [23], and reducing sugars were measured using the DNSA method mentioned above. Some of the operating conditions used in the assays were maintained constant: 4 h total time, with samplings each hour, and pH = 8.2, while the enzyme: substrate ratio (E:S) was adapted to simulate the expected changes correlated to the different feeding patterns. This physiological E:S ratio (Table 2) was estimated only for protease. It was determined considering, on the one hand, the total enzyme production calculated for the average size of the individuals used in the experiment, and, on the other hand, the average intake of protein ingested when the fish received the amount of feed corresponding to the daily ration either in one or three meals. Hence, in the assays, a fixed amount of enzyme extracts was combined with two different amounts of the substrate assuming that, in the live fish, enzyme secretion as a response to intake is independent of the quantity of feed ingested.

Table 2. Enzyme: substrate ratios used in the in vitro assays.

Species	Total Ux10³/Fish	Meals/Day	Food Intake Per Meal (mg)	Protease E:S Ratio (U/mg Food)	Amylase E:S Ratio (U/mg Food)
L. aurata	7.22 ± 3.65	1	350	21.82 ± 13.85	0.01 ± 0.00
		3	120	50.18 ± 21.15	0.03 ± 0.00
C. labrosus	15.72 ± 5.51	1	350	40.79 ± 2.27	0.02 ± 0.00
		3	120	142.08 ± 54.26	0.06 ± 0.00

Data expressed as means ± standard deviation (SD).

2.5. Statistical Analysis

After completing normality and homoscedasticity tests, data obtained in the different experiments were evaluated using a two-way ANOVA (species (S) and feeding frequency (F)) followed by a "least significant difference" (LSD) test or a Kruskal–Wallis's test in cases where the data did not meet normality or homoscedasticity. All analyses were carried out using the program IBM® SPSS Statistic 20 (New York, NY, USA). In addition, the statistical power analysis program G*power [24] was used to determine the minimum number of fish required to run the experiment.

3. Results

3.1. Somatic Indexes

The results of somatic indexes are shown in Table 3. Significant differences were evidenced between both species, with a higher liver and digestive tract size measured in *C. labrosus* and a higher percentage of eviscerated weight for *L. aurata*. Additionally, mullets fed once daily presented a higher weight percentage of the digestive tract.

Table 3. Somatic indexes expressed by species (S) and feeding frequency (F) (n = 18).

	L. aurata		*C. labrosus*		*p* Values		
	One Meal	Three Meals	One Meal	Three Meals	S	F	SxF

%Eviscerated weight	87.38 ± 0.17	90.24 ± 1.58	86.55 ± 2.55	86.51 ± 0.64	0.02	0.10	0.10
HSI	0.83 ± 0.11	0.82 ± 0.03	1.04 ± 0.07	0.97 ± 0.04	0.00	0.30	0.41
% Digestive weight	7.02 ± 0.64	5.99 ± 0.37	11.16 ± 0.38	9.96 ± 1.11	0.00	0.04	0.82

Data expressed as means ± standard deviation (SD).

3.2. Biochemical Assays

The proximate composition of the muscle and liver is detailed in Table 4. The muscle lipid content was significantly higher in *L. aurata* than in *C. labrosus*. The liver lipid content was significantly higher in fish receiving their daily ration in only one meal.

Table 4. Proximal composition (% of dry weight) of the muscle and liver expressed by species (S) and feeding frequency (F) (n = 9).

	L. aurata		***C. labrosus***		***p* Values**		
	One Meal	**Three Meals**	**One Meal**	**Three Meals**	**S**	**F**	**SxF**
Muscle							
Lipid	16.49 ± 0.69	18.97 ± 3.19	13.18 ± 1.58	13.15 ± 1.06	0.00	0.29	0.21
Protein	80.83 ± 1.89	83.26 ± 4.82	84.46 ± 0.40	87.52 ± 2.40	0.08	0.08	-
Ash	6.89 ± 0.05	6.13 ± 0.53	6.00 ± 0.72	6.18 ± 0.62	0.08	0.26	-
Liver							
Lipid	40.22 ± 4.56	31.53 ± 5.53	44.14 ± 3.37	38.94 ± 5.33	0.61	0.03	0.52

Data expressed as means ± standard deviation (SD).

3.3. In Vitro Digestion Assays

Activities of intestinal alkaline protease and amylase are presented in Figure 1 for both species. The results showed significantly higher values for both enzymes in samples from *C. labrosus* ($p \leq 0.01$). The effect of the number of daily meals and sampling moments on the enzyme activities of each species is shown in Table 5. In *L. aurata*, the protease activity was significantly decreased ($p = 0.01$) with time spent after the first meal. In contrast, the intestinal protease activity in *C. labrosus* did not show significant variations related to the feeding frequency or sampling moment. This was also the case for amylase activity in both species.

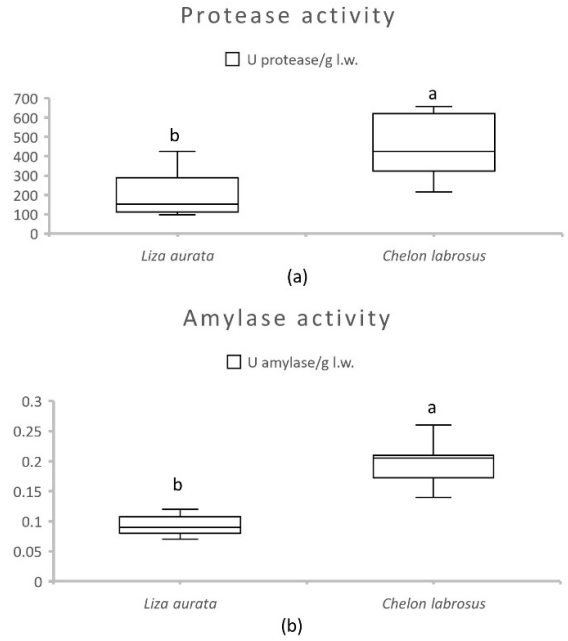

Figure 1. Average activity of total alkaline protease (**a**) and amylase (**b**) (U/g live weight) in the intestines of *C. labrosus* and *L. aurata* juveniles (n = 36). Data expressed as means ± standard deviation (SD). Significant differences between species ($p < 0.05$) are indicated using lowercase letters (a, b).

Table 5. Protease and amylase activities (U/g live weight) of *C. labrosus* and *L. aurata* as a function of the number of daily meals and sampling moment, expressed as sampling moment (SM) and feeding frequency (F) (n = 18).

	Feeding Frequency (F)		**Sampling Moment (SM)**		***p* Values**		
	One Meal	**Three Meals**	**15 h**	**40 h**	**F**	**SM**	**F x SM**
C. labrosus							
Protease	411.35 ± 129.03	487.13 ± 185.88	500.68 ± 176.49	397.80 ± 130.95	0.25	0.13	0.01
Amylase	0.18 ± 0.03	0.21 ± 0.03	0.20 ± 0.03	0.20 ± 0.04	0.16	0.99	0.65
L. aurata							
Protease	218.22 ± 133.47	172.05 ± 72.52	270.25 ± 101.8	120.02 ± 21.01	0.26	0.01	0.13
Amylase	0.09 ± 0.03	0.10 ± 0.01	0.11 ± 0.03	0.09 ± 0.02	0.69	0.15	0.12

Data expressed as means ± standard deviation (SD). Data on enzyme activities as a function of feeding frequency is calculated irrespectively of the sampling moment and vice versa.

The results obtained with the in vitro assays are shown in Figures 2 and 3 and reflect the specific differences observed in the enzyme activity levels for both species. Hence, after hydrolysis, the release of amino acids was significantly higher ($p = 0.04$) when simulating digestion in *C. labrosus* than in *L. aurata*. On the other hand, the total release of amino acids was not significantly affected by the different E:S ratios used to simulate the conditions associated with the two feeding patterns in both species. In contrast, a significantly lower release of reducing sugars was obtained when testing an E:S ratio adapted to simulate three meals in *L. aurata*, but this was not the case in *C. labrosus*.

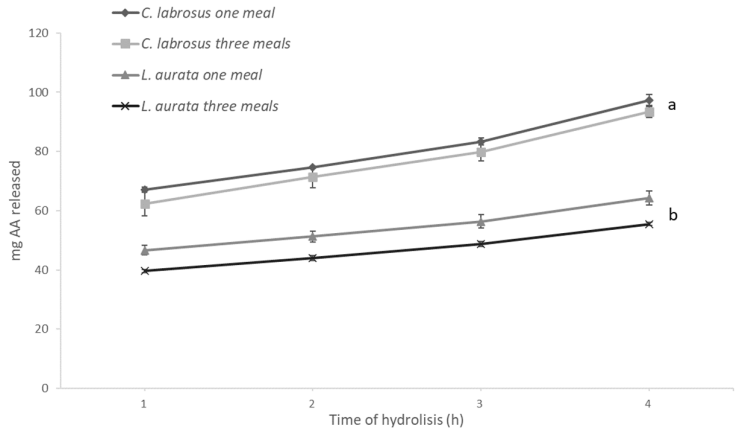

Figure 2. Release of amino acids (AA) from *L. aurata* and *C. labrosus* when simulating the different feeding frequencies in vitro (n = 18). Data expressed as means ± SD. Significant differences between species ($p < 0.05$) are indicated using lowercase letters (a, b).

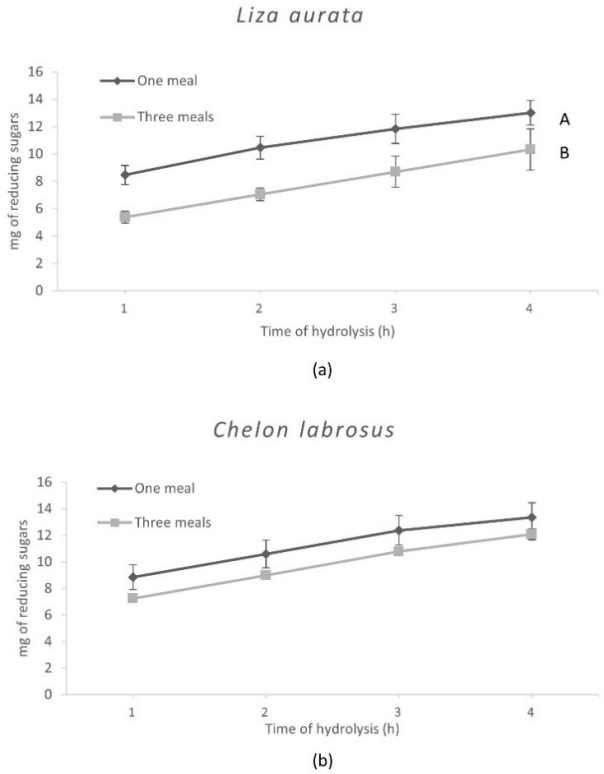

Figure 3. Release of reducing sugars from *L. aurata* (**a**) and *C. labrosus* (**b**) when the different feeding frequencies in vitro (n = 18). Data expressed as means ± SD. Significant differences between feeding patterns ($p < 0.05$) are indicated using uppercase letters (A, B).

4. Discussion

The low voluntary intake in a single meal observed in both species can be partially explained by considering that mullets are continuous feeders. Their stomachs are formed by a thin-walled cardiac and a thick-walled pyloric portion [25], which is adapted to function as a mill for feed particles, similar to the gizzards of birds. For this reason, such a stomach is much less distensible than that present in other fish species with a more

discontinuous feeding pattern, which limits to a great extent the maximum amount of feed that mullets can ingest in one meal [26]. This could explain why, in the present experiment, the maximum intake in one single meal used as a reference for the two selected feeding patterns accounted for only 1% of the body weight.

With regards to biochemical composition, interestingly, muscle from *L. aurata* juveniles presented a higher percentage of lipids than *C. labrosus*, which is a valuable feature due to the beneficial role of marine products from aquaculture in human nutrition and health [27,28], especially for its polyunsaturated fatty acid content. In the case of the livers, many fish species are known to store lipids in this organ, which is sensitive to feeding management [29]. In the present trial, fish fed only once per day presented a higher amount of liver lipids, indicating that these animals could accumulate energy reserves in the form of fat when food is offered infrequently. Furthermore, the higher weight percentage of the digestive tract (produced probably by a higher amount of perivisceral fat) in mullets fed once per day supports this hypothesis. On the other hand, the lower proportion of viscera in relation to the carcass of *L. aurata*, in addition to a more stylized body, gives an interesting benefit to increasing the culture of these animals.

Essential differences in the use of nutrients are determined by specific features of the digestive biochemistry of fish species [30,31]. In the present study, significantly higher total activities of both protease and amylase were measured in *C. labrosus* compared to *L. aurata*. This suggests a higher potential for the hydrolysis of essential nutrients in the former species that could reflect differences in the proximate composition of the food items that constitute its diet under natural conditions. In the present study, two factors may influence the measured total activities of digestive enzymes: the number of daily meals and the moment of sampling. In this sense, lower enzyme activities could be expected when fish are fed under suboptimal feeding frequencies [32]. On the other hand, since digestive enzymes are partially hydrolysed and eliminated with faeces as digestion progresses [33], samplings done at different moments after a meal should evidence a decreasing trend in the activities. In the present study, the number of daily meals and sampling moments influenced enzyme activities differently in both species. In *C. labrosus*, no significant variations in the total activities of protease or amylase were detected irrespective of the amount of food ingested or the sampling moment. This suggests the existence of a pattern of enzyme production frequently described in continuous feeders, in which the maintained presence of food in the gut is associated with a continuous secretion of the enzymes, ensuring suitable hydrolysis of the primary substrates [34]. This maintained production of intestinal proteases is also a typical feature described in stomachless fish as a compensatory mechanism for the absence of acid hydrolysis of proteins [35]. In the case of *L. aurata*, the observed reduction in the total activity of alkaline protease linked to the digestion time points suggests an enzyme production pattern less identifiable compared to that of a continuous feeder. However, the observed variations cannot be fully assigned to an occasional feeder.

Both species have been widely described as omnivorous and opportunistic fishes having high trophic overlap [36], analogous trophic niche breadths [37], and similar relative gut lengths [27]. However, for different seasons and estuarine systems, a slightly higher trophic level has been described for *L. aurata* (3.02–3.30) than for *C. labrosus* (2.16–3.29) [9]. In addition to the differences found in digestive biochemistry, this point contributes to differentiating physiological strategies in each species that can be driven by the differences in the composition of the food items they preferably consume in the wild.

Regarding the results obtained with the in vitro simulation of digestion, the higher protease activity measured in the digestive tract of *C. labrosus* determined a significantly higher release of amino acids from the same amount of substrate than that of *L. aurata*. Surprisingly, no significant differences were obtained in the total amount of amino acids released when simulating the two different enzyme: substrate ratios theoretically present in the guts of fish ingesting their daily ration either in one or three meals. In the first case, the expected net release of amino acids from the feed should have been higher than when

three times less substrate was present. This points out that in the case of intestinal proteases, an optimal enzyme: substrate ratio is reached when a very low amount of substrate is present and suggests that, from a practical point of view, it should be better to distribute the daily ration into several meals. Results obtained for carbohydrate hydrolysis in *C. labrosus* confirm this suggestion and are in concordance with those obtained in juveniles of *Mugil liza*, in which a higher feed efficiency was obtained when fish were fed three times per day [38]. In addition, Solovyev and Gisbert [39] recorded inappropriate enzyme/substrate ratios and lower digestive efficiency and growth rates in *Mugil cephalus* fry fed one or two times per day, compared to mullet fry provided a continuous regime. Similar results have also been described in other species such as the snapper (*Lutjanus johnii*) [40] or the doctor fish (*Garra rufa*) [41].

5. Conclusions

To summarize, concerning the comparison between feeding frequencies, the results obtained in the digestion model support the observed benefits for three daily meals instead of one. Regarding the comparison between the two species, *L. aurata* showed higher muscle lipid content compared to *C. labrosus*, in addition to a greater eviscerated weight and lower HSI. *C. labrosus* instead presented higher protease and amylase activities which determined an increased release of amino acids and reducing sugars in the in vitro assays. The obtained results suggest the different potentialities of these similar fish species when reared under diverse circumstances and open the future to species-specific nutrition and feeding research among others.

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3.4. Evaluation of *Aloe vera* by-product against cereals in feeds for golden mullet (*Liza aurata*)

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Evaluation of *Aloe vera* by-product against cereals in feeds for golden mullet (*Liza aurata*)

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A B S T R A C T

Among of the actual challenges for the sustainable aquaculture development are to lower the trophic level of the cultured species, and to increase the use of by-products from the primary sector. The golden mullet (*Liza aurata*) is a marine low trophic level consumer present in the Mediterranean and in the Canary Islands, where there was a significant consumption in the past. On the other hand, *Aloe vera* plant, which production in Canary Islands is highly world representative, contains more than 70 biologically active components which have aroused the interest for its use in aquaculture, although the enormous quantity of the generated by-products has no use at all, and has never been tested for aquaculture purposes. The objective of this study was to run the first controlled feeding test with *Liza aurata* in the Canary Islands, as a target species to promote its cultivation, and to evaluate the use of pure Canarian *Aloe vera* against different levels of the by-product, to determine the effects in growth, health, and quality parameters. Therefore, 5 diets were formulated to contain 0% of aloe inclusion (diet control), 2% of pure form of aloe (diet P2), and 2, 4 and 6% of aloe by-product (diets BP2, BP4 and BP6). At 91 days of feeding, growth, proximal and fatty acid composition of liver, muscle and whole body, serum lysozyme, serum antibacterial activity, and malonaldheyde content of liver and whole body, were measured. According to obtained results, up to 6% of the aloe by-product could be included in diets for this species, without any rejections in growth or quality parameters, although no improved results compared to the control fish could be observed. Further studies are on the way to determine the sustainability and bioeconomic impact of present results, to gain knowledge on their direct industrial applications.

1. Introduction

The over-exploitation and lack of terrestrial and fishery resources have turned aquaculture into the main subject to respond to the world's increasing demand for food, which is considered the animal production sector with the fastest growth in the last 40 years (Tveterås et al., 2012).

One of the various measures for the sustainable expansion of aquaculture is species diversification, which helps maintain natural stocks and prevents the spread of diseases, offering the opportunity to expand further the sector (Abellan and Basurco, 1999). Besides, diversification of the culture species is gaining in importance as it helps to satisfy the increasing demand in quantity and variety of aquatic products by consumers (Nielsen et al., 2017).

The Mugilidae family is characterized by being in a low level of the marine trophic chain, mainly as primary and opportunistic consumers, being able to feed on a wide range of products such as plant material, macroalgae, detritus and small macrofauna (Lebreton et al., 2011). For this reason, the feed formulas for this species could presumably include high percentages of plant by-products, compared to species with higher trophic levels, of which it has been widely described low digestibility from feeds with high inclusion of vegetal sources (Mundheim et al., 2004; Zhang et al., 2018). The total global production of mullets was

Abbreviations: BP, by-product of *Aloe vera*; BP2, BP4, BP6, diets with 2, 4 and 6% of by-product of *Aloe vera*; DHA, docosahexaenoic acid; FCPCT, University Technological Scientific Park; GIA-ECOAQUA, aquaculture research group belonging Ecoaqua Institute of the University of Las Palmas de Gran Canaria; GIFT, genetically improved farmed tilapia; HUFA, highly unsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; MDA, malonaldehyde; P, product of *Aloe vera*; P2, diet with a 2% of pure form of *Aloe vera*; SD, standard deviation; Tbars, thiobarbituric acid reactive substances.

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728,546 metric tons in 2014, of which 140,187 were from aquaculture (FAO 2016 in Oliveira et al., 2019).

The golden mullet (*Liza aurata*) has been cultivated for centuries extensively or semi-intensively, being also a key species for artisanal fishing in some Mediterranean countries (Katselis et al., 2007). Furthermore, it is one of the most widely available mullet species in the Canary Island's geographical area. Nevertheless, very little work has been done related to *Liza aurata* nutrition, even less in indoor facilities. Richard et al. (2010) evaluated the effects of periphyton substrates, stocking density, and supplemental feeding on the growth and production of *Liza aurata* juveniles reared in marine ponds. Karapanagiotidis et al. (2014) tested the effects in growth and feed utilization of dry feeds containing different levels of protein in *Liza aurata* juveniles reared in outdoor hapas, while Hotos and Avramidou (2020) compared growth and use of food of *Liza aurata* fry with other mullet species, proving great values of weight increase and food conversion ratio for this species. However, there are no works that evaluate the use of vegetal by-products in diets for *Liza aurata* or the effects of added-value ingredients on this species' health, quality, or antioxidant status.

Aloe vera is a perennial plant belonging to the family Liliaceae, which lives in tropical and subtropical regions, and that contains more than 70 biologically active components (Langmead et al., 2004). These benefits have aroused the interest for its use in aquaculture, having shown some positive effects in fish such as the promotion of the survival against pathogens (Kim et al., 1999), improvement of growth and immune response (Lu et al., 2013), hypocholesterolemic effects (Palermo et al., 2013), effects against oxidative stress (Kang et al., 2014), reduction in the burden of gill parasites (Dotta et al., 2014), activation of the not–-specific immune response (Dotta et al., 2015), effects on haemato-biochemical parameters (Gabriel et al., 2015a), improvements in post-spawning epidermal healing (Zanuzzo et al., 2015), and improvement of the innate immune response after post-transport stress (Zanuzzo et al., 2017).

The Canary Islands is the leading European region in the cultivation and production of *Aloe vera* due to its particular climatic conditions. Although there are no updated data for Aloe vera's production in the Canary Islands, the annual production is growing every year, helped by national and European funding to the sector. According to the producer's personal communication, the generation of value-added products from the *Aloe vera* plant causes an important proportion of waste in the form of mush, from the pressing procedure for the *Aloe vera* gel extraction. Those by-products do not have any use, being a logistical problem to the producers that must eliminate them mainly by natural degradation on the land.

As the need for a "turn" towards a sustainable production strategy in the global political agenda grows, taking advantage of by-products and promoting reusable biomass makes sense. For this reason, we tested for the first time the by-product of Canary *Aloe vera* as a novel ingredient in the diet of *Liza aurata* due to its potential to be a sustainable and pro-health novel ingredient for aquaculture.

To summarize, this study aims to get valuable knowledge for the golden mullet as an alternative and more sustainable species for aquaculture and value the local *Aloe vera* by-product for its inclusion in feeds for this low trophic level marine species. Furthermore, this is the first study of the family Mugilidae in the Canary Islands under aquaculture conditions and required fishing, adaptation to captivity, and standardization of this species' quarantine protocols in this geographical area. The results add relevant new information to the knowledge gap of this species and could be extrapolated to the entire Macaronesian geographical area.

2. Materials and methods

2.1. Experimental animals

Three hundred mullets were carefully fished from the Las Palmas de Gran Canaria sports pier and transported to the research aquaculture group facilities at the Ecoaqua Institute of the University of Las Palmas de Gran Canaria (GIA-ECOAQUA). The mullets were subjected to a quarantine period of one month, in tanks with flow-through natural filtered seawater. Preventive oxytetracycline treatment was carried out (Gisbert et al., 2016), using a dose of 20 mg/ liter of water for five consecutive days. After this period, fish presented well appearance and well adaptation to controlled feeding.

2.2. Experimental design

Two hundred and forty juveniles were distributed in 15 tanks (triplicated by treatment) with an average weight and size of 8.93 ± 1.88 g and 10.08 ± 0.95 cm, respectively. Cylindro-conical PVC tanks of 500 L (n = 16) were used in open circuit with natural seawater in the GIA-ECOAQUA facilities of the University Technological Scientific Park (FCPCT) of Taliarte, Telde.

The average water temperature determined during the feeding period was 20.1 ± 1.22 °C, with an average oxygen content of 6.4 ± 0.37 mg/l. An automatically controlled photoperiod of 12 h of light and 12 h of darkness was used to simulate indoor conditions close to Canary's latitude.

Fish were manually fed, twice a day and six days a week, to apparent satiation. The feeding was performed carefully to avoid the loss of feed, offered until only a few pellets fell to the bottom of the tanks, given that un-eaten feed was not observed between meals.

2.3. Diets

Aloe vera of Canarian origin was tested in the form of both pure commercial product (aloe gel) and by-product (remained after compression of the product). Both were ceded by a Canarian company in the form of gel, so for its evaluation and inclusion in the diets, the following processing was carried out: a) Product of *Aloe vera* (P), lyophilization (IMA Telstar Model 50 Hz, Spain); b) By-product of *Aloe vera* (BP), lyophilization (IMA Telstar Model 50 Hz, Spain) and drying in an airflow oven at a temperature below 40 °C, in the GIA-ECOAQUA Pilot Plant of Products and Processes. The polyphenol content, due to its recognized bioactive antioxidant role (Gabriel et al., 2015b), was taken as a reference to decide the end quality of the by-product in relation with the pure aloe and to decide the different levels of by-product inclusion in diets, also according to reported results for aloe in fish diets (Gabriel et al., 2015b).

Five isoproteic (40% protein) and isolipidic (15% lipid) diets were formulated, with different raw materials and different percentages of inclusion of *Aloe vera* in the form of pure product and by-product (Table 1). The mixed ingredients were pelleted through a 1.6 mm die in a compressed pellet machine (California Pellet Mill, USA, sourced by Eriez Magnetics, UK). The obtained pellets were stored at 12 °C before use.

Samples of raw materials, aloe and feed, were taken for the corresponding biochemical (Tables 1 and 2) and polyphenol analyzes. Samples for biochemical analysis of whole fish and tissues were taken at the beginning and end of the experiment. The experiment's final sampling was carried out after 91 days of feeding when the weight of the fish was duplicated.

2.4. Growth and feed utilization parameters

The weight gain was obtained by the formula: Weight gain = Final weight (g)- Initial weight (g).

Specific growth ratio was calculated by the formula: $SGR = (lnWf - lnWi)/ days\ of\ experiment\ x100$, where:

lnWf: Final weight neperian logarithm

lnWi: Initial weight neperian logarithm

Feed intake (voluntary daily amount of feed eaten by the animals,

Table 1
Experimental diets formula (%) and proximal composition (mean ± SD).

Ingredients (%)	Control	P2	BP2	BP4	BP6
Fish meal (Peruvian origin)[a]	20.0	20.0	20.0	20.0	20.0
Blood meal[b]	3.0	3.0	3.0	3.0	3.0
Ulva meal[c]	10.0	10.0	10.0	10.0	10.0
Rapeseed meal (0.0)[d]	8.0	8.0	8.0	8.0	8.0
Corn meal[e]	6.0	5.0	5.0	4.0	3.0
Wheat gluten[e]	6.0	6.0	6.0	6.0	6.0
SPC (soy protein concentrate)[f]	20.0	20.0	20.0	20.0	20.0
Wheat meal[e]	6.0	5.0	5.0	4.0	3.0
Fish oil[a]	8.5	8.5	8.5	8.5	8.5
Soy lecitin[g]	1.0	1.0	1.0	1.0	1.0
Aloe product[h]	0.0	2.0	0.0	0.0	0.0
Aloe by-product[h]	0.0	0.0	2.0	4.0	6.0
Vitamin mix[i]	2.0	2.0	2.0	2.0	2.0
Mineral mix[j]	2.0	2.0	2.0	2.0	2.0
Ca(H2PO4)2[k]	1.0	1.0	1.0	1.0	1.0
CMC[l]	0.5	0.5	0.5	0.5	0.5
Analytical composition					
Protein	41.5 ± 0.3	40.5 ± 0.3	41.2 ± 2.3	39.6 ± 0.9	39.5 ± 0.4
Lipids	14.9 ± 0.3	14.1 ± 0.3	15.0 ± 2.3	15.4 ± 0.9	14.2 ± 0.4
Ash	10.6 ± 0.0	10.9 ± 0.0	11.6 ± 0.6	11.6 ± 0.2	11.7 ± 0.2
Humidity	7.7 ± 0.4	7.1 ± 0.4	6.3 ± 0.5	6.9 ± 0.1	7.1 ± 0.2

[a] Fish meal and fish oil from South America (supplied by Skretting, Spain).
[b] Blood meal (supplied by Dibaq, España).
[c] Ulva meal (supplied by Puerto Muiños S.L., Spain).
[d] Rapeseed 0.0 (supplied by Dibaq, Spain).
[e] Flours obtained from local producers.
[f] Soy protein concentrate (Sopropeche, France).
[g] Soy lecithin (92% fat; Korott S.L., Alicante).
[h] Aloe y Aloe by-product (local production).
[i] Vitamin premix containing (mg kg-1 o IU/kg of dry feed): thiamine 40 mg, riboflavin 50 mg, pyridoxine 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, folic acid 10 mg, cyanocobalamin 0.5 mg, choline chloride 2700 mg, mio-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg, cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU. Vitamin E (DL-alpha-tocopherol acetate) 250 mg.
[j] Mineral premix containing (g/kg of dry feed): calcium orthofosfate 1.60 g, calcium carbonate 4 g, ferrous sulfate 1.5 g, magnesic sulfate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminum sulfate 0.0 g, zinc sulfate 0.24 g, copper sulfate 0.20 g, manganese sulfate 0.1 g, potassium iodate 0.0 g.
[k] Sigma-Aldrich, Munich, Germany.
[l] Carboxymethylcellulose (sodium salt, Sigma-Aldrich, Munich, Germany).

expressed as a percentage of body weight) was obtained by the formula: *Feed intake= (feed consumed (g) /fish weight average(g))/ days x100*, and the feed conversion ratio, an indicator of the efficiency of growth, was calculated by the formula: *FCR = Feed consumed (g)/Weight gain (g)*. Feed efficiency was obtained by the formula *FE = Weight gain (g) /Feed consumed (g)*.

2.5. Biochemical analyses

2.5.1. Proximal composition and fatty acid analyses

The whole-body proximal and fatty acid composition was evaluated in a pool from two fish per tank (6 fish per treatment), while the proximal and fatty acid composition of liver and muscle were evaluated in a pool of 5 fish per tank (15 fish per treatment).

Proteins were determined by the Kjeldahl technique (AOAC, 2000). Moisture and ash content were determined, according to AOAC (2000).

The determination of total lipids was carried out as described by Folch et al. (1957). The total lipids were trans-esterified with 1% sulfuric acid in methanol, following the methodology of Christie (1989). A

Table 2
Experimental diets fatty acid profile expressed in % of the total fatty acids identified.

	Control	P2	BP2	BP4	BP6
14:00	2.3	2.1	2.3	1.5	2.2
15:00	0.3	0.3	0.3	0.2	0.3
16:00	14.7	14.1	14.0	12.2	14.1
18:00	1.6	3.5	3.5	3.7	3.5
Σsaturated[1]	19.3	20.4	20.5	18.2	20.6
16:1n-7	3.0	2.9	3.0	2.6	3.0
18:1n-9	33.6	32.2	32.6	33.3	32.8
18:1n-7	2.0	3.0	2.9	3.1	3.0
20:1n-7	3.1	2.9	3.0	3.5	3.0
Σmonoenes[2]	45.2	44.4	45.0	46.6	45.5
18:2n-6	17.5	18.1	17.3	17.5	17.2
20:4n-6	0.5	0.5	0.5	0.5	0.5
Σ n-6 PUFA[3]	19.2	19.8	19.0	19.7	18.9
18:3n-3	4.4	3.8	3.7	3.8	3.7
Σ n-3 PUFA[4]	14.9	14.2	14.2	14.4	13.7
20:5n-3	2.5	2.6	2.7	2.7	2.6
22:5n-3	0.9	1.0	1.0	1.0	0.9
22:6n-3	5.5	5.3	5.4	5.4	5.1
Σ n-3 LC-PUFA[5]	9.7	9.7	9.8	10.0	9.3
Total PUFA[6]	34.7	34.4	33.8	34.6	33.2
n-3/n-6	0.8	0.7	0.8	0.7	0.7

[1] Totals include 16:OISO and 20:00.
[2] Totals include 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5, 22:1n-11 and 22:1n-9.
[3] Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:5-n6 and 22:4n-6.
[4] Totals include 18:4n-3, 20:3n-3, and 20:4n-3.
[5] Totals include 20:3n3 and 20:4n-3.
[6] Totals include 18:2n-9, 18:2n-4, 18:4n-3 18:4n-1, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 20:3n-3, 20:4n-3 and 22:4n-6.

dilution was made in hexane, and the separation, identification and quantification of the different fatty acids were carried out through gas chromatography, following the protocol described by Izquierdo et al. (1992).

All analyses were done in triplicate samples.

2.5.2. Polyphenol determination

The determination of polyphenols was carried out in 3 replicated samples of the lyophilized product of *Aloe vera* and the by-product of *Aloe vera,* lyophilized and dried, using the technique described by González-Montelongo et al. (2010).

The biochemical and polyphenol content determined for the lyophilized pure aloe and by-product showed that both lipid and protein content were remarkably low (1.57 &1.14% and 0.1 & 0%, respectively), which indicate that both, lyophilized product and by-product, had a remarkable amount of carbohydrates. The amount of ash was 15.09% and 6.51% for the lyophilized pure aloe and by-product, respectively. The lyophilized by-product presented 58.02% of the polyphenols that had the pure lyophilized product (1.88 and 3.24 mg/g, respectively), with lower differences (24.47%) found for the oven-dried by-product versus lyophilized by-product (1.42 mg/g and 1.88 mg/g, respectively), which is of interest at the time to optimize methodologies of the processing by-products on a large scale.

2.6. Immunological analyses

The determination of the serum lysozyme activity was performed in triplicates by turbidimetry, from a pool of serum of 8 fish per tank, following the technique of Anderson and Siwicki (1994). The bactericidal activity in serum was also done from the pool of 8 fish per tank, following the technique described by Sunyer and Tort (1995).

2.7. Antioxidant analyses

Thiobarbituric acid reactive substances (Tbars) were used. The determination of the malonaldehyde content in the whole body and liver

samples of 2 and 5 fish per tank (6 and 15 fish per treatment), respectively, was carried out from a dilution of 10 mg/ml of the total lipids of the sample, following the modification of the protocol of Burk (1980).

2.8. Statistical analyses

To determine normality, all results were subjected to the nonparametric Kolmorov-Smirov test. The homogeneity of variances was determined by the Levene test ($P \geq 0.05$). The variance analysis was performed using one-way ANOVA, and the means were compared by Duncan's post-hoc test ($P \leq 0.05$). Lineal and quadratic regression models were used to determine correlations between some parameters. All the analyses have been carried out using the statistical program IBM® SPSS Statistic 20 (New York, USA).

3. Results

3.1. Growth

There were no significant differences for weight or length at the end of the experiment, nor for the growth and feed utilization parameters (Table 3).

3.2. Proximal and fatty acid composition of the fish

Regarding the biochemical analysis, no statistically significant differences were found between the fish fed with the different diets no for whole fish nor for any of the studied tissues (Table 4). The fatty acid composition (Tables 5 and 6), in general, did not also differ significantly between the fish of the different treatments, neither for the control or the Aloe supplementation treatments.

In the muscle, the levels of monoenes and saturated fatty acids were lower in those treatments with BP inclusion, while levels of n-3 and total PUFA were higher in treatments BP4 and BP6 (Table 6), although, except for monoenes in the BP4 treatment and ARA in treatments BP4 and BP6, there were no statistical differences with the control group.

Regarding the liver, although there were some differences in certain fatty acids, as the higher percentage of linoleic acid in the aloe treatments, in general, no significant differences were observed between groups.

3.3. Immunological analyses

Lysozyme analysis results (U/mL) showed significant differences (Duncan post-hoc test, $p \leq 0.05$) between the fish of the BP2 diet with the lowest level of lysozyme compared with the fish fed with the other diets (Fig. 1). For bactericidal activity, no significant differences between treatments were observed (Fig. 1), with results between 35. 77 ± 7.32% and 43.37 ± 8.6%, for P2 and BP2 treatments, respectively.

Table 3
Values for parameters of growth performance and use of diets (n = 48 per treatment).

	Control	P2	BP2	BP4	BP6
Initial weight (g)	8.8 ± 0.1	8.8 ± 0.3	9.3 ± 0.5	8.9 ± 0.2	8.9 ± 0.4
Final weight (g)	19.8 ± 2.5	18.5 ± 0.9	18.0 ± 0.7	18.4 ± 0.3	18.5 ± 0.3
Weight gain (g)	10.9 ± 2.5	9.7 ± 0.8	8.7 ± 1.2	9.5 ± 0.4	9.6 ± 0.2
SGR (%/day)	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.10	0.8 ± 0.0	0.8 ± 0.0
Feed Intake (%)	1.6 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.8 ± 0.2
FCR	2.0 ± 0.2	2.4 ± 0.3	2.4 ± 0.4	2.2 ± 0.2	2.3 ± 0.2
FE	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.0

Data expressed as means± SD. Values of the means in the same row without superscripts indicate the absence of significant differences (p≥0.05).

Table 4
Proximal composition, expressed in % of dry weight, for whole-body (n = 6 per treatment) and muscle (n = 15 per treatment) of fish fed with experimental diets.

	Control	P2	BP2	BP4	BP6
Whole body					
Lipids	35.2 ± 7.6	31.6 ± 3.0	29.2 ± 7.3	27.2 ± 2.5	34.9 ± 0.9
Protein	55.6 ± 4.3	56.9 ± 3.8	58.5 ± 5.8	60.7 ± 3.2	55.5 ± 0.9
Ash	12.1 ± 2.9	11.0 ± 1.7	12.2 ± 2.9	12.7 ± 1.9	11.6 ± 0.8
Muscle					
Lipids	17.8 ± 2.5	16.4 ± 0.8	16.1 ± 1.6	16.2 ± 0.9	16.7 ± 2.3
Protein	84.8 ± 1.7	84.0 ± 2.3	84.1 ± 2.4	84.1 ± 2.8	85.1 ± 3.9
Ash	5.6 ± 1.1	5.3 ± 0.3	5.3 ± 0.6	5.4 ± 0.3	5.2 ± 0.6

Data expressed as means± SD. Values of the means in the same row without superscripts indicate the absence of significant differences (p≥0.05).

Table 5
Fatty acid profile, expressed in% of the total fatty acids identified, of the whole body of the different experimental groups (n = 6 per treatment), at the end of the experiment.

	Control	P2	BP2	BP4	BP6
14:0	2.0 ± 0.2	2.0 ± 0.5	1.5 ± 0.5	2.0 ± 0.30	1.99 ± 0.1
15:0	0.3 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.2 ± 0.1^{b}	0.3 ± 0.0^{ab}	0.4 ± 0.1^{a}
16:0	14.7 ± 0.9	15.4 ± 1.2	12.5 ± 3.1	14.8 ± 0.5	14.3 ± 0.4
18:0	3.1 ± 0.1	3.0 ± 0.3	3.1 ± 0.3	3.2 ± 0.3	2.7 ± 0.3
Σsaturated[1]	20.4 ± 1.0	21.0 ± 1.4	17.7 ± 3.5	20.7 ± 0.4	19.9 ± 5.5
16:1n-7	3.8 ± 0.0	2.9 ± 2.5	3.3 ± 0.7	4.3 ± 0.4	3.1 ± 1.2
18:1n-9	31.2 ± 2.0	32.3 ± 2.6	30.8 ± 1.0	30.4 ± 1.0	28.5 ± 4.0
18:1n-7	3.3 ± 0.1^{ab}	4.0 ± 0.6^{a}	3.2 ± 0.2^{b}	3.1 ± 0.2^{b}	3.0 ± 0.5^{b}
20:1n-7	2.6 ± 0.1	2.7 ± 0.6	2.6 ± 0.2	2.1 ± 0.3	3.0 ± 0.7
Σmonoenes[2]	9.0 ± 0.5	7.6 ± 2.4	8.3 ± 0.3	8.4 ± 0.3	9.7 ± 2.4
18:2n-6	16.8 ± 0.4	17.0 ± 0.8	18.3 ± 1.5	17.9 ± 0.8	16.8 ± 1.3
20:4n-6	0.7± 0.2	0.6 ± 0.1	0.8 ± 0.3	0.9± 0.2	0.8 ± 0.2
22:5n-6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3
Σ n-6 PUFA[3]	19.1 ± 0.5	19.3 ± 1.1	20.9 ± 2.1	20.2 ± 0.9	19.9 ± 0.1
18:3n-3	4.4 ± 0.1	4.0 ± 0.4	4.5 ± 0.7	4.3 ± 0.5	4.2 ± 0.2
Σ n-3 PUFA[4]	15.2 ± 1.7	14.4 ± 2.2	17.6 ± 3.9	15.4 ± 1.6	18.5 ± 5.0
20:5n-3	2.3 ± 0.4	2.2 ± 0.4	2.6 ± 0.6	2.8 ± 0.4	3.0 ± 1.0
22:5n-3	1.5 ± 0.4	1.3 ± 0.2	1.7 ± 0.4	1.4 ± 0.2	1.9 ± 0.8
22:6n-3	5.5 ± 0.7	5.5 ± 0.8	7.3 ± 2.1	5.5 ± 0.2	7.5 ± 2.5
Σ n-3 LC-PUFA[5]	10.2 ± 1.5	9.9 ± 1.4	12.5 ± 3.1	10.6 ± 0.6	13.5 ± 4.7
Total PUFA[6]	35.3 ± 2.1	34.4 ± 2.4	39.2 ± 4.8	36.4 ± 1.3	39.9 ± 5.9
n-3/n-6	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	1.0 ± 0.3

Data expressed as means± SD. Values with different superscripts in the same row indicate significant differences according to Duncan's post hoc test (P≤0.05).

[1] Totals include 16:OISO and 20:00.
[2] Totals include16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5, 22:1n-11 and 22:1n-9.
[3] Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:5-n6 and 22:4n-6.
[4] Totals include 18:4n-3, 20:3n-3, and 20:4n-3.
[5] Totals include 20:3n3 and 20:4n-3.
[6] Totals include 18:2n-9, 18:2n-4, 18:4n-3 18:4n-1, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 20:3n-3, 20:4n-3 and 22:4n-6.

3.4. Antioxidant analyses

Thiobarbituric acid reactive substances in the whole body and liver of fish (Fig. 2) showed no significant differences between groups.

Table 6
Fatty acid profile expressed in% of fatty acids identified, from the muscle and liver of the mullets of the different experimental groups (n = 15 per treatment), at the end of the experiment.

	Muscle					Liver				
	Control	P2	BP2	BP4	BP6	Control	P2	BP2	BP4	BP6
14:0	1.6 ± 0.5	1.7 ± 0.5	1.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.6	2.0 ± 0.8	1.9 ± 0.5	1.7 ± 0.8	1.5 ± 0.4	0.9 ± 0.7
15:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:0	15.1 ± 1.6	15.5 ± 1.5	14.9 ± 0.0	13.4 ± 0.3	13.0 ± 1.9	14.1 ± 4.9	17.3 ± 2.6	15.3 ± 4.7	14.9 ± 1.3	10.4 ± 7.5
18:0	3.5 ± 0.3^{ab}	3.7 ± 0.0^{ab}	3.8 ± 0.3^{a}	3.2 ± 0.2^{b}	3.5 ± 0.5^{ab}	2.6 ± 0.3^{b}	3.4 ± 0.3^{a}	3.1 ± 0.2^{ab}	3.3 ± 0.6^{a}	3.1 ± 0.2^{ab}
Σsaturated[1]	20.9 ± 2.0	21.6 ± 2.1	20.7 ± 0.2	19.6 ± 0.1	18.6 ± 2.6	19.4 ± 5.2	23.0 ± 2.8	20.6 ± 5.5	20.2 ± 1.2	14.8 ± 8.3
16:1n-7	3.2 ± 0.5	3.3 ± 0.4	3.3 ± 0.1	3.2 ± 0.1	2.9 ± 0.6	4.8 ± 2.4	5.0 ± 0.8	4.5 ± 1.5	4.1 ± 1.0	3.0 ± 2.0
18:1n-9	29.9 ± 1.9^{a}	30.2 ± 0.8^{a}	31.3 ± 0.6^{a}	26.0 ± 2.4^{b}	28.6 ± 1.6^{ab}	27.6 ± 5.1	31.1 ± 0.9	31.1 ± 3.6	30.2 ± 2.4	32.0 ± 1.0
18:1n-7	3.2 ± 0.2^{ab}	3.3 ± 0.1^{ab}	3.4 ± 0.1^{a}	3.0 ± 0.3^{b}	3.1 ± 0.2^{ab}	3.9 ± 0.6	4.5 ± 0.1	2.7 ± 2.3	4.3 ± 0.2	4.4 ± 0.2
20:1n-7	2.6 ± 0.4	2.6 ± 0.2	2.7 ± 0.3	2.5 ± 0.5	2.6 ± 0.6	2.7 ± 0.7^{ab}	2.3 ± 0.1^{b}	2.7 ± 0.3^{ab}	2.7 ± 0.2^{ab}	3.1 ± 0.6^{a}
Σmonoenes[2]	42.2 ± 1.3^{bc}	42.4 ± 0.8^{bc}	43.2 ± 1.3^{c}	39.4 ± 2.0^{a}	40.4 ± 0.8^{ab}	43.4 ± 6.1	45.2 ± 1.5	44.0 ± 3.4	44.2 ± 3.3	45.8 ± 1.7
18:2n-6	15.4 ± 0.5^{ab}	15.8 ± 0.6^{a}	16.4 ± 0.5^{a}	14.3 ± 0.9^{b}	15.8 ± 0.9^{a}	9.2 ± 1.7^{b}	12.0 ± 0.6^{a}	12.6 ± 1.4^{a}	12.2 ± 0.6^{a}	14.4 ± 1.7^{a}
20:4n-6	0.8 ± 0.1^{b}	0.9 ± 0.1^{b}	1.0 ± 0.0^{ab}	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.0 ± 0.3	1.3 ± 0.3	1.4 ± 0.4	1.4 ± 0.4	1.5 ± 0.5
22:5n-6	0.4 ± 0.1^{b}	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	1.0 ± 0.4^{a}	0.5 ± 0.1^{b}	1.8 ± 2.0	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.0	0.3 ± 0.1
Σ n-6 PUFA[3]	18.7 ± 0.24	19.2 ± 0.21	19.3 ± 0.4	19.3 ± 0.6	19.4 ± 0.5	16.2 ± 7.9	15.6 ± 1.2	16.4 ± 2.7	16.0 ± 1.4	18.4 ± 2.9
18:3n-3	3.8 ± 0.2	3.5 ± 0.3	3.8 ± 0.3	3.3 ± 0.2	3.5 ± 0.1	2.0 ± 0.4^{b}	2.3 ± 0.1^{ab}	2.5 ± 0.6^{ab}	2.3 ± 0.0^{ab}	3.0 ± 0.7^{a}
Σ n-3 PUFA[4]	16.2 ± 2.1	15.0 ± 3.2	15.4 ± 1.7	17.0 ± 1.2	19.2 ± 3.0	27.4 ± 12.5	23.5 ± 6.0	26.8 ± 9.5	28.2 ± 9.1	29.7 ± 10.9
20:5n-3	2.4 ± 0.4	2.3 ± 0.6	2.5 ± 0.3	3.0 ± 0.5	3.0 ± 0.3	1.8 ± 0.5	2.0 ± 0.4	2.3 ± 0.9	2.4 ± 0.4	2.6 ± 0.9
22:5n-3	1.8 ± 0.3	1.5 ± 0.5	1.6 ± 0.2	1.7 ± 0.3	2.1 ± 0.4	1.7 ± 1.4	0.9 ± 0.2	1.2 ± 0.5	1.2 ± 0.3	1.3 ± 0.5
22:6n-3	6.8 ± 1.4^{ab}	6.1 ± 2.3^{b}	6.3 ± 1.1^{ab}	7.2 ± 1.1^{ab}	9.1 ± 2.4^{a}	9.7 ± 3.8	8.7 ± 2.6	9.8 ± 3.6	10.5 ± 4.0	10.8 ± 4.2
Σ n-3 LC-PUFA[5]	11.9 ± 2.1	11.1 ± 2.9	11.2 ± 1.6	13.0 ± 1.3	15.1 ± 3.1	14.2 ± 5.9	12.1 ± 3.3	14.1 ± 5.2	15.0 ± 4.4	15.5 ± 6.0
Total PUFA[6]	35.9 ± 2.^{2ab}	35.04 ± 2.9^{b}	35.3 ± 1.4^{b}	38.7 ± 0.6^{ab}	39.7 ± 2.5^{a}	36.1 ± 10.5	31.2 ± 4.2	34.7 ± 8.2	34.9 ± 4,6	38.6 ± 9.6
n3/n6	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.4	1.1 ± 0.9	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2

Data expressed as means± SD. Values with different superscripts in the same row indicate significant differences according to Duncan's post hoc test (P≤0.05).
[1] Totals include 16:OISO and 20:00.
[2] Totals include 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5, 22:1n-11 and 22:1n-9.
[3] Totals include 18:3n-6, 20:2n-6, 20:3n-6, and 22:4n-6.
[4] Totals include 18:4n-3, 20:3n-3, and 20:4n-3.
[5] Totals include 20:3n3 and 20:4n-3.
[6] Totals include 18:2n-9, 18:2n-4, 18:4n-3 18:4n-1, 20:2n-9, 20:2n-6, 20:3n-9, 20:3n-6, 20:3n-3, 20:4n-3 and 22:4n-6.

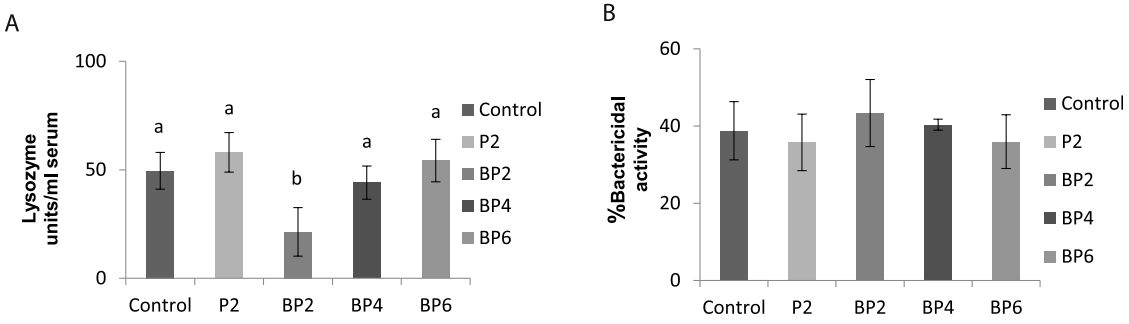

Fig. 1. Lysozyme and bactericidal activity in serum from the fish fed with de different diets (n = 8 per treatment).
Data expressed as means± SD. Columns with superscripts indicate the presence of significant differences (p≤0.05).

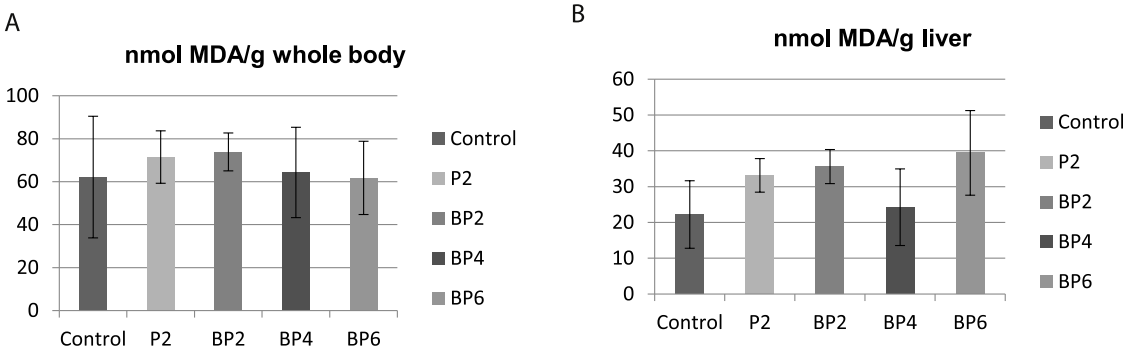

Fig. 2. Tbars content expressed in nmoles of malonaldehyde (MDA) per gram of whole body and liver (n=8 per treatment).
Data expressed as means± SD. Columns without superscripts indicate the absence of significant differences (p≥0.05).

4. Discussion

The mullets have adapted quickly to tanks and experimental feeds, with no differences in appetite for the five tested diets from the beginning, nor among the increasing BP levels up to 6%. Daily intake ranged between 2.11–2.40% at the beginning and 1.33–1.54% at the end of the trial. These results are in concordance with voluntary feeding rates around 2.4% reported for *Liza aurata* fry (0.15–4 g) (calculated data from Hotos and Avramidou, 2020), and also with those reported by Karapanagiotidis et al. (2014), who described feeding rates under 2% for medium-size animals (50−100 g approximately).

In terms of growth, 2% of *Aloe vera* supplementation were related to improved growth performance in species like the common carp (*Cyprinus carpio*) (Mahdavi et al., 2013) and the GIFT-tilapia (Gabriel et al., 2015a), although other authors described no growth improvement in the goldfish (*Carassius auratus*) (Palermo et al., 2013). In the present study, with no improved results for the P2 group, it was found that up to 6% of *Aloe vera* by-product in the diet gave comparable growth results in the golden mullet to the other groups.

The specific growth rate and the feeding efficiency were markedly better than those previously described for *Liza aurata* raised in hapas in a coastal lagoon (51.1 ± 5.2 g of initial weight) (Karapanagiotidis et al., 2014). Vagner et al. (2014) also reported lower SGR values for *Liza aurata* juveniles reared in indoor recirculation tanks (26.1 ± 0.4 g of initial weight). Thus, the smaller fish size, proper diets or rearing conditions may promote the improved results obtained in this assay. However, growth parameters and feeding efficiency in the present trial were lower than those described for smaller fish of this species (Hotos and Avramidou, 2020), which is in concordance with general better growth of fish at early life stages.

The administration of the different diets has also not caused significant differences in terms of the proximal composition. Although very few studies have considered the relation between *Aloe vera* supplementation and body composition changes, it has been reported that the administration of *Aloe vera* nanoparticles in feeds for the Siberian sturgeon (*Acipenser baerii*) neither produced changes in body composition (Sharif Rohani et al., 2017). However, other studies do have reported modifications in the body composition due to dietary *Aloe vera* supplementation at similar doses, as the reduction in the muscle moisture and protein contents in GIFT tilapia juveniles (Gabriel et al., 2017), or the reduction in the whole body lipid content in African catfish (*Carias gariepinus*) juveniles (Gabriel et al., 2020).

It is remarkable that in the case of muscle, the fat content in the present trial was five times higher than that described for this species by other authors. Vagner et al. (2014) reported total lipid contents in the muscle around 30 mg/g (around 3.0 expressed in % of dry weight) in *Liza aurata* juveniles (26.1 ± 0.4 g of initial weight), reared in indoor tanks (19.9 ± 0.5 °C and 33.4 ± 0.1 g/l of temperature and salinity values, respectively), and fed diets containing around 11% of lipid content, at 2% of daily feed intake. Also, Karapanagiotidis et al. (2014) described low fillet lipid values (around 7.0% in dry weight) in fish of approximately 100 g of final weight, fed with diets containing 6.6–9.2% of crude fat, which were reared in hapas in the Lafra Lagoon, Greece (12−26 °C and 27.0–32.8 g/l of temperature and salinity values, respectively). This aspect gives an idea of this species' high capacity to accumulate fat in the muscle, probably depending on age, lipid content in the diet, and rearing conditions.

Regarding the fish fatty acid profile, although no significant effects were found in general, an interesting correlation between the percentage of aloe by-product in the diets and DHA concentration in both muscle ($R^2 = 0.998$, $p = 0.042$, quadratic regression model) and liver ($R^2 = 0.930$, $p = 0.036$, lineal regression model) was found, and also higher levels of ARA in muscle of the fish fed with the by-product diets and higher levels of linoleic acid in the liver of the fish fed with all aloe diets. Although no studies have been performed in fish before which related *Aloe vera* supplementation with changes in the fatty acid profile, Rajasekaran et al. (2006) related the administration of oral *Aloe vera* with the restoration of the PUFA composition in diabetic rats, presumably, due to the elimination of free radicals and the control of lipid metabolism. All this data may suggest that the *Aloe vera* by-product could play a positive role in the accumulation of some essential fatty acids in the fish, which increases with dietary inclusion of the by-product up to 6%.

Lysozyme activity variations have been associated with a wide range of factors as sex, age, size, season, toxicants, pH, water temperature, infections or stressors. However, lysozyme increases also had been related to increased protection against various diseases in various fish species (Saurabh and Sahoo, 2008). In the present study, plasma lysozyme content showed no clear tendencies, since the only significant difference was for fish fed BP2 diet, which presented the lower concentration of lysozyme, being the higher observed in P2, followed by BP6 treatment. A similar trend was described for tilapia (*Oreochromis niloticus*) by Gabriel et al. (2015a), in whose study they did not find statistical differences among groups, but also higher concentrations in fish fed with *Aloe vera* powder supplementation. The lysozyme values of fish in the present study were similar or even higher than those described for mullets in captivity (*Mugil cephalus*) by Akbary and Jahanbakhshi (2016); this data could suggest that, regarding lysozyme and in general terms, the fish in the present study presented a good immunological status, regardless of the feeding diets used. Similarly, bactericidal activity results, without statistical differences due to aloe product and by-product supplementation, were comparable with those reported by Dotta et al. (2014), which documented non-statistical differences in bactericidal activity among tilapia (*Oreochromis niloticus*) groups fed with aloe and propolis mixtures in comparison with control ones.

Regarding oxidative status, in the present experiment, no significant differences were found in malonaldehyde concentration either for the liver or the whole body, without showing any tendency between the different treatments. This coincides with no results found in rainbow trout (*Oncorhynchus mykiss*) fed with *Aloe vera* (Golestan et al., 2015). If we compare liver MDA results with those reported for *Mugil cephalus* (Ben Ameur et al., 2012), around 100 nmol/g tissue, it is found that in the present experiment, the animals demonstrated lower MDA rates. Compared with the values reported for seabass by this same article (around 200 nmol/g of tissue), this difference is even more noticeable, so it can be deduced that the mullets analyzed in this test had a good antioxidant status as regards lipid peroxidation.

To sum up, the administration of dietary *Aloe vera* and its by-product, in general, has not produced an improvement in either the immunological or in the antioxidant status of *Liza aurata* juveniles, due probably to a good basal general status of the animals, which may have dissembled possible beneficial effects of both pure *Aloe vera* and *Aloe vera* by-product.

5. Conclusions

The growth and feed utilization by the *Liza aurata* have not been affected by the inclusion of aloe product and by-product, being the biochemical composition of the animals similar between treatments.

It has been the first time observed for the *Aloe vera* by-product an interesting tendency in the fish fatty acid metabolism, promoting mostly ARA muscle content. Further studies must be done to better understand the effects of these ingredients on fish lipid metabolism.

Aloe vera's by-product up to 6% can be used in diets for *Liza aurata* against cereals, without rejections in growth or quality parameters of the muscle tissue, which promote alternative use of Aloe vera's by-product and the subsequent circularity by producers.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.5. Effects of fish oil replacement and environmental salinities on fatty acid profile and expression of fatty acid desaturase and elongase genes in the thick-lipped grey mullet (*Chelon labrosus*)

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Abstract

The global increase in marine aquaculture and the overall collapse in the fish meal (FM) and fish oil (FO) market have turned the search for suitable alternatives into one of the main challenges aquaculture faces today. One of the most significant FO substitution bottlenecks is that marine fish generally have a limited capacity to convert 18C fatty acids into long-chain highly unsaturated fatty acid (LC-HUFA). However, the family Mugilidae (mullets) is a great candidate to explore this physiological potential, as it has demonstrated LC-HUFA biosynthesis capacity in some mullet species. Besides, although mullets are euryhaline species, salinity may affect their fatty acid utilization.

The present study aimed to analyze the dietary effects on the LC-HUFA biosynthesis capacity of *Chelon labrosus* as mullet target species and to explore the effects on that by culture salinity conditions. For that reason, triplicate groups of juveniles of this species were fed on two different diets including (FO) or linseed oil (LO), each of them being also reared at three different salinities (46 ppt, 35 ppt and 16 ppt). The fish were analysed to determine the proximate and fatty acid composition of the whole body, liver and muscle, as well as the relative gene expression of elongase (*Elovl5*) and desaturase (Fads2) enzymes in the liver.

The two diets (FO & LO) performed equally regarding growth, the whole body and liver DHA and n-3 LC-HUFA levels and biochemical composition. Regarding environmental salinity, fish reared in brackish water (BW, 16ppt) had better growth and higher expression of *Elovl5* in the liver. Additionally, muscle DHA, and n-3 LC-HUFA contents

were similar between the mullets fed the LO diet reared in BW, and the fish fed the FO diet in all salinities. In conclusion, the present results support the feasibility of FO-free diets for *Chelon labrosus* juveniles, mainly when reared in BW.

Keywords: Mugilidae, biochemistry, fatty acid, LC-HUFA, sustainable aquaculture

1. Introduction

Aquaculture is widely regarded as the fastest-growing animal food production sector globally, with a total production of fish, crustaceans, and molluscs of 87.5 million tonnes in 2020, which reached a total value of 264 800 million USD (United States Dollar) (FAO, 2022). This meaningful expansion has also increased the demand for ingredients for aquafeeds, being fish meal (FM) and fish oil (FO), protein and lipid sources, with still high demand to produce commercial diets. This aspect, the imminent outstrip of the supply, and escalating prices make dietary FM and FO a renewed bottleneck for further sector expansion, increasing the urgency within the global aquafeed industry to find suitable alternatives. Regarding FO substitution, vegetable lipids are being years back widely implemented in the aquaculture industry (Turchini *et al.*, 2019). However, although these sources are abundant in precursors of 18C (fundamentally linoleic and α-linolenic fatty acids), the use of plant oils in marine aquaculture is limited due to the lack of n-3 highly unsaturated fatty acids (n3 LC-HUFA) (Oliva-Teles *et al.*, 2015). Marine fish generally have limited activity of desaturase and elongase enzymes to convert 18C precursors to LC-HUFA. Therefore, those long-chain fatty acids are considered essential in most marine fish's diets, directly impacting fish health and welfare (Montero and Izquierdo, 2010; Oliva-Teles *et al.*, 2015).

Δ6 desaturase (Δ6D), the main limiting enzyme involved in LC-HUFA biosynthesis, is modulated by nutritional and environmental conditions in fish (Vagner and Santigosa, 2011). The gene expression and activity of Δ6D is higher in fish fed vegetable oils (VO) than those fed FO, although in general this compensatory mechanism is insufficient to maintain tissues fatty acid profile (Sarker *et al.*, 2011; Rosas *et al.*, 2019). In addition, although data on the modulation of metabolic pathways and fatty acid requirements by salinity is limited, culture practices at different salinities have been shown to affect the long chain poly-unsaturated fatty acid (LC-PUFA) metabolism in fish (Izquierdo and Koven, 2010; Marrero *et al.*, 2021).

In this sense, the thick-lipped grey mullet (*Chelon labrosus*) has a great potential for sustainable aquaculture diversification due to its worldwide distribution and its omnivore and euryhaline nature (Lebreton *et al.*, 2011), which will allow to implement more sustainable and profitable nutritional practises by producers. Phylogeny of the species seems to play a crucial role in the LC-HUFA biosynthesis capacity of fish species, having been demonstrated though radiolabeled [1-^{14}C] fatty acids, the enzymatic capacity to biosynthesize docosahexaenoic acid (DHA, 22:6n-3) from linolenic acid (18:3n-3) for *Chelon labrosus* (Galindo *et al.*, 2021). Additionally, it has been

hypothesised for mullets that a decrease in salinity can lead to an improvement in the potential of PUFA biosynthesis (Khériji *et al.*, 2003; Imen *et al.*, 2013; Rabeh *et al.*, 2015). However, there are no studies which directly evaluate the combined effect of VO diets and salinity on the expression of Δ6 and Δ5 desaturases, and elongases enzymes, neither of the effects because the combination of these two factors in the growth, the proximal composition, and the fatty acid profile of this species.
On the other hand, there is a traditional and gastronomic added value of fish reared in salines and estuaries, which also are nowadays enclaves with an interesting potential for revalorization and implementation of novel practises as aquaponics or eco-tourism. As mullets have a traditional baggage in these environments, the present study not only aimed to evaluate the combined effect between diet and brackish or seawater, but also including high salinity water and its interactions with the above-mentioned parameters.

Therefore, two diets (FO and linseed oil diet (LO)) were evaluated at three different salinities (brackish water (BW) (16ppt), seawater (SW) (35 ppt) and high salinity water (HS) (46 ppt)) in *Chelon labrosus* juveniles. The aim of the present study was to assess the effects of the combination diet-salinity on growth, whole body, liver and muscle proximal and fatty acid composition, and the liver gene expression of two of the main enzymes involved in the biosynthesis of n-3 LC-HUFA, the fatty acid elongase (*Elovl5*) and the fatty acid desaturase 2 (*Fads2*). Present results highlight the capability of these species to biosynthesize HUFA from precursors, which potential is maximized under an optimal rearing salinity, placing *Chelon labrosus* as a promising species for the establishment of free fish oil diets in aquaculture.

2. Material and methods

2.1. Experimental animals

Thick-lipped grey mullet juveniles, previously captured by a local authorized fisherman at the east coast of Gran Canaria (Canary Islands, Spain), were used after being carefully transported and acclimated to the facilities of the GIA-Ecoaqua Institute belonging to Marine Scientific Technological Park of the University of Las Palmas de Gran Canaria (ULPGC), where they were maintained in an open-flow system and fed twice a day to apparent satiation with a commercial extruded feed (R2, Skretting, Burgos, Spain).

2.2. Experimental design

One hundred-eighty mullets (26.74±4.92 g and 13.13±0.89 cm of initial total weight and length, respectively) were distributed in 18 aquaria of 60 L in recirculating aquaculture system (RAS). Six aquaria were filled with local seawater (35 ppt); 6 aquaria were filled with brackish water (16 ppt), mixing natural seawater with fresh spring water, and the remain 6 aquaria were filled with high salinity water (46 ppt), mixing sea water with natural salt from a local saline. The salinity was monitored every day with a

refractometer, being the evaporated water from the different reservoirs weekly renewed using the correspondent stocked water. Temperature and oxygen were also monitored daily with a digital probe (OxyGuard, Handy Polaris, Acuitec S.L. Gipuzkoa, Spain) (average values of 25.43±0.25 ºC and 5.71±0.18 mg/L, respectively), while ammonia, nitrites, and nitrates were measured weekly with a spectrophotometer (YSI 9500, Xylem Analytics, USA), being maintained within the safe levels reported for mullets by Sampaio *et al.* (2002) and for marine fish in general by Boyd (2014). Fish were hand fed at apparent satiety two times per day and 6 days per week being the feeding performed carefully to minimize the loss of feed.

All the procedures were rigorously conducted according to the European Union Directive (2010/63/EU) on animal welfare protection for scientific purposes.

2.3. Diets

For each salinity, two isoproteic (42% protein) and isolipidic (13% fat) diets (Tables I and II) were provided in triplicate groups; a FO diet, adapted from the one previously tested in the same species by Quirós-Pozo *et al.* (2023), and a Linseed Oil (LO) diet from which the fish oil added was substituted by linseed oil. The corresponding 3 mm size pellets were produced in the Pilot Plant of Products and Processes at the GIA-Ecoaqua Institute (ULPGC).

2.4. Growth and feed utilization parameters

At the end of the trial, growth and productive parameters were measured according to the next equations:

Weight gain (WG) (%) = 100*(Final weight (g) -Initial weight (g)) / Initial weight (g).

Specific growth ratio (SGR) (%/day) = ((lnWf –lnWi)/ days of experiment) x100

where:

lnWf: Final weight neperian logarithm

lnWi: Initial weight neperian logarithm

Feed intake (FI) (%) = (feed consumed (g) / fish weight average (g)) / days x 100

Feed conversion ratio (FCR) = Feed offered (g) / Weight gain (g)

2.5. Biochemical analyses

Once sacrificed by immersion in iced-cold water, 3 fish per tank were stored at -80ºC for whole body proximal and fatty acid composition (9 fish and 3 pools in total per treatment). To determine moisture, lipids and protein from the whole fish, the pool was homogenized and devoted to proximal analysis by a Food Scan™ (FOSS, Hillerød, Denmark). Ash content of the whole-body homogenate was determined according to

AOAC (2000). A total of 4 fish per tank were dissected to obtain samples of muscle and liver, being these latter stored at -80ºC until analysed. The proximate composition of muscle and liver was evaluated in pools of four fish per tank (12 fish and 3 pools in total per treatment). Proximate composition of these tissues and the experimental diets were done following the techniques described in AOAC (2000), thus proteins being determined by the Kjeldahl technique, and the total lipids, following the protocol described by Folch *et al*. (1957). For fatty acid determination (whole body, tissues, and diets), after lipid extraction, the total lipids were trans-esterified (Christie, 1989), and the fatty acids obtained were quantified by gas chromatography (Izquierdo *et al*., 1992).

2.6. Gene expression analyses

Expression of *Elovl5* and *Fads2* genes in liver were determined by quantitative real time PCR (qPCR). Elongation factor-1α (*ef1a*), β-actin (*actb*) and *18S r-RNA* were tested as potential reference genes to normalize the expression of *Elovl5* and *Fads2*. *18S r-RNA was* selected for that purpose since it was the most stable genes according to the "Repeated Pair-Wise Correlation Analysis" (Pfaffl *et al*., 2004). Total RNA of 3 fish livers per aquaria was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, USA) and the extraction kit RNAeasy® MiniKit from Quiagen. 1 µg of RNA of each sample was reverse transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California) in 20 µl reaction volume according to the manufacturer's instructions, to later be diluted 1/10 with miliQ water. Real time PCRs were realized using a l-cycler with optical module (Bio-Rad Hercules, Ca, USA) in a final volume of 15 µl containing 7.5 µl of iQTM-SYBER® Green Supermix (Bio-Rad Hercules, Ca, USA), 5 µl of cDNA, 0.75 µl of each primer (forward and reverse), and 1.0 µl of miliQ water. The conditions for the real time reactions were adapted from referenced protocols to the conditions of the reagents used in the present study, being resumed in Table III. All reactions were performed in duplicate for each sample and blank control reactions were implemented replacing the cDNA for miliQ water. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The statistical analyses were carried out using the program R Project for Statistical Computing. After normality and homoscedasticity test, the variance analyses were performed using a two-ways ANOVA to determine the individual and combined effects of both diet and salinity. In cases when data did not meet normality or homoscedasticity, the medians were compare using a Kruskal-Wallis non-parametric test. Significant differences were considered when $p<0.05$.

3. Results

3.1. Growth and feed utilization parameters

The two diets evaluated performed equally in terms of growth parameters and use of feed, however, the salinity did cause significant effects in fish performance, having the mullets reared in BW the best values for final weight, weight gain, SGR and FCR (Table IV).

3.2. Biochemical analyses

The proximate composition of the whole body was not affected by either diet or salinity (Table V). However, the muscle proximal composition (Table VI) was affected by the diet, with higher lipid and ash content for mullets fed LO, this difference produced primarily by the treatment C-SW, which had lower muscle lipids than the treatments FO-BW, LO-SW, and LO-HS. Additionally, the higher lipid and ash content of fish fed the LO diet produced a slightly lower muscle protein content in these mullets. Ash content was also higher for fish reared in BW in comparison with fish reared at HS. The proximate composition of liver it was not affected neither by diet nor by salinity (Table VII).

As regards the results of the fish fatty acid profile, the different salinities evaluated did not caused effects on the fatty acid profile of the fish, however, the two diets evaluated did determine considerable effects on the fatty acid composition of the mullets in whole body, muscle and liver.

Additionally, the results of whole body fatty acid profile (Table VIII) showed that fish fed FO diet presented higher levels of 20:4n-3, arachidonic acid (ARA, 20:4n-6), saturated, monoenoics and n9 fatty acids, while the fish fed the LO diet had higher levels of linoleic acid (18:2n-6), linolenic acid (18:3n-3), 20:3n-3, total n3 fatty acids, total PUFA, n3/n6 ratio and n3-PUFA. Eicosapentaenoic acid (EPA, 20:5n-3), DHA, n3 LC-HUFA, and DHA/EPA ratio were equivalent in all treatments. Muscle fatty acid profile (Table IX) of mullets fed the FO formula presented higher levels of ARA, EPA, DHA, monoenoics, and n3 LC-HUFA, although, for DHA and n3 LC-HUFA, the interaction diet-salinity determined that the mullets fed the LO diet reared in BW were like those fed the FO diet in all salinities ($p>0.05$). The muscle of mullets fed the LO diet had, on the other hand, higher levels of 18:2n-6, 18:3n-3, total n3, total n6, total PUFA, n3/n6 ratio and n3 PUFA. ARA/EPA, DHA/EPA, and DHA/ARA ratios were equivalent between treatments. In addition, the liver of the mullets (Table X) fed the FO diet showed higher levels of 20:2n-6, 20:4n-3, ARA, EPA, 22:5n-3 and monoenoics, while mullets fed the LO diet had higher levels of 18:3n-3, 18:3n-6, 18:4n-3, 20:3n-3 and ARA/EPA, DHA/EPA and DHA/ARA ratios. The hepatic levels of DHA and n3 LC-HUFA were equivalent between treatments.

3.3. Gene expression analyses

Respecting the liver gene expression of *Elovl5* and *Fads2*, there were no statistical differences between the fish fed the two diets evaluated (Figure 1), however, for both enzymes, there was a tendency of higher gene expression in the fish fed the LO diet. The different rearing salinities did cause higher gene expression of *Elovl5* in the liver of fish reared in brackish water ($p<0.01$) (Figure 2), with similar values between the fish reared at HS and SW. However, the expression of *Fads2* was not affected by salinity,

although the fish reared in SW showed a trend of higher gene expression of this enzyme.

4. Discussion

The low trophic and euryhaline nature of mullets makes them highly adaptable species to be cultured under different salinities and feeding formulas; however, to maximize the potential of the former species to optimized profitability and sustainability, these conditions must be assessed for each species.

In the present study, regarding the effect of VO diets in fish growth, the mullets fed the LO diet, with a 100% of the FO in the formula replaced by LO, presented similar growth than those fed the FO formula. Similarly, Sarker *et al*. (2011) and Yu *et al*. (2021) recorded equal growth between juveniles of red seabream (*Pagrus major*) and red tilapia (*Oreochromis mossambicus*♀ × *O. niloticus*♂), respectively, fed vegetable oil diets vs a FO diet. However, high substitution of FO has produced a poorer growth response in comparison with the FO diet in other marine species such as European seabass (*Dicentrarchus labrax*) or black seabream (*Acanthopagrus schlegeli*) (Montero *et al*., 2005; Peng *et al*., 2008) or other marine tropical carnivore species reviewed by Alhazzaa *et al*. (2019). Present results highlight the potential of *Chelon labrosus* of utilizing efficiently diets with low LC-HUFA content, which is of great interest at the time of chose more sustainable feds for this species.

To achieve better growth at intermediate salinities was reported quite often in cultured fish. This fact can be explained by the higher energetic cost necessary to maintain the osmotic regulation at extreme salinities, but also by factors like feed intake and feed conversion regulation (Bœuf and Payan, 2001; Laiz-Carrión *et al*., 2005). In the present study, although there were no differences in the feed intake between the different treatments, the rearing salinity did cause statistical differences with the fish reared in brackish water obtaining the best performance in terms of final weight, weight gain, SGR and FCR. This is in concordance with other studies in *Mugil cephalus* juveniles which recorded improved growth in brackish water (Barman *et al*., 2005; Olukolajo and Omolara, 2013), and with the study of Pujante *et al*. (2018) in *Chelon labrosus* juveniles, that reported a decreased digestive efficiency in fish reared at high salinities (55 ppt).

No differences in the proximal composition of whole body and liver were caused by the different diets or salinities, which agreed with the results of Loi *et al*. (2022). These authors neither found differences in liver lipid deposition between *Mugil cephalus* fry reared in fresh water and SW. What is more, our results agreed with those reported by Sarker *et al*. (2011) in red sea bream (*Pagrus major*), who did not found differences neither in whole body proximatl composition between the diets (FO 100% vs 67% FO replacement diet), nor between salinities (15, 20 and 33ppt). However, Raso and Anderson (2003), Shapawi *et al*. (2008) or Ghosi Mobaraki *et al*. (2020) recorded differences in the carcass composition of the juvenile barramundi (*Lates calcarifer*), the humpback grouper (*Cromileptes altivelis*) or the pearl gourami (*Trichogaster leeri*), respectively, fed diets with different vegetal lipid sources.

In the case of the muscle, in the present study, the proximate composition did was significantly affected both by the diet and salinity and by the interactions among them. Hence, the fish fed the LO diet showed higher lipid deposition than the fish fed the FO diet, which was accompanied in the first case by a relative reduction in protein and by higher ash content. These results are in concordance with the increase in fillet fat in some marine species fed plant ingredients in substitution of FM and FO, such as the turbot (*Psetta maxima*) or the Senegalese sole, as reviewed by Grigorakis *et al*. (2017). However, in other freshwater species such as the pacu (*Piaractus mesopotamicus)* no differences were recorded in proximate muscle composition of fish fed VO diets in comparison with the FO ones (Gonçalves *et al*. 2021). So, these differences, could be related to the fish environmental origin (freshwater vs salt water). Besides, the rearing salinity affected the ash muscle content with higher values for fish reared at BW in comparison with those reared at HS, and intermediate values for fish reared in SW. Therefore, ash muscle content was inversely correlated to rearing salinity. Rahman et al. (2020) also reported the influence of environmental factors like salinity in the mineral concentration of fish, as minerals are involved in several osmoregulatory physiological functions (Njinkoue *et al*., 2016). Interestingly, ash content has being shown to increase fillet flavour and texture of fish (Ersoy and Celik, 2010 in Ahmed *et al*., 2022), which together with the higher percentage of muscle lipids of fish fed LO, may be a valuable feature by consumers.

It has been widely described how the dietary fatty acid profile may be reflected in the marine fish tissues (Izquierdo *et al*., 2001; Grigorakis *et al*., 2017), however, in this study, *Chelon labrosus* juveniles showed important variations in their fatty acid composition in comparison to the diet provided. Thus, fish fed the FO diet reproduced the dietary levels of saturated and monoenoic fatty acids in their tissues, however, its content of EPA, DHA and n-3-LC-HUFA were slightly lower than the dietary values. On the other hand, fish fed LO diet presented higher levels of 18:2n-6 and 18:3n-3 than fish fed the FO diet, reflecting the higher content of these fatty acids in the LO diet. However, the levels of both fatty acids were remarkable diminished in the LO fed fish whole body in comparison with the diet. Also, although levels of ARA, EPA, DHA and n3-LC-HUFA in the LO diet were relatively low (0.23%, 0.59%, 1.64% and 2.53%, respectively), LO fish showed remarkably higher levels of those fatty acids (~0.40%, 3%, 5%, 9-10%, respectively) in the whole body than those present in the diet, suggesting the capacity to physiologically equalize the levels of EPA, DHA and n3 LC-HUFA to those of the fish fed the FO diet.

Although the levels of 20:3-n3, an intermediate fatty acid during the biosynthesis pathway of both EPA and DHA, were quite low in the LO diet (0.07%), the levels of this fatty acid in the LO fish whole body (~0.40%), higher than those of the FO fish, give an idea of the elongation activity in the animals fed the LO diet. The levels of other intermediate fatty acids such as 20:4n-3 and 20:2n-6 were higher in the FO than in the LO fish, but it seems that these differences could be primarily driven by the higher concentration of these fatty acids in the FO diet, more than by a biosynthesis activity by itself, nevertheless, in the LO fish, the levels of these two fatty acids were remarkable higher than those of the diet, which may suggest a certain LC-HUFA biosynthesis activity for those fish. These results are in concordance with the higher expression of

both *Elovl5* and *Fads2* obtained in the liver of the fish fed the LO diet, which suggest that this rise in the expression, although not statistically significant compared to those of the FO fish, was enough to compensate notably the levels of LC-HUFA in the LO fish whole body.

Despite, salinity is considered a modulative environmental factor on the LC-HUFA biosynthesis, the composition of the whole body did not presented variations caused by this factor, furthermore, there were some interactions between diet and salinity for certain fatty acids such as EPA, DHA and total n3 LC-HUFA, but post-hoc analyses did not show significant differences at the comparison between groups ($p>0.05$). The absence of differences due to the environmental salinity in *Chelon labrosus* agree with the results of Loi *et al*. (2022), which recorded similar fatty acid profile of n3 LC-HUFA for *Mugil cephalus* juveniles reared in both fresh water and SW.

Additionally, although ARA/EPA, DHA/EPA and DHA/ARA ratios were quite different between the two diets evaluated, these variations were minor between the fish of the different treatments, suggesting a sort of compensatory mechanism of the fish to conserve the physiological LC-HUFA's ratios, as it has been previously described for other marine species (Montero *et al*., 2010; Benítez-Dorta *et al*., 2013). On the contrary, present results contrast with those of Sarker *et al*. (2011), who reported that fish fed the diet with a 67% of the FO replacement showed signs of LC-HUFA biosynthesis, the levels of EPA and DHA reflected those of the diet, in contrast with the present study in which the levels of both fatty acids in the whole body were similar between treatments irrespectively of the diet, suggesting the potential of this species to utilize efficiently diets with limited content of LC-HUFA.

In muscle fatty acid profile, it is remarkable how the levels of DHA and n3 LC-HUFA are higher in the fish muscle than those in the two diets evaluated. For LO fish, the levels of ARA, EPA, DHA and n3 LC-HUFA are quite higher than those present in the LO diet, while levels of 18:2n-6 and 18:3n-3, although higher than levels in the muscle of the FO fish, are diminished in comparison with levels present in the LO feed. Additionally, there were interactions between diet and salinity for some fatty acids as DHA and n3 LC-HUFA, allowing the fish fed the LO diet and reared in BW to equalize the levels of these fatty acids to those of the FO fish in all salinities ($p>0.05$), which is in concordance with the results of highest *Elovl5* expression in liver of the fish reared in BW ($p<0.05$), and with those obtained for other species as the Senegalese sole (Marrero *et al*., 2019). Additionally, ARA/EPA, DHA/EPA, and DHA/ARA ratios were equivalent in the muscle of the fish from all the treatments, with is consistent with the hypothesis of the conservancy of the physiological ratios of these fatty acids, as discussed above. Rosas *et al*. (2019), tested different levels of substitution of FM and FO with spirulina and LO in juveniles of *Mugil liza*, however, although they recorded signs of elongation from 18:3n-3 to 20:3n-3 in the treatments with higher substitution, there were not a compensation of the levels of LC-HUFA in the muscle of the fish in any of the replacement levels evaluated, suggesting these authors a feasible partial but not total substitution of the FO and FM of the formula. The data mentioned above suggest that the capacity of LC-HUFA biosynthesis of Mugilidae may depend significantly on the diverse mullet species

but also on the environmental and nutritional conditions, which may be optimized in each species.

The liver of the fish fed the LO diet presented high levels of "intermediate" fatty acids as 18:3n-6, 18:4n-3 and 20:3n-3, which were remarkably higher than levels present in the diet and that levels in the livers of FO fish, pointing the important role of this tissue for LC-HUFA biosynthesis activity. As for the case of whole body, DHA and n3 LC-HUFA levels were similar between fish fed the FO and the LO diet, confirming the markedly potential of these species to synthesize n3 LC-HUFA when these fatty acids are limited in the diet, but a source of precursors is provided. In contrast with the other tissues under study, ARA/EPA, DHA/EPA, and DHA/ARA ratios were higher in the liver of the LO fish, which highlights the preferential conservancy of DHA in comparison with EPA or ARA for these mullets, as it has been suggested also for marine fish eggs (Izquierdo *et al.*, 2001; Fernández-Palacios *et al.*, 2011; Quirós-Pozo *et al.*, 2022), due to the essential role of DHA for different physiological functions in fish.

Present results corroborate the potential found for *Chelon labrosus* to synthetize LC-HUFA from their precursors as were reported by Garrido *et al.* (2019) and by Galindo *et al.* (2021), which studied the tissue expression of *Fads2* and *Elovl5*, respectively, in both cases in *Chelon* juveniles from wild origin. Also, present results are in concordance with the reported higher percentages of ARA, EPA, and DHA in mullets (*Mugil cephalus*) reared at low salinities in comparison with those reared in SW (Khériji *et al.*, 2003).

Although the obtained results of *Elovl5* and *Fads2* gene expression in liver are not statistically significant in function of the diets, they show a clear tendency of higher expression of both enzymes in fish fed the LO diet, being this tendency even more evident for *Elovl5* in fish fed the LO diet and reared in BW. This is in concordance with the results of *Fads2* and *Elovl5* gene expression obtained for the Senegalese sole (*Solea senegalensis*) by Marrero *et al.* (2021), which suggested an improved potential of LC-HUFA biosynthesis under conditions of reduced LC-HUFA intake and low salinity. Rearing salinity do produced effects in liver gene expression of *Elovl5*, with higher expression in the liver of the fish reared in BW, which is in concordance with similar results obtained for other species as the genetically improved farmed tilapia (GIFT) (*Oreochromis niloticus*) (You *et al.*, 2019), the rabbitfish (*Siganus canaliculatus*) (Xie *et al.*, 2014), or the Senegalese sole (Marrero *et al.*, 2021).

In conclusion, *Chelon labrosus* juveniles were able to utilize efficiently diets with no added fish oil, obtaining similar growth, proximate body composition and LC-HUFA fatty acid profile in many cases that FO fed fish. n3 LC-HUFA levels in the muscle of LO fed fish achieved similar values of DHA and n3 LC-HUFA when the fish were reared in brackish water, evidencing an interaction between diet and salinity which produced an optimized LC-HUFA biosynthesis capacity when the juveniles of *Chelon labrosus* were fed the LO diet and reared in BW. Furthermore, for LO fed fish there was evidence of LC-HUFA biosynthesis from precursors in all salinities. Enzyme gene expression of *Elovl5* and *Fads2* was not statistically different between the groups fed the two experimental diets; however, a clever tendency of higher expression for both enzymes was observed in the fish fed the LO diet. Concerning gene expression in the function of salinity, fish reared in BW showed the highest levels of hepatic *Elovl5* expression.

Overall, the present results suggest the feasibility of using diets with no fish oil for this species, which results can be optimized when fish are reared in brackish water.

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Tables and Figures

Table I. Experimental diets formula (%) and proximal composition (means± SD).

Ingredients (%)	PERCENT	
	FO	LO
Fish meal[a]	20	20
Blood meal	3	3
Ulva meal[b]	10	10
Rapeseed meal[c]	8	8
Corn meal[d]	6	6
SPC (soy protein concentrate)[e]	20	20
Wheat meal[d]	6	6
Wheat gluten[d]	12	12
Fish oil[a]	9.5	0.0
Lineseed oil[d]	0	9.5
Vit mix[f]	2	2
Min mix[g]	2	2
Ca(H2PO4)2[h]	0.5	0.5
CMC[i]	1	1
Analytical composition		
Protein	41.80±0.30	42.38±0.47
Lipids	13.27±0.16	12.89±0.30
Ash	10.30±0.24	10.38±0.22
Moisture	6.89±0.31	7.39±0.29

[a] Fish meal and fish oil from South America (supplied by Skretting, Spain); [b] Ulva meal (supplied by Puerto Muiños S.L., Spain); [c] Rapeseed 0.0 (supplied by Dibaq, Spain); [d] Flours and oils obtained from local suppliers; [e] Soy protein concentrate (Sopropeche, France); [f] Vitamin premix containing (mg kg-1 or IU/kg of dry feed): tiamine 40 mg, riboflavin 50 mg, pyridoxine 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, folic acid 10 mg, cyanocobalamin, 0.5 mg, choline chloride 2700 mg, myo-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg, cholecalciferol 2000 IU, etoxyquine 100 mg, retinol acetate 5000 IU. Vitamin E (DL-alpha-tocopherol acetate) 250 mg; [g] Mineral premix containing (g/kg of dry feed): calcium orthophosfate 1.60 g, calcium carbonate 4 g, ferrous sulfate 1.5 g, magnesic sulfate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminum sulfate 0.0 g, zinc sulfate 0.24 g, copper sulfate 0.20 g, manganese sulfate 0.1 g, potassium iodate 0.0 g; [h] Sigma-Aldrich, Munich, Germany; [i] carboxymethylcellulose (sodium salt, Sigma-Aldrich, Munich, Germany).

Table II. Fatty acid profile of diets.

	FO	LO
14:0	2.71	0.63
15:0	0.33	0.13
16:0	14.36	9.86
16:1n-7	4.13	1.02
16:1n-5	0.13	0.05
16:2n-4	0.28	0.04
17:0	0.20	0.02
16:3n-4	0.24	0.12

16:3n-1	0.03	0.02
16:4n-3	0.25	0.02
18:0	4.04	5.52
18:1n-9	28.47	23.43
18:1n-7	3.25	1.54
18:1n-5	0.13	0.04
18:2n-9	0.05	0.01
18.2n-6	13.95	18.16
18:2n-4	0.16	0.03
18:3n-6	0.16	0.03
18:3n-4	0.16	0.03
18:3n-3	4.07	34.43
18:4n-3	0.80	0.14
20:0	0.32	0.26
20:1n-9	0.31	0.09
20:1n-7	2.86	0.64
20.1n-5	0.21	0.05
20:2n-6	0.57	0.09
20:3n-6	0.19	0.04
20:4n-6	0.64	0.23
20:3n-3	0.23	0.07
20:4n-3	0.64	0.07
20:5n-3	4.17	0.59
22:1n-11	2.08	0.33
22:1n-9	0.48	0.15
22:4n-6	0.14	0.05
22:5n-6	0.25	0.10
22:5n-3	1.41	0.17
22:6n-3	7.00	1.64
Saturated	21.95	16.42
Monoenoics	42.19	27.38
n-3	18.68	37.16
n-6	16.15	18.79
n-9	29.43	23.70
n-3 HUFA	13.45	2.53
ARA/EPA	0.15	0.38
DHA/EPA	1.68	2.78
DHA/ARA	10.97	7.28
Total PUFA	37.44	56.42
n-3/n-6	1.16	1.98
n3PUFA	18.32	37.10
n6PUFA	15.90	18.70

Contains: 14:1n-7, 14:1n-5, 15:1n-5, 16:0ISO, 16:2n-6, 16:3n-3, 16:4n-1, 18:3n-1, 18:4n1, 20:2n-9, and 20:3n-9.

Table III. Primer sequences and RT-PCR conditions of the different genes analysed.

Gene	Access. Number		Primer Sequence 5'-3'	Initial denaturation (°C) (duration in min)	*Denaturing temperature (°C) (duration in s)	*Anneling T (°C) (duration in s)	*Extension temperature (°C) (duration in s)	*Number of cycles	Reference
Elovl5	MT019563	F	AGAACGGCTCCTCCCTATCA	95 (3)	95 (15)	58.5 (45)	72 (30)	35	Galindo *et al.*, 2021
		R	CAGCATTAGCTAACACGCTACA					35	
Fads2	MH293504	F	GTGTCAAGGCTTCGCTGATG	95 (3)	95 (15)	58.5 (30)	72 (30)	35	Garrido *et al.*, 2019
		R	AACGTCACTCCTTTCGCATACA					35	
18S-rRNA		F	CACATCCAAGGAAGGCAGCA	94 (2)	94 (30)	60 (30)	68 (30)	30	Raingeard *et al.*, 2006
		R	AAGATACGCTATTGGAGCTG						

Table IV. Values for parameters of growth performance and use of diets (n = 30 per treatment).

	FO			LO					
	BW	SW	HS	BW	SW	HS	Diet (D)	Salinity (S)	SxD
Initial weight (g)	26.01±0.94	27.17±0.50	27.84±0.26	27.94±1.45	27.19±1.39	26.73±1.39			
Final weight (g)	48.33±4.54	37.18±4.84	37.20±4.39	46.67±9.68	41.73±3.90	35.78±2.42	0.82	p≤0.01	0.16
Weight gain (%)	$71.69±4.86^{A}$	$35.68±18.30^{B}$	$41.72±11.10^{B}$	$68.98±27.14^{A}$	$49.20±13.91^{B}$	$30.12±4.17^{B}$	0.97	0.01	0.39
SGR (%/day)	$0.74±0.04^{A}$	$0.41±0.18^{B}$	$0.47±0.11^{B}$	$0.71±0.23^{A}$	$0.54±0.13^{B}$	$0.36±0.04^{B}$	0.96	0.01	0.33
Feed intake (%)	2.23±0.20	2.20±0.24	2.18±0.12	2.41±0.67	2.11±0.35	2.03±0.01	0.89	0.52	0.69
FCR	$2.29±0.05^{B}$	$4.32±1.15^{A}$	$3.49±0.74^{A}$	$2.66±0.43^{B}$	$2.95±0.23^{A}$	$4.06±0.53^{A}$	0.63	0.01	0.04

Values in a row followed by a different uppercase superscript (A, B) indicate significant differences ($p < 0.05$) between salinities.

Table V. Proximate whole body composition (% of dry weight) for fish maintained under the different feeding conditions and salinities (n = 9 per treatment).

	FO			LO			p values		
	BW	SW	HS	BW	SW	HS	D	S	SxD
Lipids	37.01±8.35	32.30±6.57	32.19±1.76	33.77±1.14	32.49±3.36	27.64±1.19	0.32	0.19	0.61
Protein	36.19±1.89	46.03±6.61	41.38±10.59	42.96±12.18	35.14±2.27	40.40±2.78	0.58	0.93	0.10
Ash	14.89±3.32	16.60±2.60	18.66±2.36	13.95±2.40	17.67±2.80	17.47±2.03	0.77	0.07	0.70

Values in a row without different superscripts indicate the absence of significant differences ($p \geq 0.05$).

Table VI. Proximate muscle composition (% of dry weight) for fish maintained under the different feeding conditions and salinities (n = 12 per treatment).

	FO			LO			p values		
	BW	SW	HS	BW	SW	HS	D	S	SxD
Lipids	$13.29±0.61^{b}$	$8.68±1.20^{b}$	$11.05±1.45^{b}$	$11.17±0.85^{a}$	$14.11±1.70^{a}$	$12.68±1.77^{a}$	0.02	0.57	p≤0.01
Protein	$84.70±1.46^{a}$	$89.10±5.63^{a}$	$92.63±6.79^{a}$	$84.87±1.98^{b}$	$81.66±2.25^{b}$	$79.84±2.72^{b}$	p≤0.01	0.82	0.05
Ash	$7.20±0.53^{Ab}$	$5.20±1.40^{ABb}$	$6.07±1.12^{Bb}$	$9.04±1.34^{Aa}$	$8.32±2.25^{ABa}$	$6.51±0.77^{Ba}$	p≤0.01	0.02	0.11

Values in a row with different superscripts indicate the presence of significant differences ($p<0.05$). Significant differences between diets are expressed by lowercase letters, while significant differences between salinities are expressed by uppercase letters.

Table VII. Proximal liver composition (% of dry weight) for fish maintained under the different feeding conditions and salinities (n = 12 per treatment).

	FO			LO			p values		
	BW	SW	HS	BW	SW	HS	D	S	SxD
Lipids	46.46±1.12	32.61±3.60	34.00±5.71	33.94±2.43	37.69±5.75	38.63±8.20	0.50	0.90	0.01
Ash	2.77±0.88	3.64±1.12	4.42±0.99	2.11±1.05	2.60±0.50	3.51±1.30	0.07	0.05	0.94

Values in a row without different superscripts indicate the absence of significant differences ($p \geq 0.05$).

Table VIII. Whole body fatty acid profile, as % of the total fatty acids identified, for the different experimental groups (n = 9 per treatment) at the end of the experiment.

	FO			LO			p value		
	BW	SW	HS	BW	SW	HS	D	S	SxD
14:0	2.80±0.17[a]	2.94±0.16[a]	2.76±0.14[a]	2.16±0.14[b]	2.19±0.33[b]	2.31±0.27[b]	p≤0.01	0.94	0.49
15:0	0.34±0.01[a]	0.36±0.01[a]	0.36±0.02[a]	0.28±0.01[b]	0.28±0.02[b]	0.29±0.02[b]	p≤0.01	0.36	0.57
16:0	16.47±0.59[a]	16.85±1.21[a]	16.63±0.37[a]	16.08±0.20[b]	14.77±0.14[b]	14.77±0.64[b]	p≤0.01	0.52	0.13
16:1n-7	5.53±0.34[a]	5.54±0.41[a]	5.29±0.08[a]	4.22±0.35[b]	4.16±0.63[b]	4.26±0.25[b]	p≤0.01	0.89	0.72
16:1n-5	0.14±0.00[a]	0.16±0.01[a]	0.18±0.02[a]	0.12±0.02[b]	0.13±0.01[b]	0.12±0.01[b]	p≤0.01	0.13	0.03
16:2n-4	0.30±0.02[a]	0.31±0.01[a]	0.30±0.02[a]	0.21±0.03[b]	0.24±0.05[b]	0.25±0.04[b]	p≤0.01	0.64	0.59
17:0	0.23±0.02	0.23±0.02	0.20±0.02	0.18±0.03	0.21±0.04	0.22±0.03	0.35	0.84	0.24
16:3n-4	0.32±0.01[a]	0.32±0.03[a]	0.33±0.01[a]	0.26±0.00[b]	0.26±0.02[b]	0.26±0.00[b]	p≤0.01	0.64	0.64
16:3n-1	0.08±0.02	0.11±0.03	0.12±0.05	0.07±0.00	0.07±0.01	0.08±0.01	0.38	0.38	0.51
16:4n-3	0.21±0.02	0.18±0.04	0.15±0.03	0.17±0.01	0.21±0.04	0.21±0.03	0.19	0.46	0.02
18:0	2.80±0.23	3.07±0.26	3.21±0.17	3.44±0.20	3.20±0.44	3.31±0.10	0.05	0.54	0.21
18:1n-9	29.39±0.77[a]	29.89±0.93[a]	31.16±0.89[a]	27.92±0.55[b]	27.73±0.23[b]	27.29±0.22[b]	p≤0.01	0.28	0.04
18:1n-7	3.70±0.09[a]	3.74±0.06[a]	3.59±0.32[a]	2.82±0.22[b]	2.79±0.40[b]	3.03±0.08[b]	p≤0.01	0.92	0.35
18:1n-5	0.15±0.01[a]	0.15±0.01[a]	0.14±0.01[a]	0.10±0.01[b]	0.11±0.01[b]	0.11±0.01[b]	p≤0.01	0.8	0.78
18:2n-9	0.21±0.05	0.23±0.10	0.19±0.04	0.30±0.03	0.24±0.07	0.19±0.05	0.42	0.35	0.49
18:2n-6	12.90±0.48[b]	13.42±0.47[b]	13.66±0.36[b]	14.41±0.05[a]	14.22±0.48[a]	14.14±0.51[a]	p≤0.01	0.54	0.2
18:2n-4	0.18±0.00[a]	0.18±0.00[a]	0.18±0.01[a]	0.12±0.02[b]	0.13±0.03[b]	0.13±0.01[b]	p≤0.01	0.54	0.84
18:3n-6	0.17±0.01	0.17±0.01	0.16±0.00	0.18±0.01	0.18±0.02	0.17±0.01	0.13	0.27	0.95
18:3n-4	0.16±0.00[a]	0.14±0.02[a]	0.15±0.01[a]	0.09±0.01[b]	0.10±0.02[b]	0.10±0.02[b]	p≤0.01	0.92	0.73
18:3n-3	3.17±0.03[b]	3.22±0.16[b]	3.56±0.57[b]	11.80±2.74[a]	11.00±4.37[a]	10.37±1.73[a]	p≤0.01	0.91	0.76
18:4n-3	0.76±0.06	0.67±0.03	0.59±0.10	0.63±0.01	0.76±0.04	0.73±0.02	0.18	0.21	0.01
20:0	0.27±0.03	0.29±0.04	0.31±0.01	0.26±0.00	0.25±0.02	0.28±0.02	0.07	0.09	0.59
20:1n-9	0.32±0.03[a]	0.33±0.03[a]	0.34±0.01[a]	0.21±0.03[b]	0.23±0.05[b]	0.25±0.02[b]	p≤0.01	0.24	0.89
20:1n-7	2.85±0.24[a]	3.05±0.29[a]	3.17±0.21[a]	1.92±0.24[b]	2.14±0.47[b]	2.34±0.31[b]	p≤0.01	0.2	0.96

20:1n-5	0.35±0.03^{a}	0.37±0.03^{a}	0.39±0.03^{a}	0.26±0.04^{b}	0.26±0.05^{b}	0.30±0.02^{b}	p≤0.01	0.23	0.92
20:2n-6	0.64±0.06^{a}	0.69±0.07^{a}	0.70±0.03^{a}	0.47±0.04^{b}	0.52±0.09^{b}	0.55±0.05^{b}	p≤0.01	0.16	0.99
20:3n-6	0.16±0.00^{a}	0.16±0.01^{a}	0.15±0.00^{a}	0.10±0.00^{b}	0.11±0.02^{b}	0.12±0.02^{b}	p≤0.01	0.67	0.26
20:4n-6	0.58±0.06^{a}	0.56±0.08^{a}	0.54±0.04^{a}	0.43±0.03^{b}	0.48±0.09^{b}	0.50±0.04^{b}	0.02	0.99	0.44
20:3n-3	0.27±0.02^{b}	0.29±0.02^{b}	0.30±0.01^{b}	0.37±0.05^{a}	0.39±0.05^{a}	0.38±0.04^{a}	p≤0.01	0.67	0.85
20:4n-3	0.56±0.02^{a}	0.50±0.03^{a}	0.46±0.05^{a}	0.35±0.04^{b}	0.42±0.09^{b}	0.43±0.04^{b}	p≤0.01	0.75	0.02
20:5n-3	3.58±0.23	2.99±0.30	2.62±0.53	2.60±0.30	3.26±0.65	3.27±0.13	0.89	0.58	0.02
22:1n-11	1.22±0.10^{a}	1.28±0.17^{a}	1.38±0.14^{a}	0.77±0.09^{b}	0.86±0.22^{b}	1.00±0.15^{b}	p≤0.01	0.16	0.92
22:1n-9	0.35±0.03^{a}	0.37±0.04^{a}	0.40±0.05^{a}	0.24±0.01^{b}	0.26±0.04^{b}	0.30±0.03^{b}	p≤0.01	0.12	0.93
22:4n-6	0.12±0.01^{a}	0.12±0.02^{a}	0.11±0.01^{a}	0.08±0.00^{b}	0.09±0.02^{b}	0.10±0.00^{b}	p≤0.01	0.65	0.22
22:5n-6	0.26±0.03	0.24±0.04	0.21±0.02	0.21±0.01	0.23±0.05	0.25±0.02	0.91	0.94	0.15
22:5n-3	1.69±0.05	1.41±0.16	1.19±0.25	1.11±0.20	1.37±0.40	1.41±0.12	0.35	0.57	0.04
22:6n-3	6.14±0.33	4.85±0.63	4.16±1.20	4.45±0.39	5.67±1.43	5.67±0.40	0.48	0.56	0.03
Saturated	22.89±0.50^{a}	23.73±1.06^{a}	23.47±0.52^{a}	22.51±0.19^{b}	20.89±0.09^{b}	21.18±0.92^{b}	p≤0.01	0.83	0.03
Monoenoics	44.17±0.66^{a}	45.06±0.77^{a}	46.21±1.59^{a}	38.72±1.51^{b}	38.79±1.79^{b}	39.13±1.06^{b}	p≤0.01	0.28	0.59
n-3	16.49±0.54^{b}	14.21±1.23^{b}	13.14±2.15^{b}	21.55±1.87^{a}	23.16±1.76^{a}	22.55±1.39^{a}	p≤0.01	0.32	0.09
n-6	15.09±0.62^{b}	15.59±0.71^{b}	15.75±0.32^{b}	16.10±0.13^{a}	16.07±0.25^{a}	16.08±0.54^{a}	0.03	0.45	0.53
n-9	30.43±0.80^{a}	30.98±0.93^{a}	32.25±0.99^{a}	28.79±0.58^{b}	28.58±0.25^{b}	28.17±0.24^{b}	p≤0.01	0.28	0.04
n-3 LC-HUFA	12.25±0.52	10.04±1.14	8.73±2.03	8.87±0.87	11.11±2.52	11.16±0.66	0.76	0.57	0.03
ARA/EPA	0.16±0.02^{a}	0.19±0.01^{a}	0.21±0.04^{a}	0.17±0.01^{b}	0.15±0.00^{b}	0.15±0.00^{b}	0.01	0.29	0.04
DHA/EPA	1.72±0.09	1.62±0.06	1.57±0.14	1.71±0.04	1.73±0.09	1.74±0.08	0.06	0.42	0.32
DHA/ARA	10.61±1.02^{b}	8.66±0.09^{b}	7.65±1.74^{b}	10.23±0.18^{a}	11.74±0.77^{a}	11.36±0.49^{a}	p≤0.01	0.23	p≤0.01
Total PUFA	33.41±0.45^{b}	31.74±2.01^{b}	30.98±1.73^{b}	38.92±1.64^{a}	40.50±1.71^{a}	40.04±1.65^{a}	p≤0.01	0.65	0.19
n-3/n-6	1.10±0.07^{b}	0.91±0.04^{b}	0.84±0.15^{b}	1.34±0.13^{a}	1.44±0.10^{a}	1.40±0.07^{a}	p≤0.01	0.17	0.04
n3PUFA	16.18±0.53^{b}	13.93±1.21^{b}	12.88±2.12^{b}	21.31±1.88^{a}	22.87±1.81^{a}	22.26±1.43^{a}	p≤0.01	0.33	0.1
n6PUFA	14.82±0.60^{b}	15.35±0.67^{b}	15.53±0.34^{b}	15.88±0.12^{a}	15.83±0.28^{a}	15.82±0.54^{a}	p≤0.01	0.57	0.44

Data expressed as means+ SD. Contains: 14:1n-7, 14:1n-5, 15:1n-5, 16:0ISO, 16:2n-6, 16:3n-3, 16:4n-1, 18:3n-1, 18:4n1, 20:2n-9, and 20:3n-9. Values in a row with different superscripts indicate the presence of significant differences ($p<0.05$). Significant differences between diets are expressed by lowercase letters, while significant differences between salinities are expressed by uppercase letters.

Table IX. Muscle body fatty acid profile, as % of the total fatty acids identified, for the different experimental groups (n = 12 per treatment) at the end of the experiment.

	FO			LO			p value		
	BW	SW	HS	BW	SW	HS	D	S	SxD
14:0	2.16±0.21	1.93±0.26	2.02±0.30	1.73±0.61	1.67±0.17	2.07±0.38	0.22	0.51	0.52
15:0	0.30±0.01[a]	0.28±0.01[a]	0.29±0.02[a]	0.24±0.06[b]	0.24±0.02[b]	0.27±0.03[b]	0.01	0.71	0.45
16:0	17.01±0.82	15.47±0.76	16.18±0.50	15.05±1.85	15.16±0.52	15.45±0.68	0.05	0.45	0.35
16:1n-7	4.65±0.29[a]	4.39±0.67[a]	4.36±0.32[a]	3.38±1.00[b]	3.49±0.27[b]	3.92±0.53[b]	0.01	0.83	0.48
16:1n-5	0.15±0.03[a]	0.15±0.01[a]	0.14±0.01[a]	0.12±0.03[b]	0.12±0.01[b]	0.12±0.02[b]	0.01	0.58	0.99
16:2n-4	0.23±0.02[a]	0.24±0.04[a]	0.23±0.03[a]	0.16±0.05[b]	0.16±0.02[b]	0.22±0.05[b]	0.01	0.42	0.38
17:0	0.17±0.03	0.18±0.03	0.19±0.03	0.14±0.04	0.14±0.01	0.19±0.04	0.16	0.18	0.66
16:3n-4	0.32±0.02[a]	0.29±0.02[a]	0.28±0.02[a]	0.24±0.03[b]	0.25±0.01[b]	0.25±0.02[b]	p≤0.01	0.42	0.06
16:3n-1	0.17±0.04	0.19±0.08	0.23±0.05	0.17±0.09	0.16±0.01	0.19±0.03	0.37	0.34	0.81
16:4n-3	0.22±0.03	0.30±0.12	0.27±0.03	0.30±0.01	0.19±0.02	0.21±0.04	0.31	0.83	0.04
18:0	3.43±0.34	3.80±0.68	3.79±0.36	4.24±0.16	3.91±0.18	3.99±0.28	0.06	0.97	0.26
18:1n-9	28.39±0.27	26.85±0.87	26.76±0.61	25.74±0.57	27.06±0.38	26.97±1.20	0.05	0.89	0.01
18:1n-7	3.15±0.12[a]	2.91±0.31[a]	2.95±0.08[a]	2.29±0.32[b]	2.60±0.07[b]	2.86±0.26[b]	p≤0.01	0.32	0.03
18:1n-5	0.14±0.01[a]	0.14±0.01[a]	0.13±0.00[a]	0.09±0.01[b]	0.09±0.00[b]	0.11±0.01[b]	p≤0.01	0.4	0.17
18:2n-9	0.25±0.05[b]	0.17±0.02[b]	0.24±0.04[b]	0.27±0.09[a]	0.30±0.04[a]	0.28±0.06[a]	0.03	0.76	0.29
18:2n-6	12.44±0.34[b]	12.40±0.54[b]	12.21±0.25[b]	13.85±0.76[a]	14.56±0.35[a]	13.53±0.46[a]	p≤0.01	0.13	0.29
18:2n-4	0.17±0.00[a]	0.16±0.01[a]	0.17±0.01[a]	0.11±0.02[b]	0.11±0.00[b]	0.13±0.03[b]	p≤0.01	0.49	0.27
18:3n-6	0.17±0.01	0.16±0.02	0.17±0.01	0.16±0.01	0.18±0.01	0.17±0.00	0.44	0.87	0.16
18:3n-4	0.14±0.00[a]	0.14±0.00[a]	0.13±0.01[a]	0.11±0.03[b]	0.08±0.03[b]	0.10±0.02[b]	p≤0.01	0.41	0.57
18:3n-3	3.22±0.39[b]	2.95±0.50[b]	2.84±0.19[b]	10.88±3.42[a]	12.43±1.23[a]	8.66±3.11[a]	p≤0.01	0.24	0.35
18:4n-3	0.62±0.07	0.64±0.10	0.64±0.08	0.54±0.06	0.55±0.05	0.60±0.07	0.06	0.66	0.78
20:0	0.26±0.02	0.27±0.05	0.26±0.03	0.24±0.02	0.26±0.00	0.29±0.02	0.93	0.4	0.43
20:1n-9	0.25±0.02[a]	0.23±0.06[a]	0.24±0.03[a]	0.19±0.01[b]	0.18±0.00[b]	0.23±0.03[b]	0.02	0.3	0.38
20:1n-7	2.46±0.18[a]	2.55±0.17[a]	2.42±0.03[a]	1.76±0.04[b]	1.76±0.05[b]	2.26±0.44[b]	p≤0.01	0.16	0.04

20:1n-5	0.32±0.02[a]	0.34±0.03[a]	0.34±0.02[a]	0.23±0.00[b]	0.26±0.01[b]	0.31±0.04[b]	p≤0.01	0.03	0.17
20:2n-6	0.67±0.06[a]	0.73±0.06[a]	0.69±0.03[a]	0.56±0.03[b]	0.56±0.01[b]	0.61±0.05[b]	p≤0.01	0.3	0.18
20:3n-6	0.21±0.04[a]	0.21±0.05[a]	0.20±0.01[a]	0.14±0.01[b]	0.12±0.01[b]	0.15±0.01[b]	p≤0.01	0.92	0.49
20:4n-6	1.08±0.07[a]	1.22±0.21[a]	1.22±0.15[a]	0.90±0.10[b]	0.76±0.02[b]	0.86±0.10[b]	p≤0.01	0.7	0.17
20:3n-3	0.28±0.04[b]	0.28±0.02[b]	0.27±0.00[b]	0.47±0.14[a]	0.49±0.06[a]	0.43±0.07[a]	p≤0.01	0.29	0.16
20:4n-3	0.51±0.04[a]	0.52±0.01[a]	0.51±0.01[a]	0.36±0.03[b]	0.33±0.03[b]	0.41±0.07[b]	p≤0.01	0.27	0.16
20:5n-3	4.05±0.23[a]	4.55±0.28[a]	4.66±0.16[a]	3.35±0.77[b]	2.83±0.38[b]	3.40±0.53[b]	p≤0.01	0.35	0.18
22:1n-11	0.92±0.08[a]	0.94±0.14[a]	0.89±0.00[a]	0.63±0.02[b]	0.64±0.03[b]	0.88±0.22[b]	p≤0.01	0.24	0.07
22:1n-9	0.29±0.02[a]	0.31±0.03[a]	0.31±0.03[a]	0.26±0.03[b]	0.24±0.01[b]	0.31±0.03[b]	0.02	0.1	0.08
22:4n-6	0.14±0.01[a]	0.15±0.02[a]	0.14±0.01[a]	0.11±0.01[b]	0.10±0.01[b]	0.11±0.02[b]	p≤0.01	0.97	0.18
22:5n-6	0.41±0.03[a]	0.50±0.09[a]	0.49±0.05[a]	0.41±0.02[b]	0.35±0.04[b]	0.38±0.08[b]	0.01	0.8	0.09
22:5n-3	1.76±0.10[a]	2.08±0.18[a]	1.98±0.03[a]	1.52±0.17[b]	1.25±0.16[b]	1.50±0.35[b]	p≤0.01	0.66	0.06
22:6n-3	8.29±0.55[a]	10.80±1.25[a]	10.60±0.30[a]	8.57±0.75[b]	5.98±1.26[b]	7.07±1.39[b]	p≤0.01	0.7	p≤0.01
Saturated	23.33±0.94	21.94±0.63	22.72±0.52	21.65±2.50	21.38±0.70	22.26±0.78	0.14	0.42	0.64
Monoenoics	40.86±0.44[a]	38.93±1.32[a]	38.66±0.84[a]	34.83±0.51[b]	36.54±0.62[b]	38.09±2.03[b]	p≤0.01	0.59	p≤0.01
n-3	19.05±0.55[b]	22.23±1.38[b]	21.88±0.61[b]	26.06±2.38[a]	24.11±1.26[a]	22.36±2.51[a]	p≤0.01	0.55	0.01
n-6	15.54±0.56[b]	15.88±0.30[b]	15.62±0.45[b]	16.55±0.69[a]	16.99±0.34[a]	16.20±0.38[a]	p≤0.01	0.18	0.6
n-9	29.31±0.24[a]	27.71±0.81[a]	27.69±0.65[a]	26.57±0.66[b]	27.89±0.33[b]	27.93±1.17[b]	0.04	0.93	0.01
n-3 LC-HUFA	14.90±0.82[a]	18.23±1.70[a]	18.03±0.45[a]	14.26±1.57[b]	10.88±1.77[b]	12.81±2.19[b]	p≤0.01	0.56	0.01
ARA/EPA	0.27±0.03	0.27±0.03	0.26±0.02	0.28±0.04	0.27±0.03	0.26±0.03	0.88	0.8	0.9
DHA/EPA	2.05±0.06	2.37±0.14	2.28±0.05	2.62±0.40	2.10±0.20	2.08±0.26	0.72	0.5	0.01
DHA/ARA	7.74±0.88	8.92±0.50	8.74±0.83	9.51±0.50	7.87±1.49	8.16±0.70	0.92	0.89	0.04
Total PUFA	35.89±0.67[b]	39.18±1.24[b]	38.62±0.97[b]	43.37±3.04[a]	42.04±1.29[a]	39.81±2.65[a]	p≤0.01	0.44	0.03
n-3/n-6	1.23±0.06[b]	1.40±0.09[b]	1.40±0.01[b]	1.57±0.09[a]	1.42±0.08[a]	1.38±0.13[a]	0.02	0.9	0.01
n3PUFA	18.74±0.51[b]	21.83±1.27[b]	21.51±0.58[b]	25.68±2.40[a]	23.85±1.24[a]	22.07±2.47[a]	p≤0.01	0.54	0.01
n6PUFA	15.12±0.53[b]	15.37±0.32[b]	15.12±0.40[b]	16.14±0.69[a]	16.63±0.34[a]	15.82±0.36[a]	p≤0.01	0.73	0.19

Data expressed as means+ SD. Contains: 14:1n-7, 14:1n-5, 15:1n-5, 16:0ISO, 16:2n-6, 16:3n-3, 16:4n-1, 18:3n-1, 18:4n1, 20:2n-9, and 20:3n-9. Values in a row with different superscripts indicate the presence of significant differences ($p<0.05$). Significant differences between diets are expressed by lowercase letters, while significant differences between salinities are expressed by uppercase letters.

Table X. Liver fatty acid profile, as % of the total fatty acids identified, for the different experimental groups (n = 12 per treatment) at the end of the experiment.

	FO			LO			p value		
	BW	SW	HS	BW	SW	HS	D	S	SxD
14:0	1.81±0.22[a]	1.57±0.15[a]	1.85±0.46[a]	1.41±0.39[b]	1.43±0.05[b]	1.38±0.14[b]	0.03	0.73	0.56
15:0	0.24±0.02[a]	0.25±0.00[a]	0.24±0.02[a]	0.18±0.03[b]	0.17±0.01[b]	0.21±0.02[b]	p≤0.01	0.26	0.19
16:0	17.31±0.89	15.37±2.84	17.07±3.24	16.45±3.41	17.71±1.98	14.53±0.60	0.76	0.74	0.25
16:1n-7	4.97±0.25[a]	4.16±0.30[a]	4.85±1.11[a]	3.47±0.23[b]	3.27±0.14[b]	2.98±0.05[b]	p≤0.01	0.24	0.26
16:1n-5	0.22±0.04	0.21±0.02	0.20±0.04	0.21±0.23	0.19±0.05	0.14±0.02	0.16	0.13	0.49
16:2n-4	0.14±0.01[a]	0.16±0.04[a]	0.15±0.03[a]	0.07±0.02[b]	0.08±0.03[b]	0.13±0.02[b]	p≤0.01	0.21	0.19
17:0	0.12±0.01[a]	0.13±0.03[a]	0.13±0.02[a]	0.08±0.02[b]	0.09±0.02[b]	0.12±0.02[b]	0.02	0.26	0.42
16:3n-4	0.39±0.03[a]	0.32±0.06[a]	0.33±0.05[a]	0.28±0.02[b]	0.28±0.02[b]	0.25±0.01[b]	p≤0.01	0.14	0.25
16:3n-1	0.04±0.00	0.03±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.14	0.12	0.51
16:4n-3	0.08±0.01	0.09±0.03	0.09±0.03	0.05±0.02	0.06±0.03	0.09±0.02	0.08	0.25	0.41
18:0	2.92±0.17	4.12±0.78	3.70±0.67	4.27±1.16	3.83±0.68	4.46±0.04	0.09	0.47	0.16
18:1n-9	31.66±1.61	27.28±2.26	29.21±3.93	27.72±4.08	31.01±0.68	25.86±1.39	0.36	0.37	0.05
18:1n-7	4.25±0.19[a]	3.93±0.20[a]	4.01±0.16[a]	3.13±0.10[b]	3.02±0.17[b]	2.99±0.19[b]	p≤0.01	0.11	0.6
18:1n-5	0.12±0.00[a]	0.14±0.01[a]	0.13±0.01[a]	0.09±0.01[b]	0.08±0.00[b]	0.09±0.00[b]	p≤0.01	0.49	0.15
18:2n-9	0.64±0.01[b]	0.36±0.15[b]	0.57±0.33[b]	1.12±0.62[a]	1.28±0.54[a]	0.74±0.14[a]	0.01	0.57	0.25
18:2n-6	11.46±0.21	11.95±1.37	11.30±1.77	12.46±1.66	11.72±1.42	13.49±0.12	0.13	0.74	0.3
18:2n-4	0.17±0.00[a]	0.17±0.01[a]	0.16±0.03[a]	0.07±0.03[b]	0.07±0.02[b]	0.10±0.02[b]	p≤0.01	0.43	0.18
18:3n-6	0.31±0.01[b]	0.24±0.02[b]	0.26±0.07[b]	0.46±0.08[a]	0.48±0.10[a]	0.37±0.02[a]	p≤0.01	0.19	0.19
18:3n-4	0.15±0.00[a]	0.14±0.00[a]	0.14±0.00[a]	0.08±0.02[b]	0.09±0.01[b]	0.08±0.01[b]	p≤0.01	0.67	0.28
18:3n-3	2.56±0.20[b]	2.57±0.75[b]	2.30±0.41[b]	10.51±2.80[a]	10.58±1.64[a]	10.44±1.62[a]	p≤0.01	0.95	0.97
18:4n-3	0.49±0.02[b]	0.49±0.09[b]	0.47±0.05[b]	0.65±0.19[a]	0.74±0.03[a]	0.67±0.08[a]	p≤0.01	0.65	0.75
20:0	0.20±0.01	0.24±0.02	0.22±0.04	0.23±0.05	0.23±0.01	0.25±0.01	0.34	0.48	0.34
20:1n-9	0.43±0.02[a]	0.39±0.03[a]	0.35±0.03[a]	0.17±0.05[b]	0.17±0.04[b]	0.20±0.03[b]	p≤0.01	0.56	0.06
20:1n-7	2.78±0.29[a]	2.75±0.30[a]	2.75±0.17[a]	1.43±0.14[b]	1.66±0.32[b]	1.87±0.28[b]	p≤0.01	0.42	0.33

20:1n-5	0.52±0.07[a]	0.52±0.05[a]	0.55±0.07[a]	0.28±0.01[b]	0.33±0.03[b]	0.32±0.06[b]	p≤0.01	0.62	0.65
20:2n-6	0.73±0.07[a]	0.85±0.09[a]	0.80±0.23[a]	0.52±0.14[b]	0.52±0.07[b]	0.64±0.12[b]	p≤0.01	0.46	0.55
20:3n-6	0.16±0.01[a]	0.20±0.02[a]	0.19±0.02[a]	0.11±0.03[b]	0.10±0.01[b]	0.13±0.01[b]	p≤0.01	0.16	0.08
20:4n-6	0.94±0.13[a]	1.51±0.10[a]	1.22±0.44[a]	0.98±0.56[b]	0.68±0.15[b]	0.92±0.15[b]	0.03	0.75	0.09
20:3n-3	0.28±0.03[b]	0.30±0.07[b]	0.28±0.08[b]	0.58±0.17[a]	0.61±0.05[a]	0.65±0.06[a]	p≤0.01	0.84	0.81
20:4n-3	0.47±0.05[a]	0.49±0.11[a]	0.45±0.09[a]	0.23±0.04[b]	0.25±0.06[b]	0.35±0.03[b]	p≤0.01	0.43	0.2
20:5n-3	2.80±0.40[a]	3.71±0.46[a]	4.17±0.79[a]	1.74±0.88[b]	1.47±0.35[b]	2.49±0.28[b]	p≤0.01	0.28	0.09
22:1n-11	1.40±0.13[a]	1.34±0.23[a]	1.25±0.11[a]	0.50±0.17[b]	0.48±0.22[b]	0.67±0.12[b]	p≤0.01	0.88	0.25
22:1n-9	0.47±0.05[a]	0.44±0.03[a]	0.43±0.02[a]	0.39±0.13[b]	0.34±0.04[b]	0.36±0.09[b]	0.03	0.61	0.87
22:4n-6	0.11±0.01[a]	0.15±0.02[a]	0.12±0.02[a]	0.10±0.02[b]	0.09±0.01[b]	0.11±0.01[b]	0.01	0.63	0.11
22:5n-6	0.18±0.02	0.31±0.05	0.24±0.08	0.25±0.03	0.20±0.03	0.24±0.01	0.58	0.24	0.01
22:5n-3	1.37±0.19[a]	1.88±0.66[a]	1.58±0.54[a]	0.79±0.26	0.79±0.25[b]	1.32±0.24[b]	0.01	0.29	0.23
22:6n-3	6.56±1.38	10.74±2.05	8.65±3.85	8.48±6.22	5.44±0.37	9.90±1.90	0.65	0.65	0.15
Saturated	22.60±1.12	21.67±3.32	23.21±3.15	22.63±3.43	23.46±2.69	20.96±0.69	0.91	0.93	0.44
Monoenoics	46.90±2.10[a]	41.26±2.15[a]	43.81±4.77[a]	37.46±3.68[b]	40.62±0.64[b]	35.56±0.62[b]	p≤0.01	0.33	0.04
n-3	14.70±2.13[b]	20.37±4.16[b]	17.09±5.74[b]	23.11±5.15[a]	20.00±1.89[a]	25.97±1.06[a]	0.01	0.5	0.1
n-6	14.08±0.40	15.50±1.55	14.38±2.53	15.14±2.21	13.98±1.19	16.15±0.34	0.57	0.76	0.21
n-9	33.39±1.68	28.63±2.32	30.74±4.27	29.52±4.50	32.97±1.13	27.31±1.39	0.48	0.35	0.05
n-3 LC-HUFA	11.48±1.92	17.12±3.32	14.14±5.31	11.83±7.32	8.56±0.44	14.70±2.53	0.22	0.53	0.14
ARA/EPA	0.34±0.02[b]	0.41±0.08[b]	0.38±0.05[b]	0.56±0.06[a]	0.49±0.19[a]	0.37±0.03[a]	0.04	0.25	0.12
DHA/EPA	2.33±0.18[b]	2.88±0.19[b]	2.64±0.58[b]	4.55±1.02[a]	3.87±0.99[a]	3.95±0.33[a]	p≤0.01	0.93	0.28
DHA/ARA	6.94±0.52[b]	7.19±1.75[b]	6.96±0.83[b]	8.13±1.32[a]	8.26±1.44[a]	10.75±0.83[a]	p≤0.01	0.16	0.13
Total PUFA	31.53±2.43[b]	38.08±5.71[b]	33.88±7.99[b]	40.21±6.89[a]	36.12±2.81[a]	43.87±1.48[a]	0.04	0.61	0.13
n-3/n-6	1.04±0.12[b]	1.31±0.15[b]	1.17±0.20[b]	1.52±0.15[a]	1.43±0.01[a]	1.61±0.03[a]	p≤0.01	0.36	0.07
n3PUFA	14.52±2.13[b]	20.18±4.14[b]	16.91±5.72[b]	23.00±5.14[a]	19.88±1.87[a]	25.81±1.04[a]	0.01	0.51	0.1
n6PUFA	13.89±0.38[b]	15.19±1.50[b]	14.13±2.47[b]	14.89±2.19[a]	13.78±1.21[a]	15.90±0.33[a]	0.01	0.56	0.09

Data expressed as means+ SD. Contains: 14:1n-7, 14:1n-5, 15:1n-5, 16:0ISO, 16:2n-6, 16:3n3, 16:4n-1, 18:3n-1, 18:4n1, 20:2n-9, and 20:3n-9. Values in a row with different superscripts indicate the presence of significant differences ($p<0.05$). Significant differences between diets are expressed by lowercase letters, while significant differences between salinities are expressed by uppercase letters.

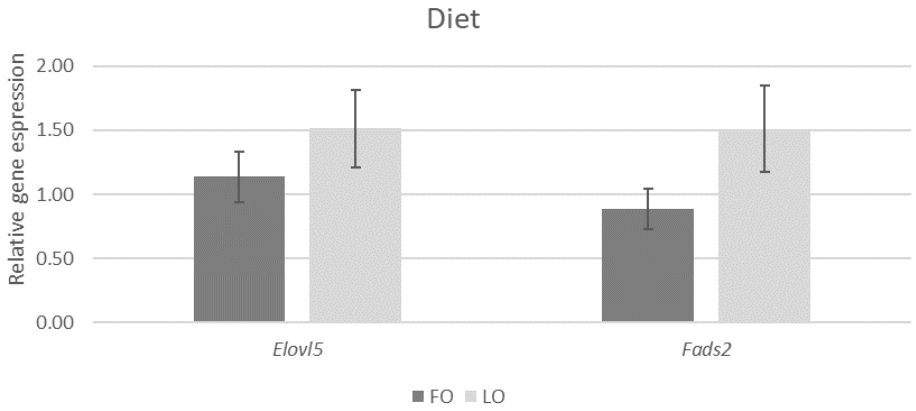

Fig 1. Liver relative gene expression of *Elovl5* and *Fads2* for the fish fed the different diets at the end of the experiment. Data are presented as normalized expression ratios ± standard error. Columns without superscripts indicate the absence of significant differences (p≥0.05).

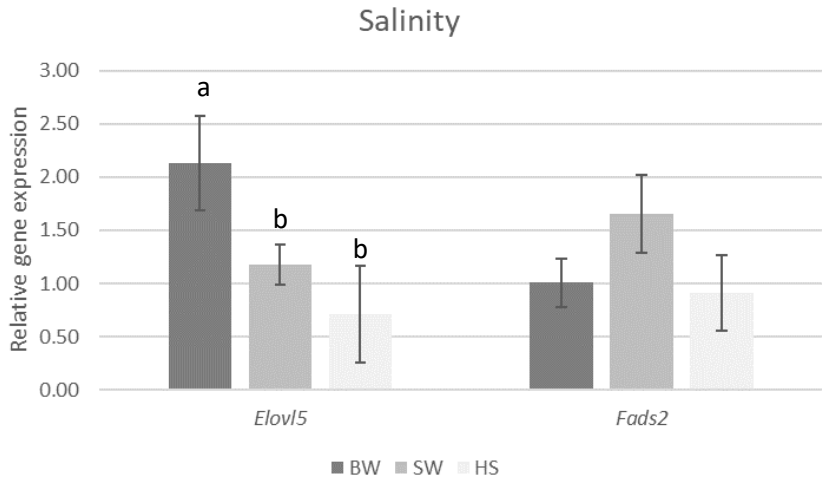

Fig 2. Liver relative gene expression of *Elovl5* and *Fads2* for the fish reared under the different salinities at the end of the experiment. Data are presented as normalized expression ratios ± standard error. Columns with different superscripts indicate the presence of significant differences (p<0.05).

2. General conclusions

1. Zootechnical protocols and conditions for capture, transport, and acclimatization of *Liza aurata* and *Chelon labrosus* were successfully improved in Gran Canaria both for juveniles and broodstock, being survival after captured improved with large size of the acclimatization tanks and utilization of preventive treatments at arrival.

2. Species identification was successfully performed both with external/internal morphological features and confirmed with molecular tools.

3. Natural spawnings of *Liza aurata* broodstock acclimated to culture conditions were obtained for the first time in Gran Canaria, validating zootechnical protocols.

4. It has been confirmed the importance of adequate broodstock feeding for these species since the FA composition of the eggs of *Liza aurata* maintained in captivity varied significantly from that found in the wild female gonads.

5. The comparative analysis of LC-PUFA content in gonads, muscle and liver from wild fish suggested that precursors of LC-PUFA and n-3 LC-HUFA play an essential role in the reproductive metabolism of *Liza aurata*. Specifically linoleic acid (18:2n-6) as ARA precursor in wild female gonads (13%) and DHA in male gonads (34%).

6. Other FA contents such as ARA and EPA must be maintained at dietary levels around 1.35% and 4%, respectively, to ensure a similar fatty acid profile in the gonads of broodstock cultured populations to wild ones.

7. LC-HUFA precursors and enzymatic capacity of the species seem to play an important role in the reproductive fatty acid metabolism of *Liza aurata*, however, more studies are needed in this field to optimize the broodstock formulas of this species.

8. The weaning of *Mugil cephalus* larvae can be successfully achieved by 32 dph (10 mm of total length) only with rotifers administered as single live feed.

9. The utilization of rotifers as live prey for *Mugil cephalus* larvae need to be extended until 32 dph (10 mm of total length) to improve larval survival and fatty acid profile.

10. When enriched *Artemia* sp metanauplii is utilized, it is recommended to be introduced from day 26-29 post-hatching (8-9 mm of total length) to improve growth and minimize size dispersion in *Mugil cephalus* larvae.

11. In *Liza aurata* and *Chelon labrosus* juveniles, three daily meals instead of one led to a more efficient enzyme: substrate ratio and, therefore, to a better utilization of the fed, as revealed by *in vitro* assays with amylase and alkaline protease extracts.

12. Present results demonstrated higher amylase and alkaline protease activities for *Chelon labrosus* than for *Liza aurata*, which determined an increased release of amino acids and reducing sugars in the *in vitro* assays.

13. The species *Liza aurata* presented higher lipid muscle content and percentage of eviscerated weight than *Chelon labrosus*, when cultured in the same conditions.

14. Although *Chelon labrosus* and *Liza aurata* are considered close trophic level species, they present different physiological and productive potentialities, which specificities must be considered to optimize the rentability and sustainability of their production.

15. *Liza aurata* juveniles can efficiently utilize up to 6% of dietary *Aloe vera* by-product, with no rejections in the growth or quality parameters.

16. The utilization of *Aloe vera* by-product in diets for *Liza aurata* juveniles promoted a higher ARA muscle content in the animals. No effects on health or productive parameters studied were observed.

17. Growth, proximate body composition and n3 LC-HUFA levels of *Chelon labrosus* were similar when a source of HUFA precursors was provided in a vegetable oil diet against a FO diet.

18. There was found an interaction ($p \leq 0.01$) between diet and salinity, being DHA and n3 LC-HUFA muscle levels of *Chelon labrosus* juveniles fed a linseed oil diet and reared in brackish water similar to those fed a FO diet in all salinities studied.

19. The highest level of hepatic *Elovl5* gene expression in *Chelon labrosus* juveniles was obtained in brackish water (16 ppt of salinity).

20. The three species studied during this PhD thesis have shown different potentialities in aspects such as reproduction and quality, being great candidates for sustainable aquaculture diversification.

Overall, an integrated approach which adapted global knowledge to local specificities, technical conditions and production objectives has shown to be a good exercise for developing the culture of the selected species in the context of the expected growth of Mugilidae aquaculture.

5. Resumen en español

5.1. Crecimiento sostenible de la acuicultura y agenda 2030

Se predice que la población mundial alcance los 10 billones de personas para 2050 (UN, 2022a). En este escenario, el cambio climático, el agotamiento de recursos, la inseguridad alimentaria, y la pérdida de biodiversidad son, cada vez más, las amenazas más urgentes que la humanidad debe enfrentar.

En septiembre de 2015, los líderes de las Naciones Unidas se reunieron para proponer una serie de objetivos globales para hacer frente a la pobreza, proteger el planeta y promover la prosperidad, como parte de la nueva agenda de desarrollo sostenible (Agenda 2030). Sin embargo, actualmente, más de un 30% de la población mundial sufre de inseguridad alimentaria, mientras que la crisis del Covid 19 y la guerra de Ucrania han agravado la problemática del acceso a alimentos dando lugar a una de las mayores crisis de alimentación desde la segunda guerra mundial (UN, 2022b).

Frente a este escenario, la transformación azul nació como una estrategia para hacer frente a los retos de seguridad alimentaria y sostenibilidad ambiental, poniendo en el punto de mira, entre otros, la expansión sostenible de la acuicultura. Mientras que la producción pesquera disminuyó un 4,4% en 2020, la acuicultura creció de manera continua, alcanzando un récord histórico en 2020 con 122,6 millones de toneladas totales y 87,5 millones de toneladas de animales acuáticos producidos, que alcanzaron un valor de 264 800 millones de dólares estadounidenses. Además, para 2030, se prevé que la producción de organismos acuáticos aumente en más del 15%, principalmente mediante la intensificación y expansión de la producción acuícola. Sin embargo, dicho crecimiento debe conservar la salud del ecosistema acuático, minimizar la contaminación y salvaguardar la biodiversidad y la equidad social (FAO, 2022a).

Por esas razones, el desarrollo de la acuicultura sostenible ayuda directamente a lograr al menos 5 de los diecisiete objetivos de la Agenda 2030: hambre cero, salud y bienestar, trabajo decente y crecimiento económico, ciudades y comunidades sostenibles y uso sustentable de los océanos, mares y recursos marinos (UN, 2022b), e indirectamente, a todos los demás hitos del desarrollo sostenible (Troel *et al.*, 2023).

Sin embargo, la alimentación de los organismos acuáticos representa más del 90% del impacto ambiental de la acuicultura (Little *et al.*, 2018), dado que los ingredientes marinos continúan siendo esenciales para esta industria. La harina de pescado sigue siendo una importante fuente de proteínas y micronutrientes, contribuyendo a la digestibilidad y palatabilidad de los piensos. Por otro lado, el aceite de pescado sigue jugando un importante papel en la acuicultura marina, ya que sigue siendo la fuente más utilizada de ácidos grasos omega 3 de cadena larga, fundamentales para la salud de los peces y la calidad del producto final (Kok *et al.*, 2020).

Además de los precios crecientes, el uso de estas materias primas para alimentos acuícolas es cada vez más controvertido debido al previsto colapso de las pesquerías para el año 2050 si se mantienen las tendencias de captura actuales (Worm *et al.*, 2016).

Por esas razones, la investigación de las últimas décadas se ha centrado en encontrar ingredientes alternativos. Sin embargo, aunque se han logrado avances significativos, la sustitución completa de estos ingredientes en especies marinas es poco común debido a limitaciones nutricionales, tecnológicas o económicas (Oliva-Teles *et al.*, 2015, Glenncross *et al.*, 2023).

En este contexto, el cultivo de peces herbívoros y omnívoros, al ser menos dependiente de estos recursos (Tacon y Metian; 2008), ofrece un interesante potencial para el crecimiento de la acuicultura sostenible.

5.2. Diversificación, el rol de las especies de bajo nivel trófico

Los avances en nutrición acuícola y la implementación de ingredientes alternativos han alejado las dietas de las especies cultivadas de aquellas sus contrapartes silvestres, influyendo el nivel trófico efectivo de estas. No obstante, las especies carnívoras pueden sufrir efectos adversos en el crecimiento o la salud a niveles altos de sustitución de harina de pescado (Chen *et al.*, 2019; Pham *et al.*, 2020; Liu *et al.*, 2021) mientras que en general, en los carnívoros no obligados, la inclusión baja o nula de ingredientes de origen animal se alinea con los hábitos dietéticos naturales y, por lo general, es mejor tolerada. De esa manera, las políticas para el crecimiento sostenible acuícola a menudo promueven la producción de especies de bajo nivel trófico (Santillo y Dorey., 2013; Cottrell *et al.*, 2021); aunque no solo el nivel trófico sino también otros aspectos fisiológicos como la eficiencia alimentaria, son factores determinantes para definir la sostenibilidad de una especie (Cottrell *et al.*, 2021).

Las especies omnívoras de agua dulce representan más del 80% de la producción piscícola (Tacon, 2019); sin embargo, la producción de peces marinos de bajo nivel trófico es aún poco común y pocas especies se cultivan en cantidades significativas. Por ejemplo, el sabalote (*Chanos chanos*) se produce en aguas cálidas de los océanos Pacífico e Índico, alcanzando una producción total de 1167,8 mil toneladas en 2020 (FAO, 2022a). Sin embargo, su producción es limitada dada la necesidad de una producción de semilla bien establecida y su bajo precio en el mercado internacional (Mirera *et al.*, 2019).

Los múgiles se sitúan en la segunda posición, con 291,2 mil toneladas producidas en 2020 (FAO, 2022a). A pesar del alto precio de algunos de sus productos procesados, como las huevas secas y saladas, su cultivo en sistemas intensivos aún es limitado. Entre los factores limitantes para una mayor intensificación está el bajo precio de mercado del pescado entero y la ausencia de producción de semilla a nivel comercial, lo que hace que su cultivo aún se sustente en la captura de alevines del medio (Crosetti, 2015). Sin embargo, su carácter omnívoro, eurihalino y cosmopolita, ha despertado el interés por esta familia, provocando un aumento de la producción en los últimos años (FAO, 2022a).

5.3. Estado actual del cultivo de mugílidos

La acuicultura de mugílidos comenzó hace siglos en base a sistemas de captura de los peces aprovechando las migraciones naturales desde el mar hacia las lagunas costeras o viceversa, y su confinamiento en áreas restringidas (cultivo extensivo o semi-intensivo en la mayoría de los casos) (Crosetti, 2015).

La producción acuícola mundial de mugílidos fue de 291,2 mil toneladas en 2020, lo que representa el 3,5% del total de la acuicultura marina y costera de peces y es comparable a producciones de especies tan apreciadas como la dorada (282,1 mil toneladas, 3,4% del total) o la lubina (243,9 mil toneladas, 2,9% del total) (FAO, 2022a). En los últimos años la producción se ha incrementado significativamente, aumentando un 125,4 % en solo 5 años (2015 a 2020) (Figura 1.5), probablemente debido a los recientes avances en el cierre del ciclo biológico en cautividad (Besbes *et al.*, 2020; Vallainc *et al.*, 2021; Ramos-Júdez; 2022; Vallainc *et al.*, 2022) y al creciente interés en estas especies para el desarrollo sostenible de la acuicultura y el crecimiento azul (Tacon *et al.*, 2009; Neori y Nobre., 2012; Krause *et al.*, 2022).

La producción acuícola de múgiles se ha registrado en al menos 15 países (Crosetti, 2015), siendo Egipto el primer productor con 250 mil toneladas producidas en 2018 (El-Son *et al.*, 2021). Los otros países que lideran la acuicultura de estas especies son la República de Corea, Italia, la provincia china de Taiwán e Israel. La producción acuícola se basa principalmente en la recolección de alevines silvestres, lo que conlleva altas mortalidades (hasta un 96%) (Crosetti, 2015) y solo en algunos países como Israel, Hawái e Italia, éstos se producen a pequeña escala en condiciones controladas (Sukumaran *et al.*, 2022).

5.4. Reproducción y cultivo larvario

Los múgiles son teleósteos bisexuales ovíparos sin dimorfismo sexual, con fecundación y desarrollo externos. Las gónadas, tanto masculinas como femeninas, son órganos internos pares que se encuentran a ambos lados de la vejiga natatoria y se unen cerca del poro urogenital en la papila genital.

Las hembras son reproductoras totales (Hunter, 1992) con ovarios sincrónicos por grupos (Wallace y Selman, 1981); por lo tanto, generalmente solo desovan una vez al año. Aunque los múgiles son peces eurihalinos, la mayoría de las especies solo se reproducen en agua salada.

Por otro lado, la coexistencia habitual en los mismos hábitats ha determinado comportamientos biológicos diferenciales que ayudan a evitar la competencia por los recursos (Salvarina *et al.*, 2016). Así, es habitual que especies de mújol que comparten hábitat tengan diferentes épocas de puesta a lo largo del año. Por ejemplo, cuatro de las especies cultivadas con mayor frecuencia en la región mediterránea tienen temporadas de desove consecutivas y diferenciadas, *Mugil cephalus* (julio-septiembre) (Besbes *et al.*, 2020), *Liza aurata* (septiembre-noviembre) (Hotos *et al.*, 2000; Ghaninejad *et al.*, 2010), *Liza ramada* (noviembre-enero) (Mousa *et al.*, 2018; Vallainc *et al.*, 2022) y *Chelon labrosus* (febrero -abril) (Besbes *et al.*, 2020), lo que también puede

ser una valiosa ventaja para los productores de alevines, que pueden producir durante todo el año.

Principales disfunciones reproductivas de los mugílidos en cautividad

Como muchos otros teleósteos marinos en cautividad, algunas especies de mugílidos como *Mugil cephalus* (Aizen *et al*., 2005; Ramos-Júdez *et al*., 2022) o *Liza ramada* (Mousa *et al*., 2018), fallan durante la etapa final de maduración y las hembras no completan la maduración final del ovocito, mientras que los machos fallan en la producción de esperma. Por ello, algunos métodos para la producción de alevines se basan en la recolección de adultos durante la época de desove y la inducción hormonal en ejemplares muy maduros (Vallainc *et al*., 2021; Vallainc *et al*., 2022). Sin embargo, otras especies como *Chelon labrosus* si logran madurar en condiciones de cultivo (Sarasquete *et al*., 2014, datos propios, GIA-Ecoaqua); sin embargo, aunque la reproducción controlada comenzó hace más de 30 años (Cataudella *et al*., 1988), sigue siendo común la necesidad de altas dosis hormonales para lograr el desove (Besbes *et al*., 2020).

Los huevos fertilizados son generalmente pelágicos, transparentes, esféricos y flotantes. El diámetro del huevo de *Chelon labrosus* es uno de los mayores en esta familia (1,3-1,4 mm) y contiene de 1 a 13 gotas lipídicas (Boglione *et al*., 1992; datos propios, GIA-Ecoaqua), mientras en otras especies como en *Mugil cephalus* el tamaño es menor (0,88-0,99 mm) y generalmente los huevos contienen una única gota lipídica (Kuo *et al*., 1973; Meseda y Samira., 2006).

Al igual que otras especies de peces marinos, las larvas de mugílidos nacen con órganos y sistemas poco desarrollados que maduran durante la fase larvaria. El tamaño a la eclosión varía mucho entre especies, desde 4 mm en larvas de *Chelon labrosus*, lo que permite implementar la primera alimentación directamente con *Artemia* sp. (Cataudella *et al*. 1988; de las Heras *et al*., 2012), 2,9 mm para larvas de *Liza aurata* (datos propios, GIA-Ecoaqua), 1,97 mm para *Mugil cephalus* (Meseda y Samira, 2006), y hasta 1,43 mm en larvas de *Mugil macrolepis* (Crosseti, 2015). Además, otras diferencias fisiológicas como el tamaño del saco vitelino, el tamaño de la boca y su momento de apertura, o la ontogenia visual y esquelética, pueden determinar diferentes protocolos de manejo y nutricionales que deben ser estudiados en cada caso para optimizar la supervivencia, el costo-beneficio y el esfuerzo de trabajo.

5.5. Engorde y parámetros de cultivo

La acuicultura se basa en el engorde de peces en condiciones controladas, lo que determina la rentabilidad final. El número de parámetros a controlar es enorme y depende, principalmente, del tipo de sistema acuícola empleado y de las especies utilizadas. Algunos ejemplos de parámetros que pueden influir en el crecimiento son la calidad del agua, la temperatura, el fotoperíodo, la densidad de cultivo, la salinidad y el manejo de la alimentación. En cuanto a los factores ambientales, la calidad del agua, la temperatura y la salinidad dependen principalmente de las características inherentes al agua de entrada cuando se trata de acuicultura en flujo abierto, pero son parámetros que deben controlarse y pueden modificarse fácilmente cuando se trata de sistemas acuícolas de recirculación (RAS).

En este sentido, la salinidad es un factor crucial que considerar para el cultivo de mugílidos, ya que tiene un papel fundamental en su fisiología natural y comportamiento que afecta a varios factores de su productividad, desde el crecimiento y la reproducción hasta la calidad del producto final.

Los múgiles son peces eurihalinos, y muchas especies pueden vivir en una amplia gama de salinidades, desde agua dulce hasta aguas a alta salinidad. La mayoría de las especies desovan en aguas salobres o marinas, y los alevines migran a lagunas costeras, estuarios o incluso a agua dulce (Nordlie *et al.*, 2015). Sin embargo, existe una gran variabilidad en el grado de eurihalinidad según la especie, y la propia salinidad afecta a la distribución de especies en las mismas regiones. Por ejemplo, *Liza aurata* evita los ambientes de agua dulce y tiene preferencia por aguas polihalinas (15,1-30 ppt) y euhalinas (30,1-40 ppt). Por el contrario, *Mugil cephalus* suele seleccionar hábitats con salinidades inferiores a 15 ppt, mientras que las preferencias de *Chelon labrosus* varían mucho a lo largo del año. No obstante, las tres especies se pueden encontrar en agua dulce (Cardona *et al.*, 2006), aunque en crecimiento suele mejorar a salinidades intermedias (Barman *et al.*, 2005; Olukolajo y Omolara, 2013; Loi *et al.*, 2022).

5.6. Alimentación y engorde

Las postlarvas de mújol son principalmente zooplanctívoras, produciéndose el cambio a la dieta bentónica a partir de los 20-30 mm de longitud total. A partir de ese momento, la proporción de proteínas en el contenido del estómago disminuye mientras que la cantidad de detritus y materia vegetal aumenta continuamente, teniendo los peces de 40-50 mm una dieta similar a la de los adultos. Tras esta transición, los estómagos de los juveniles y adultos silvestres se suelen encontrar con una mezcla de arena, detritos, macroalgas, zooplancton, fauna béntica, cianobacterias, y diatomeas (Cardona, 2015).

El estómago posee un número especie-específico de ciegos pilóricos y una capa muscular muy bien desarrollada para procesar la comida. El intestino es excepcionalmente largo, aunque la longitud relativa varía significativamente entre especies y poblaciones. Aunque la longitud relativa del intestino se ha considerado tradicionalmente un buen indicador de la capacidad de digerir materia vegetal, la actividad enzimática puede ser más relevante para definir mejor los diferentes potenciales de estas especies para digerir fuentes vegetales (Cardona, 2015). Los hidratos de carbono suelen representar la mitad de la materia orgánica de la dieta, aunque la eficiencia de asimilación parece relativamente baja (alrededor del 50%) (Cardona *et al.*, 2001; Cardona *et al.*, 2015).

Existe una gran controversia sobre el nivel trófico de las especies de mújol, ya que la relevancia de las presas animales en las dietas naturales varía mucho entre especies e incluso geográficamente, habiéndose sugerido un nivel trófico entre 2 y 3 para la mayoría de las especies estudiadas de Mugilidae. (Cardona *et al.*, 2015; Salvarina *et al.*, 2016). En este sentido, los estudios enzimáticos son cruciales para cubrir las lagunas de conocimiento sobre este tema.

Los precios crecientes y la escasez de harina y aceite de pescado para la nutrición animal han promovido la investigación de ingredientes alternativos para satisfacer la

demanda de producción de alimentos acuícolas. En este sentido, diferentes materias primas como el alga verde *Ulva lactuca* al 20% de inclusión, la levadura de cerveza hasta el 40% de inclusión (Wassef *et al.*, 2001), la espirulina (*Arthrospira platensis*) hasta el 22,5% (Rosas *et al.*, 2019), la biomasa de zooplancton hasta un 30 % (Abo-Taleb *et al.*, 2021a; Abo-Taleb *et al.*, 2021b), y fermentados de subproductos vegetales hasta un 80 % de inclusión (De *et al.*, 2018) se han evaluado en esta familia con resultados exitosos.

Además del potencial sugerido de las especies de mújol para digerir carbohidratos y proteínas de fuentes vegetales en comparación con otras especies acuícolas marinas, los mugílidos ofrecen un interesante potencial para la utilización de dietas libres de aceite de pescado debido a la capacidad de transformar los ácidos grasos de 18C en ácidos grasos poliinsaturados de cadena larga (LC-PUFA) (Galindo *et al.*, 2021). Los peces marinos generalmente tienen una actividad limitada de las enzimas implicadas en la elongación (enzimas *Elovl*) y desaturación (enzimas *Fads2*) de ácidos grasos de 18 carbonos, siendo las actividades Δ4 y Δ6 las últimas responsables de la formación de LC-PUFA (Figura 1.9). Por esas razones, los LC-PUFA se consideran esenciales en las dietas de la mayoría de los peces marinos, afectando directamente a su bienestar y salud (Montero e Izquierdo, 2010). Sin embargo, en el caso de *Chelon labrosus* se han descrito actividades tanto Δ6 como Δ8 en *Fads2*, sugiriéndose que la filogenia de las especies es un factor más relevante para la capacidad de biosíntesis de LC-PUFA que el nivel trófico en sí mismo (Garrido *et al.*, 2019, Galindo *et al.*, 2021). Estudios en otras especies de mújoles respaldan este potencial para biosintetizar LC-PUFA (Mourente y Tocher 1993; Khériji *et al.*, 2003; Imen *et al.*, 2013; Rabeh *et al.*, 2015); sin embargo, se necesita más investigación en este campo para una mejor comprensión del potencial de los mugílidos para la utilización de piensos sin aceite de pescado añadido.

5.7. Objetivos

El objetivo general de esta tesis es el de incrementar el conocimiento para la optimización del cultivo de tres importantes especies de mújol presentes en la costa mediterránea y atlántica, *Liza aurata*, *Chelon labrosus* y *Mugil cephalus*.

Para lograr este objetivo general, se propusieron 2 objetivos específicos:

1. Profundizar el conocimiento sobre la reproducción controlada y la cría larvaria de la familia Mugilidae. Para ello, por un lado, se realizó la pesca, aclimatación y establecimiento de un stock de reproductores de *Liza aurata* en cautividad, además de una primera aproximación al metabolismo reproductivo lipídico y de la obtención final de puestas viables (primer capítulo). Por otro lado, se trabajó la mejora del manejo alimentario para optimizar las prácticas de cría larvaria durante la fase de destete en *Mugil cephalus* (segundo capítulo).

2. Incrementar el conocimiento del manejo nutricional bajo un enfoque sostenible, incluyendo un estudio combinado que evalúe el manejo alimentario de ambas especies

criadas bajo las mismas condiciones (tercer capítulo), la valorización del subproducto local de *Aloe vera* en dietas para *Liza aurata* (cuarto capítulo) y la valoración del efecto dietético y de la salinidad sobre el potencial de síntesis de HUFA en *Chelon labrosus* (quinto capítulo).

5.8. Metodología general

Para lograr los objetivos de esta tesis, se ha realizado la pesca, el transporte y la aclimatación a cautividad de ejemplares silvestres de *Chelon labrosus* y *Liza aurata*, tanto en la fase juvenil como adulta, en posesión de los permisos gubernamentales correspondientes.

Los experimentos con animales, tanto con larvas como con juveniles, se realizaron por triplicado, considerando el tanque como unidad experimental. Se han utilizado tanto sistemas abiertos de agua de mar (capítulos uno, tres y cuatro) como sistemas acuícolas de recirculación (RAS) (capítulos dos y cinco). La manipulación y sacrificio de los peces se realizó siguiendo rigurosamente la Directiva de la Unión Europea (2010/63/UE) y la legislación española (RD 1201/2005) sobre protección del bienestar animal con fines científicos, habiéndose enviado los protocolos experimentales correspondientes al Comité de Bioética de la Universidad de Las Palmas de Gran Canaria.

La parte experimental del capítulo uno se realizó en colaboración con el Centro Integrado de Formación Profesional (CIFP) Marítimo Zaporito, en San Fernando (España). El segundo capítulo se realizó en colaboración con el "International Marine Center" (IMC) en Oristano, Italia, como parte de la estancia en un centro de investigación extranjero contemplada en el programa de doctorado de la ULPGC para la mención internacional. Los capítulos tercero, cuarto y quinto se llevaron a cabo en las instalaciones del instituto GIA-Eco-aqua de la Universidad de Las Palmas de Gran Canaria (ULPGC).

5.9. Resultados y discusión. Publicaciones científicas

5.9.1. Manejo reproductivo del mugílido *Liza aurata* y caracterización de la composición proximal y de ácidos grasos de tejidos reproductores y puestas

La lisa dorada (*Liza aurata*), es una especie prometedora para la expansión sostenible de la acuicultura. sin embargo, la falta del control de la reproducción en cautividad, y la consecuente falta de una provisión estable de alevines, es uno de los cuellos de botella más significativos para la expansión del cultivo de esta especie. En muchos casos, el manejo de la reproducción de mugílidos requiere la aplicación de tratamientos hormonales para inducir la maduración gonadal o el desove. Sin embargo, no existen trabajos directamente relacionados con la recolección de reproductores, aclimatación y manejo reproductivo de esta especie.

Por otro lado, el conocimiento de los requerimientos y patrones de movilización de ácidos grasos esenciales (AGE) por los reproductores es uno de los primeros pasos para diseñar fórmulas y protocolos de alimentación apropiados, cruciales para el éxito de la reproducción y del desarrollo larvario.

Por estas razones, este estudio tuvo como objetivo 1) describir por primera vez el manejo reproductivo de la especie *Liza aurata* en condiciones controladas de cultivo y 2) ofrecer una primera aproximación del metabolismo lipídico reproductivo de esta especie.

Para ello, se tomaron muestras de tejidos (gónadas, hígado y músculo) de animales recién capturados de una población silvestre (hembras, machos y animales inmaduros), para evaluar la composición proximal y de ácidos grasos. Además, se seleccionaron 22 reproductores y se aclimataron en un tanque de 10 m^3 en sistema abierto con agua de mar, adaptándolos a la alimentación con un pienso comercial (R5 Europe, Skretting, Burgos, España).

Los resultados han evidenciado la posibilidad de obtener desoves naturales a partir de reproductores con una media de peso de 787 g y 604 g (hembras y machos, respectivamente), en una proporción de sexos de 2:1 (hembras/machos), en condiciones naturales de fotoperíodo y del agua marina con temperaturas en descenso de 20,4 ± 0,3 ◦C a 18,8 ± 0,4 °C.

Por otra parte, se ha constatado el papel crucial de los precursores de HUFA (ácidos grasos altamente insaturados) en el desarrollo gonadal de *Liza aurata*, principalmente en las hembras, en las que se ha encontrado notorios niveles de ácido linoleico. Por otro lado, las gónadas de los machos salvajes presentaron un notable contenido de HUFA, predominantemente de DHA (ácido docosahexaenoico) (34% del total de ácidos grasos). En los huevos obtenidos en condiciones de cautividad, aparecieron variaciones significativas en la composición en comparación con la gónada femenina salvaje, con niveles más bajos de ARA (ácido araquidónico) y EPA (ácido eicosapentaenoico) y mayores proporciones de EPA/ARA y DHA/ARA.

Además, se evidenció el importante papel del hígado como reservorio fisiológico de HUFA, que parecen ser movilizados a la gónada durante el proceso de maduración.

Los resultados actuales pueden ayudar a obtener una mejor perspectiva para ajustar las condiciones de manejo y alimentación de los reproductores, contribuyendo de esta manera a un crecimiento acuícola más sostenible.

5.9.2. Efectos de diferentes protocolos de co-alimentación en el destete temprano de larvas de múj ol (*Mugil cephaus*)

La expansión sostenible de la acuicultura depende de un suministro suficiente de huevos y larvas, que son el primer paso para la gestión del ciclo biológico. Sin embargo, la cría de larvas de peces marinos generalmente depende de la producción de zooplancton, lo que requiere una importante inversión en instalaciones y mano de obra. El múj ol (*Mugil cephalus*), una especie prometedora para la diversificación de la acuicultura, tiene un desarrollo precoz del sistema digestivo, lo que respalda la viabilidad de estrategias de destete temprano.

Por estas razones, este estudio evaluó tres protocolos de co-alimentación (destete) desde el día 22 al 36 post-eclosión. Se diseñaron 2 protocolos con distintas concentraciones de *Artemia* sp., A100, A50 (2 y 1 *Artemia* sp. inicial ml^{-1} $día^{-1}$, respectivamente) y un protocolo que solo administró rotíferos como presa viva durante todo el periodo (A0). Las dosis iniciales de alimento vivo fueron reducidas progresivamente para ser totalmente sustituidas por la microdieta al día 32 post-eclosión. Los parámetros evaluados fueron la supervivencia, el crecimiento, la composición proximal y de ácidos grasos, y la expresión génica de las larvas.

El tratamiento A0 produj o mej or supervivencia (64,79±7,40%) que el protocolo A100 (32,46±12,82%). Por el contrario, las larvas del tratamiento A100 presentaron una longitud final significativamente mayor (15,51±0,86 mm) que el tratamiento A0 (12,19±1,45 mm) y un peso final (41,28±1,48 mg) mayor que los tratamientos A50 y A0 (31,23±3,65 mg y 24,03±7,99 mg, respectivamente).

En cuanto a la composición proximal y de ácidos grasos, las larvas reflej aron el perfil del alimento, presentando las larvas del tratamiento A100 mayor contenido lipídico, y de ácidos oleico y linolénico, en comparación con las larvas del tratamiento A0, que presentaron mayores niveles de ácidos grasos de cadena larga como el EPA, DPA y DHA.

Por otro lado, la expresión génica de enzimas digestivas y factores de crecimiento (hormonas gh e igf1) no mostró diferencias entre tratamientos, aunque si se observó un aumento de la expresión de las enzimas relacionadas con la digestión de proteínas y grasas desde el punto inicial (22 días post-eclosión) al final (36 días post-eclosión), lo que refleja el aumento de la capacidad de los animales durante este periodo para asimilar tanto las grasas como las proteínas de la dieta.

En conclusión, los tratamientos con *Artemia* sp. promovieron un mayor crecimiento, mientras que el tratamiento A0 produj o una mayor supervivencia y dispersión de tallas, ya que mantener los rotíferos por más tiempo favoreció la supervivencia de las larvas más pequeñas. En base a los resultados anteriores, para optimizar la supervivencia se recomienda mantener los rotíferos hasta el día 30-32 dph (al menos 10 mm de longitud total). Además, para minimizar la dispersión de tallas y promover una transición más suave entre presas vivas, se pueden administrar nauplios de Artemia sp. previamente a los metanauplios durante 1 o 2 días, sin embargo, la administración de estos últimos no se recomienda hasta el día 26–29 post-eclosión (8,0–8,8 mm de longitud).

5.9.3. Evaluación del efecto de dos frecuencias de alimentación en la bioquímica digestiva de dos especies de mugílidos (*Chelon labrosus* y *Liza aurata*)

Los mugílidos (Mugilidae) presentan un gran potencial para la diversificación de la acuicultura sostenible debido a su naturaleza euriterma, eurihalina y de bajo nivel trófico. Sin embargo, las diferencias fisiológicas y las condiciones óptimas de cultivo para las diversas especies de mújoles siguen siendo bastante desconocidas, lo que dificulta un manejo nutricional óptimo.

Por estas razones, el presente estudio busco abordar dos objetivos principales: 1) caracterizar las diferencias en la bioquímica digestiva, los índices somáticos y la composición corporal entre dos especies de mugílidos (*Liza aurata* y *Chelon labrosus*); y 2) evaluar las interacciones de dos frecuencias de alimentación (una contra tres tomas diarias) en los parámetros mencionados anteriormente, y también en la biodisponibilidad de nutrientes determinada mediante ensayos *in vitro*.

Para esto, 36 juveniles de cada especie fueron distribuidos en 6 tanques en sistema abierto de agua de mar. Los animales fueron alimentados con un pienso control al 1% de su biomasa, siendo esta dosis distribuida en una alimentación diaria o en 3 tomas, a 3 tanques por especie y por frecuencia de alimentación. Tras 6 semanas de alimentación, los animales fueron muestreados en dos momentos, a las 15 h y 40 h post-alimentación, para evaluar las diferencias enzimáticas entre los momentos de digestión y ayuno.

Los resultados evidenciaron mayor actividad de proteasa alcalina y amilasa para *Chelon labrosus* que para *Liza aurata*, además de una producción de enzimas más sostenida pese al tiempo de ayuno. Por otro lado, los juveniles de *Liza aurata* presentaron un mayor porcentaje de peso eviscerado y de lípidos en músculo, además de un menor índice hepato-somático.

Los resultados de los ensayos *in vitro* fueron en concordancia con la mayor actividad enzimática de *Chelon labrosus,* con una liberación mayor de aminoácidos y azúcares reductores en esta especie.

En cuanto a los patrones de alimentación, los resultados de los ensayos *in vitro* simulando las relaciones enzima:sustrato (E:S) correspondientes a una o tres tomas diarias indicaron un claro aumento de la biodisponibilidad de nutrientes cuando la ración diaria se divide en varias tomas.

Los presentes resultados mejoran el conocimiento de la fisiología de estas especies y ayudan a definir criterios para desarrollar mejores protocolos de manejo por parte de los productores.

5.9.4. Evaluación del subproducto de *Aloe vera* frente a cereales en piensos para la lisa dorada (*Liza aurata*)

Entre los desafíos actuales para el desarrollo de la acuicultura sostenible se encuentra bajar el nivel trófico de las especies cultivadas, además de aumentar el uso de subproductos del sector primario. La lisa dorada (*Liza aurata*) es un consumidor marino de bajo nivel trófico presente en el Mediterráneo y en Canarias, donde existió un consumo tradicional importante. Por otro lado, el *Aloe vera*, cuya producción en Canarias es altamente representativa a nivel mundial, contiene más de 70 componentes biológicamente activos que han despertado el interés para su uso en acuicultura. Sin embargo, la enorme cantidad de subproductos generados por esta industria carece de uso, y estos nunca han sido probados en acuicultura.

Por estos motivos, el objetivo del presente estudio fue realizar el primer ensayo de alimentación controlada con *Liza aurata* en Canarias, y evaluar el uso de *Aloe vera* puro local frente a diferentes niveles del subproducto, para determinar los efectos en parámetros de crecimiento, salud y calidad.

De esta manera, se formularon 5 dietas: 0% de inclusión de aloe (dieta control), 2% de de aloe puro (dieta P2) y 2, 4 y 6% de subproducto de aloe (dietas BP2, BP4 y BP6). A los 91 días de alimentación, se evaluaron el crecimiento, la composición proximal y de ácidos grasos de hígado, músculo y pez entero, las actividades lisozima y bactericida en suero, y el contenido de malonaldehído en el hígado y el pez entero.

En general, no se encontraron diferencias por la suplementación dietética con aloe puro o su subproducto en el crecimiento, composición proximal, o en los parámetros serológicos y de peroxidación estudiados. Sin embargo, se observó un aumento en los niveles de ácido araquidónico en el músculo de los peces alimentados con los mayores porcentajes de subproducto de aloe (BP4 y BP6).

Según los resultados, se puede incluir hasta un 6% del subproducto de aloe en las dietas de esta especie, sin que se produzcan efectos adversos en los parámetros de crecimiento o calidad, aunque en general no se observaron mejores resultados en comparación con los peces control.

Otros estudios sobre este campo siguen en camino para determinar la sostenibilidad y el impacto bioeconómico de los resultados actuales, para aumentar el conocimiento sobre sus aplicaciones industriales directas.

5.9.5. Efectos del reemplazo del aceite de pescado y la salinidad ambiental sobre el perfil de ácidos grasos y la expresión génica de enzimas de desaturación y elongación en el mújol (*Chelon labrosus*)

El incremento global de la acuicultura marina, junto con el colapso general del mercado de harina y aceite de pescado, ha convertido la búsqueda de ingredientes alternativos en uno de los desafíos más significativos a los que se enfrenta la acuicultura en la actualidad. Uno de los cuellos de botella más significativos es la sustitución del aceite de pescado en dietas de peces marinos, los cuales generalmente tienen una capacidad limitada para biosintetizar ácidos grasos altamente insaturados (HUFA) a partir de los precursores de 18 carbonos. Sin embargo, la familia Mugilidae (mújoles) es una gran candidata para explorar este potencial fisiológico, ya que se ha demostrado la capacidad de biosíntesis de HUFA en algunas especies (Garrido *et al.*, 2019; Galindo *et al.*, 2021).

Por estas razones, este estudio tuvo como objetivo evaluar dos dietas (dieta control vs dieta con aceite de lino (LO)) en juveniles de *Chelon labrosus* cultivados a tres salinidades (46 ppt, 35 ppt y 16 ppt), para explorar la capacidad de biosíntesis de HUFA de esta especie en función de la dieta y de la salinidad ambiental.

Para ello, 180 juveniles de *Chelon labrosus* fueron distribuidos en 18 acuarios de 60 L en recirculación (RAS), de manera que cada combinación de dieta y salinidad se evaluara en triplicado. Tras 73 días de experiencia, se analizó la composición bioquímica y de ácidos grasos del hígado y músculo y pez entero, así como la expresión génica relativa en el hígado de enzimas de elongación (*Elovl5*) y desaturación (*Fads2*) de ácidos grasos.

Las dos dietas funcionaron por igual en términos de crecimiento, composición proximal y niveles de n3 LC-HUFA en pez entero e hígado. Sin embargo, los peces alimentados con la dieta LO presentaron mayor contenido en lípidos y cenizas en el músculo. Además, pese a la ausencia de diferencias significativas, se observó una tendencia de mayor expresión hepática tanto de *Elovl5* como de *Fads2* en los animales alimentados con la dieta LO.

En cuanto a la salinidad ambiental, los peces cultivados en agua salobre (16ppt) tuvieron un mejor crecimiento, un mayor nivel de cenizas en el músculo, y una mayor expresión de *Elovl5* en el hígado. Además, los niveles de ácido araquidónico (ARA), ácido docosahexaenoico (DHA) y de n3 LC-HUFA fueron similares en el músculo de los múgiles criados con la dieta LO en agua salobre y los peces alimentados con la dieta de control en todas las salinidades.

En conclusión, los presentes resultados respaldan la viabilidad de dietas libres de aceite de pescado para juveniles de *Chelon labrosus*, principalmente cuando estos se crían en agua salobre.

5.10. Conclusiones generales

1. Los protocolos zootécnicos y las condiciones para la captura, transporte y aclimatación de *Liza aurata* y *Chelon labrosus* se llevaron a cabo con éxito en Gran Canaria tanto para juveniles como para reproductores, mejorando la supervivencia post-captura por medio del aumento del gran tamaño de los tanques de aclimatación y la utilización de tratamientos preventivos a la llegada.

2. La identificación de especies se realizó con éxito tanto con características morfológicas externas/internas y herramientas moleculares.

3. Se constató por primera vez la reproducción natural de *Liza aurata* de individuos aclimatados, validando los protocolos zootécnicos usados.

4. Se ha confirmado la importancia de una alimentación adecuada de los reproductores de estas especies, ya que la composición de ácidos grasos de los huevos de *Liza aurata* mantenidos en cautividad varió significativamente de la encontrada en las gónadas de las hembras silvestres.

5. El análisis comparativo del contenido de LC-PUFA en gónadas, músculo e hígado de peces silvestres sugiere que los precursores de LC-PUFA y n-3 LC-HUFA desempeñan un papel esencial en el metabolismo reproductivo de *Liza aurata*. Concretamente el ácido linoleico (18:2n-6) como precursor de ARA en gónadas femeninas salvajes (13%) y el DHA en gónadas masculinas (34%).

6. Otros contenidos de AG como el ARA y el EPA deben mantenerse en niveles dietéticos de alrededor de 1,35% y 4%, respectivamente, para garantizar un perfil de ácidos grasos similar de las gónadas de reproductores en cautividad a las silvestres.

7. Los precursores de LC-HUFA y la capacidad enzimática de la especie parecen jugar un papel importante en el metabolismo reproductivo de ácidos grasos *de Liza aurata*, sin embargo, se necesitan más estudios en este campo para optimizar las fórmulas para reproductores.

8. El destete de las larvas de *Mugil cephalus* se puede lograr con éxito a los 32 dph (10 mm de longitud total) solo con rotíferos administrados como alimento vivo.

9. La utilización de rotíferos como presa viva para las larvas de *Mugil cephalus* debe extenderse hasta los 32 dph (10 mm de longitud total) para mejorar la supervivencia y el perfil de ácidos grasos de las larvas.

10. Cuando se utiliza *Artemia* sp metanauplii enriquecida, se recomienda introducirla a partir del día 26-29 después de la eclosión (8-9 mm de longitud total) para mejorar el crecimiento y minimizar la dispersión de tamaño en las larvas de *Mugil cephalus*.

11. En juveniles de *Liza aurata* y *Chelon labrosus*, la división de la ración diaria en tres tomas en vez de en una condujo a una relación enzima:sustrato más eficiente y, por

lo tanto, a una mejor utilización del alimento, tal como muestran los ensayos *in vitro* con extractos de amilasa y proteasa alcalina.

12. Los resultados demostraron actividades de amilasa y proteasa alcalina más altas para *Chelon labrosus* que para *Liza aurata*, lo que determinó una mayor liberación de aminoácidos y azúcares reductores en los ensayos *in vitro*.

13. La especie *Liza aurata* presentó un mayor porcentaje de peso eviscerado y contenido lipídico en músculo que *Chelon labrosus*, bajo las mismas condiciones de cultivo.

14. Si bien *Chelon labrosus* y *Liza aurata* se consideran especies de nivel trófico cercano, presentan potencialidades fisiológicas y productivas diferentes cuyas especificidades deben ser consideradas para optimizar la rentabilidad y sostenibilidad de su producción.

15. Los juveniles de *Liza aurata* pueden utilizar eficientemente hasta un 6% del subproducto de *Aloe vera* en dietas, sin afectación de los parámetros de crecimiento o calidad.

16. La utilización de subproducto de *Aloe vera* en dietas para juveniles de *Liza aurata* promovió un mayor contenido de ARA en el músculo de los animales. No se observaron efectos sobre la salud ni sobre los parámetros productivos estudiados.

17. El crecimiento, la composición proximal y el perfil de ácidos grasos n3 LC-HUFA corporales de *Chelon labrosus* fueron similares cuando se suministró una fuente de precursores de HUFA en una dieta con aceite vegetal frente a una dieta con aceite de pescado.

18. Se encontró una interacción ($p \leq 0.01$) entre dieta y salinidad. Los niveles de DHA y n3 LC-HUFA en músculo fueron similares entre de los juveniles de *Chelon labrosus* alimentados con una dieta con aceite de lino y cultivados en agua salobre a los. alimentados con una dieta con aceite de pescado en todas las salinidades en estudio.

19. El nivel más alto de expresión hepática del gen *Elovl5* en juveniles de *Chelon labrosus* se obtuvo en agua salobre (16 ppt de salinidad).

20. Las tres especies estudiadas durante esta tesis doctoral han mostrado diferentes potencialidades en aspectos como la reproducción y la calidad, siendo grandes candidatas para la diversificación de la acuicultura sostenible.

En general, un enfoque integrado que adapte el conocimiento global a las especificidades locales, las condiciones técnicas y los objetivos de producción, ha demostrado ser un buen ejercicio para promover el desarrollo exitoso del cultivo de las especies seleccionadas en el contexto del crecimiento esperado de la acuicultura de mugílidos.

6. Annex

6.1. International oral and poster presentations of the PhD project

-Poster presentation (presenting author) at the International Symposium on Fish Nutrition and Feeding, 2018 with the work: "*Aloe Vera* product and by-product for aquaculture feeds: preliminary study on mugilids (*Liza aurata*)".

-Poster presentation (presenting author) at the RETI 2019 Symposium, 2019 with the work: "*Aloe vera* by-products as an example to promote circularity in islands".

-Oral presentation (presenting author) at the 2020 European Aquaculture Congress: "Evaluation of growth, biochemistry, immunological status, and activity of digestive enzymes in two mullet's species (*Liza aurata* and *Chelon labrosus*), feed at two different feeding frequencies".

-Poster presentation (presenting author) at the European Aquaculture Congress 2021 (Funchal, Madeira): "First attempts of ontogeny development in the golden mullet (*Liza aurata*)".

-Poster presentation (presenting author) at the European Aquaculture Congress 2022 (Rimini, Italy): "Comparative acclimatization and reproductive management of two mullet species *Chelon labrosus* and *Liza aurata*".

6.2. National oral and poster presentations of the PhD project

-Poster presentation (presenting author) at the "Congreso Nacional de Acuicultura 2021", Cartagena (Spain): "*Aloe vera* y subproducto de *Aloe vera* en piensos para lisa dorada (*Liza aurata*)". Organizing entity: Sociedad Española de Acuicultura.

-Oral presentation (presenting author) at the "Congreso Nacional de Acuicultura 2022", Cádiz (Spain): " El potencial de una especie de bajo nivel trófico (*Chelon labrosus*) para el establecimiento de dietas sin aceite de pescado en acuicultura marina ". Organizing entity: Sociedad Española de Acuicultura.

6.3. Collaborative works (other activities, co-authorships, teaching)

-Venia docendi 2018/2019 of 15 hours in the subject "Marine Living Resources" of the degree of Marine Sciences (University of Las Palmas de Gran Canaria).

-Venia docendi 2018/2019 of 24 hours in the subject "Aquaculture" of the degree of Veterinary Sciencies (University of Las Palmas de Gran Canaria).

-Venia docendi 2019/2020 of 9 hours in the subject "Marine Biotechnology and Aquaculture" of the degree of Marine Sciences (University of Las Palmas de Gran Canaria).

-Venia docendi 2019/2020 of 14 hours in the subject "Marine Living Resources" of the degree of Marine Sciences (University of Las Palmas de Gran Canaria).

-Venia docendi 2021/2022 of 5 hours in the subject "Marine Biotechnology and Aquaculture" of the degree of Marine Sciences (University of Las Palmas de Gran Canaria).

-Venia docendi 2021/2022 of 3 hours in the subject "Aquaculture" of the degree of Veterinary Sciences (University of Las Palmas de Gran Canaria).

-Oral presentation (presenting author) in the 46th Larvae Conference at Lisboa, Portugal, 2023: "Evaluation of different taurine levels on the weaning of the greater amberjack (*Seriola dumerili*)".

-Participation as a co-author at the 2019 European Aquaculture Congress (Berlin, Germany), in the oral presentation.: "Novel banana by-products in seabass *Dicentrarchus labrax* diets".

-Participation as a co-author at the 2019 European Aquaculture Congress (Funchal, Madeira), in the poster presentation: "In vivo bioactivity test towards bioprospection in aquaculture ingredients and additives".

- Participation as a co-author in the publication "Determination of heavy metals from *Aloe vera* by- product in golden mullet (*Liza aurata)*; A consumer health risk assessment", 2022, Food and Chemical Toxicology.

- Participation as a co-author in the publication "Assessing the growth and physiological performance of juvenile tilapia (*Oreochromis niloticus*) with the inclusion of new banana by-products in starter diets", 2023, Aquaculture Reports.

-Participation as a co-author in the chapter "Start Circular" Erasmus+. Diseñando la docencia universitaria europea en economía circular. Objetivos y primeros resultados. in "Avances en Educación Superior e Investigación". Vol. 2, 2021.

- Participation as a co-author in the XIX International forum on evaluation of the quality of research and higher education (FECIES), with the oral presentation "Competencias para la sostenibilidad y aprendizaje basado en problemas. La percepción del alumno", 2022.

- Participation as a co-author in the XIX International forum on evaluation of the quality of research and higher education (FECIES), with the oral presentation "Start circular" Erasmus+. Diseñando la docencia universitaria europea en economía circular. Objetivos y primeros resultados", 2022.

6.4. Scholarships during the PhD

-Predoctoral grant from the University of Las Palmas de Gran Canaria (01/09/2018-31/08/2022).

-Erasmus + scholarship for traveling and maintenance expenses of a 3-month internship at the International Marine Center in Oristano, Italy (14/09/2020 to 14/12/2020).

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