Universidad del País Vasco / Euskal Herriko Unibertsitatea
eman ta zabal zazu

ZIENTZIA ETA TEKNOLOGIA FAKULTATEA
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Biologiako Gradua / Grado en Biología

# Differential growth and health situation of thicklip grey mullets (*Chelon labrosus*) fed on different diets

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Leioa, 2019ko ekainaren 21a /Leioa, 21 de junio de 2019

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## 1. Abstract

Aquaculture produces fish organisms for food, aquarium or scientific purposes, whose one of the major challenges have always been feed costs. *Chelon labrosus* has been proposed as an aquacultural species in the Basque Country. They may be promising due to their extensive and abundant distribution, omnivorous (and increasingly herbivorous) diet, eurythermal and euryhaline nature and gastronomical value. The AKURA project aims to design process for their farming, including a proper diet that avoids less sustainable and more expensive compounds like fishmeal. In this study, *C. labrosus* individuals were exposed to 4 different diets in order to determine the biometrical and histological effects of the different nutritional compositions, for which some indices were also calculated. This was related to the health condition of mullets. Feed containing a high amount of protein and lipids resulted in the highest growth rates for both length and weight. Fish fed on the lowest amount of protein had the lowest growth rates but presented the highest efficiency in utilising protein. High dietary carbohydrate concentrations were related to bigger livers and higher vacuolisation levels. Therefore, it was suggested that plant-based carbohydrates could be a suitable compound of feed for *C. labrosus*, but only if proteins were to be added. Good quality fats would also be necessary for being the principal energy source of fishes. Other methods like the biofloc system were proposed as well.

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Akuakulturak oganismo itsastarrak ekoizten ditu elikagaigintza, akuario edo asmo zientifikoetarako, eta haren erronka nagusietako bat betidanik izan da pentsuen kostu altua. Euskal Herrian *Chelon labrosus* akuikulturarako espezie bezala sartzea proposatu da. Etorkizun oparoa izan dezakete, besteak beste, haien banaketa zabala eta ugaria, dieta-eredu omniboroa (eta gero eta herbiboroagoa), hedapen euritermiko eta eurihalinoa eta balio gastronomikoa direla medio. AKURA proiektuak haien hazkuntzarako prozesu bat diseinatzea du helburu, horren barne arrain-irina bezala osagai ez hain sostengarri eta garestiak saihesten dituen dieta bat egonez. Ikerketa honetan, *C. labrosus*en banakoak 4 dieta ezberdinen bidez elikatu eta haietako bakoitzaren konposaketa nutrizionalak zituen eragin biometriko eta histologikoak aztertu ziren, horretarako zenbait indize ere kalkulatuz. Hori korrokoien osasun-egoerarekin erlazionatu zen. Proteinen eta lipidoen portzentai handiak zeuzkan pentsuari egokitu zitzaizkion luzera zein pisurako hazkunde-tasa altuenak. Proteina-kopuru txikiena ingeritu zuten arrainek hazkunde-tasa baxuenak aurkeztu zituzten, baina efizienteenak izan ziren proteina erabiltzeko orduan. Karbohidratoen kontzentrazio altuak gibelen tamainak eta haien bakuolizazio-maila areagotu zituen. Ondorioz, jatorri begetaleko karbohidratoak *C. labrosus*entzako pentsuaren konposatu egokia izan daitezkeela iradoki da, betiere proteinak gehitzen

badira. Kalitate oneko gantzak beharrezkoak izango lirateke ere arrainen energia-iturri nagusia izateagatik. Biofloc sistema bezalako beste metodo batzuk ere proposatu ziren.

## 2. Introduction

Aquaculture is a growing way of producing aquatic organisms for food, aquarium or scientific purposes that has risen from providing just 28% in 1995 to over 50% worldwide. One of the most cultured organisms is fsh for human consumption. From the 80 million tonnes of food fish in was farmed in 2016, 54.1 million belonged to finfish, and it is expected that it increases in the next years, as the consumption of seafood is increasing and the amount of captured fish declining (Hixson, 2014). The success of aquaculture is primarily due to the possibility of controlling production and predicting supply and the obtainment of more stable prices. However, it also faces a number of challenges, such as increasing regulatory and voluntary import requirements and feed costs and sustainability. Fish feed is the most expensive input, which often accounts for 60-70% of total expenditures (FAO, 2018). Though fishmeal and oil account for the 30-50% of the carnivorous diet, they are not indispensable, but provide fish with the essential nutrients they need to be healthy and grow. Most of the times, this is made from harvested small, pelagic fish -anchovies, sardines, herring, mackerel, etc.-, as their short cycles give them the capability to reproduce and restore their populations rapidly; the other 30%, diversely, is manufactured from the scraps of fish that are processed for human consumption. In spite of the fact that aquaculture is a net producer of fish protein, it still requires half a metric ton of wild for each ton of farmed seafood (NOAA, 2019).

Fishes use energy from food much more efficiently than other farmed animals like cows and pigs (NOAA, 2019). This is meant to consider aquaculture as a more sustainable alternative of producing more protein for human consumption, while its environmental impact could still dramatically be reduced if fish feed was composed of more plant-based proteins. This could be achieved by a broader farming of herbivorous or omnivorous species. Herbivorous species are typically given a feed based on plant proteins, vegetable oils, minerals, and vitamins, which are obtained both more sustainably and at lower costs than fish oils and proteins that are normally destined for carnivores. A fish family that could potentially be adapted to this kind of compounds would be that formed by mullets. These fish species have an omnivorous diet, and, what is more, they have a tendency to become herbivorous over time, since juveniles only feed on zooplankton and adults feed mostly on benthic diatoms, epiphytic algae, small invertebrates and detritus (De las Heras *et al.*, 2012; FishBase, 2019). Furthermore, their euryhaline and eurythermal nature allows to culture them in either fresh, brackish or marine water and in broad temperature ranges (Liao, 1981). As for their gastronomical value, grey mullets' meat and roes are highly valued in the food industry. Roes are traditional product since 3,000 years ago, and they are sold after being salted and dried, and their cost can be as high as 230 €/kg (Vallainc *et al.,* 2017). This is gastronomically known as "bottarga" in Italy, "avgotaracho" in Greece and ''karasumi" in Japan, an international market which is nowadays expanding around the Mediterranean Sea (Rosa, 2018). Thus, they come to be very suitable for aquaculture due to both their ecological and economical value. Wild mullets are captured by fisheries to commercialise them in the food industry using gill nets, seines and hooks, or in some regions of the Mediterranean and the Black Sea, principally in extensive ponds or confined coastal lagoons (Turan, 2015). However, no specific feed for this species group has been commercialised yet, and little is known about their life cycle in captivity. These fish species are extensively and traditionally cultured in the Iberian Peninsula by capturing wild juvenile fishes, mostly in the southeast coast, though their production is very scarce (De las Heras *et al.*, 2012).

The fish family Mugilidae comprises approximately 70 mullet species, which inhabit coastal and brackish waters of all tropical and temperate regions of the world (Hett *et al.*, 2011). Mullets have a specially uniform external morphology and a scarcely less so internal anatomy. Hence, the number of scales, gill rakers, fin spines, fin rays and measurements of body proportions are usually used to identify them (González-Castro & Ghasemzadeh, 2015). They have a V-shape stomach and a closely coiled intestine. Their gizzard helps mechanic digestion, and mineral particles ingested with plant detritus and microalgae act as grinding paste (Olsen & Ringø, 1997).

*Chelon labrosus* (Risso, 1927) is the species that has been examined in this research. This mullet species can reach up to 70 cm long (females are larger than males). It possesses an elongated body, which widens at the middle lacking a lateral line, and a characteristically thick upper lip, with numerous papillae. It is grey-coloured, darker at the dorsal side and white at the ventral side. It has two dorsal fins, the first one with 4 spiny rays, and the second one with only one spiny and 8 soft rays. Its caudal fin has 3 spiny rays and 8-11 soft rays (Ictioterm, 2019) (Fig. 1).

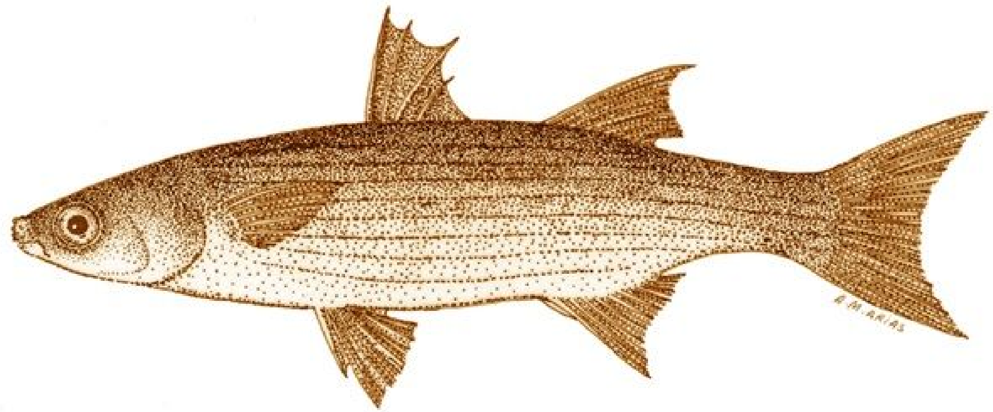

**Figure 1.** Drawing of a thicklip grey mullet (*Chelon labrosus*) individual (Ictioterm, 2019).

*C. labrosus* is widely distributed inshore in schools, often entering brackish lagoons and freshwater. It can be found in the Mediterranean Sea, South-western Black Sea, the British Isles, North Sea, Barents Sea, Baltic Sea, Bay of Biscay, the Canary Islands, Azores, Madeira and coasts of West Africa (Turan, 2015) (Fig. 2). It has amphidromic life cycles, migrating to estuarine areas for growth and feeding, and adults returning to the sea for reproduction. This is characterised by some periods of starving and recovering after feeding (De las Heras *et al.*, 2012).

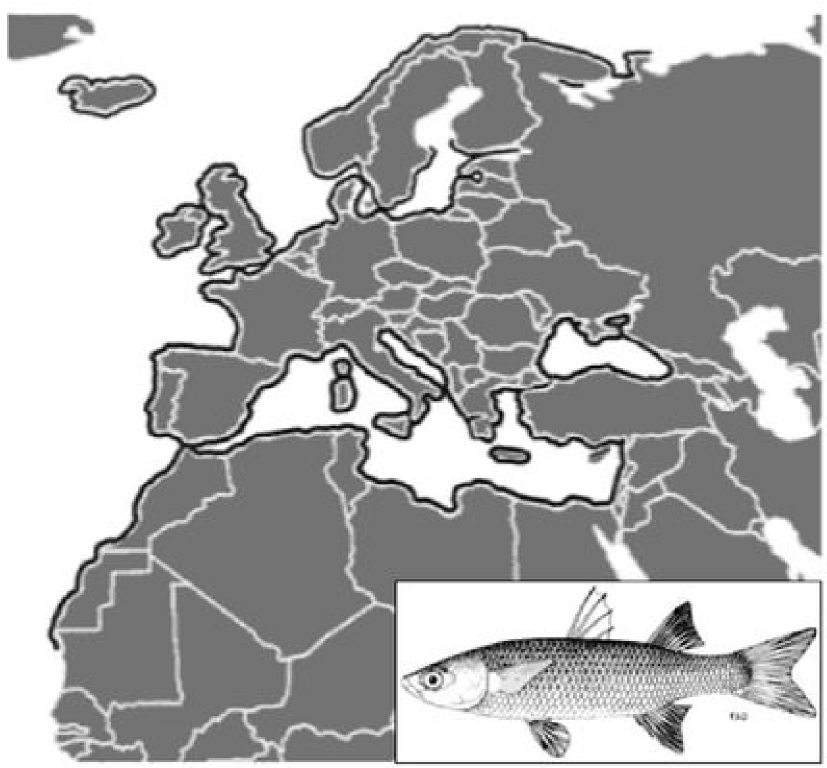

**Figure 2.** Distribution of *Chelon labrosus*, indicated by thick black lines (Turan, 2015).

Aiming both a sustainable farming and a good-quality nutritional value of *C. labrosus*, the Department of Economic Development and Infrastructures of the Basque Government has financed the AKURA project, with the collaboration of the Technological Centre GAIKER-IK4, the Kardala Aquaculture School and the University of the Basque Country (UPV/EHU). During the 36 months (2017-2019) it lasts for, a process for their farming is to be designed, based on their biology, developing and applying diets composed of microalgae and improving the Recirculating Aquaculture Systems (RAS), hence developing a method and a product pioneers in aquaculture (Gaiker, 2019).

In this framework, the objective of present study was to assess the growth and histological changes in juvenile *C. labrosus* when exposed to different diets. It was proposed that mullets fed on diets with different nutritional compositions have diverse growth rates and organ and tissue development, affecting their general condition. In order to test this hypothesis, juvenile mullets were separated into groups and fed on different diets. These feeds were selected so that each of them contained very different proportions of carbohydrates, proteins and lipids. Commercial feeds for both carnivorous and herbivorous fishes were used to check whether they had positive effects on the growth of mullets. Two of the groups were fed on a diet typically employed for carnivorous fishes like sole, sea bream and sea bass (high in protein and fat). A third group was fed on a commercial feed for tilapia (herbivorous), made of plant-based ingredients. This would allow to identify whether the overall growth performance of juvenile mullets is further favoured by a carnivorous or a herbivorous diet. Additionally, a fourth group was fed on bread as a way of basing the obtainment of feed on a circular economic system. This is expected to be a suitable carbohydrate source, as mullets have traditionally been fished using bread for bait.

In order to achieve the above mentioned objectives, weights and lengths of the fish were recorded at different time periods for growth assessment, and length and weight gain percentages, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated with this data, which are typically used to assess how growth is with a given feed (Yong *et al.*, 2015). Health and nutritional conditions were examined by Fulton's condition factor (K) and the histological analysis of liver, spleen and intestines (Raskovic *et al.*, 2011). Weight of livers and viscera were also recorded and the hepatosomatic index (HSI) and viscerosomatic index (VSI) calculated to further contribute to this evaluation.

## 3. Materials and methods

### 3.1. Collection of specimens

A total of 124 juvenile grey mullets (*Chelon labrosus*) used in this study were caught in August in the Butron Estuary in Plentzia, Biscay (43º40’17.33’’N 2º94’41.66’’W) (Fig. 3), under the permission from the Department of Agriculture, Fisheries and Food Policy, Basque Government.


**Figure 3.** Butron Estuary. The red circle indicates the place where samples were caught (GeoEuskadi, 2019).

### 3.2. Maintenance of the specimens

The fish were transported to the Research Center for Experimental Marine Biology and Biotechnology (PiE-UPV/EHU). Until the beginning of the study, they were kept in a 250 L tank fed by hand on around 4.5 g of grinded MAR-Perla MP-M feed under a continuous water flow, for acclimation. The weight of the feed was determined by 1.5% of the average weight of 15 random fish (1.491 g x 0.015= 0.022 g/fish). Water temperature was 17.5ºC. Feed weight was considered to be scarce, so it was doubled to 9 g two weeks later, when the fish were already used to eating it.

### 3.3. Experimental set-up

The study was held in the PiE-UPV/EHU. Room temperature was at 18ºC before and 17ºC during the study. In November, 31 fish were moved to each of the 4 tanks used for the study.

The fish were kept under a continuous water flow of 150-200 L/hour (i.e. a rate of water renewal of about 7-10 renovations/day) in 4 different water tanks of 35 L. Water quality was monitored daily for temperature (T), salinity (S), pH and dissolved oxygen (DO). These parameters were maintained in T= 15-17.5ºC, S= 31.2-34‰, pH= 7.8-7.9 and DO= 90-98% during the whole experiment. A 12/12 photoperiod was maintained during the whole study. Transparent covers were placed atop of the tanks and held with pegs for the maintenance of a correct photoperiod.

The study began the 16th of November. Fish in each tank were fed by hand each on a different diet (Table 1). All feeds were grinded and their weights were defined at 5% of the mean weights of the fish in each of the tanks. Water in each of the 4 tanks was siphoned everyday to remove the organic matter deposited from feces and surplus feed.

**Table 1.** Composition of the 4 different feeds that *C. labrosus* individuals were exposed to.

| TANK | DIET | Carbohydrates (%) | Proteins (%) | Lipids (%) |
|---|---|---|---|---|
| 1 | MAR-Perla MP-M *Skretting* S.A. | 27.8 | 56 | 15 |
| 2 | Bread | 51 | 7.9 | 1.1 |
| 3 | TI-3 *Tilapia* (3.2 mm) *Skretting* S.A. | 58.1 | 32 | 6 |
| 4 | GEMMA 0.3 *Skretting* S.A. | 25 | 58 | 17 |

All experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD53/2013 and law 32/2007) for the handling and use of laboratory animals under the supervision and acceptance of the Ethics for experimentation and animal welfare committee of the University of the Basque Country and provincial authorities (CEEA M20-2018-133- Diputación de Bizkaia).

### 3.4. Biometric/physiological measurements

Biometric data was taken at the beginning of the study (t0) and after 1 month (t1), 3 months (t2) and 5 months (t3). All fish were anesthetised using 300 µL/L of tricaine mesylate (MS-222) to obtain their fork length (FL) and weight. Feed weight was again adapted to the new weight of the fish after each measurement time, maintaining feed percentage at 5% relative to the total weight of fish.

Some indices were calculated using the data obtained in the four time periods. To determine how much had the fish grown in length and weight, the following formulas were used:

Length gain (%)= $\frac{Final\ length - Initial\ length}{Initial\ length}$ x 100

Weight gain (%)= $\frac{Final\ weight - Initial\ weight}{Initial\ weight}$ x 100

The specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) of the fish were calculated using the following formulae. The SGR determines how much weight a fish gains in a day. The FCR indicates which proportion of the feed given is converted into biomass, and the PER relates the increase in biomass with the protein consumed (Yong *et al.*, 2015):

SGR (%growth/day)= $\frac{Ln\ Final\ weight - Ln\ Initial\ weight}{Days\ of\ experiment}$ x 100

FCR= $\frac{Feed\ consumed\ (g)}{Wet\ weight\ gained\ of\ fish\ (g)}$

PER= $\frac{Wet\ weight\ gained\ of\ fish\ (g)}{Protein\ intake\ (g)}$

Fulton's condition factor (K) was calculated to determine the general condition under which the fish were growing in terms of feed quality, water quality and diseases in the culture system, using this formula (Jin *et al.*, 2015):

K= $\frac{W}{FL^3}$ x100, where W is the body weight (g) and FL the fork length (cm).

Total visceral weights and liver weights were taken in order to calculate the viscerosomatic index (VSI) and the hepatosomatic index (HSI), which were used as a measure of the energy reserves of the fish (Ighwela *et al.*, 2014; Yong *et al.*, 2015):

HSI (%)= $\frac{Liver\ weight\ (g)}{Body\ weight\ (g)}$ x 100

VSI (%)= $\frac{Visceral\ weight\ (g)}{Body\ weight\ (g)}$ x 100

### 3.5. Histological analysis

In t2, 10 mullets from each tank were dissected for histological analysis. Samples were extracted from their digestive tract, liver and spleen. The weight of the liver and the total visceral weight of each fish were taken. A part of the liver and muscle was kept in cryovials and deep-frozen in liquid nitrogen and stored in a freezer at -80ºC for analytical chemistry. The fixed samples were left in formalin. One piece from each tissue was put in labelled cassettes and immediately fixed in neutral buffered 4% formalin. 24 h later, they were moved into 70º ethanol until their processing.

The tissue samples were passed through graded ethanol, cleared with xylene and impregnated using an automated tissue processor Leica ASP 300S (Leica Microsystems Nussloch GmbH, Germany) for their processing. Samples were embedded in paraffin wax with arrangements to obtain both sagittal and transversal sections. Sections of 5 μm thick were cut with a Leica RM2125RTS microtome. They were stained with hematoxylin and eosin (H&E) using an Autostainer XL (Leica Microsystems), mounted with DPX and air-dried. The slides were examined using a Nikon Eclipse E200 microscope.

### 3.6. Statistical analyses

Data was mathematically analysed with the statistical computer programme SPSS (IBM, 2017) to calculate the significance of differences between samples by one-way ANOVA and Tukey's range test as a posthoc in order to establish differences between experimental groups. A 95% significance level ($p < 0.05$) was established for all statistical analyses carried out.

## 4. Results

During the experiment there was no mortality in any of the 4 tanks, except for 2 fish from tank 2 that jumped out. In general terms, the fish adapted properly to every diet. Recorded water temperature, dissolved oxygen, salinity and pH were all within established ranges for fish culture. Morphological and anatomical differences were found between the fish, depending on their diet.

### 4.1. Growth performance

#### 4.1.1. Length, weight and feed efficiency

Significant differences were found over time between the weight and length of fish fed on different diets. Growth percentages were remarkably different for both length and weight among fish from the different groups. Fish fed on diet 4 grew the most in length and weight, gaining on average 3.115 cm in length and 8.629 g in weight. On the contrary, fish fed on diet 2 had the smallest increase in both length and weight (Fig. 4), gaining on average just 0.780 cm and 2.015 g. Fish having diets 1 and 3 had a mean increase of 2.876 cm and 2.070 cm in length and 7.885 g and 5.464 g in weight, respectively.

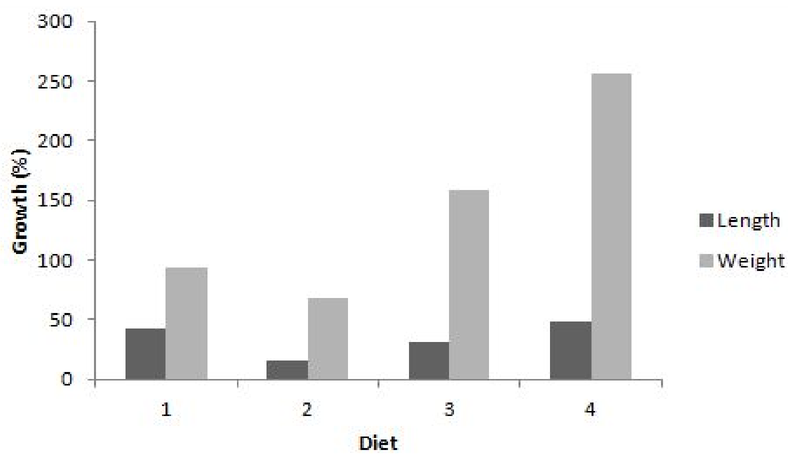


**Figure 4.** Length and weight gained by fish from the 4 different diets during the 5 months of the study.

The highest SGR mean value belonged to mullets fed on diet, and the lowest those fed on diet 2. Fish maintained with diet 2 had notably the highest FCR, around 4 times higher than the others, with those fed on diets 1 and 4 fish having the lowest FCRs. Similar values of PER were obtained for animals under diets 1 and 4. These values were around 0.3 higher than in those mullets fed on diets 2 and 3. Fish under diet 2 showed notably the highest PER (Table 2).

**Table 2.** Mean values of initial and final lengths and weights, specific growth rates (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) for fish of each of the 4 diets.

| DIET | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| **Initial body weight (g)** | 3.635 ± 1.884 | 4.091 ± 1.637 | 3.454 ± 1.224 | 3.367 ± 1.507 |
| **Final body weight (g)** | 11.520 ± 3.430 | 6.105 ± 1.923 | 8.918 ± 2.992 | 11.996 ± 2.284 |
| **SGR (%/day)** | 0.703 | 0.244 | 0.578 | 0.775 |
| **FCR** | 2.484 | 11.135 | 3.069 | 2.320 |
| **PER** | 0.720 | 1.385 | 1.026 | 0.777 |

### 4.1.2. Fish condition index

According to the Fulton condition index, no significant differences were observed between the different dietary interventions over time. However, a slight increase is observed in all the experimental groups, which is stabilised over after $t_1$ for all the diets, except for 4 (Fig. 5).

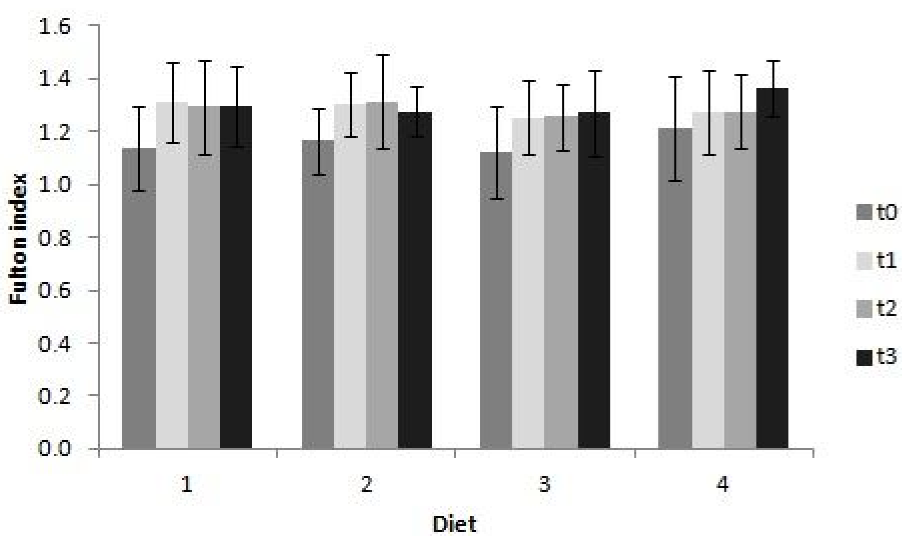


**Figure 5.** Fulton indices found for fish fed on the 4 different diets throughout time. Bars indicate the mean and intervals represent the standard deviation.

### 4.2. Histology

#### 4.2.1. Macroscopical observations

Big amounts of perivisceral fat was observed during the dissection in fish having diets 1 and 4 after 3 months. Fish fed on diet 2 had a remarkably white and soft liver, with a specially dark green-coloured bile. In fish having diet 3, bile was found to be yellow.

#### 4.2.2. Fish hepatosomatic and viscerosomatic indices

Significant differences were found for the HSI values between fish having diet 2 and fish having the rest of the diets. Maximum HSI values were found fish fed on diet 2, while fish under diet 1 showed the minimum mean value (Fig. 6).

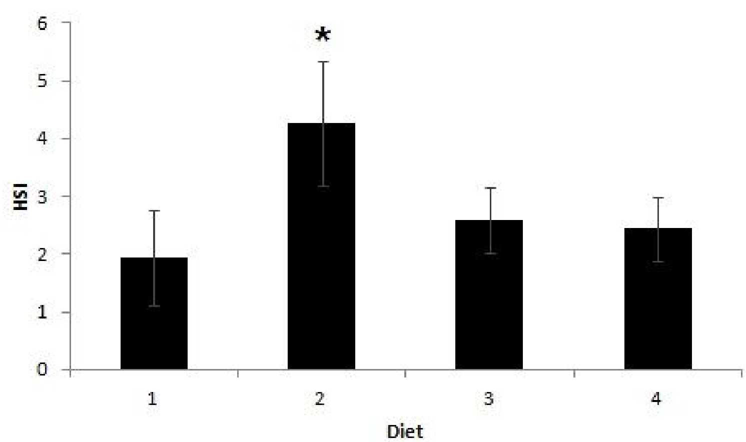


**Figure 6.** Hepatosomatic index (HSI) values for fish fed on the four different diets. The asterisk indicates significant differences between diet 2 and the rest of the experimental groups. Bars indicate the mean and intervals represent the standard deviation.

The same pattern was observed for the VSI, for which significant differences were found between the animals maintained with diet 2 and with the rest of the diets. Maximum values were again obtained for fish fed on diet 2 (Fig. 7).

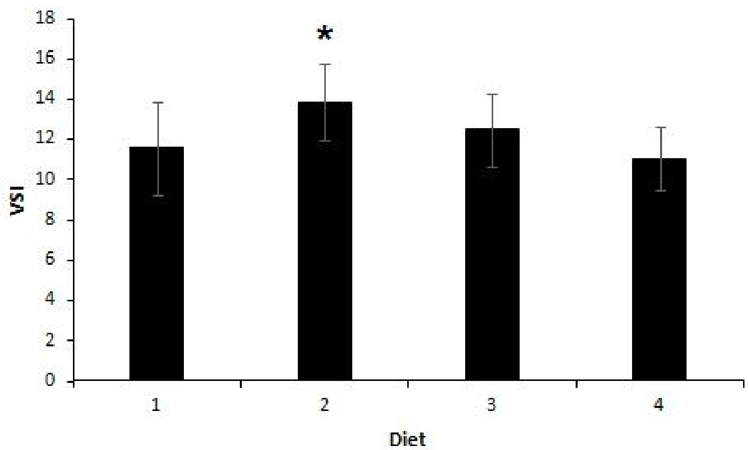


**Figure 7.** Viscerosomatic Index (VSI) values for fish fed on the four different diets. The asterisk indicates significant differences between diet 2 and the rest of the experimental groups. Bars indicate the mean and intervals represent the standard deviation.

### 4.2.3. Histopathological observations

The histological analysis of the intestines showed no remarkable differences between fish fed on the four different diets in the number of goblet cells  and structure of villi.

No histological differences were found between the abundance, size and pigmentation of the melanomacrophage centers (MMCs) in the spleens from fishes of the 4 tanks, except for an individual in tank 1, which had more notable and abundant melanocytes.

The histological analysis of the livers of fish fed with different diets showed diverse vacuolization levels. The livers of fish fed on diet 2 showed a higher degree of vacuolisation than the fish fed with the other diets, followed by diet 3 (tilapia). The vacuolisation was confirmed to be mostly lipidic after the histochemical demonstration of neutral lipid (data not shown). On the other hand, the lowest vacuolisation degree was observed in livers from fish fed on diet 1. Hepatic cords were well arranged in all the cases, nevertheless, sinusoidal space was lost in the liver of fish maintained with diets 2 and 3 due to the dilatation of the hepatocytes (Fig. 8).

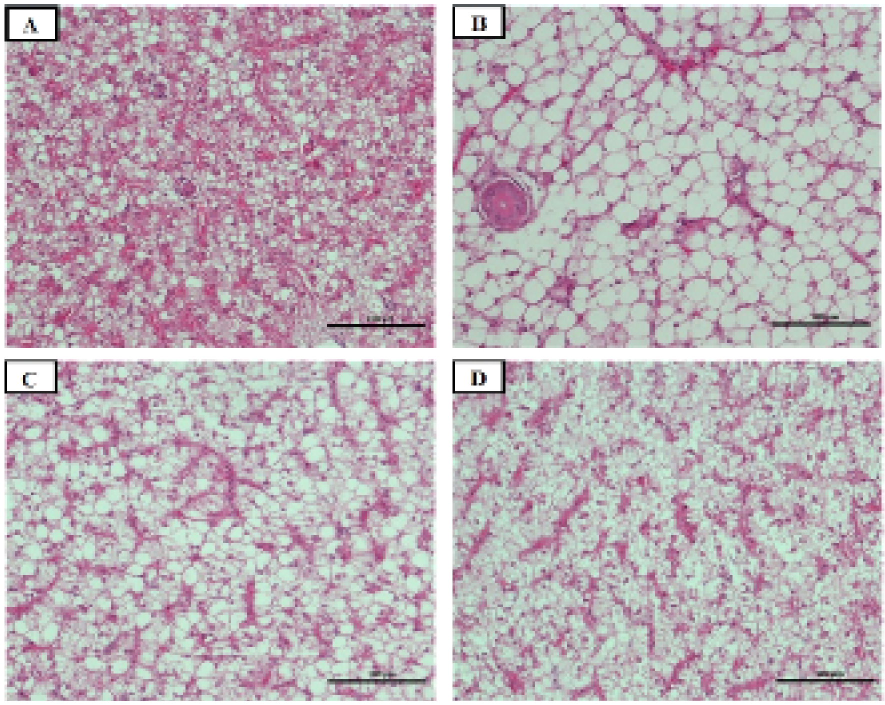


**Figure 8.** Hepatocytes from *C. labrosus* fed experimental diets. (A) Diet: MAR-Perla. Large and spherical nucleus centrally located in each small-diametre cell (H&E). (B) Diet: bread. High vacuolisation level, with nucleus displaced to the periphery of the cell (H&E). (C) Diet: TI-3 *Tilapia*. Notable vacuolisation, though not as high as in B (H&E). (D) Diet: GEMMA. Large and spherical nucleus and cells not as densely packed as in A (H&E).

## 5. Discussion

As a pioneer study on the farming of mullets in Basque Country aquaculture, we applied different diets in order to determine their effect in these animals' growth and health condition. Different conditions were established in terms of nutritional composition of feeds, including a more restrictive diet such as bread.

Grinded feeds came to be suitable to feed the mullets. Traditionally, feed granulometry is selected depending on mouth size (Mosig & Fallu, 2004). But these fish did not properly fed on feed pellets proportional to their mouth size, most likely due to the small size of their oesophagus. The original size of the tilapia feed (3.2 mm) was too big for the juvenile mullets used in this study, so all feeds were grinded in order to homogenise the sizes and correct the error that the difference in sizes could induce.

All fish fed on the 4 different diets grew in both length and weight. No mortality was recorded (apart from the two fish that jumped out of one of the tanks) during the study, and the immune system of fish was not altered, as the MMCs in the spleen have shown. These are good indicators of several factors like pathogens (Sales *et al.*, 2017), nutritional status (Wolke *et al.*, 1985) and general stress condition and tissue damage (Caballero *et al.*, 2004)., but juvenile individuals were used in this study, which have a lower probability of suffering from pathologies (Wolke *et al.*, 1985; Fournie *et al.*, 2001). Fulton's condition factor was over 1 in all fish regardless of the diet they were fed on and time periods, which means that fish were healthy and every feed supported their growth, reflecting a sufficient environmental and nutritional quality (Sogbesan *et al.*, 2017). Moreover, they had a tendency to have similar values over time. Differences in other parameters such as vacuolisation of the liver and the lipidic accumulation in the intestine indicate that their health can be affected by diet. In addition to this, as length and weight gain percentages have shown, there are differences in the proportional growth of fish between these two parameters, not detected by Fulton's condition factor either. Therefore, it can be observed that this index does not detect notable differences in either health condition or relative growth of length and weight, thus requiring the use of other parameters to determine a more precise situation of fish. Though an increase in length and weight was experienced by every fish, each diet resulted in differential growth.

Mullets fed on diet 1 (MAR-Perla feed) obtained the highest SGR in the first month, which could be explained by the fact that the same feed was used at the acclimation period. These animals had an elevated increase in length, as protein is the nutrient responsible for muscular growth, although diets 3 and 4 resulted in a higher increase. Hence it is clear that this increase in length depends on the dietary protein utilisation and the balance between proteins and non-protein energy (Altunok & Özden, 2017). As a consequence, the ingestion of an excessive amount of proteins can result in either protein catabolism to energy or a decline in growth (especially at protein levels up to 50% in juvenile mullets) because of higher energy requirements (Carvalho *et al.*, 2010; Yong *et al.*, 2015), which could have been the case of diet 1 fish. These results are confirmed by the observed FCR and PER values. Their lower FCR means that a smaller amount of diet 1 was necessary for the growth of these fish, or, in other, words, that they destined a higher proportion of the feed to the development of biomass. Their PER was the lowest one compared with the rest of the diets. Several studies performed with different fish species (Sweilum, 2005; Siddiqui & Khan, 2009; Amoah, 2012; Altunok & Özden, 2017) have reported that the PER decreases with increased dietary protein level, in agreement with the values observed for fish under diet 1 (56% protein). On the other hand, the HSI was the lowest one for these fish, which may be related to the fact that diet 1 had the lowest carbohydrate content.

As expected, fish fed on diet 2 (bread) had the lowest growth rate since it was the diet containing the lowest amount of protein. It is known that young individuals need a higher amount of protein for the development of their muscle tissue, growing in measure and weight (Soderberg, 2017). On the contrary, the use of carbohydrates in fishes is very limited and they are rather used for reducing feed costs and for their binding activity during feed manufacturing (Craig & Helfrich, 2002). The presence of less lipid content in the muscle can be due to the fact that fishes digest lower proportions of carbohydrates, thus gaining less weight (Gümüs & Ikiz, 2009). The FCR value was remarkably the highest among the 4 tested diets, which means that much of the food was not converted into biomass. According to (Zhou *et al.*, 2013), high dietary carbohydrate intake caused a lower growth rate and a higher FCR in Wucham bream. The reason for being able to grow up from an extremely low protein intake may be explained by their high PER value. This means that they may have directed a larger proportion of the ingested proteins towards muscular growth, making a more effective use of the scarce amount of proteins ingested. Accordingly, bread might be a good base for the production of a specific feed for mullets, but more protein would be necessary, which would successfully be utilised. Their HSI was the highest one, since this index increases together with the carbohydrate intake, as reported by Ahmad *et al.* (2012) in the common carp, Gümüs & Ikiz (2009) in rainbow trout and Ighwela *et al.* (2014) in Nile tilapia. Excess hepatic carbohydrate is either converted to glycogen or fat (Zhou *et al.*, 2013). In the case of the mullets fed on bread, lipogenesis seems to be the process happening in the liver, as the accumulation of fat in this organ demonstrated (Duncan, 2019). This means that the digested carbohydrates were metabolised to fat in the liver. The VSI was also the highest for fish fed on diet 2, but this was due to the weight of the liver. In fact, VSI without livers showed a similar value for every fish, regardless of their diet. These two indices therefore indicate that most lipids were stored as hepatic and perivisceral fat. The histological analysis of the liver showed a very high level of hepatocytes vacuolisation. Those vacuoles were identified as lipid droplets after the confirmation through the demonstration of neutral lipids (Duncan, 2019). The accumulation of triglycerides and other neutral lipids could be signal of an inadequate food (Raskovic *et al.*, 2011) and is known as hepatic steatosis (Turola *et al.*, 2015).

Mullets fed on diet 3 (tilapia feed) had a big increase in weight, but low in length. In fact, this feed was greatly based on carbohydrates, which, as aforementioned, seem to be deposed as fat. Their low increase in length may also be related with the high carbohydrate amount, but higher protein quantities in the diet would allow a higher tissular growth when compared to bread-fed fish. Hence, the length-weight balance was more disproportionated in fish fed on diet 3 than in those fed on diet 2. The HSI was not particularly high, in contrast to the VSI. Liver vacuolisation also occurred but it was not as abundant as in fish under diet 2. In spite of it, some studies have reported that lipids are deposited in a lower extent with carbohydrate-rich diets when compared to lipid-rich ones (Hutchins *et al.*, 1998; Shapawi *et al.*, 2011). This would explain why mullets under diet 3 gained less weight than those in tank 4. With a 30% protein diet, Altunok & Özden (2017) found that the FCR was of 1.61 in juvenile *C. labrosus*, while in this study an almost threefold higher value was obtained. This can be explained by the fact that the individuals used by these authors were farmed at a higher temperature, which increases feed intake of mullets (Durand & Whitfield, 2015) and growth rate (Handeland *et al.*, 2008). The powdered state of the feed and the use of the amount of feed given for the calculations (instead of the actual amount of feed eaten) might as well have caused a deviation from the real FCR value. Therefore, these factors should be taken into account for future studies. According to the PER value, the assimilation of protein was higher in fish fed in diet 3 than in those fed on diets 1 and 4, most likely due to a smaller proportion of proteins found in the tilapia diet.

Mullets fed on diet 4 (GEMMA feed) presented the highest growth rates in both weight and length. Though this was not the diet containing the highest protein amounts, the presence of lipids may enhance the effectiveness of nutrients related with muscle development, as lower FCR values have shown. This was confirmed by Hixson (2014), who stated that the use of highly digestible nutrient-density feeds employed in commercial fish farming and containing 46 to 50% protein and 20 to 24% fat improved the effectiveness of protein assimilation for growth; this would result in a FCR value of about 0.9-1.2 for rainbow trout. This effect was also noted by Lee *et al.* (2002), who saw a more effective protein utilisation and weight gain in the Korean rockfish when they were fed with 42% protein and 14% lipid instead of 49% protein and 7% lipid. The mean SGR of fish fed on diet 4 was especially high in comparison with the other ones. This may be due to the fact that this was the feed containing the highest lipid content. Moreover, a high amount of perivisceral fat was observed when dissecting these mullets. This might happen because lipids were directly used as storage and they possess about twice the energy density of proteins and carbohydrates (Craig & Helfrich, 2002). The proximal part of the intestine seems to be the main area involved in lipid absorption in fish (Olsen & Ringø, 1997), and hence overaccumulation of fat in and around the enterocytes of the intestine's villi could avoid the absorption of nutrients. As a result, excess lipid ingestion causes not only a lower rate of muscular growth, but also an inefficient use of nutrients. Additionally, the lack of lipases may have resulted in a large proportion of the feed not being digested and being expelled as fecal matter, as stated by Duncan (2019), who employed tilapia and trout feed. The trout feed, which, as the MAR-Perla and GEMMA contains high levels of protein, and also elevated concentrations of fat, caused a remarkably higher deposition of neutral lipids in the intestinal epithelium and lamina propia, reducing both nutrient transport and growth rate. The low PER value of fish fed on diet 4 can be related with their high dietary protein intake, as aforementioned.

Consequently, a feed composed of bread would be a suitable alternative for aquaculture, but only if a proper amount protein source is added, especially during the early stages, in which animals mostly convert proteins into biomass. As observed by Carvalho *et al.* (2010), the optimum protein would be 35% for juvenile mullets. Lipids, especially good quality omega 3 fatty acids, would also be necessary for fishes since they are their main energy source, but in low proportions, because they may affect negatively their body composition, nutrient digestibility and diet costs (Gümüs & Ikiz, 2009). In addition, they may also affect the health of the animals due to an excessive fat deposition in the liver. In fact, fat is poorly catabolised in the liver, and, as fish are less active in captivity, spending less energy than in nature and therefore tending to store these lipids. This would lower growth, feed utilisation and the commercial value of the final product (Yong *et al.*, 2015). Regarding protein, it could be acquired in several different ways in order to leave fishmeal aside. One of the possible options would be the use of larvae of insects. Larvae of the black soldier fly (*Hermetia illucens*) are already being used, contain almost 50% protein and are characterised by their efficiency to recycle wastes. After being cleaned, dried and ground into powder, they are mixed with other ingredients like the aquatic fern *Azolla* -which adds essential amino acids, vitamins and growth promoter intermediaries, amongst others- and manufactured as pellets for fish (Bhaskaran & Kannapan, 2015; FAO, 2018). Fishmeal might also be replaced at least partially by algae, which might provide fish with the necessary proteins and/or omega 3 fatty acids, apart from antioxidants (Algae Tech Conference, 2018). Another possible alternative would be the use of bioflocs. The biofloc system is based on the microbial transformation of feces and wasted feed into less complex organic products, which would be consumed by fish, returning to the food chain this way. Thus, it would be a good manner of improving the FCR and reducing the maintenance costs, as it acts like a retention trap for the nutrients in the pond (Castro-Nieto *et al.*, 2012).

As stated by Altunok & Özden (2017), an increase in the dietary energy level at lower dietary protein levels improves the efficiency of protein utilization and retention. Additionally, lipids are known to slow down all nutrients' passage through the gastrointestinal tract, giving the enzymes more time for hydrolysis (Kroghdal *et al.,* 2005), which in sufficiently small quantities could facilitate the utilisation of dietary carbohydrates, as reported by Lee & Lee (2004) in starry flounders and Tan *et al.* (2009) in gibel carps. Lee & Lee (2004) concluded that starch did not impede growth when dietary lipid was replaced by starch with equal energy, while Tan *et al.* (2009) found that the presence of appropriate dietary cellulose

(6–10%) can stimulate the movement of the intestine, improving the absorption of nutrients in the intestine. Due to the fact that grey mullets are omnivores, they need protein in proportionally lower quantities (Craig & Helfrich, 2002), thus cheapening their feed and making them a good choice as fishes for aquaculture. Besides, though carbohydrates are not fishes' main energy source, they serve to fasten growth, as they avoid the catabolism of proteins and/or lipids to provide energy (Leung & Woo, 2012). This would not only lower economic investment, but it would also be more sustainable, as lower quantities of protein would be required for the same growth rate, thus diminishing the amount of feed needed. The inclusion of polysaccharides have boosted feed utilisation and growth in omnivorous species such as tilapia (Lin & Shiau, 1995) and Chinese longsnout carp (Tan *et al.*, 2009). Anyway, further studies should be conducted to assess whether adult mullets can more efficiently use plant-based feeds, as expected.

In conclusion, every feed used in this study resulted in growth of fish, even bread, and *C. labrosus* is a suitable species for aquaculture. Mullets fed on commercial feeds had a higher increase, especially in length, but accumulated bigger amounts of fat. Protein feeds have more positive effects on growth, and this necessity for proteins is more notable because juvenile individuals were used. But a feed based on carbohydrates is effective in *C. labrosus*, since they are increasingly herbivorous with age and have less lipases than other fish species. The use of bread could be sustainable material as a base of the generation of new specific feed for mullets and is towards circular economy. Bread could actually be acquired from surplus bread that has not been sold for human consumption, thus being an ideal way of reducing food waste. Nevertheless, large proportions of protein and a small fatty acid addition would be vital for their contribution in growth. This diet would give them a high value in the gastronomical industry, since remarkably less fat would be deposited in fish fillets, one of the problems that farmed fishes present nowadays.

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