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SPAWNING INDUCTION, EGG MANAGEMENT, LARVAL REARING AND WEANING OF THE FLATHEAD GREY MULLET (*MUGIL CEPHALUS*) ANDOTHER RELEVANT MUGILIDAE

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Abstract

The cosmopolitan flathead grey mullet (*Mugil cephalus*) like other Mugilidae species is a catadromous, detritivore teleost. It is an oviparous gonochoristic species, characterized by a synchronous ovarian development and one spawning period observed from July to October, depending on geographical location. Males mature between 1 and 2 years of age, while females reach maturity between 2 and 4 years of age.

Recently, the family of Mugilidae has been identified as a promising species for aquaculture. This species has fast growth, does not require high amounts of dietary fish meal and oil and can be reared in a wide range of salinities and culture systems, making this species the perfect candidate to boost sustainable aquaculture production in the Mediterranean region. In Italy, mullet farming is still almost entirely based on extensive techniques, with wild juveniles being reared in coastal lagoons and semi-intensive ponds.

Throughout the Mediterranean region and beyond, salted and dried *M. cephalus* roe, is a traditional, highly valued product considered as a luxury food in many regions of the world (>270€ kg^{-1}). Traditionally, adult females at advanced vitellogenesis stages, are captured in lagoonal waters for 'bottarga' production prior to spawning, during their reproductive migration towards the sea. This has led to an overfishing of sexually mature adults, with a significant negative impact on *M. cephalus* wild populations.

In this context, closing the life cycle under captive conditions, producing hatchery reared juveniles and their subsequent farming has been suggested as a suitable strategy for the enhancement of depleted wild populations and for boosting the local lucrative production of bottarga.

From a detailed analysis of the scientific literature and of the industrial context, it is clear that to unlock the potential for produced captive animals, a number of key bottlenecks must be overcome, including:

- Difficulties in closing the biological cycle.
- Unspecific and underperforming larval feeding protocols.

These general objectives have lead and inspired the specific objectives of the thesis which focused on:

a) Optimisation of spawning induction and larval rearing protocols in *M. cephalus* (Chapter II)

b) Application of the production protocols developed for *M. cephalus* to *C. ramada* (Chapter III).

c) Development of a protocol for *M. cephalus* embryos handling and transport (Chapter IV).

d) Development of new larval weaning protocol for *M. cephalus* and evaluation of its physiological and productive performance (Chapter V)

Over a period of three years, working in constant collaboration with colleagues at the International Marine Centre (IMC), the Università di Bologna, and through frequent consultation with international partners (IRTA, HCMR, Universidad de Las Palmas Gran Canaria) we have successfully completed investigations into all specific objectives and achieved a new level of understanding of Mugilids biology in captivity.

In brief, objective a) was achieved by the success and excellent performance of the breeding induction protocol resulting in very promising fertilisation and hatching rates. This is also due to the improvement in broodstock management with consequent stress reduction. In addition, the rearing protocols applied to the larvae resulted in good growth and larval survival rates. Objective b) was achieved by the successful transfer of the breeding induction protocol developed for *M. cephalus*, to *C. ramada*, due to the remarkable ease of domestication of this species. The fertilisation, hatching rates and larval survival results were very encouraging. Objective c) was achieved by good results obtained during a simulated transport experiment of *M. cephalus* embryos. Objective d) was chieved by the successful early weaning under co-feeding regime, showing potential to reduce the amount of Artemia sp. fed daily while still ensuring excellent larval growth and survival. This protocol brought forward the weaning of the larvae compared to the previous observations.

Taken together these results demonstrate that species belonging to the Mugilidae family (particularly if multiple species are considered) are excellent candidates for their development in aquaculture, due to the successful breeding induction protocols applied that can ensure a constant annual production of different Mugilidae species during the different periods of the year. The breeding protocol developed here, thanks to its easy applicability, can be transferred to those working in the industry and ensure annual production of fries without the complications involved in keeping breeders in captivity. Obviously, this very pragmatic approach has significant limitations, particularly around the lack of control over gamete quality, biosecurity and it negates the future advantages offered by selective breeding. The results obtained from the simulated transport of *M. cephalus* embryos tell us that they can be transferred from the egg-laying and fertilisation sites for hundreds of kilometres, guaranteeing a good quality of the embryos. These results, although good, give us an indication that an improvement is possible by testing embryo manipulation at other stages of embryonic development. As for larval rearing and weaning protocols, despite very encouraging results due to increased rearing densities and good growth and survival of larvae, there is still a lot of work to be done so that these results can be transferred to an industrial production scale.

Furthermore, the new knowledge generated by this PhD Thesis can open new avenues for the scientific investigations into many aspects of the general physiology of the species under captive conditions, including: the nutrition and reproduction biology and the potential for the identification of key phenotypic commercial traits for future selection. Furthermore, by translating the current and future knowledge into applicable farming protocols for the species it will be possible to unlock the full potential of one of the most sustainable aquaculture species globally.

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Chapter I: General Introduction

1. A global analysis of aquaculture production

Aquaculture, the controlled cultivation of aquatic organisms encompassing fish, molluscs, crustaceans, and aquatic flora, has exhibited exceptional growth within the past two decades, ranking as one of the fastest-expanding sectors in global food production. In the period 1990–2020, total world aquaculture expanded by 609 percent in annual output with an average growth rate of 6.7 percent per year. The average annual growth rate had decreased gradually from 9.5 percent during the period 1990–2000 to 4.6 percent during 2010–2020. The growth rate reduced further to 3.3 percent per year in the most recent years (2015–2020). Its contribution to the combined production of finfish and shellfish has risen significantly from 25.7% in 2000 to approximately 49.2% of total production in 2020, with an even more substantial share, i.e., 52%, destined for human consumption. World aquaculture production of animal species to grow by 2.7 per cent in 2020 compared to 2019, an annual growth rate never so low in over 40 years (The state of world Fisheries and aquaculture 2022).

In 2020, worldwide aquaculture production of finfish and shellfish (excluding algae and ornamental shells and pearls) reached a peak of 87.5 million tons. This comprised 57.5 million tons of fish, with 49.1 million tons stemming from freshwater aquaculture and 8.3 million tons from marine and brackish water aquaculture. Additionally, molluscs contributed 17.7 million tons, crustaceans accounted for 11.2 million tons, aquatic invertebrates 525 000 tonnes and 537 000 tonnes of semi-aquatic species including turtles and frogs (FAO, 2022).

Regionally, Asia dominated aquaculture, constituting 88.43 % of total finfish and shellfish production, followed by Americas (2.57%), Europe (3.74 %), Africa (2.57%), and Oceania (0.26%). China, as the largest producer, held a commanding 59.75% share of global production. Despite the vast diversity of species cultivated (exceeding 600 species), the finfish aquaculture sector is dominated by a select few,

with the top 15 species commanding 77% of total production. Among these, predominantly freshwater species such as cyprinids, tilapia, salmonids, and catfish are prevalent. In Europe, 45.57% of finfish aquaculture is concentrated in Norway, the European Union (33.45%), and Rest of Europe (20.98%). Atlantic salmon (*Salmo salar*) (2,719,600 tons), rainbow trout (*Oncorhynchus mykiss*) (329,751 tons), common carp (*Cyprinus carpio*) (168,351 tons), gilthead seabream (*Sparus aurata*) (282,100 tons), and sea bass (*Dicentrarchus labrax*) (243,900 tons) were the dominant species in European finfish aquaculture production, primarily consisting of carnivorous species.

Notably, approximately 12% of global fish production (including fisheries) is currently channelled into the production of fishmeal and fish oil for aquafeeds, or as raw materials for direct aquaculture feeding (FAO, 2022). The availability of fishmeal and fish oil represents a constraining factor, particularly for carnivorous species in aquaculture. Long-term projections indicate a diminishing supply of fishmeal and oil due to factors such as heightened societal pressure to enhance aquaculture sustainability, escalating fishing costs, and production-related concerns, possibly linked to the depletion of natural fish stocks, with 34.2% of species already declared as overfished.

While there is a growing trend in freshwater aquaculture towards species requiring minimal or no fish meal in their diets, such as carps and tilapia, it is imperative to focus research efforts on exploring novel feed sources and diversifying cultured species. Particularly, attention should be directed towards herbivorous or omnivorous marine species, such as the family Muglilidae including the flathead grey mullet (*Mugil cephalus*), as a means to foster greater sustainability in the field of aquaculture for the future.

2. The flathead grey mullet (*Mugil cephalus*) and other Mugilidae

Among the extensive taxonomic group of the family Mugilidae, encompassing 26 genera and 80 species, only three species hold significant importance in the realm of aquaculture (Crosetti et al., 2016). Notably, the flathead grey mullet stands out as the most frequently cultivated among these species. It is regarded as a promising candidate for diversifying European marine aquaculture owing to its favourable attributes (Nordlie, 2016). This species exhibits versatility in terms of its habitat, thriving in seawater, brackish water, and freshwater environments. It adapts well to various aquaculture systems and exhibits rapid growth (Khemis et al., 2019).

Furthermore, the flathead grey mullet holds a strong position in the market due to its high desirability as a source of premium-quality protein. Its flesh properties are highly regarded in many countries. Additionally, the salted and dried roe, known as “bottarga”, commands a high market price exceeding 270 euros per kilogram in the southern Mediterranean and Asia (Crosetti et al., 2016). This adds significant value to the cultivation of this species, which is anticipated to be a cost-effective and resource-efficient endeavour. Furthermore, the flathead grey mullet demonstrates significant suitability for sustainable and environmentally-friendly aquaculture practices. It has been successfully reared using fishmeal-free feeds, aligning with its natural omnivorous detritivore dietary preferences (Gisbert, E. et al., 2016, Koven, W. et al., 2020). In light of these attributes conducive to aquaculture, it was postulated by Nash and Shehadeh in 1980 that *"the Mugilidae have the brightest future of all marine and brackishwater finfish in the developing technology of aquaculture."*

2.1. Biology and characteristics

Flathead grey mullets, scientifically classified as members of the Order Mugiliformes and the Family Mugilidae, belong to the group of Actinopterygian teleosts. Within the species *Mugil cephalus*, a complex is observed, comprising 14 distinct mitochondrial lineages, often referred to as cryptic species (Durand, et al., 2012). These lineages are distributed globally, inhabiting coastal regions with temperate and tropical climates, situated between latitudes 42 ºN and 42 ºS (82). Their distribution, however, is characterized by a discontinuous pattern (as depicted in Figure 1). Each of these mitochondrial lineages exhibits a regional distribution, yet it is noteworthy that different lineages can coexist within a single geographical locality. Consequently, precisely delineating their geographical boundaries remains a challenging task.

Despite being primarily classified as a marine species, flathead grey mullets display euryhaline characteristics, enabling them to thrive across a wide spectrum of estuarine salinity levels. These demersal fish typically inhabit water depths of approximately 20 meters, although they can also be encountered in offshore areas or at greater depths in the ocean (Whitfield, et al., 2012).

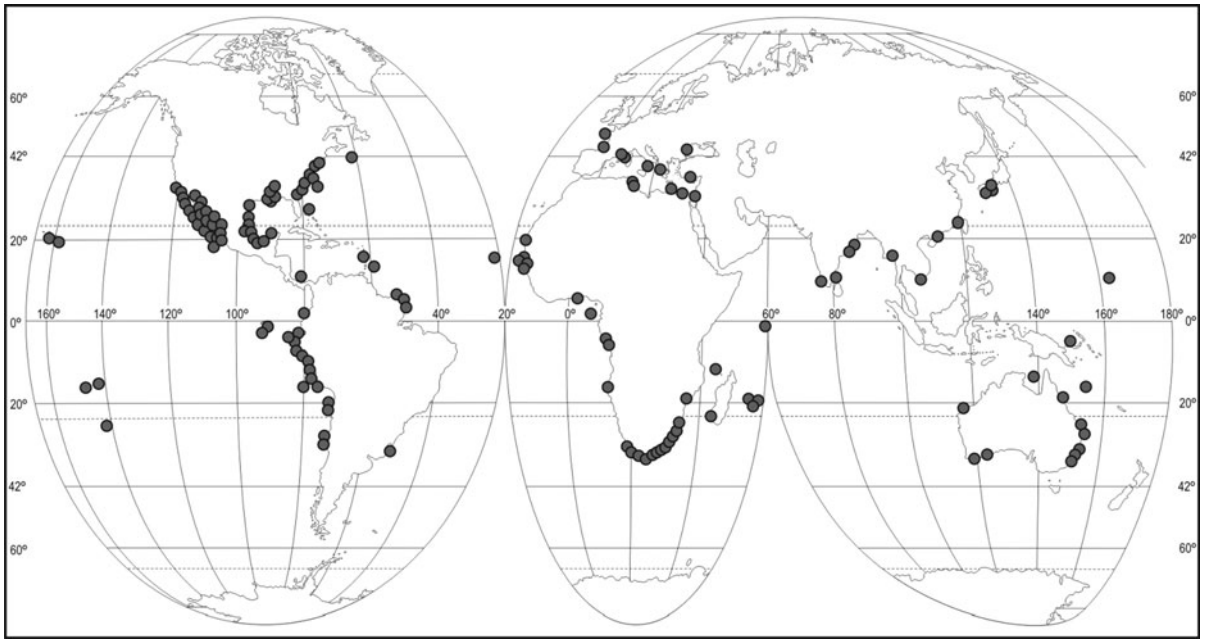

Figure 1. Global distribution records of flathead grey mullet (*Mugil cephalus*). From Whitfield *et al*. (2012).

Flathead grey mullets, categorized as ray-finned fish, possess a sub-cylindrical body shape characterized by an oval cross-section with a smoothly curving profile, rendering them visually uniform in appearance (Fig. 2) (Crosetti et al,2016).

Figure 2. Pictures of (A) a flathead grey mullet (*Mugil cephalus*), (B) and (C) detail of the transparent adipose eyelid which covers part of the eye and has a vertical elliptical opening. Scale Bars = (A) 5 cm, (B, C) 0.5 cm. Foto from IMC – International Marine Centre, Italy,

Typically, the species exhibit a dorsal colouring ranging from greyish-green to blue, while their flanks are silvery, adorned with horizontal dark stripes. Notably, they lack a lateral line. On the ventral side, their coloration tends to be pale or yellowish. These mullets can attain lengths of up to 120 centimetres, making them the largest among mullet species. While they are gonochoric with distinct sexes, no external sexual dimorphism is observed (González-Castro & Ghasemzadeh, 2016).

Flathead grey mullets are distinguished by their broad head, which is dorsally flattened. They possess a distinct feature in their thick, soft, and transparent adipose eyelid, which appears to be exceptionally developed among mullet species (refer to Fig. 2B and 2C) (González-Castro, & Ghasemzadeh, 2016). This eyelid largely covers the eye and features a vertical elliptical opening. The species presents two separate dorsal fins, with the first bearing four spines and the second comprising one spine and eight branched rays. Pelvic fins are positioned sub-abdominally and consist of one spine and five branched rays, while the anal fin exhibits three spines and eight branched rays. However, it is important to note that counts of spines and rays cannot be used to differentiate *M. cephalus* from other mugilids (González-Castro, & Ghasemzadeh, 2016). Their scales exhibit the typical percomorph type, transitioning from cycloid in early juveniles to ctenoid later on. Flathead grey mullets are equipped with an oral and branchial filter-feeding mechanism, featuring gill rakers and a denticulate pharyngo-branchial organ employed for filtering ingested material (Cardona, 2016).

Whilst it is relatively easy to discriminate between species in the muglilidae family based on morphological features when adults, the species allocation present some challenges and require a more invasive observation of anatomical features in juveniles. The correct species identification of juvenile is however important for fisheries management purposes (recruitment) and for aquaculture practices when juveniles are still caught in the wild for on growing (Crosetti et al., 2016).

In terms of behaviour, this species is primarily diurnal. Larvae primarily subsist on zooplankton, while juveniles and adults are primarily detritivores and benthic microalgal feeders, ingesting and filtering organic matter. They are also known to consume invertebrates and plankton, with algae forming a part of their diet when residing in freshwater environments (Cardona, 2016).

Regarding sexual maturity, males typically reach it at a standard body length (SL) of 25-30 cm, while females achieve maturity at 27-35 cm SL, corresponding to an age between 1 and 2 years for males and between 2 and 4 years for females. The spawning season varies depending on geographical location, with Eastern Mediterranean spawning occurring between June and October during the

warmest months (20 ºC – 28 ºC), and the Atlantic coast of South Carolina (USA) where spawning is typically observed from October to April, coinciding with the coldest months (20 ºC – 25 ºC) (Whitfield, et al., 2012).

Adult flathead grey mullets engage in large-scale migrations from inshore waters and estuaries to the open sea for spawning, performing a single spawning event per season. Fecundity is notably high, with estimates ranging from 500,000 to 3,000,000 eggs per female, contingent on adult size (González-Castro and Minos, 2016). Using the formula established by Mcdonough et al. (2003), *Ln Fecundity = 6.95 + 1.05 (Ln BW without the ovaries)*, it can be deduced that individuals weighing 1 kg would yield approximately 1,473,488 eggs. These mullets are oviparous, releasing pelagic eggs into the water for fertilization. Upon reaching sizes of 16-20 mm, the larvae undertake a migration back to rivers and estuaries.

2.2. State of mullets culture

Many mullet species are farmed for human consumption, with traditions dating back centuries. The extensive cultivation of mullet dates to ancient times and has played an important role in several countries around the world. In the Mediterranean, the ancient Egyptians, as evidenced by bas-reliefs more than 4000 years ago, and the Romans reared this species (Crosetti et al., 2016).

It must be considered that in most countries, the reported data on the production of Mugilidae are generic and do not identify the species, so it is difficult to know the actual production (Crosetti et al., 2016).

The first data on flathead mullet production were published in 1950 with 1040 tonnes, of which 10% was produced in Europe and 90% in Asia, with more than half of the total production coming from China (FAO 2021). The 1970s, 1980s and 1990s saw a great deal of enthusiasm for the cultivation of

this species, but this was not followed up in the following years. This is certainly due to the difficulties in closing the cycle of this species (Crosetti et al., 2016).

In the 2021, the total world production of Mugilidae was 816,802 tonnes. 55 % of this wasfrom capture fisheries and the remaining 45 % from aquaculture (FAO 2021). It should be considered that in most countries, 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, 2016). Asia produced 60% of global captures. Africa ranked second, contributing with 20%, thanks especially to the Egyptian production, followed by the Americas (13%), Oceania (1.7%) and Europe (1.4%) (FAO 2016). In 2021, the world-wide mullet production from aquaculture amounted to 374,029 tonnes. Mullet culture is mainly practiced in the Mediterranean, the Black Sea and in South East Asia.

In 2021, world flathead grey mullet aquaculture production was 11,939 tons, the largest amount ever produced since 1950. In 2021, Asia held more than the 77,8 % of world-wide flathead grey mullet aquaculture production. Total production is concentrated in 7 countries and includes fresh water, brackish water and salt water: Indonesia, not present as a producer in previous years, has the largest production with 5,524 tonnes, followed by Israel with 2000 tonnes, Taiwan 1738 tonnes, China 1405 tonnes, Singapore 621 tonnes, Greece 386 tonnes and Tunisia 265 tonnes. The latest data on Italian production of *M. cephalus* in aquaculture are from 2017 and report a production of 700 tonnes. The world's mullet production from aquaculture has shown large fluctuations in the last decades and depended exclusively on collection of fries from the wild (Crosetti & Blaber. 2016). In recent years, a number of production facilities have sprung up, in particular one located in Dagon, Israel, where *M. cephalus* is produced and sold (https://www.dagon-aquaculture.com/).

The majority of domestic production is derived from the capture of wild animals through structures called 'Lavorieri' that intercept the animals during their reproductive migration from the lagoon to the sea; this is recognised as extensive aquaculture.

The productivity of *M. cephalus* in Italian and Sardinian lagoons has steadily decreased in recent decades. The main cause is the reduced migration of juveniles in coastal lagoons. This is due to detrimental environmental conditions, the marinisation of lagoons, increased predation by ichthyophagous birds (especially the greater cormorant, Phalacrocorax carbo) and overfishing of sexually mature adults (Withfield et al., 2012; Crosetti, 2015). The production of 'bottarga' is a popular traditional activity in Sardinia. In the Mediterranean region, the production of dried mullet roe dates back to the Phoenicians. In the Middle Ages, it was used throughout the region by the Arabs (Monfort, 2002). In Japan, mullet roe (karasumi) has been considered a delicacy since ancient times. The traditional use of the flathead mullet *M. cephalus* to produce dried eggs in distant regions of the world can also be explained by the very high gonadosomatic index (GSI) of mature females of this species (Katselis et al., 2005; McDonough et al., 2005). Since 2002, the botarga market in Sardinia has experienced a steady annual increase of about 5% per year. Data from 2008 indicate that total production amounts to around 400 t/year, most of which is sold on the regional market. However, only about 2% of the botarga produced in Sardinia is obtained from females caught in Sardinian lagoons. To meet the growing demand for botarga, frozen female gonads of *M. cephalus* are imported in large quantities from abroad (Africa, the United States, Brazil and Australia) (Coldiretti, Impresa pesca, personal communication, 2013). In Sardinia, the province of Oristano has the largest and most popular production of *M. cephalus* botarga in Sardinia (about 3 t/year). Data recorded by the local fishermen's association 'Cooperativa Pontis' (Cabras, OR, Sardinia) report that in 2013, 2.6 t of sexually mature females of *M. cephalus* were caught in the Cabras lagoon between August and September, destined for the local production of salted and dried mullet roe. In 2014, the season started in August and lasted until November, with a total catch of 7.6 t of mature females. In 2015, around 12 tonnes were caught from August to October, while in 2016 only 3 tonnes were recorded (Cabras Fishermen's Association, personal communication). According to PSNA 21-27 (Piano Nazionale Strategico per l'acquacoltura italiana 2021-2027), in which the national production of bottarga is reported, it rose from 0.7 tonnes

in 2017 to 8.5 tonnes in 2020, a trend that is bound to increase given the considerable demand for the product.

2.3 Reproduction and life cycle

Mullets are oviparous and gonochoristic fish; individuals are either male or female without sexual dimorphism. Flathead mullets are sexually undifferentiated for the first 12 months. Differentiation begins at 13 months of age and the fish are fully differentiated at 15-19 months. Sexual maturation in male mullets first occurs at 1 year of age and 100% of males are mature at 2 years. Female flathead mullet first mature at 2 years of age and 100% maturity occurs at age 4 years (McDonough et al., 2005).

The reproductive cycle is a chain of processes that start from immature germ cells and lead to the formation of mature gametes, ova and spermatozoa. Gametes develop from primordial germ cells (PGCs) that migrate to the germinal epithelium, where the gonad is formed, during embryonic development. PGCs then proliferate by mitosis until they differentiate into oogonia or spermatogonia, in females and males respectively. In the last mitotic division, the cells enter into meiosis initiating gametogenesis (Jalabert, 2005).

The reproductive cycle can be distinguished into two relevant phases: gametogenesis with the growth of the gonads and maturation culminating in ovulation or spermiation and egg laying or sperm emission (Mañanós, et al., 2009; Lubzens, et al., 2010; Schulz, et al., 2010).

Reproduction is under neuroendocrine control, the functions of the hypothalamus and pituitary gland and the gonads are influenced by environmental factors, mainly photoperiod, water salinity and temperature. Spawning occurs once a year, and ovarian maturation is group synchronous (Kuo, 1995).

2.3.1 Ovarian development

Ovarian maturation can develop in three main modes: synchronous, group synchronous and asynchronous. In fish with synchronous ovaries, all oocytes begin vitellogenesis simultaneously and advance synchronously through the next stages of development. These include the fully spawning semelparous species, such as the coho salmon (*Oncorhynchus kisutch*), which have only one reproductive event during their entire life cycle, in which case they do not maintain a reserve of oocytes in primary growth and synchronously recruit all oocytes in secondary growth.

In fish with group synchronous ovaries, at least two populations of oocytes are present in the ovary throughout the breeding season (i.e. one population in primary growth and one in secondary growth). These fish can be distinguished into two types; single-batch species with synchronous ovaries that ovulate only once during the breeding season such as the mullet (*M. cephalus*) (Kumar et al., 2015). In contrast, multiple batch spawners ovulate and reproduce several times within a few weeks, such as sea bass (Mañanós et al., 2009) and amberjack (*Seriola dumerili*) (Mylonas et al., 2004).

In fish with asynchronous ovaries, oocytes of all developmental stages are present without a dominant population. Ovulation and spawning occurs almost daily during the annual spawning season, such as sea bream (Zohar, Y. et al., 1995) and Japanese amberjack (*Seriola quinqueradiata*).

Oogenesis is commonly divided into primary growth (PG) and secondary growth (SG). Primary oocytes go through a PG or previtellogenesis stage, characterised by an increase in size, the presence of clear material in the cytoplasm and follicle formation. At this stage, two phases of oocyte maturation are observed, in the first phase of the nucleolus the germinal vesicle has a central position, in the second phase multiple nucleoli appears in the peripheral position around the inner membrane of the germinal vesicle. Oocytes may remain in this stage throughout the juvenile period.

This is followed by secondary growth, which can be divided into three developmental stages: cortical alveolus stage, vitellogenesis and oocyte maturation (OM). The cortical alveolus stage is characterised

by the presence of cortical alveoli, and lipid droplets in the ooplasm leading to a significant increase in oocyte diameter. However, not all species have cortical alveoli. In the vitellogenesis phase, the oocyte increases in size due to the accumulation of yolk granules, consisting mainly of vitellogenin (VTG), (Wallace, & Selman, 1981; Wallace, 1985; LaFleur, 1999). During the maturation process, the germinal vesicle migrates to the oocyte periphery with the formation, in some species, of large oil droplets and the coalescence of the yolk.

The final OM is characterised by the complete migration of the germinal vesicle and the dissolution of the nuclear membrane, a process called germinal vesicle break down (GVBD). At this point, the mature oocyte remains arrested until hydration, which occurs shortly before ovulation. During the hydration phase the oocyte incorporates water with a 2 to 3-fold increase in oocyte volume. At the moment of ovulation, the follicular layers surrounding the oocyte rupture and release the ova into the lumen of the ovary. Following egg laying, the empty follicular envelopes form post-ovulatory follicles (POF), which are reabsorbed a few days after ovulation (Brown-Peterson, et al., 2011; Lubzens, et al., 2010; Mañanós, et al., 2009; Mylonas, et al., 2013).

2.3.2 Testicular development

The testicular development was classified in four stages by Ramos et al. (2022). Immature testes present a compact connective tissue (stroma), with an initial presence of spermatogonia in the seminiferous tubules. Testes in development show thickened acinus walls due to spermatogonia, spermatocytes and spermatids differentiate; few spermatozoa in the lumen are sometimes present. Mature testes acini and the efferent duct are normally filled with spermatozoa; during early spermiation, a little presence of spermatocytes and spermatids are also observed in the acinus wall. Post-spawned testes still show spermatozoa in the acini but they were less dense and packed, areas devoid of spermatozoa in the acini are also often visible.

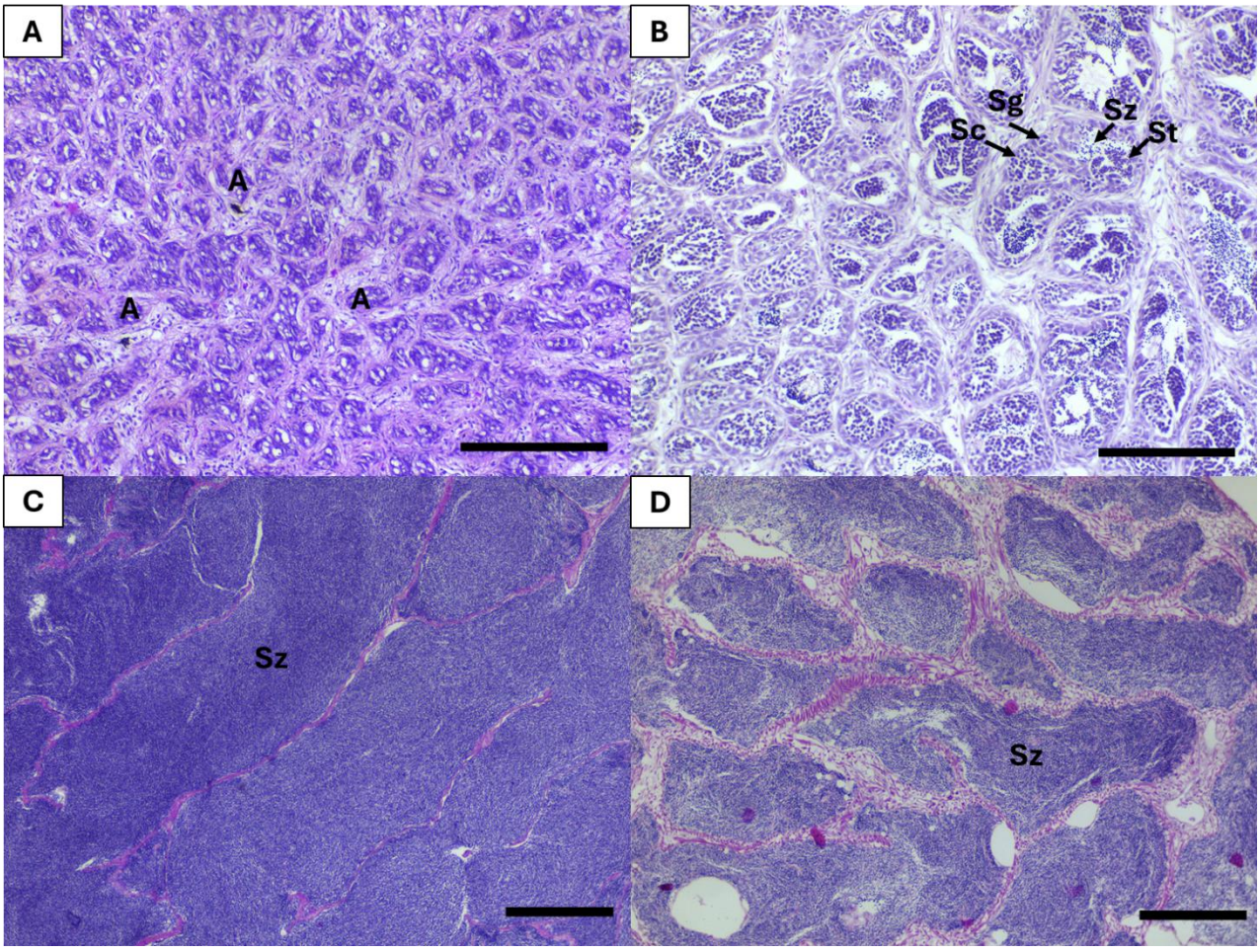

Fig. 3 - Gonad stages observed in males of *M. cephalus*. A) Immature test. Acini were visible. B) Developing test; in the acini, gametes at several stages of development are present. C) Mature test packed with spermatozoids. D) Post – spawned test; the acini still showed spermatozoids, but less packed than in the previous stage. Areas devoid of gametes are visible. A: acini; Sp: spermatogonia; Sc: spermatocytes; St: spermatids; Sz: spermatozoids. Hematoxylin - Eosin, bar = 100 µm. Photo by IMC-International marine centre

2.4 Hormonal treatments

Reproductive dysfunction in captive fish are common and often caused by several factors associated with captivity; such as: confinement, absence of natural environmental entraining cues, sub-optimal water quality, and/or rearing conditions. These can therefore cause stress which can interfere with the normal physiological functions typical of the hypothalamus-hypophysis-gonadal (HPG) axis (Corriero et al., 2021). Females can present three main reproductive disfunction linked to the three main stages

of maturation and spawning: First, oocytes can cease to mature at the pre-vitellogenic or vitellogenic stages; Second, oocytes that have stopped maturation can undergo a degenerative process known as atresia; lastly, even when all maturation is completed and atresia is avoided, mature oocytes can still get stuck in the oviduct generating a syndrome commonly called “egg-bound”. In vertebrates, gonadotropins (Gths) such as the follicle-stimulating hormone (Fsh) and the luteinizing hormone (Lh), normally constitute the main hormonal components responsible for the normal functioning of the Hypothalamus-Hypophysis-Gonadal axis and, therefore, these are the main players driving gonadal maturation. The main role of Fsh is to promote initial stages of maturation, whilst Lh is mostly involved with the final gametes maturation including ovulation and sperimiation (Lubzens, et al., 2010; Mañanós, et al., 2009). In many species belonging to the Mugilidae family, injection with gonadotropin releasing hormone agonists (GnRHs), normally only promotes very limited maturation and spawning in captivity. This is due to the presence and activity of endogenous inhibiting factors in the dopaminergic system, which disrupts the physiological function of the HPG axis (hypothalamus–pituitary–gonad) by suppressing endogenous release of Fsh and Lh (Schreck, 2010). Over the years several hormonal treatments have been tested on several mugilids (Apekin et al., 1979; Cataudella et al., 1988; Delas Heras et al., 2012; Vazirzadeg et al., 2015; Besbes et al., 2020) with mixed results, generally characterised by high dosage and repeated injections being required and therefore not likely applicable at commercial scale hatcheries.

Such issues can be overcome by the use of species specific hormone therapies as the recombinant hormones. These new therapies have been successfully adopted by Ramos-Júdez et al., 2022, who induced gametogenesis and spawning in captive gey mullet breeders. This protocol, albeit successful and applicable at commercial scale, is likely to be fairly costly.

Probably a long permanence of the breeders under optimal rearing conditions could permit, by the reduction of stress factors and domestication of the animals, to obtain gametogenesis and spawning in captive flat head grey mullet.

Indeed, during this PhD thesis, a captive population of *Chelon labrosus* kept in Gran Canaria (Universitad de Las Palamas de Gran Canaria, ULPGC), was successfully induced to spawn. During their third year in captivity the animals had completed sexual maturation without any hormonal intervention, and once treated with the induction protocol developed at the International Marine Centre (IMC) during this PhD thesis (discussed later in Chapter 2, and within an Aquaexcell3.0 funded project - REF. PID19216) spawned in the rearing tanks and larvae had been reared until weaning.

2.5 Embryonic stage and larval ontogeny

2.5.1 Embryonic stage

This description is based on our breeding experiences and Vallainc et al. 2021. The following stages are described for eggs spawned and incubated at a temperature of 23 ± 1ºC.

1. Ripe unfertilized just spawned flathead grey mullet egg

The ripe unfertilized eggs of *M. cephalus* appear spherical, yellowish, transparent, and non-adhesive, with a diameter of about 702 ± 9 µm and a single oil globule.

2. Ripe egg at first cell division

Fertilized eggs are pelagic with a smooth chorion. Cells form at the animal pole and the first meroblastic cleavage (2-cell stage) takes place from 30 to 40 minutes after fertilization.

The yolk concentrates at the vegetal pole. Egg diameter is 785±12 µm.

4. Egg in stage 4 cells

Cell division proceeds with a single layer of four uniformly sized cells (blastomeres) forming the germinal disc (blastodisc) on the yolk. The second cleavage occurs 40 minutes after fertilization. The

individual cells of the blastodisc decrease in size with each cell division. The protoplasm is gradually differentiated from yolk to form the blastodisc at the animal pole.

5-8. From eight blastomeres (50 min. after fertilization) to morula stage (2 h after fertilization).

The third cleavage (eight-cell stage) occurs 50 minutes after fertilization at right angles to the first division and parallel to the second. The fourth cleavage (16-cell stage) occurs parallel to the first division and blastomeres are in a single plane.

Thereafter, the number of blastomeres becomes a multicellular cape (morula stage after 2 hours from fertilization). The mean total diameter of the egg is 786±15 µm.

9-12. From blastula (2 h +10' after fertilization) to gastrula stage (4h + 10' after fertilization)

The arrangement of blastomeres is irregular. Beyond the 32-cell stage, it is difficult to count the cells, and cell division is not as synchronous as earlier. The fifth cleavage (64-cell stage) occurs two hours and 25 minutes after fertilization and the blastomeres are reduced in size as division continues.

Following the blastodermal cap stage, the blastula stage occurs as the mass of cells begins to flatten and encircle the yolk (epiboly), and the edge of the cell mass becomes thickened (germ ring stage). At the same time, the blastomeres lift up in the centre of the mass, creating a central cavity called the blastocoel. The blastoderm is flattened out over the yolk to become circular. By that time the cell mass is about halfway around the yolk. During the gastrula stage (4 hours after fertilization) the single-layered blastoderm becomes a multilayered embryo. As the embryonic shield forms, there is a thickening of the caudal margin of the blastodermal cap. In this area, cells invaginate to form a gastrula. Egg diameter is 786±31 µm.

13-17. Closing of the blastopore, neurula and late neurula stage

The blastocoele, germ ring and embryonic shield appears at 10 hours after fertilization. The embryonic axis is visible. Neurula stage occurs 11 hours after fertilization. Egg diameter is 787±12 µm.

18-20. Developing embryo

At the age of about 14 hours after fertilization, the embryo begins to be noticeable. Egg diameter is 793±4 µm. Pigmentation begins, the head region of the embryo starts to be visible, and somites can be distinguished (15 hours after fertilization). The primordial fin is forming, and the embryo occupies 4/5 of the egg (26 hours after fertilization).

21-22. Developing embryo

The primordial fin is formed, and the optic capsules and eye lenses are visible (from 27 to 29 hours after fertilization). Egg diameter is 802±4 µm.

23. Hatched larva (36 h after fertilization)

The hatching occurs 36 hours after fertilization. The larva breaks the egg membrane with tail movement and the head emerges first. Newly hatched *M. cephalus* larvae measure 2.1 ± 0.4 mm TL.

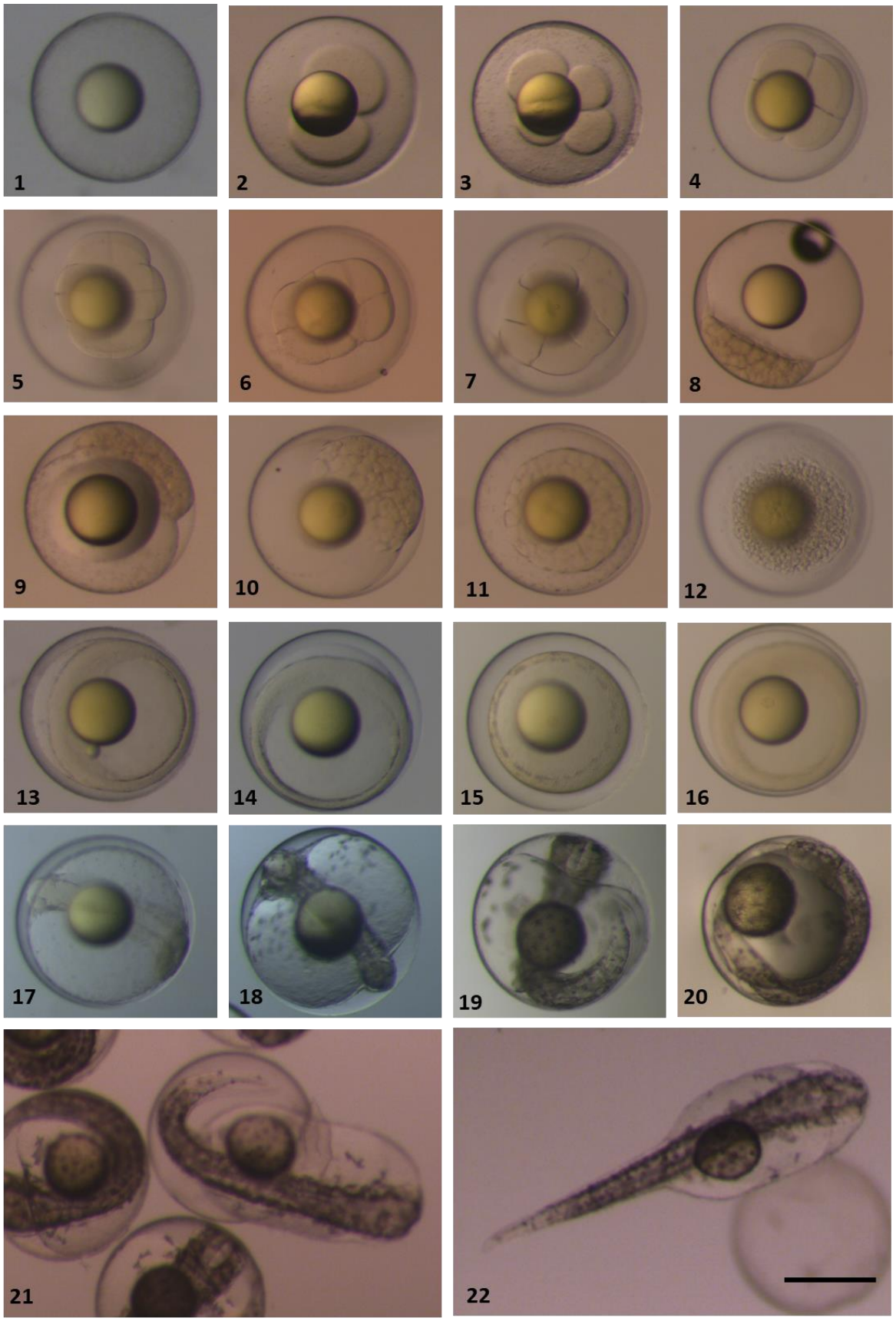

Fig. 4: Embryonic development stages of *M. cephalus* up to egg hatching. The bar corresponds to 500 µm. Photo by IMC-International marine centre

2.5.2 Larval stage

The description is based on our breeding experiences and Vallainc et al. 2021. At hatching the pre-larvae measured approximately 2.62 ± 0.1 mm TL and showed a homogeneous yolk mass with a round lipid drop at the posterior part of the yolk sac, the primordial marginal fin surrounded the body from the dorsal part of the head to the posterior margin of the yolk sac (Fig. 5). The eyes began to be pigmented between 1 and 2 dph. The pre-larval stage ended with the opening of the mouth from 3 dph onwards, this opens in the rostral part of the head and differences into lower and upper jaws (2.80 ± 0.08 mm TL, Fig 5 B), the yolk sac reserves are almost totally consumed but the lipid drop remains the pectoral fins were visible and functional at this time. Insufflation of the swim bladder began from day 4 dph positioned under the round, translucent notochord. The pre-flexion stage lasted from 4 to 11 dph (Fig. 5) and was characterised by the complete absorption of the yolk sac, followed by the lipid drop. The bending stage together with the appearance of the first caudal fin rays started at 12 DPH (Fig 5, D). The flexion stage was completed at 18 dph (3.7 ± 0.5 mm TL) (Fig 5, E), while the post-flexion stage lasted for several days (20-30 dph, 4.3-5.3 mm TL) until the caudal fin and fork were completed. At 35 dph, the juvenile stage was reached (Fig. 5).

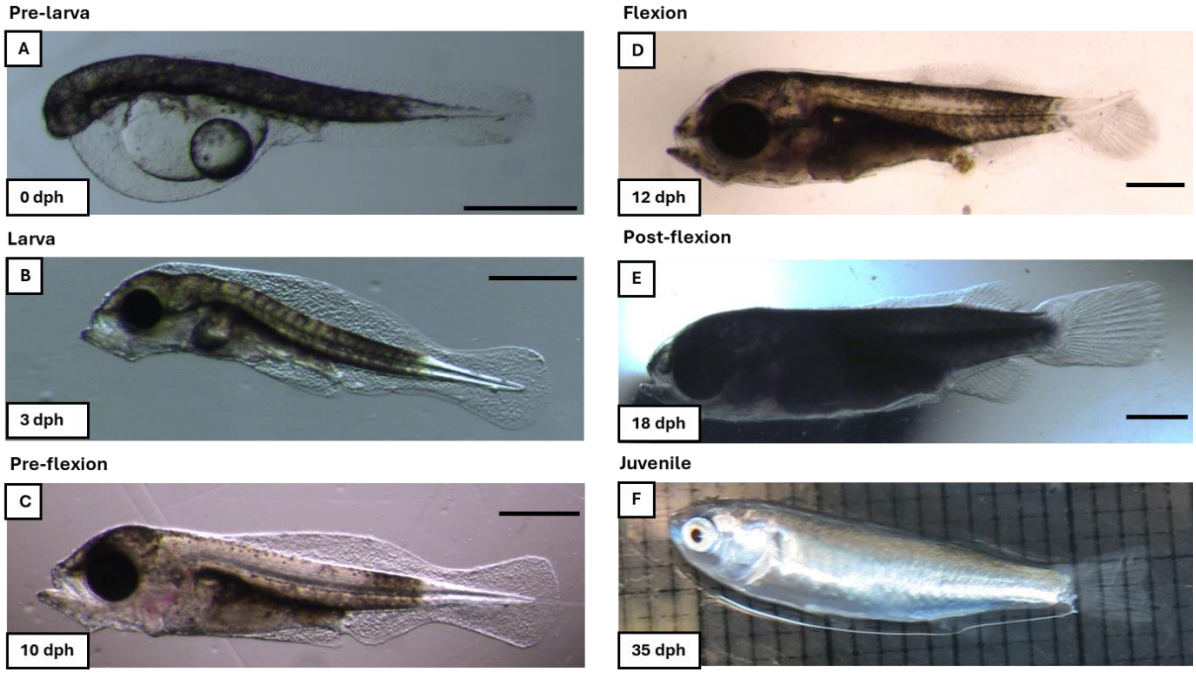

Fig. 5: *M. cephalus* larval stages from 0 dph to 35 dph. Photo IMC-International Marine Centre. The scale bar: 500 µm

As far as larval growth is concerned, rearing experiences accumulated during this PhD work have shown a significant dishomogeneity in growth, from as early as the first fifteen days of the larvae's life (Fig. 6); this has also been observed by Oz et al., 2023.

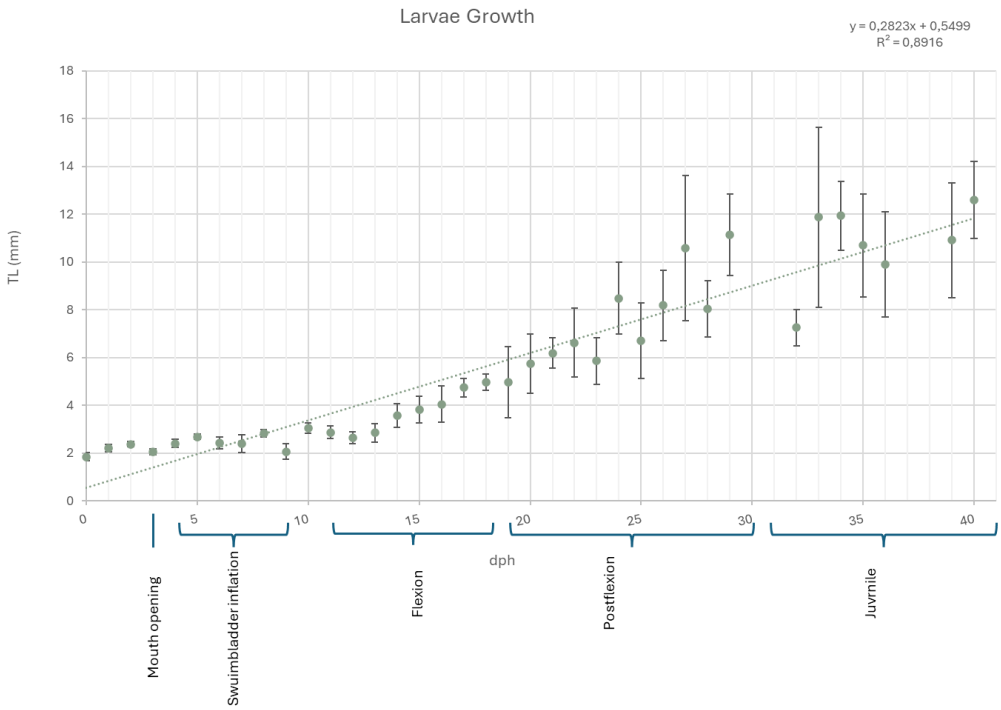

Fig. 6 Growth performance of flathead grey mullet (*Mugil cephalus*) larvae in terms of total length (mm, mean ± SD), in relation to time (days post hatch, dph) and developmental stages.

2.5.3 Digestive system

The present information was taken from Loi et al., 2020. A key step for the production of juvenile fish is linked with the ingestion and digestion of live preyes first and formulated diets later. From hatching until the mouth opening, larval nutrition is ensured by the yolk reserves and the oil globules absorbed through the syncytium layer. During the exclusively endogenous feeding phase, the digestive tract of

newly hatched larvae appears as a closed, straight, and undifferentiated tube located between the notochord and the yolk sac (Fig. 7A).

The liver is the first accessory gland that develops rapidly from 1 dph (TL 2.86 ± 0.09 mm, 44-degree days). Positioned behind the yolk sac, the liver develops to surround the anterior part of the intestine (Fig. 7B). It is a central organ for nutrient metabolism and is involved in lipid storage and in the conversion and transfer of substances to the peripherical tissues.

At 3 dph (TL 2.79 ± 0.08 mm, 88-degree days), simultaneously to the mouth and the anus opening, the intestine coils and forms a loop to accommodate its increasing size in the visceral cavity (Fig. 7C). Few intestinal villi with absorptive function are visible in the inner side of the intestinal wall, progressively increasing in number and size (Fig. 7C). In concomitance with the start of the exogenous feeding, the intestinal segmentation takes place, with the formation of the ileo-rectal valve, the sphincter that separates the antero-median and posterior intestine, whose function consists in keeping digestive enzymes in the anterior intestine for reuse (Fig. 7C). The antero-median intestine is the main site for lipid absorption, while the posterior part mainly absorbs proteins. In the same day, the pancreas appears around the middle part of the intestine, just behind the liver, ensuring the early ability to produce pancreatic enzymes such as trypsin and amylase (Fig. 7C).

At 5 dph (TL 2.80 ± 0.04 mm, 132-degree days), the longitudinal folds of the oesophageal mucosa appear providing the distensibility of the oesophagus during food intake (Fig. 7D).

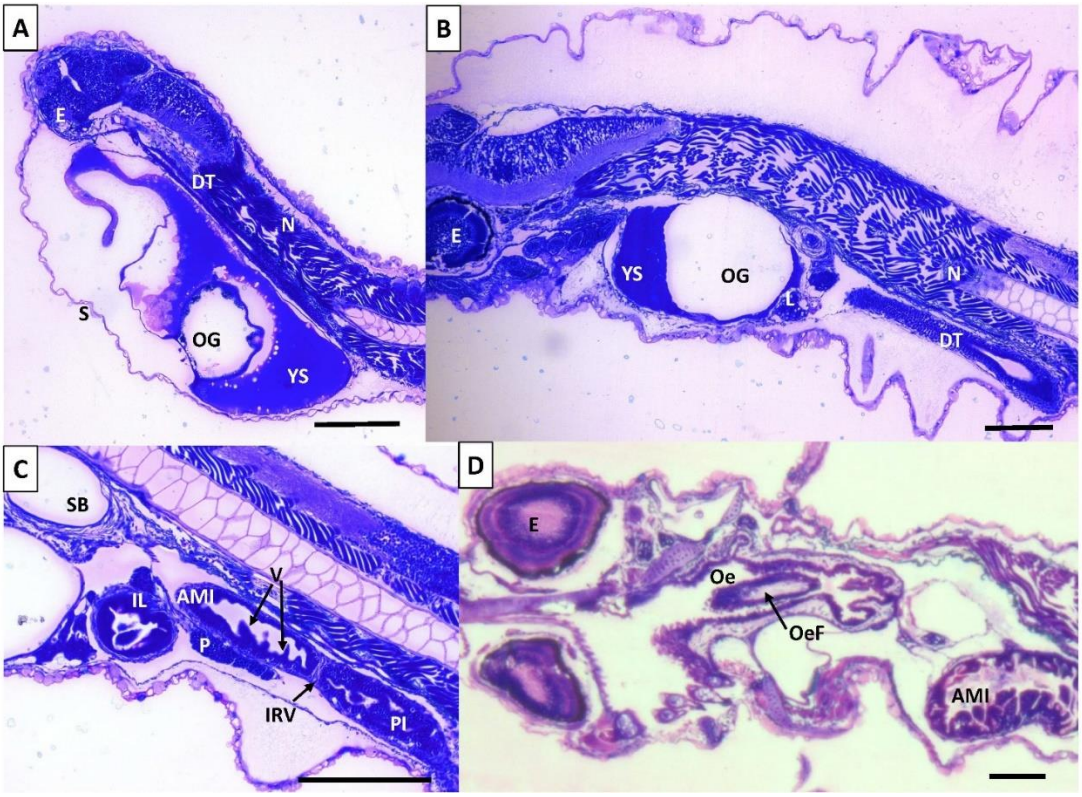

Fig. 7 – A: Newly hatched larvae, with undifferentiated digestive tube; the mouth is closed, and the sustenance depends on the absorption of nutritive reserves by the yolk. B: Liver develops at 1 dph behind the yolk sac, and it is involved in the nutrient metabolism and lipid storage. C: Intestine coils at 3 dph due to its increasing size and is now divided into antero-median and posterior intestine by the ileo-rectal valve; absorptive villi are present in the intestinal wall, while the pancreas appears around the middle intestine. D: From 5 dph, the oesophagus can expand during food intake thanks to the formation of longitudinal folds. E: eye; S: syncytium layer; DT: digestive tube; N: notochord; OG: oil globule; YS: yolk sac; L: liver; SB: swim bladder; IL: intestinal loop; AMI: antero-median intestine; P: pancreas; V: villi; IRV: ileo-rectal valve; PI: posterior intestine; Oe: oesophagus; OeF: oesophageal fold. Histological sections are stained with Methylene blue/Azure II/Basic Fuchsin. Scale bar: 100 µm. Photo credits: B. Loi.

At 7 dph (TL 2.95 ± 0.08 mm, 176-degree days), larvae acquire the sense of taste with the formation of the first taste buds in the lower part of the mouth (Fig. 8A). The taste buds are crucial to the process of food recognition and selection. At the same time, the stomach begins to delineate with the appearance of the cardiac and pyloric sphincters that separate it from the oesophagus and the intestine, respectively (Fig. 8B).

At 9 dph (TL 3.14 ± 0.32 mm, 220-degree days), goblet cells are visible at the oesophagus area, and facilitate swallowing of food through the production of mucosubstances, while protecting the epithelium against mechanical damage or bacterial invasion (Fig. 8C).

At 13 dph (TL 4.24 ± 0.30 mm, 307-degree days), the first pharyngeal teeth appear, indicating that the larvae have developed the ability to process live prey mechanically (Fig. 8D). In combination with the enzymatic production of the pancreas, the mechanical processing of prey by the pharyngeal teeth positively influences the level of digestion, even before the development of the gastric glands.

The final step of the gut differentiation is the formation of the gastric glands with the consequent production of acidity in the stomach at 17 dph (TL 5.98 ± 0.40 mm, 395-degree days) (Fig. 8E). From this moment onwards, with the secretion of HCl and digestive enzymes (e.g., pepsin), larvae can digest several types of live prey and complex substrates such as inert food items.

By 22 dph (TL 7.81 ± 0.33 mm, 502-degree days), goblet cells in the intestinal mucosa are numerous and clearly visible and protect the digestive tract through the production of mucosubstances rich in glycoproteins, also facilitating the absorption of nutrients and lubricating faeces (Fig. 8F).

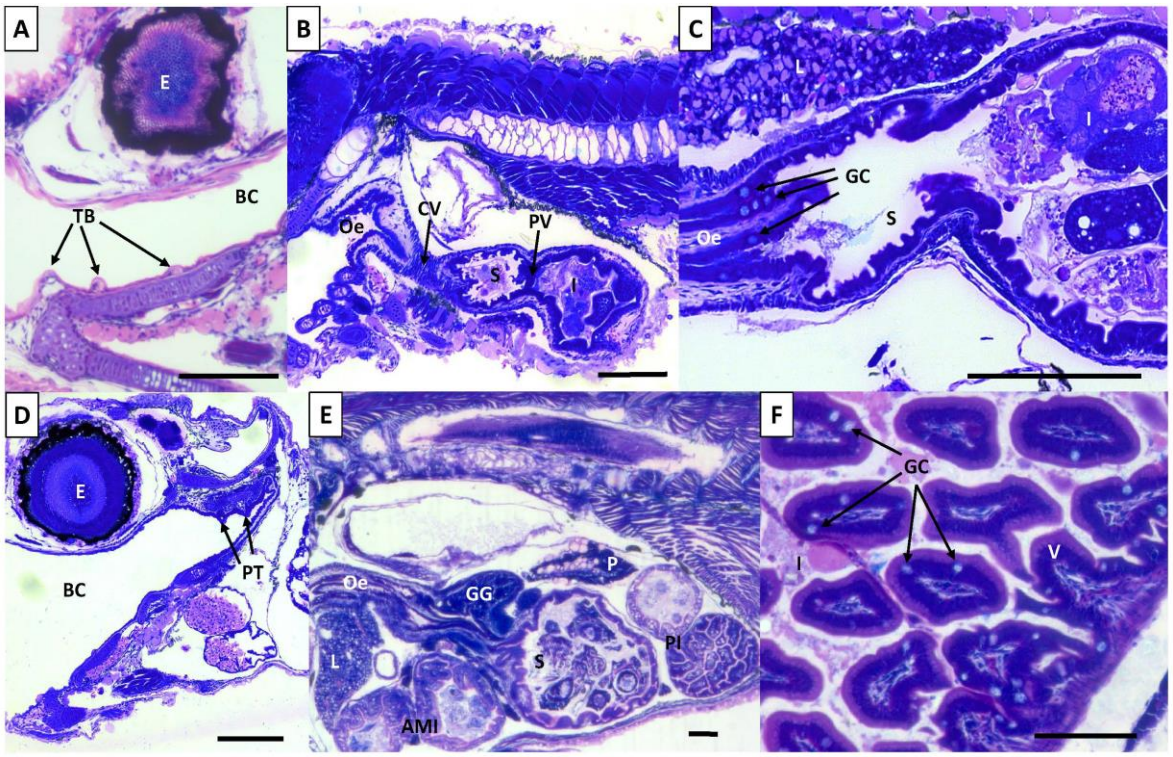

Fig. 8 - A: Taste buds develop by 7 dph in the buccal cavity to provide larvae with the sense of taste. B: The cardiac and pyloric sphincters separate the stomach from the oesophagus and the intestine. C: The first oesophageal goblet cells appear at 9 dph and produce mucosubstances that facilitate the swallowing of food: D: The pharyngeal teeth enable the 13 dph larva to process live prey mechanically. E: The gut differentiation can be considered accomplished at 17 dph, after the formation of the gastric glands all around the stomach; from now on, the larva can digest complex foods. F: The intestinal goblet cells facilitate the absorption of nutrients and lubricate faeces. E: eye; TB: taste buds; BC: buccal cavity; Oe: oesophagus; CV: cardiac valve; S: stomach; PV: pyloric valve; I: intestine; GC: goblet cells; L: liver; PT: pharyngeal teeth; AMI: antero-median intestine; GG: gastric glands; P: pancreas; PI: posterior intestine; V: villi. Histological sections are stained with Methylene blue/Azure II/Basic Fuchsin. Scale bar: 100 µm. Photo credits: B. Loi.

2.5.4 Visual system

The present information was taken from Loi et al., 2020. A key step for the larvae, immediately after the opening of the mouth, is represented by their ability to clearly identify and recognize preys. Mugilids are in fact visual predators during their early life stages. At hatching, larvae have undeveloped eyes, the lens appears as a spherical structure surrounded by the retina, which consists in undifferentiated neuroepithelium (Fig. 9A).

Between 1 and 2 dph, the initial pigmentation of the eye suggests the beginning of the differentiation of the morphological structures involved in the vision process.

At 3 dph (TL 2.79 ± 0.08 mm, 88-degree days), concurrently to the mouth opening, the first photoreceptor cells, the cones appear and progressively increase in number (Fig. 9B, C). Cones are responsible for photopic vision, ensuring that the larvae have a diurnal vision ability.

At 17 dph (TL 6.59 ± 0.57 mm, 437-degree days), the first rod nuclei appear ensuring the scotopic vision, and the larvae develop the ability to detect food items in low light conditions (Fig. 9B, C).

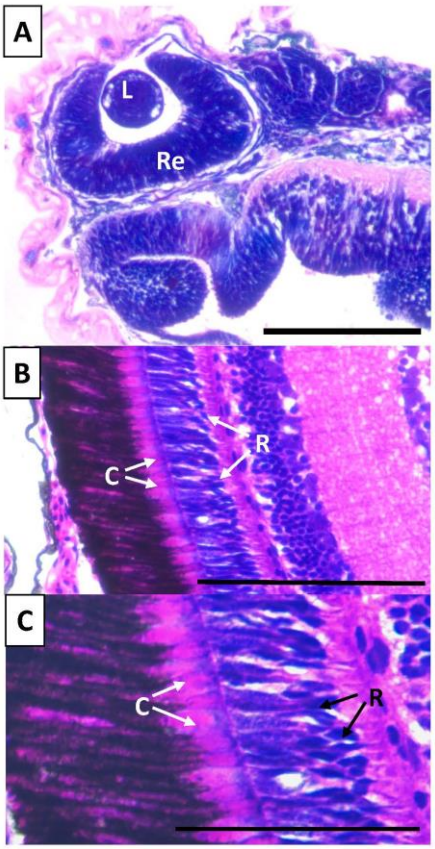

Fig. 9 – A: Newly hatched larvae have an undeveloped eye, with a central lens surrounded by undifferentiated retina. B: The cones ensure the diurnal vision ability by 3 dph, while by 17 dph larvae can detect food items in low light conditions thanks to the appearance of the rods. C: Magnification of cone and rode cells. L: lens; Re: retina; C: cone cells; R: rod cells. Histological sections are stained with Methylene blue/Azure II/Basic Fuchsin. Scale bar: 50 µm. Photo credits: B. Loi.

3. Objectives

As highlighted above, global aquaculture is constantly growing and therefore there is a need to identify new species that are more sustainable in production. Due to its remarkable adaptability to different farming conditions and its omnivorous detritivorous diet, the family Mugillidae has been identified as one of the most promising for sustainable aquaculture. Despite this, there are still a number of bottlenecks preventing the progress of aquaculture of these species; among these is the difficulty of closing the life cycle in captivity unless hormone treatments induce spawning or affect gonadal maturation. Breeding protocols for these species are difficult to transfer to the production industry, at the moment the stocking densities of larvae reported in the literature are low and report survival rates

that are not in line with industrial production. The basic nutritional requirements of juveniles to ensure rapid growth are poorly known.

In addition to this, as also reported in PSNA 21-27 (Piano Nazionale Strategico per l'acquacoltura italiana 2021-2027) data, an increase in demand and production of roe is followed by a decrease in the production of *M. cephalus*. This can be interpreted as a decrease in fish stocks that are overexploited, and a consequent import of gonads from other producing countries to meet the demand. Against this background, it is essential to have a programmable production of *M. cephalus* that does not deplete natural resources, and to do this, the doctoral work had the following objectives:

- Testing the effectiveness of breeding induction and optimisation of larval rearing protocols in *M. cephalus* (Chapter II).
- Application of production protocols developed for *M. cephalus* in C. ramada (Chapter III).
- Development of a protocol for embryo transport and handling of *M. cephalus* (Chapter IV).
- Development of new larval weaning protocol for M. cephalus and evaluation of its physiological and productive performance (Chapter V)

4. References

Besbes R., Benseddik A.B., Kokokiris L., Hamza A., Kammoun F., 2020. Thicklip (*Chelon labrosus*) and flathead (*Mugil cephalus*) grey mullets fry production in Tunisian aquaculture. Aquaculture Reports, 17 (2020) 100380.

Brown, C. and Day, R. L., 2002. The future of stock enhancements: lessons from hatchery practice to conservation biology. Fish and Fisheries 3, 79-94. https://doi.org/10.1046/j.1467-979.2002.00077.x.

Brown-Peterson, N. J., Wyanski, D. M., Saborido-Rey, F., Macewicz, B. J. & Lowerre-Barbieri, S. K., 2011. A Standardized Terminology for Describing Reproductive Development in Fishes. Mar. Coast. Fish. 3, 52–70.

Cardona, L., 2016. Food and feeding of Mugilidae. in Biology, Ecology and Culture of Grey Mullets. (ed. Crosetti D, B. S.) 165–195 (Boca Raton, FL: CRC Press, 2016).

Coldiretti, Impresa pesca, pers. comm., 2013.

Corriero, A., Zupa, R., Mylonas, C. C. & Passantino, L., 2021. Atresia of ovarian follicles in fishes, and implications and uses in aquaculture and fisheries. Journal of Fish Diseases, 44(9), 1271-1291. https://doi.org/10.1111/jfd.13469

Crosetti D., 2016 Current State of Grey Mullet Fisheries and Culture. Pp. 398–450. In: Crosetti D, Blaber S 2016, Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA.

Crosetti D., Blaber S., 2016. Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA.

Durand, J.D., Chen, W.J., Shen, K.N., Fu, C. & Borsa, P., 2012. Genus-level taxonomic changes implied by the mitochondrial phylogeny of grey mullets (Teleostei: Mugilidae). C. R. Biol. 335, 687–697.

FAO. 2023. Fishery and Aquaculture Statistics. Global production by production source 1950-2021 (FishStatJ). In: FAO Fisheries and Aquaculture Division [online]. Rome. Updated 2023. www.fao.org/fishery/en/statistics/software/fishstatj

FAO. 2022. The State of World Fisheries and Aquaculture. (SOFIA). Rome: Food and Agriculture Organization of the United Nations, 1-230.

FAO-FIGIS. 2021. FIGIS Integrated web-based dissemination system. FI Institutional Websites. In: FAO Fisheries Division [online]. Rome. (2021).

Gisbert, E., Mozanzadeh, M. T., Kotzamanis, Y. & Estévez, A., 2016. Weaning wild flathead grey mullet (*Mugil cephalus*) fry with diets with different levels of fish meal substitution. Aquaculture 462, 92–100.

González-Castro, M. & Ghasemzadeh, J., 2016. Morphology and Morphometry Based Taxonomy of Mugilidae. in Biology, Ecology and Culture of Grey Mullets. (eds. Crosetti, D. & Blaber, S.) 1–21 (Boca Raton, FL: CRC Press, 2016).

González-Castro, M. and Minos, G., 2016. Sexuality and reproduction of Mugilidae. In Crosetti, D. and Blaber, S. (eds) Biology, ecology and culture of mullets (Mugilidae). Boca Raton, FL: CRC Press, 227–263. https://doi.org/10.1201/b19927.

Jalabert, B., 2005. Particularities of reproduction and oogenesis in teleost fish compared to mammals. Reprod. Nutr. Dev. 45, 261–279.

Kagawa, H., 2013. Oogenesis in Teleost Fish. Aqua-BioScience Monogr. 6, 99–127

Katselis, G., Koutsikopoulos, C., Rogdakis, I., Dimitriou, E., Lachanas, A. and Vidalis, K., 2005. A model to estimate the annual production of roes (avgotaracho) of flathead mullet (*Mugil cephalus*) based on the spawning migration of species. Fish Res. 75, 138–148.

Khemis, I. B., Hamza, N. & Sadok, S., 2019. Nutritional quality of the fresh and processed grey mullet (Mugilidae) products: a short review including data concerning fish from freshwater. Aquat Living Resour. 32.

Koven, W., Gisbert, E., Meiri-Ashkenazi, I., Nixon, O., Israeli, D., Tandler, A., Nolasco Soria, H., Solovyev, M. M. & Rosenfeld, H., 2020. The effect of weaning diet type on grey mullet (*Mugil cephalus*) juvenile performance during the trophic shift from carnivory to omnivory. Aquaculture. 518, 734848.

Kumar, P., Arasu, A. R. T., Kailasam, M., Sukumarran, K., Subburj, R., Tyagraj, G. & Natarajan, M., 2015. Gonadal development and steroid hormone profile of wild caught grey mullet (*Mugil cephalus*). Biol. Rhythm Res. 46, 601–610.

Kuo C.M. (1995). Manipulation of ovarian development and spawning in grey mullet, *Mugil cephalus* L. Israeli Journal of Aquaculture/Bamidgeh. 47, 43–58.

Kuo C.M., Nash C.E. and Shehadeh Z.K., 1974. A procedural guide to induce spawning in grey mullet (*Mugil cephalus* L.) females by injection of human chorionic gonadotropin. Aquaculture 3, 1–14.

Kuo C.M., Shehadeh Z.H. and Nash C.E., 1973). Induced spawning of captive grey mullet (*Mugil cephalus* L.) females by injection of human chorionic gonadotropin (HCG). Aquaculture 1, 429–432.

LaFleur, G. J., 1999. Vitellogenins and vitellogenesis. Encyclopedia of reproduction, vol 4 985.

Lubzens, E., Young, G., Bobe, J. & J, C., 2010. Oogenesis in teleosts: How fish eggs are formed. Gen Comp Endocrinol. 165, 367–389.

Mañanós, E., Duncan, N. & Mylonas, C., 2009. Reproduction and control of ovulation, spermiation and spawning in cultured fish. in Methods in Reproductive Aquaculture: Marine and Freshwater species. (eds. Cabrita, E., Robles, V. & Herráez, M.) 3–80 (Taylor and Francis Group LLC, CRC Press, 2009).

McDonough C.J. and Wenner C.A., 2003. Growth, recruitment and abundance of juvenile striped mullet (*Mugil cephalus*) in South Carolina estuaries. Fish Bull. 101, 343–357.

McDonough C.J., Roumillat W.A. and Wenner C., 2005. Sexual differentiation and gonad development in striped mullet (*Mugil cephalus*) from South Carolina estuaries. Fish Bull. 103, 601–619.

Mylonas, C. C., Fostier, A. & Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516–534.

Mylonas, C. C., Papandroulakis, N., Smboukis, A., Papadaki, M. & Divanach, P., 2004. Induction of spawning of cultured greater amberjack (*Seriola dumerili*) using GnRHa implants. Aquaculture. 237, 141–154.

Monfort, M.C., 2002. Fish roe in Europe: supply and demand conditions. FAO/GLOBEFISH Research Programme, Vol. 72. Rome, FAO. 47p.

Nash C.E. and Shehadeh Z.H., 1980. Review of breeding and propagation techniques for grey mullet, *Mugil cephalus* L. ICLARM Studies and Reviews 3, International Center for Living Aquatic Resources Management, Manila, Philippines. 87pp.

Nordlie, F. G., 2016. Adaptation to Salinity and Osmorregulation in Mugilidae. in Biology, Ecology and Culture of Grey Mullets. (eds. Crosetti, D. & Blaber, S.) 293–323 (Boca Raton, FL: CRC Press, 2016).

PSNA 21-27 (Piano Nazionale Strategico per l'acquacoltura italiana 2021-2027)

Ramos Júdez, S., 2021. Control of reproduction in the flathead grey mullet *Mugil cephalus*: Broodstock management, hormonal therapies and transcriptomic signature. Ph.D. Thesis, Universidad Autónoma de Barcelona, Spain, 2021.

Ramos-Júdez, S. & Duncan, N., 2022. Feeding habits and the influence of pellet diameter on the feeding responses of the flathead grey mullet (*Mugil cephalus*) in captivity. Animal Feed Science and Technology, 290, 115368. https://doi.org/10.1016/j.anifeedsci.2022.115368

Ramos-Júdez, S., Giménez, I., Gumbau-Pous, J., Arnold-Cruañes, L. S., Estévez, A. & Duncan, N., 2022. Recombinant FSH and LH therapy for spawning induction of previtellogenic and early spermatogenic arrested teleost, the flathead grey mullet (*Mugil cephalus*). Scientific reports, 12(1), 1-15. https://doi.org/10.1038/s41598-022-10371-0

Schulz, R. W., de França, L. R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R. H. & Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Saleh, M., 2008. Capture-based aquaculture of mullets in Egypt. pp. 109–126. In: A. Lovatelli and P.F. Holthus (eds.). Capture-based Aquaculture. Global overview. FAO Fisheries Technical Paper. n. 508. Rome, FAO.

Shehadeh Z.H., Kuo C.M. and Milisen K.K., 1973. Validation of method for monitoring ovarian development in the grey mullet (*Mugil cephalus*). J. Fish Biol. 5, 489–496.

Schreck, C. B., 2010. Stress and fish reproduction: the roles of allostasis and hormesis. Gen. Comp. Endocrinol. 165, 549–556.

Whitfield A.K., Panfili J. and Durand J.D., 2012. A global review of the cosmopolitan flathead mullet *Mugil cephalus* Linnaeus 1758 (Teleostei: Mugilidae), with emphasis on the biology, ecology, genetics and fisheries aspects of this apparent species complex. Reviews in Fish Biology and Fisheries. 22, 641-681.

Wallace, R. A. & Selman, K., 1981. Cellular and Dynamic Aspects of Oocyte Growth in Teleosts. Am. Zool. 21, 325–343.

Wallace, R. A., 1985. Vitellogenesis and oocyte growth in non mammalian vertebrates. Dev. Biol. (N. Y. 1985) 1, 127–177.

Zohar, Y., Harel, M., Hassain, S. & Tandler, A., 1995. Gilthead seabream (*Sparus aurata*). in Broodstock Management and Egg and Larval Quality. (eds. Bromage, N. R. & Roberts, R. J.) (Blackwell Science, 1995).

Chapter II: Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons

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This chapter is the continuation of previous work. The candidate has significantly contributed to the field work, data curation, analysis and manuscript preparation.

1. Introduction

The Flathead grey mullet *Mugil cephalus* is a euryhalinic detritivorous finfish with a worldwide distribution. It is an oviparous gonochoristic species, with no external sexual dimorphism (González-Castro et al., 2012), characterized by a synchronous ovarian development and one spawning season per year, in autumn (Shehadeh et al., 1973; Kuo, 1995). Sexual maturation in males first occurs between 1 and 2 years of age, while females mature for the first time between 2 and 4 years of age (McDonough et al., 2005).

Mugil cephalus has been identified as a suitable species for feeding populations in developing countries (U.S. Agency for International Development 2007), and it is a species which is traditionally harvested and consumed in various European countries. In Italy, salted and dried *M. cephalus* roe, known such as '*bottarga di muggine*', is a luxury item sold in various regions, like Tuscany and Sardinia.

Adult females at advanced vitellogenesis stages are captured for '*bottarga*' production prior to spawning, during their reproductive migration towards the sea. This leads to an overfishing of sexually mature adults, with an impact on *M. cephalus* populations (Crosetti and Blaber, 2016).

In this context, the production of juvenile individuals in laboratory conditions and their following release into the wild has been suggested as a suitable strategy for the enhancement of depleted wild populations (Brown and Day, 2002). Production in hatcheries is considered necessary to maintain a steady supply of fry (Liao et al., 2015) and the expansion of *M. cephalus* aquaculture. The main setbacks in *M. cephalus* fry hatchery production are: i) the high hormone dosages required to induce spawning in captivity, ii) the high larval mortality and iii) the slow growth rates in captivity (Crosetti, 2016).

Mugil cephalus does not spawn spontaneously in captivity but requires hormonal treatment (Crosetti and Blaber, 2016). Many experimental reproduction trials employing different hormonal treatments have been carried out in different countries, mainly based on two or more hormone injections to achieve ovulation. The first published experiences on hormonal treatments for *M. cephalus* date back to the 1980s (Nash and Shehadeh, 1980), but the experimental findings have failed on a commercial scale (Liao et al., 2015). High larval mortality, mainly during the first 15-20 days post-hatching (U.S. Agency for International Development, 2007), is considered one of the main obstacles which prevent more widespread use of *M. cephalus* in aquaculture (Eda et al., 1990; Tamaru et al., 1992). Few authors report the operational procedures for rearing *M. cephalus* larvae (Nash and Shehadeh, 1980) and juveniles. Little data on growth performances of captive *M. cephalus* are reported in the literature (Crosetti and Blaber, 2016) and no data concerns growth performance in recirculating aquaculture systems (RAS).

In Italy, the culture of grey mullet is carried out in extensive systems, and grey mullets represent an important proportion of the production of coastal lagoons. Italian coastal lagoon management is an extensive culture system based on natural cycles and dynamics (Pellizzato, 2011). Production is low and based on wild fry availability. It cannot compete with intensive cage culture at sea, but aims to

combine environmental compatibility with economic sustainability. This is an advanced form of coastal lagoon management and represents one of the most interesting examples of coastal lagoon management in the world (Cataudella et al., 2001).

The aim of this paper is to present the results obtained by producing *M. cephalus* fry from sexually mature adults captured in Sardinian lagoons, based on other existing works, such as Cataudella et al., 2001, Crosetti, 2001 and Crosetti, 2016. The Sardinian regional territory is characterized by a high quantity of coastal lagoons and ponds, 27 main ones with an area of approx 10,000 hectares, suitable for the breeding of mullets.

This study illustrates an improved production process of *M. cephalus* from spawning induction to the juvenile stage, based on the data collected in 5 production cycles. The described protocols for broodstock collection, spawning induction, egg incubation and larval rearing, decrease mortality rates during the larval stage and increase growth rates up to the juvenile stage.

2. Materials and methods

2.1 Broodstock collection and spawning induction

In this paper we will refer to the experimental activities carried out in 2016 as 'phase 1' and to the subsequent trials, carried out by applying readapted spawning induction protocols, as 'phase 2' (Table 1).

Table 1 Origin of broodstock and operative conditions. * Distance from fishing sites.

Phase	Adult fishing sites	Spawning sites (km*)	Female external features	Ovarian biopsy	Male fluency check
1	Cabras and Mistras	IMC lab (0.4-9 Km)	Protruded papilla Bulging abdomen	yes	yes
2	Porto Pino and Tortolì	Porto Pino and Tortolì (0.05-0.1 Km)	Protruded papilla Bulging abdomen	no	no

Mature adults of *M. cephalus* were collected in Sardinia, Italy (Fig. 1), during their natural spawning season (August-September) in the lagoons of Cabras and Mistras (phase1) and in the lagoons of Porto Pino and Tortolì (phase 2). A traditional technique was used that takes advantage of their reproductive migration toward the sea. Migrating fish confined in traditional enclosures ('*lavorieri*'), placed next to the sea mouth of the lagoon, were gently collected one by one (by hand or with a silicone net) to avoid excessive stress and then put into 50 L volume transparent polyethylene transport bags (120 x 50 cm) filled 1/3 with lagoon water and 2/3 with oxygen and sealed up. First spawning induction trials were carried out in 2016 (phase 1*)* according to Vallainc (2017) and the results were used for the fine-tuning of the protocol.

2.1.1 Phase 1

Fish transport bags were covered with a shade cloth, kept in the dark and transported by car (air temperature: 22°C) to the International Marine Center (IMC) facilities. The IMC laboratories are located about 0.4 km far from the Cabras lagoon capture site (2 minutes transport time) and 9 km far from the Mistras lagoon capture site (15 minutes transport time). Salinity and temperature were respectively 28±5 ppt and 22±1 °C in Cabras, and 42±1 ppt and 23±1°C in the Mistras lagoon.

In the laboratory, both fish and lagoon water were transferred into 100 L volume black circular acclimation tanks equipped with overflow. Fish were acclimated by introducing 1 L min^{-1} of natural seawater (salinity 37±1 ppt, temperature 23±1 °C), previously micro filtered (0.5 µm, sand filter) and UV sterilized (80 W), into the acclimation tank. Once the acclimation tanks reached the desired salinity and temperature, water inlet was turned off. The fish were anesthetized with a solution of 0.08% clove oil (Erboristeria Magentina®) and weighed (Aizen et al., 2005).

Males were checked for fluency by applying a gentle pressure to the abdomen and only males with fluid sperm were selected. The state of ovarian maturity of females was assessed through an ovarian biopsy made by using a polyethylene cannula of 0.86 mm inner diameter and 1.52 mm outer diameter (Lee et al., 1992). Oocytes (n=100; a minimum of 5 oocytes $female^{-1}$) were checked under a light microscope (Leica MZ8), through a Leica DMC2009 and measured with LAS 4.5 software (Leica, Germany) to determine the stage of maturity and to measure their diameter. According to Meseda and Samira (2006), the 500 µm oocyte diameter has been established as the minimum size for proceeding to the spawning induction.

A single intramuscular injection of 200 µg kg^{-1} of body weight (bw) of the slow release gonadotropin-releasing hormone analog (GnRHa) preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a., $C_{59}H_{84}N_{16}O_{12}$, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) was administered to females below the dorsal fin. Males were treated with 10 µg kg^{-1} bw of the same hormone. After treatment, the fish were transferred to the broodstock system at a sex ratio of 3 or 4 males for female. The system was an indoor recirculating aquaculture system (RAS) consisting in two black 3,500 L volume fiberglass tanks, provided with a mechanical (10 µm) and biological filter, UV lamp (80 W), protein skimmer and water chiller. Each tank had a gentle air supply and an overflow egg collector equipped with a 500 µm net-mesh. Water flow was set to produce a 30% water recirculation h^{-1}.

Salinity and temperature were 36.9±0.4 ppt and 23±0.4 °C, respectively. Dissolved oxygen (DO) was kept at 8.2±0.8 mg L^{-1}. Ammonia concentration was 0.03±0.03 mg L^{-1}, nitrite 0.2±0.1 mg L^{-1}, nitrate 7±3 mg L^{-1} and pH was 8.4±0.1.

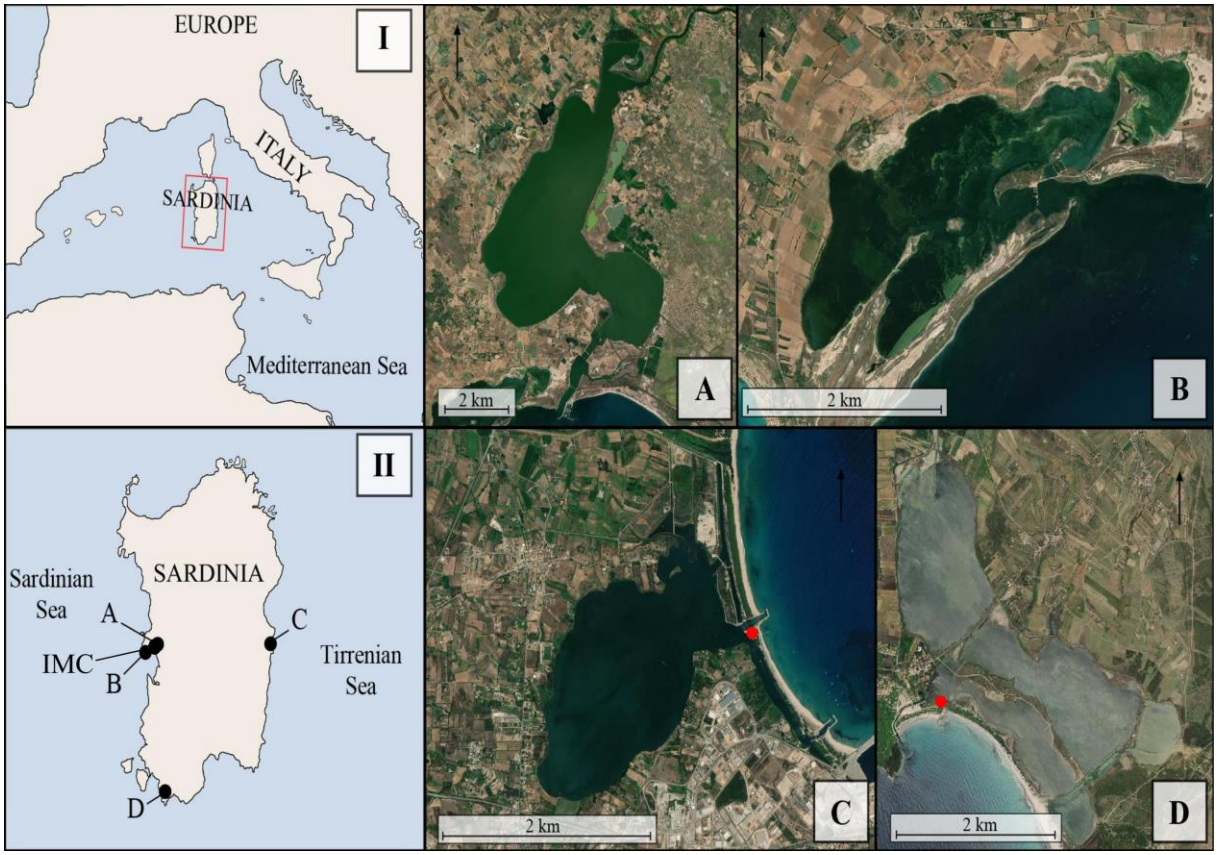

Fig. 1. Sardinian lagoons where *M. cephalus* breeders were collected. I, II: Sardinia and adult fishing site locations, respectively. Phase 1: A: Cabras, B: Mistras; Phase 2: C: Tortolì, D: Porto Pino. IMC: IMC's laboratory location. Red dot: location of the spawning sites.

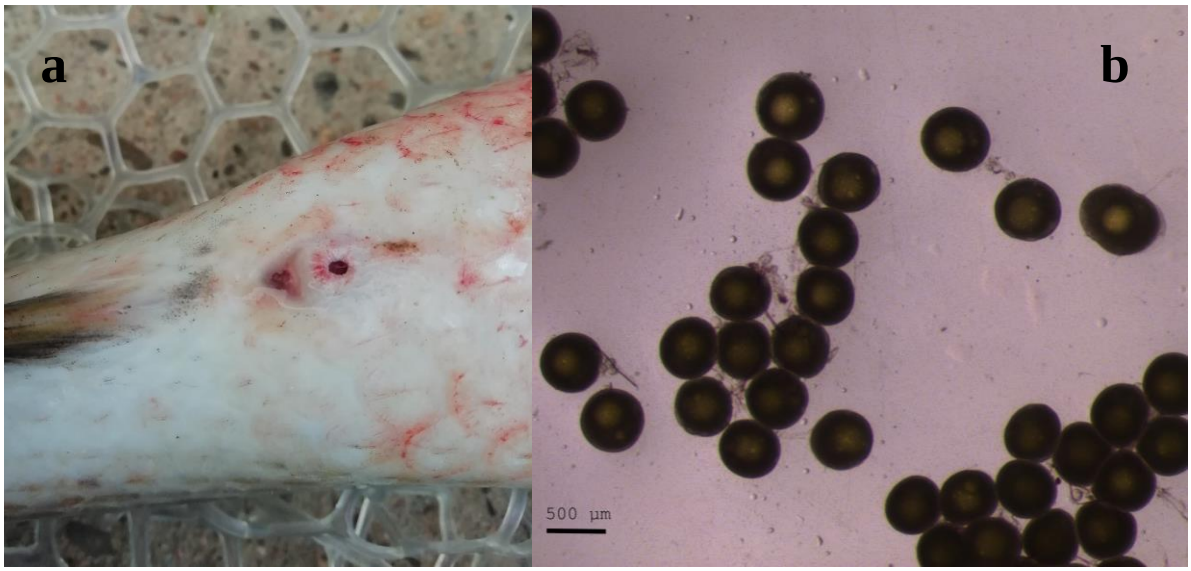

Fig. 2. a: fully mature *M. cephalus* female with red protruded papilla. b: oocytes in advanced vitellogenesis (average diameter: 578±13 µm).

2.1.2 Phase 2

During preliminary trials carried out in Phase 1 (Vallainc, 2017), we observed that it was possible to determine the sex of the breeders with good accuracy as all females undergoing sexual migration presented prominent and bulging abdomen. Furthermore, all females showing both a red, protruded papilla and a bulging abdomen resulted in fully vitellogenic oocytes (Fig. 2, Table 1). Thus, in order to reduce excessive stress and manipulation (Schreck et al. 2001), we assumed these distinctive characteristics as a signature of their maturity and in Phase 2 breeders were selected based on visual assessment alone. However, in order to check the maturity of animals, two days before collecting the broodstock, a few mullets captured by fishermen were subjected to an ovarian biopsy and/or to gentle pressure of the abdomen. Oocytes were placed on a micrometric slide and checked under a stereoscope (Carl Zeiss). All checked females (3 females and 3 males day^{-1}) presented fully mature oocytes larger than 550 µm and all the males released fluid sperm.

Fish collected were transported to the broodstock systems, placed about 50 m from the capture site in Porto Pino and about 100 m from the capture site in Tortolì (Fig. 1). Both systems involved a flow-

through set up with a 3500 L volume tank equipped with a submersible pump (0.5 hp) collecting the water from the capture site and an overflow egg collector (500 µm net-mesh). Each pool of breeders consisted of one female and four males. A mechanical scale (capacity=150 kg, division=50g) was used for determining the weight of the fish, by measuring the difference between the weight of the bag with the animal inside it and the tare weight (the bag containing 50 L of water taken from the capture site).

Eight hours after capture, the water inlet was turned off, tank volume was reduced to 350 L and the fish were partially anesthetized with a low dosage of clove oil (0.01 %). The operator gently entered the breeding tank wearing a smooth rubber diving suit and kneeled at the bottom of the tank. The head and back of each animal were covered with a wet hand towel and the animal was delicately trapped between the operator's knees. The animal tended to remain motionless. A single intramuscular injection of 200 µg kg^{-1} of body weight (bw) of ENANTONE was administered to the females, while the males were treated with 100 µg kg^{-1} bw of the same hormone.

The recirculation pump was turned on and set to produce a 30% water renewal h^{-1}. At Porto Pino, salinity and temperature for the spawning induction were 36.8±0.6 ppt and 24.5±1.2 °C, respectively. At Tortolì, salinity was 40.0±1.0 ppt and temperature was 22.3±1.0°C. Fish were exposed to a natural photoperiod and allowed to spawn spontaneously in the tank. Spawning success was monitored for a maximum of 39 hours after the hormone injection.

2.2 Egg incubation and hatching

Both in phase 1 and phase 2, eggs were checked under a light microscope (Leica MZ8) for viability, fertilization rate, measuring and counting. Fertilization rate was determined for each batch of eggs at 40 minutes from the onset of spawning (Abraham et al., 1999). Three samples of eggs (n=100; 20 eggs $female^{-1}$) were checked under a stereo microscope (Leica MZ8) for viability, for the presence of the

first cleavage stage and for the diameter measurements. Aeration was furnished to the eggs through an air stone placed in the bottom of the egg collector. A sub-sample (10 mL volume) of randomly distributed eggs was collected through a 10 mL pipette from the whole water column for counting (Panini et al., 2001).

In phase 1 both spawning and egg incubation took place at the IMC laboratories. Eggs were collected in 15 L buckets and transported to egg incubation systems. In phase 2 eggs were divided at a density of 4000 eggs L^{-1} into 50 L fish transport bags (120 x 50 cm), filled 1/3 with lagoon water and 2/3 with oxygen. Bags were kept at a constant temperature of 22 °C and transported to the IMC laboratories. Transport time was about 2 hours by car both for Porto Pino and for Tortolì.

Eggs were incubated at a density of 400−1000 eggs L^{-1} into 3 hatching systems (salinity 37±1 ppt; temperature 23±1°C). Hatching systems were 3 RAS with 3 circular, truncated, cone-shaped tanks of 300 L volume, each one equipped with a 500 µm net-mesh banjo filter, supplied with a biological and mechanical sand filters (0.5 µm), a UV lamp, a protein skimmer, water chiller and aeration produced by circular air stones. Water recirculation was set at 4 L h^{-1}. A 30% water exchange was performed every 6 h, and settled eggs were removed from the bottom of the tanks every 4 h. After 32 hours of incubation, eggs with embryos at the eye stage (Fig. 3) were collected and seeded in the larval rearing tanks at a density of 40 embryos L^{-1} and gentle aeration was furnished.

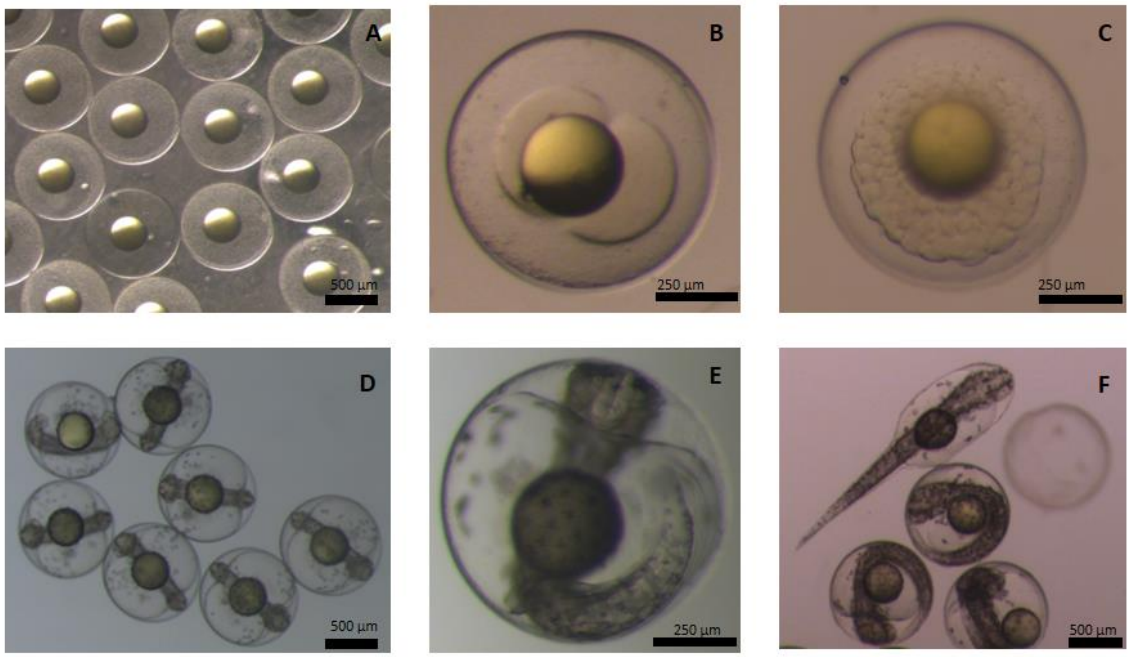

Fig. 3. A: Viable unfertilized eggs. B: Viable fertilized egg at 1st cleavage, 40 minutes after fertilization. C: Morula stage, 4 h after fertilization. D: Eggs at nerula stage, 15 h after fertilization. E: Organogenesis 2 stage, 30 h after fertilization. F: newly hatched larva, 36 h after fertilization.

After hatching was completed in each hatching tank, approximately 40 hours after fertilization, the aeration was turned off, the settled unhatched eggs were siphoned out, collected using a 500 µm net-mesh filter and counted volumetrically for the determination of the hatching percentages from the initial number of fertilized eggs seeded.

2.3 Larval rearing

As was done for reproduction, larval rearing was performed in a similar manner according to two phases. The first trials (phase 1) were used for the improvement of the rearing protocol to be applied and tested in the subsequent trials (phase 2). Larvae were reared in an indoor RAS in 6 circular fiberglass tanks of 2,000 L volume; the sides and bottom of the tanks were black and white,

respectively. The system was equipped with a biological and mechanical sand filter (0.5 µm), UV lamp, protein skimmer and water chiller. Each tank was supplied with temporized light at an intensity of 600 lx at the water surface.

During the preliminary trials, in phase 1, a total of 3 batches of fertilized eggs was obtained (Table 3). Each pool of larvae obtained from the first and second spawning was seeded into one of the tanks of the larval rearing system, the third was seeded into 2 tanks. The densities adopted were 20 larvae L^{-1} for the first pool and 40 larvae L^{-1} for the other two pools of larvae. In phase 2, on the other hand, 2 batches of fertilized eggs were obtained (Table 3). Each of the 2 batches of larvae were seeded as embryos in 6 larval rearing tanks at a density of 40 individuals L^{-1} as reported in 2.2.

In Fig. 4 the protocol used during the larviculture of *Mugil cephalus* is reported. For the first 14 days after hatching (dph), water recirculation was set at 5 % per day^{-1}, at 10% per day^{-1} from 14 to 25 dph and it was increased up to 20% from 25 to 35 dph, with further increasing steps from 35 dph onwards (Lee and Kelley, 1991). Water recirculation was turned off at each live food administration. Continuous light (24 L:0 D, 600 lx) was used until 13 dph, and then a 12 L:12 D photoperiod was adopted for the rest of the rearing phase.

Phytoplankton (*Isochrysis galbana* and *Tetraselmis suecica*, 1:1 in volume ratio) was added daily to the tanks to maintain the green medium at a concentration of 400,000 cells ml^{-1} for a period of 22 dph. The first feeding was administered at 2 dph and consisted of rotifers (*Brachionus spp.*) enriched for 12 h in a $6x10^6$ cells ml^{-1} *I. galbana* medium. Rotifer concentration was adjusted daily to 4 individuals ml^{-1} up to 22 dph. The administration of *Artemia* nauplii began at 12 dph and continued up to 22 dph. Enriched (Easy DHA Selco, INVE Aquaculture) *Artemia* nauplii began at 17 dph and continued up yo 34 dph at an average concentration of 2.5 nauplii ml^{-1}. This enrichment (0.6 gL^{-1}) was performed for 24 hours at a density of 250 nauplii mL^{-1}. Before adding them to the tanks, the nauplii were rinsed with tap water to eliminate any excess product. Artificial feed (Skretting) was added six times day^{-1}

progressively to the tanks from 22 dph onwards, and larvae were fed ad libitum. The “Gemma Wean 0.1” type (AF1, 62% protein, 14% lipid, 8% ash, 0.2% fibre) was administered from 22 to 34 dph.

Water parameters were monitored daily with a digital probe. The temperature was set at 22.1 ± 0.7 °C, salinity was 36.8 ± 0.4 ppt, and dissolved oxygen was 83 ± 14%. Once every 10 days 30% of the water was changed. Ammonia and nitrite were kept below 0.5 and 1 mg L^{-1}, nitrate below 25 mg L^{-1}. Faeces, unconsumed feed and dead individuals were siphoned out of the tanks daily from 22 dph onwards and discarded.

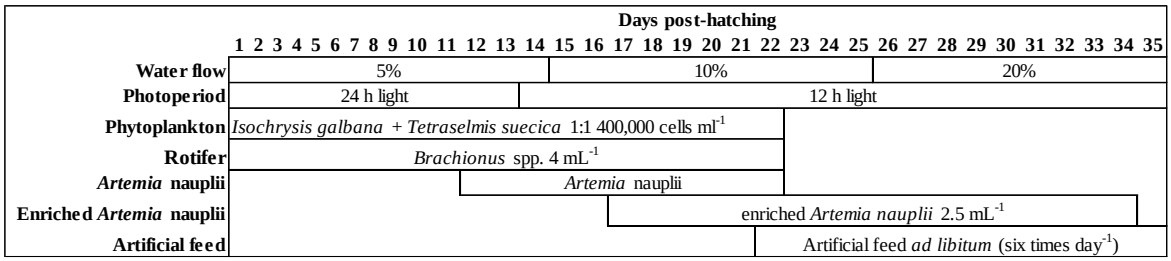

Fig. 4. Food sequence used during the larviculture of *Mugil cephalus*

2.4 Growth and survival

Larvae were randomly sampled from the 6 tanks. A total of 367 and 455 individuals, respectively from 2018 and 2019, were gradually anesthetised with clove oil up to overdose and measured in total length (TL, mm). Individuals up to 20 dph were photographed under the microscope (Leica, Germany) through a Leica DMC2009 and measured with LAS 4.5 software (Leica, Germany). Individuals older than 20 dph were photographed with a Canon G15 camera and measured through ImageJ (NIH, USA). Body weight

(bw, g) was determined by weighing randomly sampled individuals on an electronic scale. A total of 622 and 509 individuals, respectively from 2018 and 2019, were weighed.

Survival was determined at 34 and 60 dph. At 3 dph, survival was determined volumetrically (5 L volume) by counting the larvae collected from the water column of the rearing tanks. At 34 and 60 dph, the tanks were photographed from above with a Canon G15 camera and individuals were counted through ImageJ (NIH, USA). Biometrical data, TL (mm) and bw (g), were used to determine the specific growth rate (SGR%) as follows (Ricker, 1979):

$$SGR\% = [Ln(TL_t)-Ln(TL_i)] \times 100/ t$$

Where TL_t is the total length at time t and TL_i is the initial total length; t is the time (days) between TL_t and TL_i.

$$SGR\% = [Ln(bw_t)-Ln(bw_i)] \times 100 / t$$

Where bw_t is the body weight at time t and bw_i is the initial body weight; t is the time (days) between bw_t and bw_i.

2.5 Statistical analysis

Statistical comparison was carried out between the oocyte diameters of females which were successfully or unsuccessfully induced to spawn. Data were checked for normal distribution and for homoscedasticity by running the Shapiro-Wilk normality test and the LeveneTest, respectively. Data resulted normally distributed for both conditions tested (0.1449; 0.472). Since data did not satisfy the assumption of homoscedasticity (p=0.0353), the Welch Two Sample t-test was performed to verify significant differences between the two treatments. Statistical analyses were performed with R

software (R Core Team 3.X Ver). Normality was tested using the function “Shapiro.test”, homoscedasticity was tested using the package Desk tool, function “Levene Test” and the function “T.test” were used to perform the Welch Two Sample t-test.

3 Results

Table 2 summarizes the data on weight, oocyte diameter and spawning success for each induced female obtained in phase 1. A total of 19 females (2.2±0.8 kg bw) ranging from 1.3 to 4.4 kg bw were induced to spawn. No successful spawning induction was obtained from females with mean oocyte diameters ranging from 445±36 to 570±4 µm, with an average of 515.4±41.6 µm (n=65). In these individuals, 28% of the total oocytes measured were larger than 550 µm. Fish that were successfully induced had a mean oocyte diameter ranging from 566±16 to 599±5 µm with an average of 577.6±13.1 µm (n=35). In these individuals 97% of the oocytes resulted larger than 550 µm. Indeed, after statistical tests, the oocyte diameters recorded in successful spawning inductions resulted significantly larger ($p<0.001$) than those observed in unsuccessful trials (Fig. 5).

However, since in 57% of the cases males failed to fertilize the eggs, fertilized eggs were obtained only in 3 upon 7 spawning induction trials (43%) (Table 2, individuals 1*, 2* and 3*). About 70% of the individuals handled according to phase 1 protocol developed rapidly diffusing bacteriosis characterized by white stains and necrotic lesions on the body, head and fins. On the other hand, applying the phase 2 handling method, spawning induction and fertilization were obtained successfully in 100% of the trials and none of the animals developed infections.

Table 2: Successful and unsuccessful spawns after administration of 200 µg kg-1 GnRHa. *: individuals whose eggs were then successfully fertilized.

Unsuccessful			Successful		
Fish no.	Bw (kg)	Oocyte diameter (µm; mean±SD)	Fish no.	Bw (kg)	Oocyte diameter (µm; mean±SD)
1	3.3	558±33	1*	4.4	572±15
2	2.2	555±26	2*	1.3	599±5
3	1.4	566±17	3*	2.2	593±6
4	1.7	570±4	4	2.7	575±12
5	2	501±36	5	2.5	566±16
6	3.1	524±26	6	1.8	567±13
7	1.4	499±29	7	1.8	571±32
8	1.8	513±19			
9	2.1	514±20			
10	3.2	454±48			
11	2.1	486±38			
12	1.8	445±36			
average	2.2±0.7	515.4±41.6		2.4±1	577.6±13.1

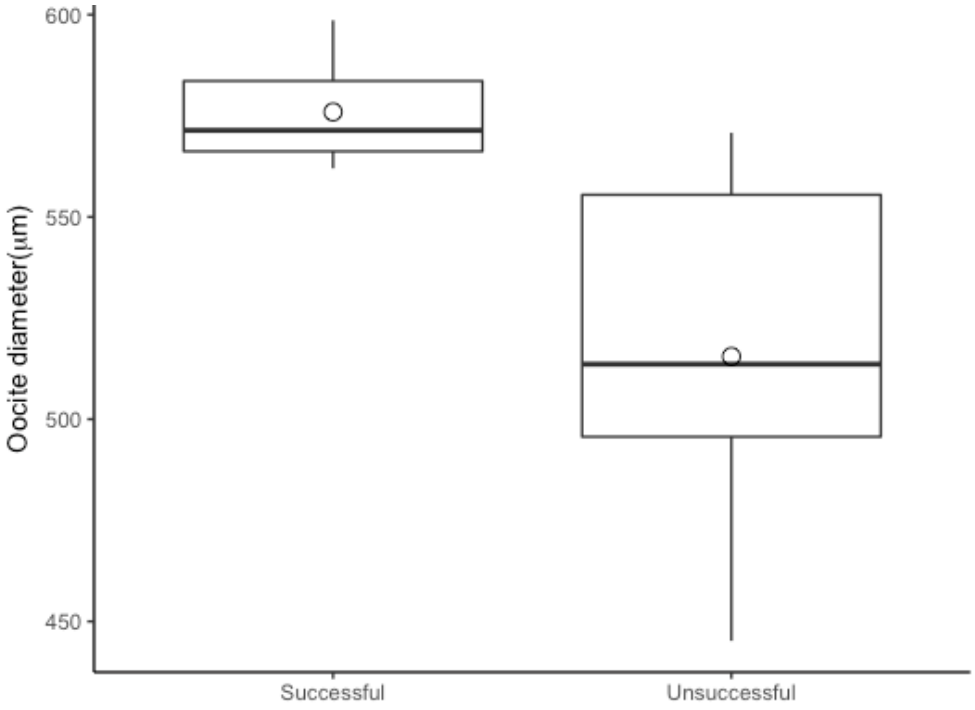

Fig. 5. Box plot (min, 25%, median, average, 75%, max) of oocyte diameters (n=100).

Spawning occurred more than 37±2 hours after the hormone injection. Successful spawning induction results are reported inTable 3. Spawning inductions carried out according to phase 1 are characterized by an higher dose of sedative (clove oil 0.08%) than those performed in phase 2 (clove oil 0.01%), as female were completely sedated and subjected to ovarian biopsy. The hormone dosage used for males was 10 times higher in phase 2 (100 µg kg bw^{-1}) than in phase 1 (10 µg kg bw^{-1}). The females used for fry production in this study ranged from 1.3 to 4.4 kg bw and spawned from 1.1 to 3.1 million eggs, corresponding to an average of 799 ±109 eggs g^{-1} bw. Males, ranging from 0.7 to 2.1 kg bw, had an average of 1.1±0.4 kg bw. Fertilization rate varied from 58±8 to 93±3 % with an average of 83±14%. Hatching rate ranged from 55±8 to 87±8%, average hatching rate was 76±13%.

Table 3 Egg production data. Phase: according to Table 1. N: number of breeders (Females-Males). *: not measured (see Table 1). Spawning inductions carried out according to phase 1 are characterized by an higher dose of sedative (clove oil 0.08%) than those performed in phase 2 (clove oil 0.01%), as females were completely sedated and subjected to ovarian biopsy. Hormone dosage for males was much higher in phase 2 (100 µg kg bw^{-1}) than in phase 1 (10 µg kg bw^{-1}). The females used for fry production in this study ranged from 1.3 to 4.4 kg bw and spawned from 1.1 to 3.1 million eggs, corresponding to an average of 799 ±109 eggs g^{-1} bw. Males, ranging from 0.7 to 2.1 kg bw, had an average of 1.1±0.4 kg bw. Fertilization rate varied from 58±8 to 93±3 % with an average of 83±14%. Hatching rate ranged from 55±8 to 87±8%, average hatching rate was 76±13%.

Phase	N (F-M)	Females bw (kg)	Sedation (clove oil %)	Oocytes diameter (µm)	Males bw (kg)	Hormone dose for males (µg kg bw^{-1})	Hormone dose for females (µg kg bw^{-1})	Eggs ($x10^6$)	Fecundity (eggs g bw^{-1})	Fertilization rate (%)	Hatching rate (%)
1	1-3	4.4	0.08	562±6	1.4±0.6	10	200	3.1	704	84±6	84±11
	1-3	1.3	0.08	599±5	1.3±0.4	10	200	1.1	842	88±7	87±8
	1-4	2.2	0.08	592±6	1.0±0.2	10	200	1.8	818	92±6	72±12
2	1-4	2.2	0.01	*	0.9±0.2	100	200	1.5	682	93±3	80±5
	1-4	2	0.01	*	1.0±0.4	100	200	1.9	950	58±8	55±8

The newly hatched *M. cephalus* larvae obtained in phase 1 were 2.2±0.2, 2.2±0.4 and 2.1±0.4 mm TL and 0.11±0.08, 0.20±0.09 and 0.13±0.03 g bw. Larvae obtained in phase 2 were 2.7±0.1 mm and 1.8±0.2 mm in TL and 0.06±0.02 mg and 0.24±0.05 mg in bw. Newly hatched larvae had a homogenous yolk mass and a round oil droplet located at the posterior part of the yolk sac. A primordial marginal fin fold surrounded the body from the dorsal part of the head to the posterior margin of the yolk sac (Loi et al., 2020). The eyes started becoming pigmented between 1 and 2 dph and the anus was clearly visible behind the intestine. The pre-larval stage lasted until 3 dph, when larvae had already consumed most of their yolk reserves while most of the oil globule remained. At 3 dph, the mouth opened at the rostral part of the head and was differentiated into lower and upper movable jaws. The pectoral fins were visible and functional at this time (Loi et al., 2020). The transition from larval to juvenile stage (Fig. 6) occurred at 34 dph (Thieme et al., 2020).

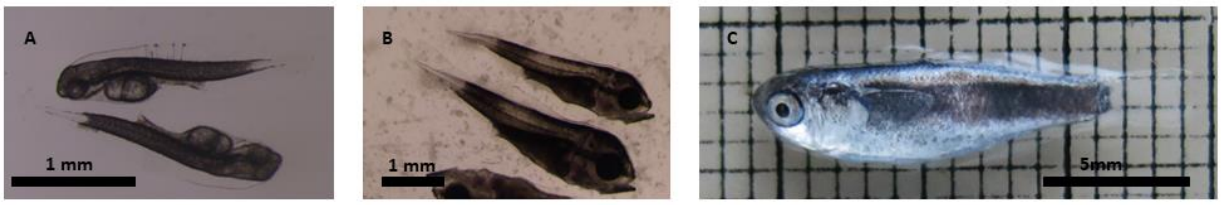

Fig. 6. Larval stages. A: larvae at 0 dph. B: larvae at 12 dph. C: fully metamorphosed juvenile at 34 dph.

During the larval rearing phase, the SGR% recorded in phase 1 were 5.6±0.1 (n=18), 2.0±0.3 (n=27) and 2.3±0.6 (n=20) in TL and 16.5±0.4 (n=18), 5.4±0.3 (n=27) and 7.5±0.6 (n=20) in BW (Table 4). Larval survival were 2%, 18% and 36%.

However, in phase 1, during the preliminary trials, the available data on larval growth were not robust enough to be considered, because of a lack of an adequate number of repetitions. Thus, we will only examine more in detail the larval growth results obtained in phase 2 (years 2018 and 2019), based on a greater number of observations.

Table 4 Growth and survival data during larval rearing trials. Phase: according to Table 1.

Phase	Larval rearing trials	Dph	N	TL SGR (%)	N	BW SGR (%)
1	1	0-46	18	5.6±0.1	18	16.5±0.4
	2	0-39	27	2.0±0.3	27	5.4±0.3
	3	0-42	20	2.3±0.6	20	7.5±0.6
2	1	0-34	247	4.8±0.7	578	17.9±0.3
	2	0-34	320	5.1±0.6	474	10.9±0.2

During the whole larval rearing phase, from 0 to 34 dph, the SGR% recorded in 2018 were 4.8±0.7 (n=247) and 17.9±0.3 (n=578) in TL and bw respectively. In 2019, SGR% were 5.1±0.6 (n=320) and 10.9±0.2 (n=474) in TL and BW respectively (Fig. 7).

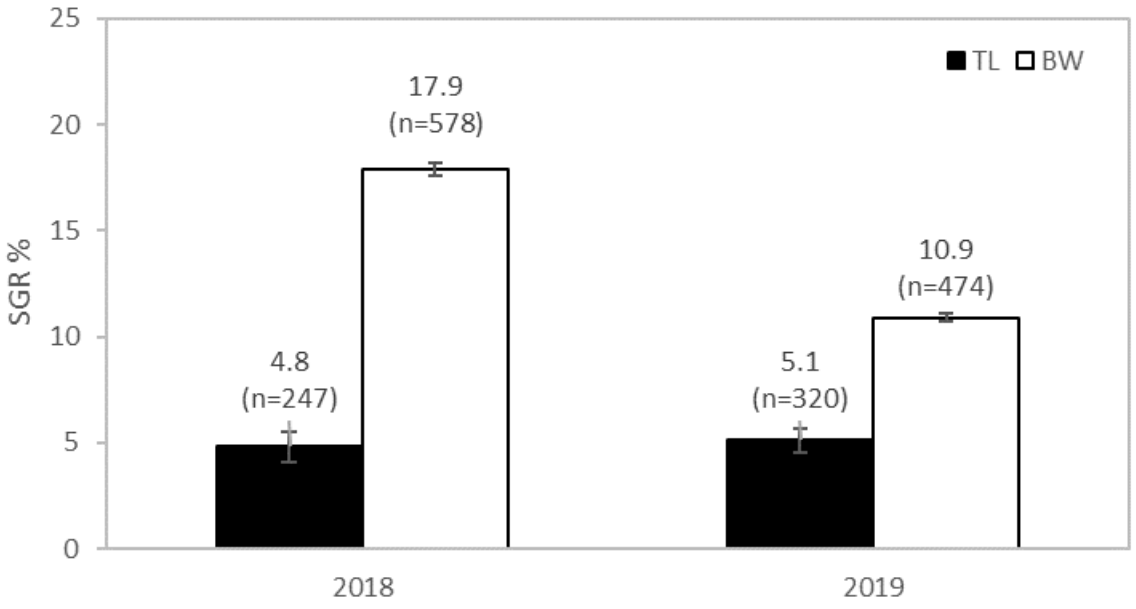

Fig. 7. Specific growth rate of flathead grey mullet during the larval stage (0-34 dph).

The growth in length of mullet larvae and juveniles was described by the equations $y = 2.58\ e^{0.043x}$ (R^2=0.94) and $y = 2.06\ e^{0.0412x}$ (R^2=0.89) for 2018 and 2019, respectively (Fig. 8). The growth in weight was described by the equations $y = 0.07e^{0.1508x}$ (R^2=0.94) and $y = 0.3\ e^{0.0947x}$ (R^2=0.95) for 2018 and 2019, respectively. Length-growth equations were obtained by measuring 367 and 455 individuals, in 2018 and 2019, respectively. Body weight equations were calculated by weighting 622 and 509 individuals from 2018 and 2019, respectively. Survival rates at 3 dph were 45±5% and 63±3%, at 34 dph they were 15±5% and 20±5% and at 60 dph they were 10±2 and 19±1, respectively in 2018 and 2019.

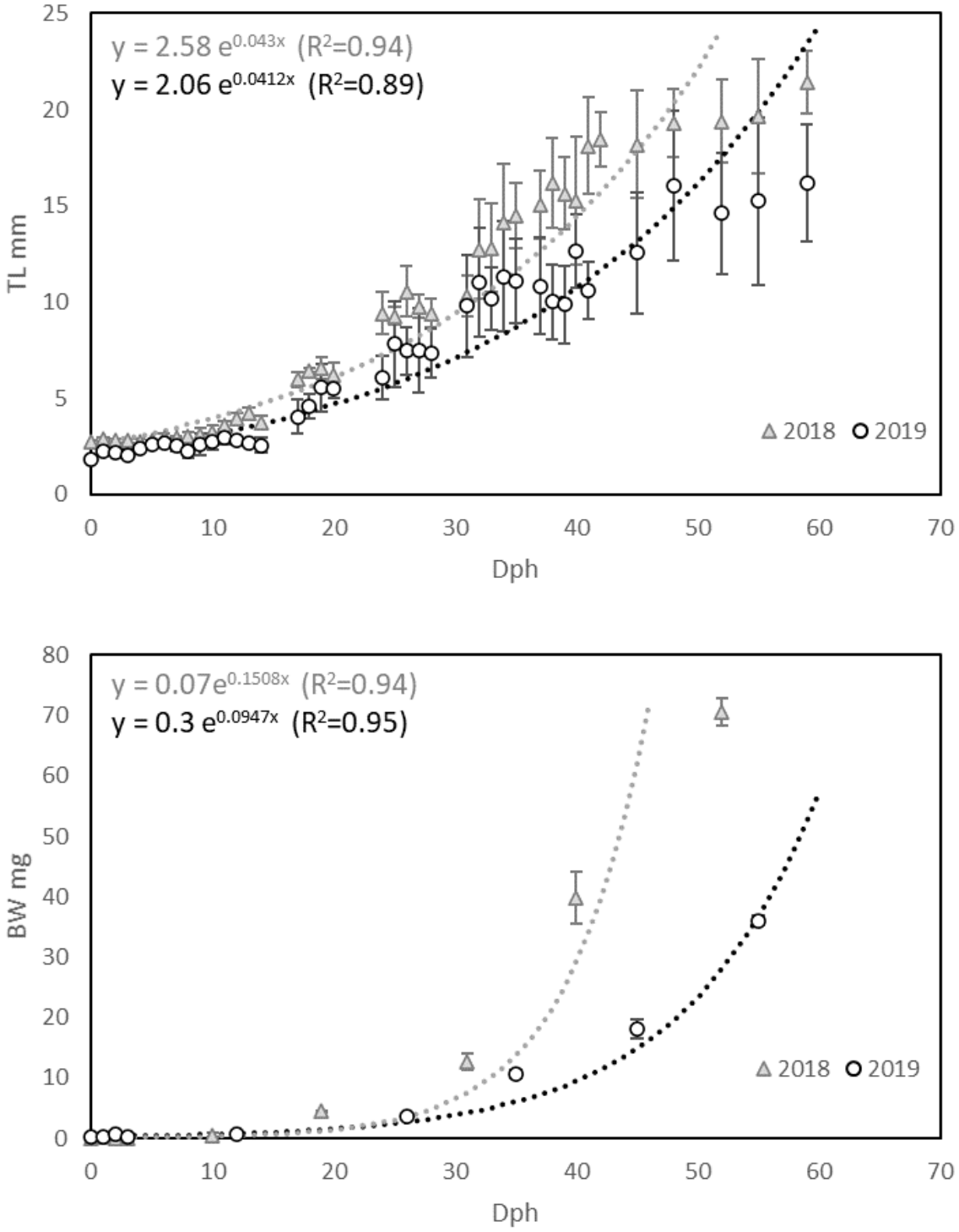

Fig. 8. Length and weight growth (average ± standard deviation) of flathead grey mullet larvae in 2018 (grey) and 2019 (black).

4. Discussion

Our results confirm that having a certain average oocyte diameter is crucial for successful spawning induction. In particular, induction was successful for the oocytes with a diameter between 570 and 600 µm, in advanced vitellogenesis at the tertiary yolk globule stage, whereas all females with oocyte diameters below 570 µm failed to spawn (Crosetti and Blaber, 2015). This agrees with the results obtained by Lee et al. (1987) in Hawaii (USA) and by Meseda and Samira (2006) in Egypt. The females used for fry production in this study spawned on average 799 ±109 eggs g^{-1} bw, thus confirming the high fecundity of the species (Liao, 1981).

Using the described protocols of capture and handling, the described results for egg quality and hatching were achieved. From our experience in phases 1 and 2, we can hypothesize that the following aspects were important: (i) improvements in the capture, transport and selection of wild adults, which was introduced into the broodstock collection protocol in order to reduce excessive stress and handling, and (ii) the selection of females on the basis of a visual assessment of their maturity , which avoided their transport far from the place of collection. All females showing a red, protruded papilla and a bulging abdomen had fully vitellogenic oocytes (diameter>570 µm) and underwent successful spawning.

Adopting lower dosages of hormones to induce spawning could represent a step towards a more economically sustainable production process of the grey mullet by reducing the costs, therefore promoting the commercial-scale production of this species (Crosetti and Blaber, 2015). Moreover, as already highlighted by Rahdari et al., (2013), identifying the minimum optimum dose of hormones to induce spawning is desirable for the broodstock's welfare and for obtaining the best breeding performance in fish. Lee and Tamaru (1988) employed CPE (20−70 mg kg^{-1}) or hCG (10.000 IU kg^{-1}) and LHRHa (200 µg kg^{-1}) to induce spawning in mullets. Crosetti and colleagues (Crosetti, 2001; Crosetti and Cordisco, 2001) used pituitary gland extract (PG) 7 + LHRH 100/200 µg + DOM 10 mg + leuprolin acetate 500/700 µg + leuprolin acetate 600/700 µg kg^{-1} bw. Vazirzadeh and Ezhdehakoshpour (2014) used CPE (20 mg kg^{-1})+ metaclopramide (20 mg kg^{-1}) + GnRHa (200 µg kg^{-1}). Mousa et al. (2018) administred carp pituitary extract (CPE) (20 mg kg^{-1} bw) or human chorionic gonadotropin (hCG) (10,000 IU kg^{-1} bw) + LHRHa (200 µg kg^{-1}). Besbes et al. (2020) treated fish with a priming

and a resolving dose of LHRHa (200 µg kg^{-1} bw). The same author used also a combination of hCG (from 5000 to 10000 IU kg^{-1}) as a priming injection, followed by resolving injections of hCG (from 5000 to 10000 IU kg^{-1}) + LHRHa (from 100 to 200 µg kg^{-1} bw).

Catching mature adults already in advanced vitellogenesis (tertiary yolk globule stage) in Sardinian lagoons is a definite advantage since it makes only one injection necessary. In most cases, two injections are necessary to induce spawning because in captivity the vitellogenesis is blocked at the secondary yolk globule stage. In our study, we successfully induced the spawning of wild female *M. cephalus* using a single injection of Enantone at a concentration of 200 µg kg^{-1} of bw. The spawning of viable eggs was achieved in 58% of the females subjected to ovarian biopsy with oocyte diameters above 550 µm, while 100% of spawning success was obtained when females were selected based solely on a visual assessment of their maturity (bulging abdomen and red papilla). By checking males for fluency and treating them with a single injection of 10 µg bw kg^{-1} of Enantone, as was done in phase 1, fertilization was achieved in 43% of the attempted trials, while with a dose of 100 µg bw kg^{-1} of the same homone, fertilized eggs were obtained in 100% of the trials. Moreover, 70% of the individuals handled according to phase 1 protocol developed rapidly diffusing bacteriosis, while none of the fish treated according to phase 2 methods, either male or female, presented bacteriosis.

Female fecundity observed in this study (Table 3), was similar to that reported by Kuo et al. (1973), 600 eggs g^{-1} bw, Nash et al. (1974), 800 eggs g^{-1} bw and Meseda and Samira (2006), 1395 eggs g^{-1} bw. Fertilization rates ranged from 58 to 93% with an average of 83% in the 5 successful reproduction trials (Table 3). The low fertilization rate observed in phase 2 in the batch of eggs obtained from Porto Pino (58%), could be due to a lower quality of gametes. Indeed, gamete quality, both in wild and in captive breeders, is influenced by many factors and is sometimes highly variable (Migaud et al., 2013; Bobe and Labbe, 2010; FAO, 2020). Furthermore, highly variable fertilization rates were reported also from Azien et al. (2005) who when inducing a total of 12 *M. cephalus* females to spawn, obtained more than 90% fertilization rate in 4 trials and values lower than 40% in the remaining 8 trials. Low hatching rates were also obtained from Abrham et al. (1999), who recorded a value of 41.6% when inducing *M. cephalus* females to spawn.

The hatching rates observed were quite satisfying, with an average of 76% within the 5 batches. The lowest hatching rate observed (55%), as for the fertilization rate, was obtained from the batch of Porto Pino, confirming the hypothesis that these values could be due to the lower quality of the gametes. However, the low hatching rate value could probably have been influenced by the high egg-incubation density tested (1000 eggs L^{-1}). Indeed, Nash and Shehadeh (1980) suggest 200 egg L^{-1} as the optimal egg-incubation density for the embryos of this species.

Moreover, also the TLs of the larvae are comparable with observations by Kuo et al. (1973), who reported that the total length of the newly hatched *M. cephalus* larvae in their study were 2.65±0.23 mm, and Meseda and Samira (2006), ~1.97±0.23 mm. Larval growth in total length reported in our study are slightly lower than those reported by Liao (1975), Lee and Kelley (1991) and Murashige et al. (1991). These authors, by rearing the larvae at lower densities (10-25 larvae L^{-1}) and testing individuals belonging to different genetic lineage of *M. cephalus* species complex (Durand and Borsa, 2015), obtained individuals ranging 3.4-5.5 mm TL at 10-15 dph and 8.8-15.0 mm TL at 25–28 dph. In our study larvae reached 2.8-3.3 mm TL at 12 dph and 6.6-7.4 mm TL at 22 dph (Fig. 8).

Few studies report the SGR of flathead grey mullet larval stages. Values reported in our study concerning the first 34 dph (4.8±0.7 and 5.1±0.6 in total length and 17.9±0.3 and 10.9±0.2 in body weight) are comparable to and slightly higher than those obtained in Besbes et al. (2020), who report an average SGR% for the first 25 dph of 5.95 in total length and 9.22 in body weight. The pools of larvae from 2018 and 2019 show similar SGR% values in TL, while the 2018 pool resulted in a higher SGR% in bw (Fig. 7). The growth differences observed could be probably due to intrinsic differences between the two batches of eggs and/or to the different rearing densities determined by the different survival rates of the two pools of larvae (45±5% for 2018 and 63±3% for 2019 at 3 dph).

The averaged survival rate observed during the study period (0-34 dph) is 18±3%, that appears quite low compared to the 72% survival rate at 35 dph observed by Ako et al. (1994). However, since Ako and colleagues reared the larvae at a much lower densities (5 larvae L^{-1}) than us (40 larvae L^{-1}), the survival rate they had is

equal to 3.6 larvae L^{-1}, whereas in our study we obtained a survival rate of 7 individuals L^{-1}, i.e. almost twice than Ako et al. (1994). Our results could be important in reaching a more profitable and economically sustainable production process. The optimal larval rearing density reported in the literature is indeed 20 larvae L^{-1}, and it dates back to Nash and Shehadeh (1980). Similarly, Yousif et al. (2010), after testing a density of 30 individuals $L^{-1,}$ observed that lower densities would produce higher survival rates. On the other hand, the final average survival rate at 60 dph that we obtained in this study (14±5%) falls within the survival range of 5-34% obtained by other authors, who reared the larvae at a maximum density of 30 individuals L^{-1} (Liao et al., 1971; Kuo et al., 1973; Eda et al., 1990; Murashige et al., 1991; Yousif et al., 2010; Besbes et al., 2020). Indeed, Eda et al. (1990), rearing the larvae at a similar density to the one adopted in this study (43 larvae L^{-1}), obtained a rate of 5.6% survival at 60 dph. Murashige et al. (1991), testing a rearing density of 17 larvae L^{-1}, observed a survival rate of 19%. Yousif et al. (2010), rearing the larvae at a density of 30 individuals L^{-1}, reported a survival of 15% at 40 dph, and Besbes et al. (2020) obtained a survival of 10% at 60 dph at the very low density of 1.5 larvae L^{-1}. However, even if results achieved by applying our protocols to obtain eggs and for larval rearing are very encouraging, further repetition is needed to consolidate them.

5. Conclusions

From our observations we can hypothesize that the handling method for reducing stress in capturing, transporting and selecting wild adults is important for a successful spawning induction.

A single dose of 200 µg kg^{-1} of bw of the slow release GnRHa preparation ENANTONE® (Takeda Italia S.p.a.) was successful in inducing spawning in flathead grey mullet females with an average oocyte diameter larger than 570 µm.

The rearing density of 40 individuals L^{-1} resulted in an average survival rate comparable to or even better than those obtained by other authors, thus indicating that the application of the protocols described here could contribute to designing a more profitable and economically sustainable production process, by

reducing costs and optimizing production. Indeed, the final average survival rate obtained in this study (14±5%) supports the possibility of producing *M. cephalus* fries in captivity from sexually mature adults captured in Sardinian lagoons.

6. References

Abraham M., Shiranee P., Kishore Chandra P., Kailasam M., Charles V.K., 1999 Embryonic and larval development of the striped mullet *Mugil cephalus* (L). Indian J Fish 46(2):123-131

Aizen J., Meiri I., Tzchori I., Levavi-Sivan B., Rosenfeld H., 2005 Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen Comp Endocrinol 142:212-221

Ako H., Tamaru C.S., Bass P., Lee C.S., 1994). Enhancing the resistance to physical stress in of *Mugil cephalus* by the feeding of enriched Artemia nauplii. Aquaculture, 122: 81–90.

Besbes R., Benseddik A.B., Kokokiris L., Hamza A., Kammoun F., 2020. Thicklip (*Chelon labrosus*) and flathead (*Mugil cephalus*) grey mullets fry production in Tunisian aquaculture. Aquaculture Reports, 17 (2020) 100380.

Bobe J., and Labbé C., 2010. Egg and sperm quality in fish. General and Comparative Endocrinology, 165: 535-548.

Brown C. and Day R. 2002. The future of stock enhancements: lessons for hatchery practice from conservation biology. Fish and Fisheries 3: 79-94.

Cataudella S., Tancioni L., Cannas A., 2001. L'acquacoltura estensiva. pp. 283–306. In: S. Cataudella and P. Bronzi (eds.). Acquacoltura Responsabile. Unimar-Uniprom, Roma.

Crosetti D., 2001. Messa a punto di una nuova tecnica di riproduzione controllata nei Mugilidi. Progetto di ricerca MiPA, Direzione Generale della Pesca e dell'Acquacoltura, 4° Piano Triennale Pesca Marittima ed Acquacoltura, L.41/82, 110p. Final report.

Crosetti D., Cordisco C.A., 2001 Prove di riproduzione controllata della volpina, *Mugil cephalus*. Conferenza Internazionale dell' Acquacoltura "Dove va l'acquacoltura del 2001 nei paesi del Sud Europa?", Verona, 26-27 april 2001, 56.

Crosetti D, 2016. Current State of Grey Mullet Fisheries and Culture. Pp. 398–450. In: Crosetti D, Blaber S (2016) Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA.

Crosetti D., Blaber S., 2016. Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA.

Durand J.D., Borsa P., 2015. Mitochondrial phylogeny of grey mullets (Acanthopterygii: Mugilidae) suggests high proportion of cryptic species. C. R. Biologies 338: 266–277.

Eda H., Murashinge R., Ooseki Y., Hagiwara A., Eastham B., Bass P., Tamaru C.S., Lee C.S., 1990. Factors affecting intensive larval rearing of striped mullet, *Mugil cephalus*. Aquaculture 91:281–294.

González-Castro M., Ibáñez A.L., Heras S., Roldán M.I., Cousseau M.B., 2012. Assessment of lineal versus landmarks-based morphometry for discriminating species of Mugilidae (Actinopterygii). Zoolog Stud 51:1515–1528

Jeney Z., Bekh V., 2020. Technical Manual on Broodstock Management of Common Carp and Chinese Herbivorous Fish. Fisheries and Aquaculture Circular No.1188. Ankara. FAO.

Kuo C.M., Shehadeh Z.H., Milisen K.K., 1973. A preliminary report on the development, growth and survival of laboratory reared larvae of the grey mullet (*Mugil cephalus* L.). J. Fish Biol. 5: 459–470.

Kuo C.M., 1995. Manipulation of ovarian development and spawning in grey mullet, *Mugil cephalus* L. Isr J Aquac 47:43–58.

Lee C.S., Tamaru C.S., Miyamoto G.T., Kelley C.D., 1987. Induced spawning of grey mullet (*Mugil cephalus*) by LHRH-a. Aquaculture 62:327–336.

Lee C.S., Tamaru C.S., 1988. Advances and future prospects of controlled maturation and spawning of grey mullet (*Mugil cephalus*) in captivity. Aquaculture 74: 63–74.

Lee C.S., Kelley C.D., 1991. Artificial propagation of mullet in the United States. In J.P. McVey, ed. Handbook of Mariculture, pp. 193–209. FL, USA, Finfish Aquaculture, Vol. II.

Lee C.S., Tamaru C.S., Kelley C.D., Moriwake A., Miyamoto G.T., 1992. The effect of salinity on the induction of spawning and fertilization in the striped mullet, *Mugil cephalus*. Aquaculture 102: 289-296

Liao I.C., Lu Y.J., Huang T.L., Lin M.C., 1971. Experiments on induced breeding of the grey mullet, *Mugil cephalus* Linnaeus. Aquaculture 1: 15–34.

Liao I.C. 1975. Experiments on induced breeding of the grey mullet in Taiwan from 1963 to 1973. Aquaculture 6:31–58

Liao I.C. 1981. Aquaculture of Grey Mullets: cultivation methods. In: O.H. Oren (ed.). Aquaculture of Grey Mullets. Cambridge University Press, Great Britain. pp. 361–389.

Liao I.C., Chao N.H., Tseng C.C., 2015. Capture and culture of Mugilidae in Taiwan. In: Crosetti D, Blaber S (2016) Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA.

Loi B., Papadakis I.E., Leggieri F., Papiol G.G., Vallainc D., 2020. Ontogeny of the digestive system and eye of reared flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758), and evaluation of lipid deposition in the liver according to the feeding protocol. Aquaculture 526: 1-13.

McDonough C.J., Roumillat W.A., Wenner C., 2005. Sexual differentiation and gonad development in striped mullet (*Mugil cephalus*) from South Carolina estuaries. Fish Bull 103:601–619.

Meseda M.E.G. and Samira S.A., 2006. Spawning induction in the Mediterranean grey mullet *Mugil cephalus* and larval developmental stages. African J. Biotechnology 5: 1836–1845.

Migaud H., Bell G., Cabrita E., McAndrew B., Davie A., 2013 Gamete quality and broodstock management in temperate fish. Rev Aquaculture 5(S1): S194–S223.

Mousa M.A., Kora M.F., Khalil N.A., 2018. Evaluation of the effectiveness and cost of different hormones in stimulating the spawning of thin lipped grey mullet, *Liza ramada*. Egypt. J. Histol. 41, 275–283.

Murashige R., Bass P., Wallace L., Molnar A., Eastham B., Sato V., Tamaru C. and Lee C.S., 1991. The effect of salinity on the survival and growth of striped mullet (*Mugil cephalus*) larvae in the hatchery. Aquaculture 96: 249-254.

Nash C.E., Kuo C.M. and Mc Connell S.C., 1974. Operational procedures for rearing larvae of the grey mullet (*Mugil cephalus* L.). Aquaculture 3: 15–24.

Nash CE, Shehadeh ZH (1980) Review of breeding and propagation techniques for grey mullet, *Mugil cephalus* L. ICLARM Studies and Reviews 3, International Center for Living Aquatic Resources Management, Manila, Philippines. 87pp.

Panini E., Mylonas C.C., Zanuy S., Carrillo M., Ramos J., Bruce M., 2001. Incubation of embryos and larvae of marine fish using microtiter plates. Aquac. Int. 9, 189 – 196.

Pellizzato M., 2011. Classic and modern valliculture. pp. 237–238. In: Cataudella, S. and Spagnolo M., (eds.). The State of Italian Marine Fisheries and Aquaculture. Ministero delle Politiche Agricole, Alimentari e Forestali (MiPAAF), Roma, Italy.

Rahdari A., Gharaei A., Ghaffari M., 2013. Spawning Latency Period in Hormonal Induced Reproduction of Snow trout (*Schizothorax Zarudnyi* (Nikolskii, 1897)). Department of fisheries, Hamoun International Wetland Research Institute, University of Zabol, Zabol, I.R. Ir

Ricker W.E. 1979. Growth rates and models. In: Hoar WS, Randall DJ, Brett (eds.), Fish physiology Vol. VIII: bioenergetics and growth. Academic Press, Flórida, pp. 677-743.

Schreck C.B., Contreras-Sanchez W., Fitzpatrick M.S., 2001 Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197:3–24.

Shehadeh Z.H., Kuo C.M., Milisen K.K., 1973. Validation of method for monitoring ovarian development in the grey mullet (*Mugil cephalus*). J Fish Biol 5:489–496

Shields R.J., Brown N.P., Bromage N.R., 1997. Blastomere morphology as a predictive measure of fish egg viability. Aquaculture 155, 1–12.

Tamaru C.S., Ako H., Lee C.S., 1992 Fatty acid and amino acid profiles of spawned eggs of striped mullet, *Mugil cephalus*. Aquaculture. 105:83–94.

Thieme P., Vallainc D., Moritz T., 2020. Postcranial skeletal development of *Mugil cephalus* L (Teleostei: Mugilidae): morphological and life-history implications for Mugiliformes. Zoological Journal of the Linnean Society, XX, 1-19.

U.S. Agency for International Development, Bureau for Global Programs, Filed Support and Research, Center for Economic Growth and Agricultural Development, 2007, Final report.

Vallainc D. 2017. Reproduction and rearing of the flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758) and of the sea urchin, *Paracentrotus lividus* (Lamark, 1816) for restocking purposes. PhD thesis. Università degli studi della Tuscia di Viterbo 110 pp.

Vazirzadeh A., Ezhdehakoshpour A., 2014. The effects of different hormonal treatments on the oocyte maturation in wild grey mullet (*Mugil cephalus*) collected from the iranian coastal waters of the Oman sea. Iran. J. Ichthyol. 1, 17–22.

Yousif O.M., Fatah A.A., Krishna K., Minh D.V., Hung B.V., 2010. Induced spawning and larviculture of grey mullet, *Mugil cephalus* (Linnaeus 1758) in the Emirate of Abu Dhabi. Aquac Asia Mag 15:41–43

Chapter III: Spawning induction and larval rearing in the thinlip grey mullet (*Chelon ramada*). The use of the slow release gonadotropin releasing hormone analog (GnRHa) preparation, leuprorelin acetate

This chapter is based on the publication: Vallainc D, Concu D, Loi B, Pitzalis A, Frongia C, Chindris A, Carboni S (2022). Spawning induction and larval rearing in the thinlip grey mullet (*Chelon ramada*). The use of the slow release gonadotropin releasing hormone analog (GnRHa) preparation, leuprorelin acetate

1. Introduction

In May 2021 the European commission has published its "Strategic guidelines for a more sustainable and competitive EU aquaculture for the period 2021 to 2030". Highlighting that *"…the sector can be made more competitive by further diversifying EU aquaculture production"* and that *"Special attention should be given to the development of aquaculture with a lower environmental impact"* (COM (2021) 236 final of 12.5.2021). Within this scenario, alternatives to carnivorous species will have to be identified and fully domesticated to support current ambitions to fulfil the ever-increasing demand for sustainable seafood products. The family *Mugilidae* has often being identified as presenting suitable biological characteristics to achieve this goal: fast growth, low trophic feeding habits, adapted to a wide range of environmental conditions (salinity and temperature) and a variety culture systems (Gonzalez-Castro and Minos, 2016) including polyculture (Saleh, 2008). Nonetheless, large scale commercial production of this group of species is still lacking being currently limited by the absence of a cost-effective and reliable juveniles production sector, indeed currently only 3 species of Mugilids are farmed and their domestication level is still very low due to several bottlenecks of their life cycle in captivity (Teletchea, 2019). Adequate production of quality seeds remains therefore a major challenge for the expansion of mullet farming industry across its geographical range (Brown and Day, 2002).As a consequence, the demand for Mugilids juveniles for on-growing has generated a gap between seed supplies and farmer's demand (El-Sayedand and El-Ghobashy, 2011). This gap is currently filled by the

collection of mullet juveniles during their seasonal migrations from the spawning grounds in the sea to fresh water and brackish coastal environments (Mousa, 2010).

Intensive juveniles' collection from the wild has, however, the potential to jeopardize the health of wild stocks. Furthermore, high mortalities linked to the transportation of the wild-caught animals to on-growing sites, as well as lack of prophylactic treatments, such as vaccines, and subsequent risks of disease outbreaks with associated use of antibiotics, increases concerns linked to animal welfare and overall sustainability of this practice. Therefore, research efforts are currently underway to optimise juvenile production techniques from broodstock management to larval and juveniles rearing. Recent advances in our knowledge of sex determination mechanisms (Dor et al., 2020; Ferraresso et al., 2021), the development of hormonal therapies to induce sexual maturation (Ramos et al., 2021; Ramos et al., 2022), as well as the identification of suitable compounds for spawning induction and synchronisation (Vallainc et al. 2021; Besbes et al., 2020; Mousa, 2010), are paving the way for a more knowledge based approach to Mugilids broodstock management. If implemented at commercial scale, these new protocols could contribute to overcome the current bottlenecks in the commercial scale-up and intensification of mullet aquaculture. Alike most Mugilids, the thinlip grey mullet (*Chelon ramada*) is a low-trophic, euryhaline species that can be farmed in sea, brackish and fresh water, thereby diversifying production in multiple environments and ensuring a lower environmental impact compared to more traditional carnivorous species. Similarly to *Mugil cephalus*, it produces ovaries that, once salted and cured, are commercialised as a highly sought after and expensive product, the *"bottarga"* (Corrias et al., 2020). Nonetheless, like females of many commercially important fishes, thin-lipped grey mullet do not undergo final oocyte maturation, ovulation or spawning in captivity without hormonal treatments (Mousa et al., 2018; Ljubobratovic et al., 2021). Previous studies on this topic (Mousa et al., 2010) have demonstrated that the use of pregnyl, human chorionic gonadotropin (hCG) as a priming injection followed by a second injection of the same compound 24 h later, proved to be effective in inducing final oocyte maturation, ovulation and spawning. In this study it was tested the use of slow release gonadotropin releasing hormone analog (GnRHa) preparation, leuprorelin acetate, as an effective single dose inducer of final oocyte maturation and spawning in *C. ramada*. The description of the induction protocols,

broodstock management, and reproductive performances is provided alongside the analysis of larval growth performances and survival.

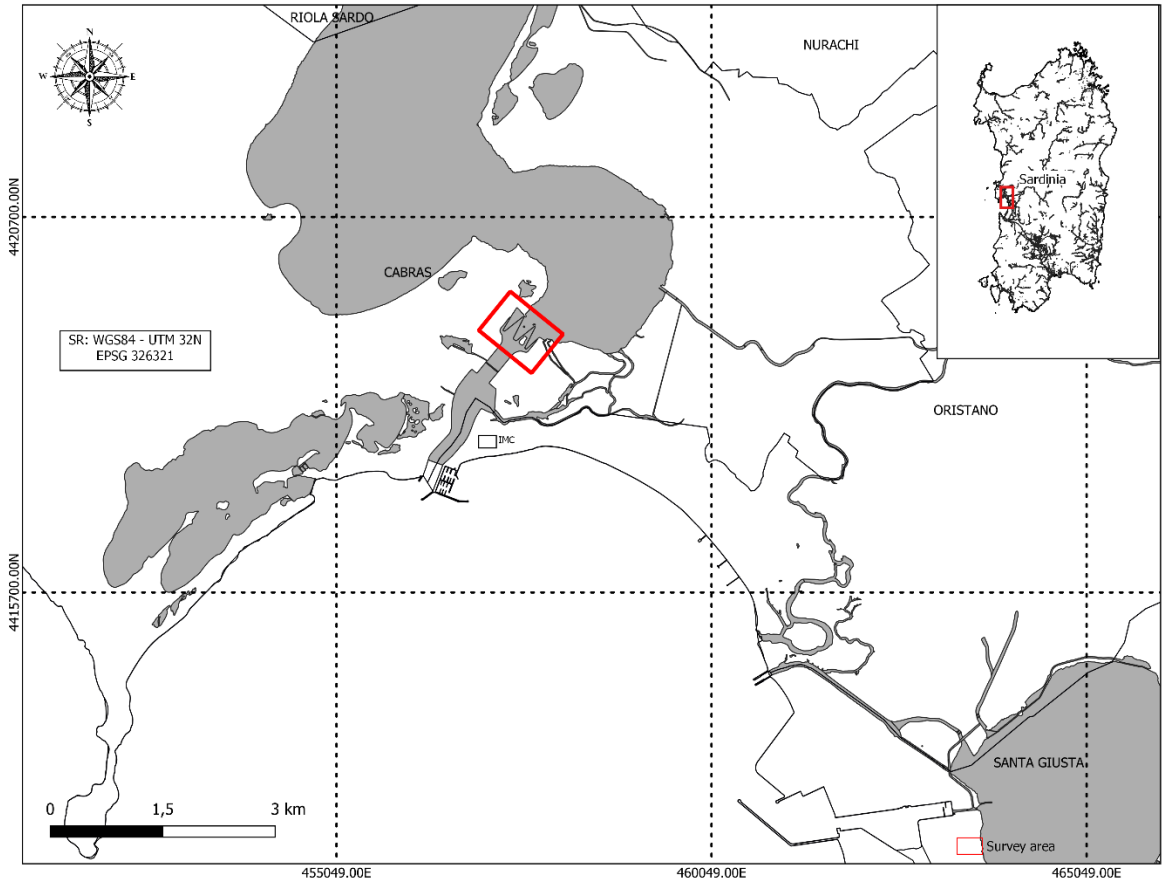

Figure 1. Cabras lagoon survey area. The red and the black rectangles highlight the capture site of the breeders and the laboratory of the IMC respectively.

Figure 2. Operational procedures to capture and induce breeders to spawn. A: a 200 L volume tank filled with lagoon sea water is placed next to the capture site. B and C: a silicon net is used to collect the individuals assembled inside the *lavorieri*. D: the animals are moved to another 100 L volume tank for anesthetization with 0.08% clove oil. E: males are checked for fluency by gentle pressure of the abdomen. F: females are checked for maturity by ovarian biopsy. G: oocytes are collected into a Petri dish and subsequently checked for maturation stage and diameter under a light microscope. H: the intramuscular injection is practiced below the dorsal fin. I: fish are included into sealed plastic bags filled with water and oxygen; L: breeders are released into the broodstock tanks; M: breeders are filmed from the bottom of the tank to observe spawning behaviours.

2 Materials and methods

2.1. Ethical statement

The present paper reports results originated from known and established protocols for the rearing of grey mullet in captivity with no experimental aim hence doesn't fall into the regulation for the use of animals for experimental procedures (DIRECTIVE 2010/63/UE art 1 comma 5 letter e; D.lvo 26/2014 art. 2 comma 1). The entire rearing has been carried out in the establishment of the IMC authorized by the Region Sardinia with authorization number IT038OR501 and under a veterinary control.

2.2. Broodstock collection and spawning induction

Broodstock collection and spawning induction were done according to the protocol developed for *M. cephalus* and described by Vallainc et al. (2021) and re-adapted for *C. ramada* as follows: mature adults were collected during their natural reproductive season (November) in the lagoon of Cabras, Sardinia, Italy (Figure 1), by taking advantage of the traditional fishing technique based on the presence of lagoon artificial enclosures ("lavorieri") which trap breeders during their reproductive migration toward the sea.

A total of 31 individuals (10 females and 21 males) were collected using a low abrasion silicone net and carefully placed into a 200 L plastic tank filled with water collected at the capture site (salinity=33 ppt; temperature=19°C) and covered with a lid to protect the fish from direct sunlight and to reduce stress. *C. ramada* females were 29±1 cm TL and 199±20 g BW, males were 28±1 cm TL and 181±80 g BW. Fish were anesthetized by transferring them to a new 200 L white rectangular tank filled with a solution of 0.08% clove oil (Erboristeria Magentina®) (Aizen et al., 2005) in groups of 3 or 4 individuals. Animals were kept in the anesthetic bath until loss of equilibrium and then quickly subjected to gentle pressure of the abdomen for sex determination. All males releasing sperm were weighted by an electronic scale (Steinberg systems, capacity=2 kg, division=0.01 g, accuracy=0.01 g). The head and back of each animal were covered with a wet hand towel and they were treated with a single intramuscular injection of 100 µg kg^{-1} body weight (BW) of the slow release gonadotropin releasing hormone analog (GnRHa) preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a., $C_{59}H_{84}N_{16}O_{12}$, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) administered intramuscularly below the dorsal fin (Vallainc et al., 2021).

Females were also weighted and a pool of them (n=3) were subjected to ovarian catheterization conducted by using a polyethylene cannula of 0.86 mm inner diameter and 1.52 mm outer diameter (Lee et al., 1992). All females (n=10) exhibited red, protruded papilla and bulging abdomen (Figure 2) and were treated with ENANTONE® with a single injection of 200 µg kg^{-1} BW dissolved in a physiological solution medium (Vallainc et al., 2021). All hormonally induced fish (n=31) were quickly transferred by car to the International Marine Centre (IMC) laboratories (about 2 minutes transport time), in dark cool condition into 6 x 50 L volume

transparent polyethylene transport bags (120 x 50 cm) filled for 1/3rd with lagoon water and for 2/3rd with oxygen.

Sampled oocytes (*n*=54; 18 oocytes female^{-1}) were collected into Petri dishes and immediately checked at IMC laboratories. Photos were taken under a light microscope (MZ8, Leica, Germany), through a Leica DMC2900 camera and measured with LAS 4.5 software (Leica, Germany) to determine the stage of maturity according to Lowerre-Barbieri et al. (2011) and to measure diameters. Fish were transferred to 2 broodstock tanks adopting a sex ratio of 2:1 males:females. The system sed consisted of an indoor recirculating aquaculture system (RAS) fitted with two black 3,500 L volume fiberglass tanks, provided with 500 µm net-mesh banjo filters, a mechanical (100 µm) and biological filter (1,000 L), UV lamp (80 W), protein skimmer and water chiller. Each tank had a gentle air supply and was provided with an overflow egg collector equipped with a 500 µm net-mesh. Tanks were covered with 1 cm mesh size nets to prevent breeders from jumping out of the tanks, and a natural photoperiod with dim illumination was used. Water flow was set to produce a 30% water recirculation h^{-1}. Salinity and temperature were kept at 37±1 ppt and 18.9±0.3 °C (means±SD), respectively. Dissolved oxygen (DO) was kept at 8±1 mg L^{-1}. Ammonia concentration was 0.2±0.1 mg L^{-1}, nitrite 0.03±0.02 mg L^{-1}, nitrate 7±3 mg L^{-1} and pH was 8.05±0.04. Breeders were allowed to spawn spontaneously in the tanks, courtship behavior and spawning activity were recorded using a submerged fotocamera GoPro Hero 4+ (GoPro, Inc., San Mateo, CA, USA) placed in the bottom of the tank 20 hours after the hormone treatment.

2.3. Eggs incubation and hatching

During spawning, egg numbers in the collection basket were estimated volumetrically every 8 hours. Eggs were removed from the collection basket, placed in a 10 L bucket and thoroughly mixed using a perforated spatula. Five replicate samples of 10 ml each were then collected from the bucket, eggs were counted under a microscope and egg density per unit volume was established (Panini et al., 2001). Additional samples were collected to establish viability, fertilisation rate, embryonic development stage and egg diameter. The latter

was measured by using the Leica image analysis software LAS 4.5 after taking a total of 23 pictures of 78 eggs from all the samples by a Leica DMC2900 digital camera.

The number of eggs g^{-1} was also determined; a sample of eggs was collected with a 500 µm mesh net, and the excess water was removed with absorbent paper. A sample of 1 g of eggs was divided into 3 aliquots of 0.33 g each, and all the eggs present in each sub-sample were counted. Once density was established, eggs were equally distributed using a 2 L volume graduated cylinder in six tanks, connected to two separated recirculation systems for incubation and hatching.

2.4. Rearing system

The larval rearing systems were indoor RAS each one consisting of 3 circular fiberglass tanks of 2,000 L volume; the walls and bottom of the tanks were black and white, respectively. Each system was equipped with a biological filter (800 L volume) and mechanical sand filter (100 µm), UV lamp (80 W), protein skimmer and water chiller. Egg incubation was performed in dark conditions. Fertilised eggs were incubated at the density of 46 eggs L^{-1}.

Water flow was set to produce a 12% water exchange h^{-1}. Dissolved oxygen and temperature were measured with a calibrated multimeter (Hach, HQ40d) while salinity was measured using a refractometer (V-RESOURCING, ATC). In the larval rearing systems, salinity and temperature were 37±1 ppt and 19.4±0.4 °C, respectively. Dissolved oxygen (DO) was kept at 9±1 mg L^{-1}. Ammonia concentration was 0.2±0.1 mg L^{-1}, nitrite 0.03±0.02 mg L^{-1}, nitrate 3±2 mg L^{-1} and pH was 7.9±0.2.

2.5. Larval rearing

Embryo development was observed and recorded every 6 h by sampling a minimum of 5 eggs from the incubation tanks. Hatching time was established when the samples from the tanks only contained hatched larvae or empty eggs.

Larvae were reared in the same two systems where hatching took place. Initial larval density was 36 larvae L^{-1}. For the first 14 days after hatching (dph), water recirculation was set at 5% day^{-1} and then increased to 10% day^{-1} until the end of the rearing phase (19 dph).

Continuous light (24 L:0 D, 600 lux) was used until 13 dph, and then a 12 L:12 D photoperiod was adopted for the rest of the rearing phase.

Phytoplankton (*Isochrysis galbana* and *Tetraselmis suecica*, 1:1 in volume ratio) was added daily to the tanks to maintain the green medium at a concentration of 400,000 cells ml^{-1} from 4 dph to the end of the rearing phase. The first feeding was administered at 5 dph and consisted of rotifers (*Brachionus spp.*) enriched for 12 h in a 6×10^6 cells ml^{-1} *I. galbana* medium. Rotifer concentration was adjusted daily to 4 individuals ml^{-1} up to 19 dph. The administration of Artemia nauplii (Great Salt Lake, Utah, US) began at 12 dph and continued up to 17 dph at the density of 0.4 nauplii ml^{-1}. Enriched (Easy DHA Selco, INVE Aquaculture) *Artemia* nauplii were given at 17 dph up to the end of rearing, at the same concentration adopted for the newly hatched nauplii. The *Artemia* enrichment (0.6 g L^{-1}) was performed for 24 h at a density of 250 nauplii ml^{-1}. Nauplii were rinsed with natural seawater (NSW) to eliminate any excess enrichment product prior to addition to the larval rearing tanks.

Water recirculation was turned off at each live food administration for 2 h. Water parameters were monitored daily. The temperature was set at 20±0.5 °C, salinity was 37±1 ppt, and dissolved oxygen was 8.1±0.5 mg L^{-1}. Every 10 days, 30% of the water was changed. Ammonia and nitrite were kept below 0.14 and 0.06 mg L^{-1}, nitrate below 3 mg L^{-1}, pH was 8.03±0.2.

Larvae were randomly sampled from the 6 tanks. From 0 to 10 dph larvae were daily sampled, subsequently the individuals were collected at 12, 16, 17 and 19 dph. A total of 353 larvae were sampled during the trial, these were euthanized using a clove oil overdose, photographed under the microscope (MZ8, Leica, Germany) through a Leica DMC2900 and total length (TL, mm) was measured with LAS 4.5 software (Leica, Germany).

TL measurements were used to determine the specific growth rate (SGR%) and to build the growth curve. SGR% was calculated at 5, 12 and 19 dph as follows (Ricker, 1979):

SGR% = [Ln(TLf)− Ln(TLi)] x100/t

Where TLf is the total length at the final time and TLi is the initial total length; t represents the number of days between TLt and TLi.

In addition, myotome height (MH, mm) was measured through the LAS software just behind the anus at 12 and 19 dph, on a total of 58 larvae.

Condition index (CI) of fish was calculated as:

CI = [myotome height (mm) / total length (mm)] x100.

Survival was determined according to Vallainc et al. (2021), at 19 dph, the tanks were photographed from above the tanks with a Canon G15 camera and individuals were counted through ImageJ (NIH, USA).

2.6. Statistical analysis

Data on oocyte diameters were analysed by Statistica 6.1 (StatSoft, Inc., USA). The normality of data and homogeneity of variance were assessed with Shapiro Wilk's W and Levene's tests, respectively. Since data resulted normally distributed (P=0.15) and variances were homogenous (P=0.41), a One-way analysis of variance (ANOVA) was employed to verify significant differences in oocyte diameters between the females (n=3). Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparison.

3. Results

3.1. Spawning induction

The diameter of the oocytes collected from the *C. ramada* females ranged from 657.1±37.4 to 665.2±45.5 µm, corresponding to advanced vitellogenic oocytes beyond the tertiary yolk stage (Figure 3).

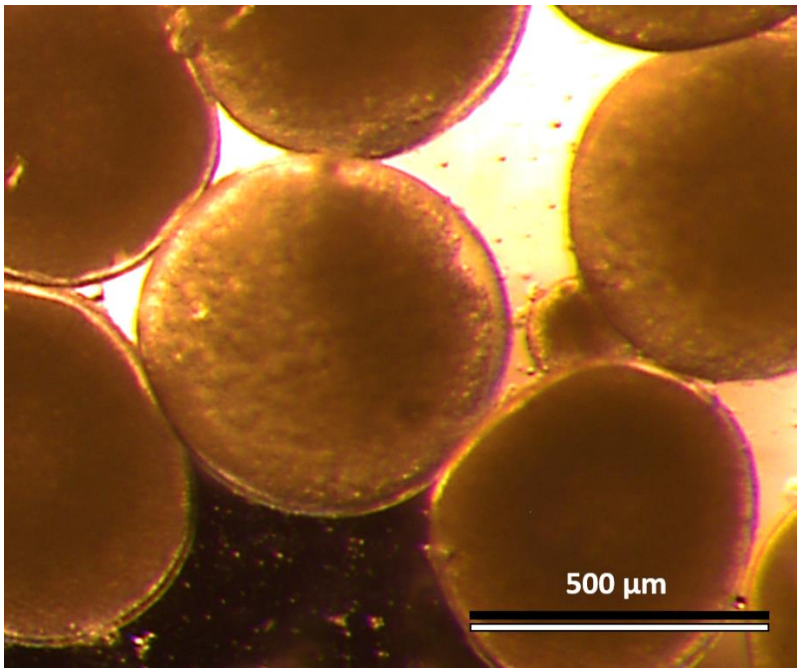

Figure 3. Vitellogenic oocytes sampled before applying the hormone treatment (Ø=665.2±45.5 µm)

No significant differences (*P*=0.84) in diameters were observed between oocytes collected from the 3 females subjected to ovarian biopsy (Figure 4).

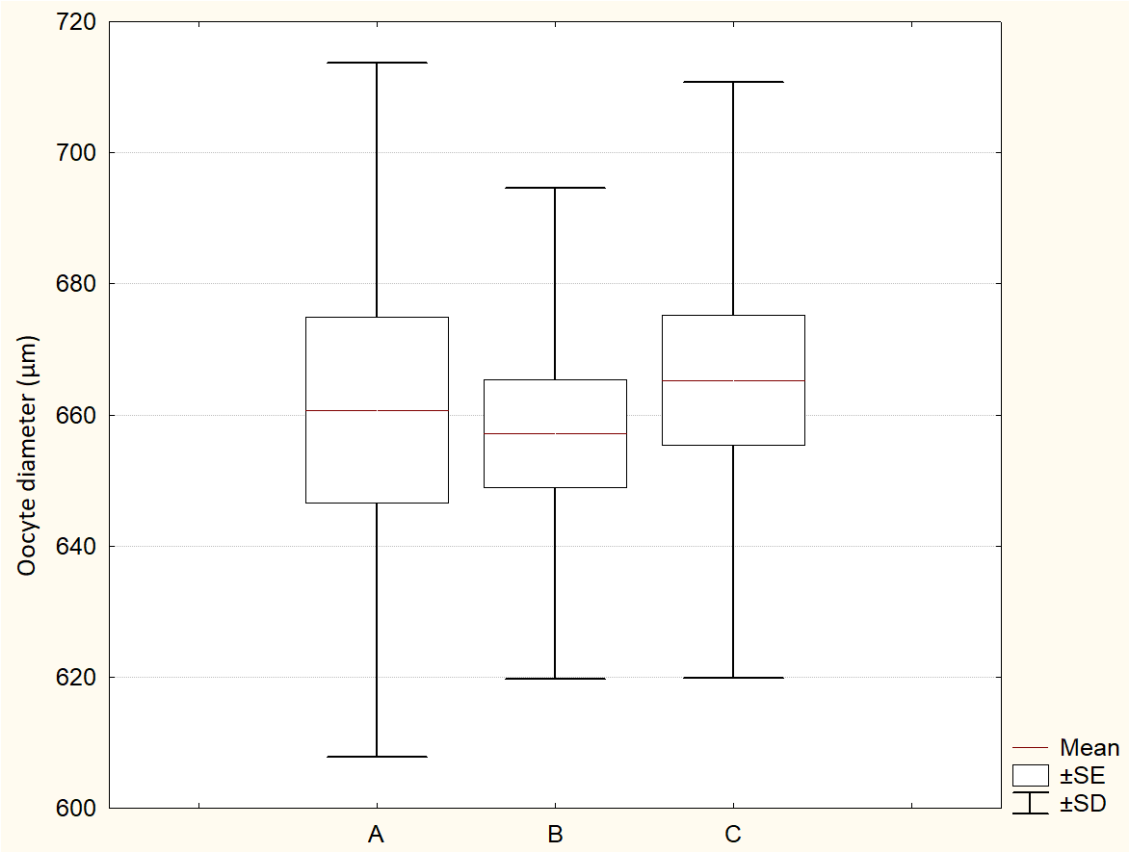

Figure 4. Box-plot of the oocyte diameters (n=19 female^{-1}) sampled from three different females (A, B and C).

First spawning occurred 38 hours after the hormone treatment and egg release continued for the subsequent 31 hours.

None of the handled animals developed visible infections and the behavior observed in the spawning tanks suggested that the breeders adapted immediately to captive conditions as they swam calmly with a classic schooling behavior (Figure 5).

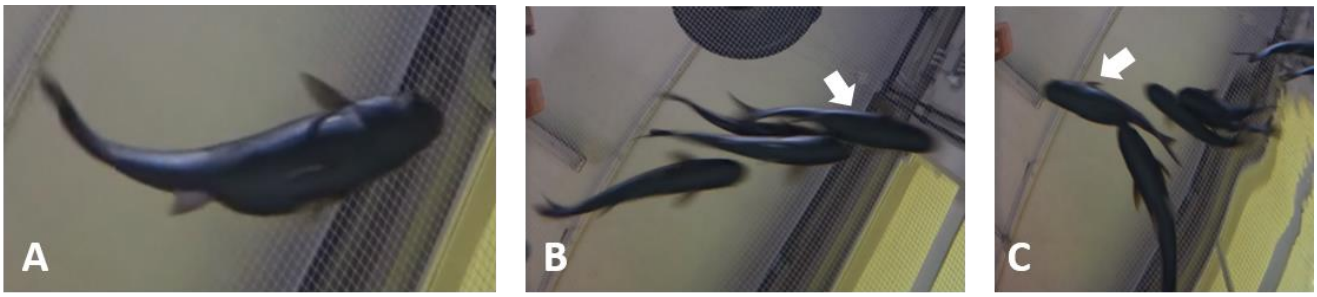

Figure 5. A: female showing pronounced abdominal swelling due to the hydration of the oocytes (24 hours after the hormone treatment); B and C: courtship behavior, males are swimming next to the female (pointed by the white arrows) abdomen and pushing her cloaca with their heads.

All breeders used in this trial have been released back to their natural environment after 5 weeks following the prescribed period to allow the hormone concentration to ware off (Regulation EC No. 470/2009).

A total of 552,000 eggs were obtained, corresponding to a fecundity of 277 eggs g BW^{-1}. The mean rates of fertilization and hatching were 92±3 and 91.4±0.4 %, respectively and the number of eggs g^{-1} was 1,600±20. Unsegmented fertilized eggs showed a diameter of 998±13 µm, and presented 2 or 3 oil drops. After 36 hours of incubation, eggs reached the embryo stage, their diameter was 979±52 µm and at this stage they presented a single oil drop. Hatching started 41 hours after spawning. Newly hatched larvae were 2.6±0.1 mm TL (Figure 6).

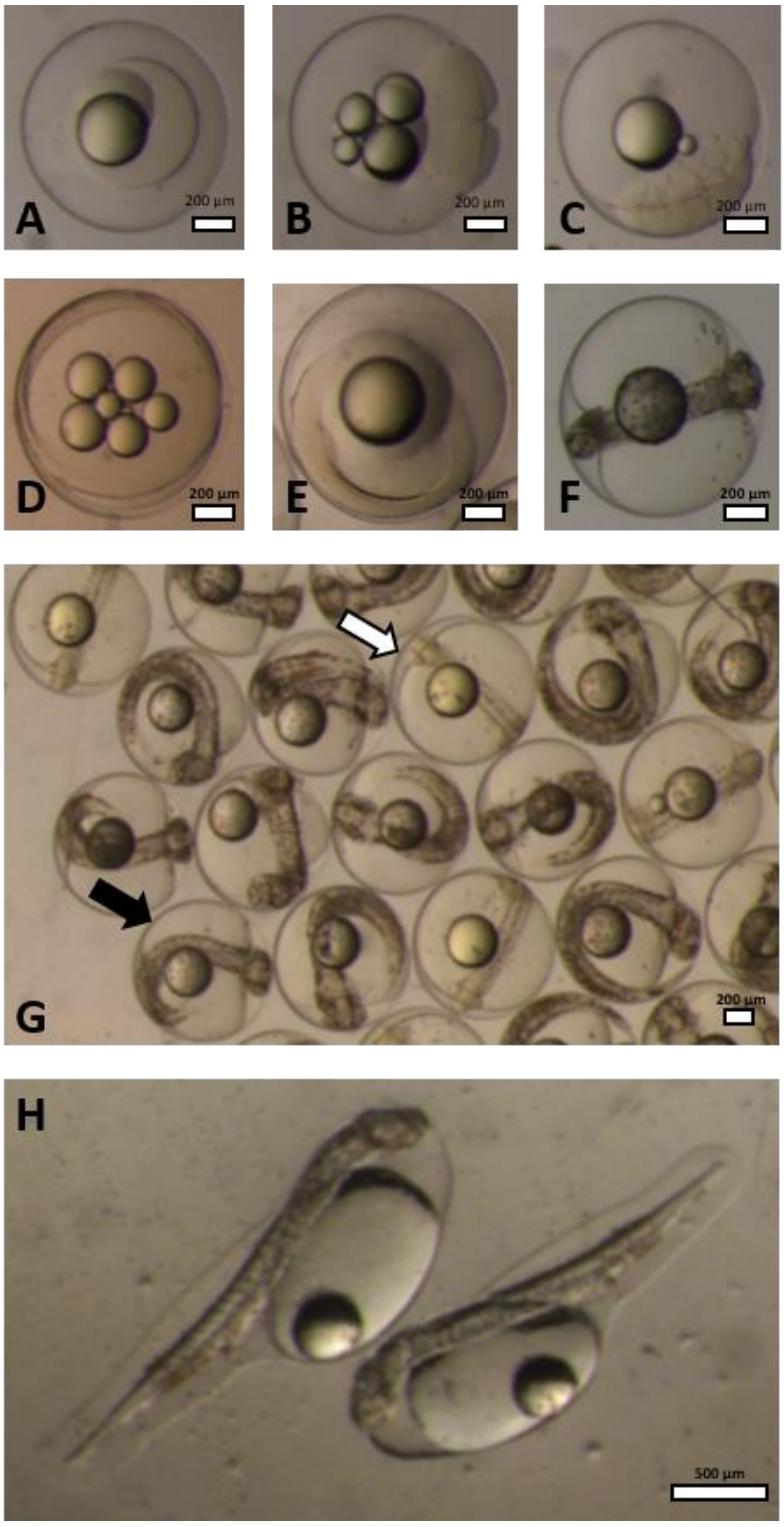

Figure 6. Embryo development. A: unsegmented fertilized egg, 30 minutes after spawning (Ø=998±13 µm). B: second cleavage, 2 hours after spawning (Ø=995±13 µm); C: fifth cleavage (32-cell stage), 5 hours after spawning (Ø=997±21 µm); D: mid-blastula stage, 9 hours after spawning (Ø=1038±51 µm); E: late blastula stage, 14 hours after spawning (Ø=1020±18 µm); F: organogenesis stage two, pigmented embryo, 30 hours after spawning (Ø=1017±12 µm); G: embryos from multiple spawning, the white arrow indicates an embryo at organogenesis stage, 30 hours after spawning; the black arrow indicates an embryo at organogenesis stage three, 37 hours after spawning (Ø=1075±19 µm). H: newly hatched larvae (2.6±0.1 mm TL), 41 hours after spawning.

3.2. Larval rearing

Larval development from 0 to 19 dph was documented (Figure 7). Larvae grew constantly according to the equation $y=2.6841e^{0.044x}$ (Figure 8). Mean SGR% day^{-1} recorded at 5, 12 and 19 dph were respectively 2.69±0.37, 2.63±0.58 and 2.07±0.33 (Figure 9). At 12 dph larvae were 4.40±0.64 mm TL, mean MH was 0.52±0.07 mm and mean CI was 11.73±1.06. At 19 dph, larvae were 6.44±0.33 mm TL, mean SGR% day^{-1} for the whole rearing period (from 0 to 19 DPH) was 2.08±0.12, MH was 0.81±0.07 mm and CI was 12.51±0.63. At the end of the rearing period the mean survival rate was 13.8±1.5%.

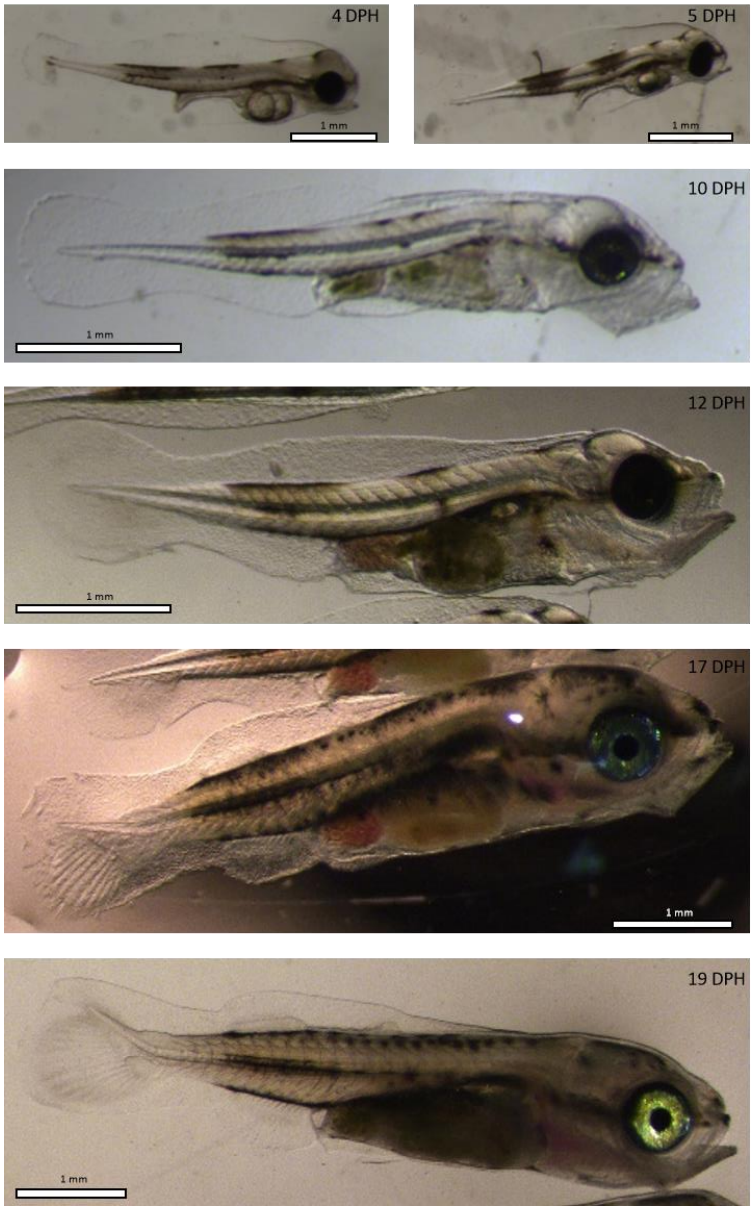

Figure 7. Larval development. 4 dph: pre-larvae were 3.54±0.08 mm TL, a primordial marginal fin fold surrounded the body from the dorsal part of the head to the posterior margin of the yolk sac. At this time, most of the yolk reserves were already consumed while the oil globule remained. The eyes were pigmented, the mouth was invaginated but not yet open. The swim bladder began to form ventrally beneath the notochord. Melanophores appeared in the dorsal contour. 5 dph: larvae were 3.53±0.55 mm. The mouth opened at the rostral part of the head differentiating into lower and upper movable jaws, the yolk sac and the oil globule were still present even if reduced, and melanophores appeared in the dorsal-lateral part of the body. 10 dph: larvae were 4.07±0.25 mm TL. The yolk sac disappeared, while the oil globule was almost completely consumed. The fin fold began to thin around the body. The eyes were relatively large. Rotifers were visible in the alimentary canal. 12 dph: larvae were 4.60±0.66 mm TL. The pigmentation was more pronounced except in the final section of the tail. The oil globule was completely resorbed. *Artemia* was recognizable in the final part of the intestine. 17 dph: larvae were 5.91±0.28 mm TL. Tail flexion has already occurred. The fins began to differentiate and the first rays were visible in the caudal fin. The pigmentation increased. 19 dph: larvae were 6.44±0.33 mm TL. Rays were developed in the tail fin, and started to be clearly visible in the anal fin. Eyes were large and highly pigmented. The body surface opacity intensified, however it was still possible to distinguish the digestive system full of live prey.

4. Discussion

Our study confirms that capturing wild mature *C. ramada* adults at the mouth of coastal lagoons by taking advantage of their reproductive migration toward the sea, represents a valid strategy to gather mature broodstock. Breeders can be easily collected limiting the capture stress and the silicon net reduces or excludes mechanical damages. None of the handled animals developed visible infections and the behavior observed in the spawning tanks suggested that the breeders adapted easily to captive conditions. Similarly to what was observed in Vallainc et al. (2021) with *M. cephalus*, *C. ramada* adults present external traits that allow for the assessment of spawning induction success. Indeed, the observation of red protruded genital papillas accompanied by bulging abdomen well correlated with the presence of advanced vitellogenic oocytes.

In this study, spawning was successfully induced in wild ripe *C. ramada* females presenting vitellogenic oocytes at tertiary yolk stage or beyond (with diameters ranging from 657.1±37.4 to 665.2±45.5 µm). This agrees with the results obtained by Mousa and Kahil (2013) which identifies 600 µm as the minimum oocyte diameter to promote final maturation, ovulation and spawning.

Previous studies on spawning induction protocols in the same species (Mousa, 2006; Mousa., 2010 and Mousa and Kahil, 2013) used pregnyl (hCG) as a priming injection at a dose of 20,000 IU kg BW^{-1} followed by a resolving dose of 40,000 IU hCG kg BW^{-1} and resulted in fertilization and hatching rate of 55±8.4 and 60±6.6%, respectively. Fahmy and El-Greisy (2014) obtained fertilization rates ranging from 52 to 75% by administrating hCG 3,500 IU + Luteinizing Hormone Releasing Hormone (LHRH) 200 + LHRH 100 µg kg BW^{-1}.

In our study we tested a simpler, less stressing protocol (as animals were anesthetised, handled and injected only once) promoting the spawning and fertilisation in *C. ramada* by administrating a single dose of ENANTONE® (200 µg kg BW^{-1} for females and 100 µg kg BW^{-1} for males). Observation of the reduction of the abdominal swelling after spawning, it is likely that all induced females contributed to the spawning.

The mean rates of fertilization and hatching observed in our study were higher than those observed in previous investigations, possibly due to minimal animal stress during the handling procedures and/or due to the hormone used. These factors could have resulted in better quality eggs compared to previous trials in which the administration of multiple doses of different hormones could have influenced the latency time between hormonal treatment and final oocyte maturation (FOM) affecting hatching rates and other egg quality parameters as described by Kucharczyk et al. (2019b; 2020) in other species.

This could, however, also be explained by the fact that unfertilized eggs may have sunk to the bottom of the spawning tanks and were therefore not included in our estimation of these reproductive performance parameters. This latter interpretation is supported by the fecundity rate observed in this trial (277 eggs g BW^{-1}), which resulted to be significantly lower than what observed in previous studies (600 to 700 eggs g BW^{-1}) (Mousa, 2010; Crosetti and Blaber, 2016). At hatching and at 5 dph, the larval TL measured in this trial was similar to what observed by Mousa et al. (2010) who reports that newly hatched larvae were 2.5 mm TL, 3.5 mm at 5 dph. The TL at hatching is however likely to be batch-dependent, as in a later paper Mousa reports larvae of 3.4 mm TL (Mousa et al., 2020). In the present study, growth proceeded comparably with previous works (Mousa et al., 2020), although some significant differences in larval rearing protocols are noteworthy

and highlight the need for more structured studies to optimize rearing conditions, particularly for feeding regimes.

In this study, we report SGR% and survival for the first time in *C. ramada* larvae. These data could therefore represent a useful benchmark for future studies on the same species. The slight decrease in SGR% day^{-1} observed here during the last phases of larval rearing (12 to 19 dph) could be explained as a larval increased investment on ontogenic development rather than on total length growth (Santamarıa et al., 2004; Khemis et al., 2006). However, the natural transition in energy allocation between ontogeny and growth during larval development is well described in many fish species. Indeed, a slower growth in total length in the last larval rearing phase could reflect the formation of definitive features such as branchial respiration and active swimming (Kupren et al., 2016; Fuiman, 1983). This interpretation is supported by the data on MH and CI which showed a rapid increase of these two morphological parameters as larvae approached the perimetamorphic period when the tail flexion occurred concomitantly with the observed decrease in growth rate (Peña and Dumas, 2009). Finally, the larval survival rate recorded in this study (13.8±1.5%) is promising considering larval survival data reported in other recently domesticated marine species (Roo et al., 2019; Vallés and Estévez, 2013; Abellán et al., 2000).

5. Conclusions

Due to their trophic positioning, their global distribution, their environmental adaptability and their high potential for added value, Mugilids could play a key role in the sustainable intensification of the global aquaculture sector, provided that significant bottlenecks in reliable juvenile production are overcome. These include, among others, the development of reliable spawning induction protocols that efficiently synchronize gamete release and minimize animal stress.

Compared to other species in the family, *C. ramada* offers significant opportunity to diversify juvenile production as its sexual maturation is achieved during the autumn months and, therefore, outwith the natural spawning time of other Mugilids such as *M. cephalus* (late summer) or *Chelon labrosus* (early spring). In theory, this would allow to extend commercial hatcheries production seasons virtually to the whole year and, at the same time, diversify production with species with very similar physiology and rearing requirements without the need for dedicated facilities for each species. Furthermore, *C. ramada* reaches sexual maturation far earlier than *M. cephalus* significantly reducing the time to maturation for bottarga production yet preserving the nutritional qualities of that produced from *M. cephalus* and a higher content of essential amino acids.

Important developments in the control of reproduction in Mugilids are being achieved by the scientific community and it is likely that the full control of sexual maturation and reproduction in Mugilids will shortly be within reach. However, until that time comes the wild capture of a limited number of sexually mature individuals, and their subsequent release after spawning in captivity, appears to be a far more sustainable proposition compared to the collection of millions of juveniles from the ecosystem.

6. References

Abellan, M. T., Adell, A., Honrubia, M. A., Mengod, G., and Artigas, F., 2000. GABAB-RI receptors in serotonergic neurons: effects of baclofen on 5-HT output in rat brain. Neuroreport 11, 941–945. https://doi.org/10.1097/00001756-200004070-00009.

Besbes, R., Benseddik, A.B., Kokokiris, L., Hamza, A., Kammoun, F., 2020 . Thicklip (*Chelon labrosus*) and flathead (*Mugil cephalus*) grey mullets fry production in Tunisian aquaculture. Aquaculture Reports, 17, 100380. https://doi.org/10.1016/j.aqrep.2020.100380.

Brown, C. and Day, R. L., 2002. The future of stock enhancements: lessons from hatchery practice to conservation biology. Fish and Fisheries 3, 79-94. https://doi.org/10.1046/j.1467-2979.2002.00077.x.

Corrias, F., Atzei, A., Giglioli, A., Pasquini, V., Cau, A., Addis, P., Sarais, G., Angioni, A., 2020. Influence of the Technological Process on the Biochemical Composition of Fresh Roe and Bottarga from Liza ramada and *Mugil cephalus*. Foods 9, 1408. https://doi.org/10.3390/foods9101408.

Crosetti, D. and Blaber, S., 2016. Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA. https://doi.org/10.1201/b19927.

Decreto Legislativo 4 marzo 2014, n. 26 (art. 2 comma 1). Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici. (14G00036) (GU n.61 del 14-3-2014). https://www.gazzettaufficiale.it/eli/id/2014/03/14/14G00036/sg.

Directive 2010/63/EU (art 1, comma 5, letter e) of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (text with EEA relevance). http://data.europa.eu/eli/dir/2010/63/oj.

Dor, L., Shirak, A., Curzon, A.Y., Rosenfeld, H., Ashkenazi, I.M., Nixon, O., Seroussi, E., Weller, J.I., Ron, M., 2020. Preferential mapping of sex-biased differentially-expressed genes of larvae to the sex-determining

region of flathead grey mullet (*Mugil cephalus*). Frontiers in Genetics, 11, 839. https://doi.org/10.3389/fgene.2020.00839.

El-Sayed, A.F.M. and El-Ghobashy A.E., 2011. Effects of tank colour and feed colour on growth and feed utilization of thinlip mullet (*Liza ramada*) larvae. Aquacult. Res . 42: 1163-1169. https://doi.org/10.1111/j.1365-2109.2010.02704.x.

European Commission. Communication from the commission to the European parliament, the council, the European economic and social committee and the committee of the regions on strategic guidelines for a more sustainable and competitive EU aquaculture for the period 2021 to 2030, 2021. https://eur-lex.europa.eu/resource.html?uri=cellar:bab1f9a7-b30b-11eb-8aca-01aa75ed71a1.0022.02/DOC_1&format=PDF.

Fahmy, A. and El-Greisy, Z., 2014. Induced spawning of *Liza ramada* using three different protocols of hormones with respect to their effects on egg quality. African Journal of Biotechnology, 13, 40, 4028-4039. https://doi.org/10.5897/AJB2013.13068.

Ferraresso, S., Bargelloni, L., Babbucci, M., Cannas, R., Follesa, M.C., Carugati, L., Melis, R., Cau, A., Koutrakis, M., Sapounidis, A., Patarnello, T., 2021. Fshr: a fish sex-determining locus shows variable incomplete penetrance across flathead grey mullet populations. iScience, 24, 1, 101886, 10.1016/j.isci.2020.101886. https://doi.org/10.1016/j.isci.2020.101886.

Fuiman, L. A., 1983. Growth gradients in fish larvae. J. Fish Biol. 23: 117-123. https://doi.org/10.1111/j.1095-8649.1983.tb02886.x

González-Castro, M. and Minos, G., 2016. Sexuality and reproduction of Mugilidae. In Crosetti, D. and Blaber, S. (eds) Biology, ecology and culture of mullets (Mugilidae). Boca Raton, FL: CRC Press, 227–263. https://doi.org/10.1201/b19927.

Khemis, I., Zouiten, D., Besbes R. and Kamoun. F., 2006. Larval rearing and weaning of thick-lipped grey mullet (*Chelon labrosus*) in mesocosm with semi-extensive technology. Aquaculture, 259, 190–201. https://doi.org/10.1016/j.aquaculture.2006.05.007.

Kucharczyk, D., Nowosad, J., Kucharczyk, D.J., Kupren, K., Targonska, ´ K., Wyszomirska, E., Kujawa, R., 2019b. Out-of-season artificial reproduction of common dace (*Leuciscus leuciscus* L.) under controlled conditions. Anim. Reprod. Sci. 202, 21–25. https://doi.org/10.1016/j.anireprosci.2019.01.003.

Kucharczyk, D., Nowosad, J., Wyszomirska, E., Cejko, B.I., Arciuch-Rutkowska, M., Juchno, D., Boron, ´ A., 2020. Comparison of artificial spawning effectiveness of hCG, CPH and GnRHa in combination with dopamine inhibitors in a wild strain of ide *Leuciscus idus* (L.) in hatchery conditions. Anim. Reprod. Sci. 221, 106543 https://doi.org/10.1016/j.anireprosci.2020.106543.

Kupren, K., Rams, I. and Żarski, D., 2016. Early development and allometric growth patterns of rheophilic cyprinid common dace *Leuciscus leuciscus* (Cyprinidae: Leuciscinae). Ichthyol Res 63, 382–390. https://doi.org/10.1007/s10228-016-0508-9

Ljubobratović U., Kwiatkowski M., Tóth F. and Demény F., 2021. Effects of hormonal treatment before water warming on synchronisation of spawning time, oocyte size, and egg quality in pikeperch (*Sander lucioperca*). Animal Reproduction Science, 226, Article 106712. https://doi.org/10.1016/j.anireprosci.2021.106712.

Lowerre-Barbieri, S.K., Brown-Peterson, N.J., Murua, H., Tomkiewicz, J., Wyanski, D.M. and Saborido-Rey, F., 2011. Emerging Issues and Methodological Advances in Fisheries Reproductive Biology. Mar. Coast. Fish. 3, 32–51. https://doi.org/10.1080/19425120.2011.555725.

Mousa, M. A. and Mousa, S. A., 2006. Involvement of corticotropin releasing factor and adrenocorticotropic hormone in the ovarian maturation, seawater acclimation and induced-spawning of *Liza ramada*. General and Comparative Endocrinology, 146: 167-179. https://doi.org/10.1016/j. ygcen.2005.10.002.

Mousa, M.A., 2010. Induced spawning and embryonic development of *Liza ramada* reared in freshwater ponds. Animal Reprododuction Science, 119, 115–122. https://doi.org/10.1016/j.anireprosci.2009.12.014.

Mousa, M.A. and Khalil, N.A., 2013. Environmental and hormonal control of induced spawning in thin-lipped grey mullet, *L. ramada* (Risso). Egyptian Journal of Histology, 36, 2, 449-458. https://doi.org/10.1097/01.EHX.0000428962.36676.05.

Mousa M.A., Ibrahim M.G., Kora1 M.F. and Ziada M.M., 2018. Experimental Studies on the Reproduction of the Thin-Lipped Mullet, *Liza ramada*. Egyptian Journal of Aquatic Biology & Fisheries, 22(3):125-138. DOI: 10.21608/ejabf.2018.9455.

Mousa, M., Khalil, N., El-Gohary, N., 2020. Morphological Study of Larval Development and the Transition to Juvenile Stage in Thin-Lipped Mullet, *Liza Ramada*. Egyptian Journal of Histology, 14, 43, 4, 1170-1176. https://doi.org/10.21608/EJH.2020.22769.1236.

Peña, R., and Dumas, S., 2009. Development and allometric growth patterns during early larval stages of the Spotted Sand Bass *Paralabrax maculatofasciatus* (Percoidei: Serranidae). Scientia Marina, 73, S1, 183-89. https://doi.org/10.3989/scimar.2009.73s1183.

Ramos-Júdez, S., Chauvigné, F., González-López, W.Á., Rosenfeld, H., Cerdà, J., Giménez, I., Duncan N., 2021a. Providing recombinant gonadotropin-based therapies that induce oogenesis from previtellogenic oocytes to produce viable larvae in a teleost, the flathead grey mullet (*Mugil cephalus*). Aquaculture, 536, 736418, 10.1016. https://doi.org/10.1016/j.aquaculture.2021.736418.

Ramos-Júdez, S., Giménez, I., Gumbau-Pous, J., Arnold-Cruañes,L. S., Estévez, A., Duncan, N., 2022. Recombinant Fsh and Lh therapy for spawning induction of previtellogenic and early spermatogenic arrested teleost, the flathead grey mullet (*Mugil cephalus*). Scientific Reports, 12, 6563. https://doi.org/10.1038/s41598-022-10371-0.

Regulation (EC) No. 470/2009 of the European Parliament and of the Council laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin.

Roo, J., Hernández-Cruz, C.M., Mesa-Rodriguez, A., Fernández-Palacios, H., Izquierdo, M.S., 2019. Effect of increasing n-3 HUFA content in enriched Artemia on growth, survival and skeleton anomalies occurrence of greater amberjack *Seriola dumerili* larvae. Aquaculture, 500, 651-659, 10.1016. https://doi.org/10.1016/j.aquaculture.2018.09.065.

Saleh, M., 2008. Capture-based aquaculture of mullets in Egypt. pp. 109–126. In: A. Lovatelli and P.F. Holthus (eds.). Capture-based Aquaculture. Global overview. FAO Fisheries Technical Paper. n. 508. Rome, FAO.

Santamaría, C.A., Marín De Mateo, M., Traveset, R., Sala, R., Grau, A., Pastor, E., Sarasqueted, C., Crespo S., 2004. Larval organogenesis in common dentex *Dentex dentex* L. (Sparidae): histological and histochemical aspects. Aquaculture, 237, 207-228, 10.1016. https://doi.org /10.1016/j.aquaculture.2004.03.020.

Teletchea, F., 2019. Fish domestication in aquaculture: reassessment and emerging questions. Cybium, 43(1): 7-15. doi: 10.26028/cybium/2019-431-001.

Vallainc, D., Concu, D., Gimenez Papiol, G., Loi, B., Leggieri, F., Brundu, G., Chindris, A., Sanna, G., Fois, N., Antognarelli, F., Rossini, P., 2021. Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons. Aquaculture Reports, Volume 21, 2021, 100844, ISSN 2352-5134. https://doi.org/10.1016/j.aqrep.2021.100844.

Chapter IV: Simulated transport of flathead grey mullet *Mugil cephalus* eggs: effects of density and duration

The present work is based on the publication: Scolamacchia M, Concu D, Loi B, Chindris A, Deiana M, Frongia C, Carboni S, Vallainc D (2023). Simulated transport of flathead grey mullet *Mugil cephalus* EGGS: Effects of density and duration. https://doi.org/10.3390/ani13101685

1. Introduction

Fish eggs transport is one key factor in the development of successful finfish aquaculture (Stuart et al., 2018). Eggs are generally more robust than yolk sac larvae, and do not show complex stress responses as the broodstock (Garcia et al., 2000; Sampaio and Freire, 2016), hence the advantage of shipping fertilized eggs when wild spawners are used, and the broodstock collection sites are remote.

Factors affecting egg quality are determined by intrinsic properties of the egg itself (Bobe, 2015), maternal gene transcripts (Reading et al., 2018), gamete ageing (Valdebenito et al., 2015) and may show considerable variability from season to season, and between batches (Reading et al., 2018; Brooks et al., 1997). Egg quality is indicated mainly by low mortalities at fertilization, eyeing, hatching and first feeding of larvae (Valdebenito et al., 2015; Kjørsvik et al., 1990). Although some degree of mortality may be acceptable after transportation, this should be kept to a minimum. Stocking density and transport time are crucial factors contributing to water quality and mechanical stress during shipment being the main causes of mortality (Stuart et al., 2018; Garcia and Toledo, 1988; Kazuyuki et al., 1988) and larval quality (Toledo et al., 1996).

The flathead grey mullet *Mugil cephalus* is an euryhaline species distributed globally between 42◦N and 42◦S (Thompson, 1963). The commercial value of flathead grey mullet is mainly related to its salted roe highly appreciated worldwide. Due to its high tolerance to salinity changes (Hotos and Vlahos, 1998) and feeding at

the lowest trophic levels, this species is also ideal for culture and represents an inexpensive source of animal protein of high commercial interest (Tamaru et al., 1993). *M. cephalus* is an isochronal single batch spawner with synchronous gamete development, a very high gonadosomatic index (GSI) and a high fecundity (Vallainc et al., 2021; Crosetti, 2015). These features may counteract a relatively slower growth compared to more established and already domesticated species. Still, the domestication process is currently limited by a lack of optimized standard protocols for fry production, a significant bottleneck in the production of this species at a commercial level due to severe reproductive dysfunctions of captive broodstock (Ramos-Júdez et al., 2022; Rosenfeld et al., n.d.; Aizen et al., 2005) and the low larval survival in hatchery conditions (Oz et al., 2022).

M. cephalus egg and larval development have been widely described (Vallainc et al., 2021; Loi et al., 2020; Thieme et al., 2021; Kuo et al., 1973), along with their thermal and salinity tolerance (Walsh et al., 1989; Sylvester and Nash, 1975). However, no recent information is available on the least impacting procedures for fertilized egg transport when no water conditioners and refrigerated vehicles are available. Indications on the best stocking density, optimal developmental stage for shipping and the evolution of water quality parameters along time, are the objectives of the present exploratory study which will provide in- formation on flathead grey mullet eggs' survival and quality after a simulated transport in a low-cost scenario.

2. Materials and methods

2.1. Experimental design

A completely 3 × 2 randomized design was applied, with two different densities (namely low density: LD, 500 eggs L−1; high density: HD, 1500 eggs L−1) and three different transport times (8, 16 and 24 h) to simulate a short, medium and long egg shipment in an entirely closed system (polyethylene, PE, bags) (Fig. 1). Densities were chosen according to the incubation ones described in Vallainc et al. (2021). Fertilized eggs of a mature wild grey mullet population were passively collected from a breeding tank set up in the lagoon of Porto Pino

(South Sardinia, Italy) in September 2021 and brought to the International Marine Centre (IMC) laboratories according to Vallainc et al. (2021).

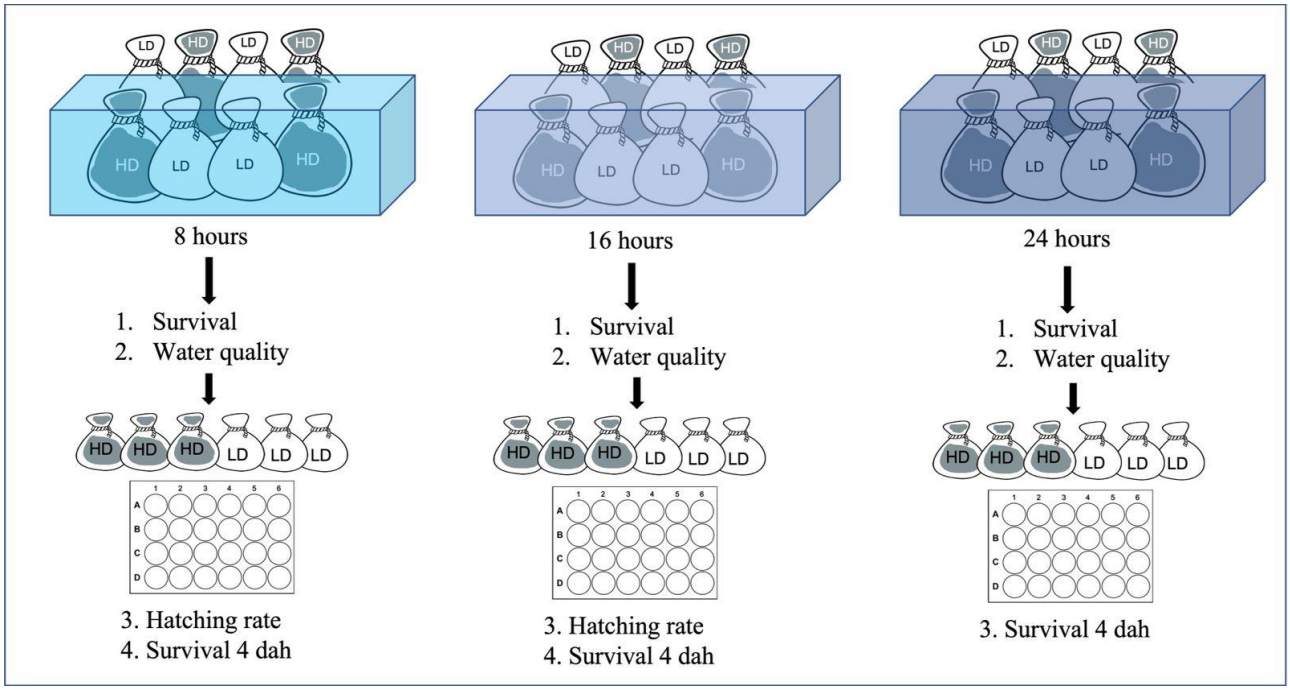

Fig. 1. Experimental design graphical abstract.

Only floating and fertilized eggs (2 cells cleavage stage) were collected. Upon arrival, eggs were at early gastrula stage, circa 10 hours post-fertilization (hpf). Eggs were incubated at 24 °C consistent with water temperature in the spawning tank, acclimated to the lab conditions and were not disinfected (Chattopadhyay et al., 2021; Koven and Rosenfeld, unpublished).

Eggs were kept under mild aeration before being bagged for the trial in sand-filtered and UV treated natural seawater. Oxygen concentration, temperature and pH were measured at the beginning and at the end of the trial with a calibrated multimeter (Hach, HQ40d) while salinity was measured using a refractometer (V-RESOURCING, ATC). Early gastrula egg concentration was estimated volumetrically, and the appropriate volume was added to 2 L double bags to reach the experimental densities plus seawater to volume and pure oxygen in a 2:3 ratio (Parazo et al., 1998). Thereafter, bags were kept still in three different polystyrene boxes (CT10 604x 425x 265 mm, 68 L) in a CT room (controlled temperature room) at 24 °C for 8, 16 and 24 hours. Each box was provided with a logger (Hobo, TidbiT v2) recording temperature every 10’ along the trial. A

total of 5 bags per density were prepared (total bags for transport time, n=10). A different blinded operator performed each of the steps of the trial.

To estimate quality after transport, a single egg was plated into one well of a 24-EIA plate (Unuma et al., 2019) for three out of the 5 bags (Giménez et al., 2006; Shields et al., 1997) for a total of 72 eggs per treatment (144 per transport time). One mL of autoclaved sea water was added to each well (Ramos-Júdez et al., 2022; Giménez et al., 2006; Mylonas et al. 2003) before being incubated in the darkness in a CT incubator at 24 °C (fig. 1).

Mortality and hatching rate were checked every 8 hours until 4 days after hatching (dah, start of the exogenous feeding for this species). Hatching rate was calculated as the number of larvae hatched over the total eggs incubated in the well plates for 8- and 16-hours transport. Embryo and larval viability were estimated according to Besbes et al. (2020) and Ramos-Júdez et al. (2021). Bags incubated for 24 hours showed mainly newly hatched larvae, hence, larvae instead of eggs were seeded in the well plates but no hatching rate was calculated for this last treatment. Larval viability was estimated via the number of surviving larvae at 4 dah and the percentage of dead larvae at hatching as a percentage over the total number of eggs incubated.

2.2 Water chemistry

Water chemistry analysis was carried out on a filtered water sample of 50 mL from each bag for ionized and unionized ammonia, nitrite and nitrate. Samples were frozen at -20 °C before analysis. An automatic sequential colorimetric analyzer was used, and the manufacturer's methods applied (EasyChem Plus, Systea S.p.A. Italy).

2.3 Statistical analysis

The impact of transport time, egg stocking density and the interaction between both independent variables upon water quality parameters (oxygen, pH, nitrogen compounds) and survival at the different opening times were analyzed using two-way ANOVA followed by a Tukey's HSD post-hoc test. Shapiro Wilk and Levene's test were used to check for the parametric analysis pre-requisites. When parametric assumptions were violated, Friedman's test with pairwise signed-rank test followed by Hochberg's correction post-hoc was applied. Percentage values were arcsine transformed before the tests. To ascertain any fit between survival and nitrogen forms concentration, a 1 tailed Pearson's correlation analysis was performed with alfa = .05 (Rossi, 2004). Data are presented as mean ± s.e.m.

3. Results

Survival rates after transport ranged from a minimum of 40% (HD, 24 hours) to a maximum of 88% (LD, 16 hours).

No significant effect on survival was due to the experimental density ($p > .05$) whereas transport time did have a significantly effect on survival explaining circa 63% of the total variation in eggs survival with values lower of circa 49% between 24 hours and 16 hours transport ($F(2,24) = 22.65$, $p = 2.98\ e^{-06}$) (fig. 2A). There was not a significant interaction between the effects of transport time and density ($p > .05$).

Hatching occurred between 29-38 hours post fertilization (hpf) varying from a minimum of 31% (HD, 8 hours) to a maximum of 54% (LD, 16 hours).

Simple main effects analysis showed that transport time did not have a statistically significant effect on hatching success ($p > .05$) and neither did density ($p > .05$). Likewise, there was not a statistically significant interaction between the effects of transport time and density ($p > .05$), however HD treatment values were always lower by 10-14% compared to the LD treatment (fig. 2B).

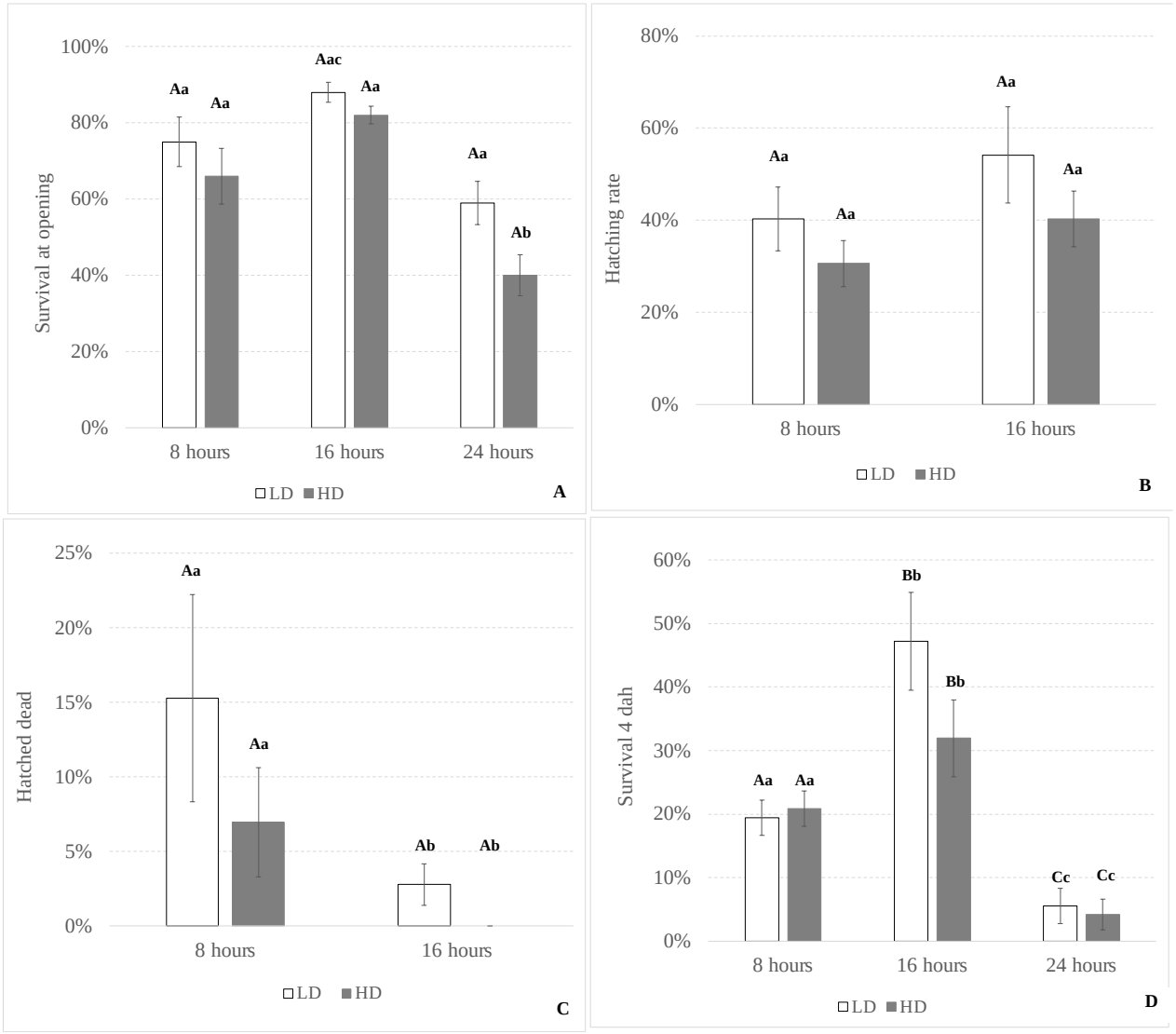

Fig.2 A) Survival after transport, B) hatching rate, C) percentage of larvae hatche dead and D) survival at the start of exogenous feeding (4 dah). Uppercase superscripts indicate significant differences between densities, lowercase between transport time ($p < .05$). Data are presented as mean ± s.e.m.

Longer term effect of egg transport at specific times was evaluated by the number of larvae which hatched dead (i.e. as soon as they hatched they died) and their later survival at the onset of exogenous feeding (4 dah for this species). Larvae hatched dead showed the lowest values after 16 hours transport (Fig. 2C). A two-way ANOVA revealed that there was not a significant interaction between the effects of density and transport time ($p > .05$). No density main effect was evident ($p > .05$) whereas there was a significant main effect of transport time ($F(1,8) = 5.85$, $p = .042$) which explained 36% of total variance, although the high variability may have had masking effects.

The lowest percentage of larvae surviving up to 4 dah was recorded for 24 hours transport time in both densities (6% and 4%, respectively), whereas the highest rate was recorded for 16 hours transport time at low density (47%). Transport time was the main factor affecting long term survival, explaining 74% of total variance ($F(2,12)= 22.98$, $p= 7.95\ e^{-05}$). Among the well plates seeded after 24 hours transport, two of them (one per density) had a mortality of 100% before 4 dah (fig. 2D).

Temperature was constant throughout the trial in all treatments, with no effects due to stocking density, transport time or the interaction of the two ($p> .05$).

All the bags at opening had an oxygen content >110% saturation regardless of the egg density. Both time and density had effect on the oxygen concentration explaining 54% ($F(2,22) = 21.46$, $p= 6.8\ e^{-06}$) and 17% ($F(1,22)= 13.33$, $p= 1.4\ e^{-03}$) of the total variance respectively, with LD showing a greater oxygen content than HD treatment (tab. 1).

pH decreased from initial values with the lowest values in HD-24 hours transport time (8.34± 0.02 vs 7.72± 0.11). Density alone wasn't responsible for the total variation in pH concentration ($p> .05$) whereas both transport time ($F(1,23) = 4.35$, $p = .025$) and the interaction of the two independent variables were ($F(2,23) = 4.60$, $p = .021$). pH in the shortest transport time was significantly higher than after 24 hours ($p= .03$). After pairwise comparisons HD treatment showed significant differences in pH between 8- and 24-hours transport ($p= .006$) (tab.1).

Initial unionized ammonia (NH_3) and ionized ammonia (NH_4) concentration were 0.015 ± 0.001 mg L^{-1} and 0.016 ± 0.001 mg L^{-1} respectively (tab. 1). Both time, density and the interaction of the two factors affected NH_3 and NH_4 concentration.

Simple main effects analysis showed that transport time did have a significant effect on NH_3 concentration ($F(2,24)=55.99$, $p= 2.55\ e^{-10}$) as well as density ($F(1,24)=1235.67$, $p= 3.72\ e^{-22}$) and the interaction of the 2 factors ($F(2,24)=15.73$, $p=4.31\ e^{-05}$). Both in LD and HD treatment, pairwise comparisons showed a significant variation between 8 hours and 16 hours (LD: p= 9.38 e-08; HD: $p= 6.34\ e^{-05}$), between 16 hours and 24 hours (LD: $p= .002$; HD: $p= .016$) and 8 and 24 hours (LD: $p= .003$; HD: $p= 2.45\ e^{-08}$).

Similarly, NH_4 was affected both by transport time ($F(2,24)= 63.6$, $p= 2.54\ e^{-10}$), stocking density ($F(1,24)= 1235.9$, $p= 3.17\ e^{-22}$) and the interaction of the two factors ($F(2,24)=15.7$, $p= 4.30\ e^{-05}$). Like for NH_3, both in LD and HD treatment, pairwise comparisons showed a significant variation between 8 hours and 16 hours with significant increase along time in both LD and HD treatment (LD: $p= 9.35\ e^{-08}$; HD: $p= 6.34\ e^{-05}$), between 16 hours and 24 hours (LD: p= .002; HD: p= .02) and 8 and 24 hours (LD: p= .003; HD: $p= 2.45\ e^{-08}$) (tab. 1). In both cases, density was the main factor explaining circa the 87% of the total variance.

Initial NO_2 concentration was 0.009 ± 0.00 mg L^{-1}. Both time ($F (2,24)= 33.1$, $p= 1.19\ e^{-07}$), density ($F(1,24)= 59.86$, $p= 5.67\ e^{-08}$) and the interaction between the two ($F(2,24)= 4.05$, $p= 3.05\ e^{-02}$) affected NO_2 trend explaining total variance in reason of 42, 38 and 5% respectively. NO_2 significantly increased in both densities from 8 to 16 hours transport (LD: $p= 6.65\ e^{-05}$; HD: $p= 5.12\ e^{-05}$) with the highest values for HD treatment (0.039± 0.003 mg L^{-1}). In the following hours, NO_2. concentration decreased significantly only for HD treatment but not for LD treatment (LD: p> .05; HD: $p= 2.76\ e^{-04}$). After 24 hours simulated transport NO_2 concentration got back to similar values between HD and LD treatments (tab. 1).

Table 1 Water quality parameters along the trial. Upper case superscripts indicate differences between stocking densities, lower case superscripts indicate differences along time (p<0.05).

		T (ºC)	O_2 (mg L^{-1})	pH	S (‰)	NH_3 (mg L^{-1})	NH_4 (mg L^{-1})	NO_2 (mg L^{-1})	NO_3 (mg L^{-1})
	INCUBATION SYSTEM	24.3 ± 0.16	8.73 ± 0.03	8.34 ± 0.02	38.63 ± 0.61	0.015 ± 0.001	0.016 ± 0.001	0.009 ± 0.00	0.594 ± 0.003
20 hpf (8 hours transport)	**LD**	24.10 ± 0.36	11.48 ± 0.86^{Aa}	7.87 ± 0.09 ^{Aa}	37.00 ± 0.00	0.060 ± 0.010^{Aa}	0.063 ± 0.010 ^{Aa}	0.022 ± 0.001^{Aa}	0.471 ± 0.001^{Aa}
	HD	24.38 ± 0.29	10.61 ± 0.74^{Ba}	8.03 ± 0.12 ^{Aa}	37.00 ± 0.00	0.223 ± 0.028^{Bd}	0.236 ± 0.030 ^{Bd}	0.027 ± 0.002^{Bd}	0.527 ± 0.003^{Aa}
28 hpf (16 hours transport)	**LD**	24.36 ± 0.05	12.92 ± 1.40 ^{Ab}	8.01 ± 0.07 ^{Aa}	37.00 ± 0.00	0.105 ± 0.04^{Ab}	0.111 ± 0.005 ^{Ab}	0.027 ±0.002^{Ab}	0.313 ± 0.004^{Ab}
	HD	24.36 ± 0.05	12.56 ± 0.54 ^{Bb}	7.82 ± 0.13 ^{Aa}	37.00 ± 0.00	0.328 ± 0.019^{Be}	0.347 ± 0.020 ^{Be}	0.038 ± 0.003^{Be}	0.239 ± 0.005^{Ab}
36 hpf (24 hours transport)	**LD**	24.13 ± 0.04	13.97 ± 0.22 ^{Ab}	7.74 ± 0.32 ^{Aa}	37.00 ± 0.00	0.078 ± 0.008^{Ac}	0.083 ± 0.09 ^{Ac}	0.028 ± 0.003^{Cb}	0.283 ± 0.005^{Ac}
	HD	24.20 ± 0.11	12.42 ± 0.90 ^{Bb}	7.72 ± 0.11 ^{Ab}	37.00 ± 0.00	0.417 ± 0.043^{Bf}	0.441 ± 0.046 ^{Bf}	0.025 ± 0.002^{Cd}	0.140 ± 0.005^{Ac}

NO_3 values consistently decreased along time with effects due to transport time ($p < .05$).

Both NH_3 and NH_4 showed a considerable negative linear correlation with survival at opening in HD treatment ($r(13) = -.51$, $p = .03$). On the contrary, no clear linear trend was observed between the other nitrogen forms and survival in both densities (NO_2(LD): $r(13) = .24$, $p = .20$; NO_2(HD): $r(13) = .48$, $p = .07$; NO_3(LD): $r(13) = .10$, $p = .37$; NO_3(HD): $r(13) = .32$, $p = .13$).

4. Discussion

Grey mullet eggs during this trial hatched between 29-38 hpf at 24 °C corroborating literature data (Ramos-Júdez et al., 2021; El-Gharabawy and Assem, 2006; Sylvester and Nash, 1975; Kuo et al., 1973). Though hatching rate was over 30% for all the conditions tested, it was generally lower than previously reported (87%, Vallainc et al., 2021) except for 16 hours simulated transport. However, temperature in the cited experiment was 2 °C lower than the present study, which may have affected the outcomes of this trial as in a Hawaiian mullet population where a peak of egg mortality at 24 °C is reported (Sylvester and Nash, 1975).

Later, survival at 4 dah showed the highest values after 16 hours (28 hpf) for both densities. Sensitivity to physical manipulation depends on one or more stages of embryonic development. Salmonids eggs shipped prior to first division, cell cleavage and during gastrulation showed low survival (Jensen and Alderice, 1983), whereas some pelagic eggs have a critical sensitive period during mid neurula stage (Hilomen-Garcia 1998; Garcia and Toledo, 1988). In our trial, 8 hours transport corresponded to 20 hpf when well plates were prepared. At this stage *M. cephalus* eggs show embryos with differentiated somites going through the blastophore closure (Abraham et al., 1999; Kuo et al., 1973). In *Pagrus major* eggs thermal tolerance drastically decreases when the embryo starts to form, and later at the blastophore closure (Kazuyuki et al., 1988), which is consistent with the present findings. Also, milkfish eggs show a lower survival when shipped in the very first stages of development with detrimental effects on hatching and larvae malformation (Toledo et al., 1996). Manipulation of this batch of eggs along this timeframe may have been reflected on their low survival at 4 dah.

Likewise, early hatched larvae (24 hours simulated transport, 36 hpf) showed a poor survival regardless of the stocking density with some replicates not even surviving at 4 dah. Although the densities tested here were lower than previously reported (4,000 eggs L^{-1}, Vallainc et al., 2021; 15,000 eggs L^{-1}, Koven and Rosenfeld, unpublished), the negative effect of a long transport at 24 °C on survival was evident at 4 dah. Manipulation of early hatched larvae, like the manipulation of eggs during the first stages of development, may have affected survival just after the simulated transport as in *C. chanos* (Hilomen-Garcia, 1998; Toledo et al., 1996). Density effects on survival of transported early hatched larvae have been associated with a drop in oxygen concentration and the accumulation of ammonia (Chattopadhyaya et al., 2021; Estudillo and Duray, 2003). In the present study, oxygen levels after transport were on average 12.33± 1.49 mg L^{-1} with minimum values of circa 10 mg L^{-1} far from the critical levels reported for grouper larvae (1.9-3.0 mg L^{-1}) (Estudillo and Duray, 2003). On the other side, ammonia levels reached the highest levels in HD treatment after 24 hours (0.417± 0.043 mg L^{-1}) which may have had an additive stress effect on the yolk sac larvae reducing further their survival at a later stage. A delayed effect of ammonia toxicity in yolk sac larvae are reported for red drum exposed to concentrations as low as 0.31 mg L^{-1} ammonia (Holt and Arnold, 1983) and for Nile tilapia at 0.45 mg L^{-1} (El-Baki El-Greisy et al., 2016) supporting the present findings. Moreover, a significant negative relation between NH_3 and NH_4 values with HD eggs and larvae survival was highlighted.

In fully sealed containers such as PE bags, CO_2 will accumulate lowering the pH and influencing, in a cascade of events, the concentration of nitrogen forms beyond the species' tolerance threshold. Usually, a pH of around 8 is recommended for optimal larvae and juvenile fish transport (Berka,1986). pH values reached in this study did not deviate significantly from 8 except for HD 24 hours treatment but there was no significant relationship with survival. Lethal pH range (4.5-5.1) for marine first-feeding larvae were indeed far from the values recorded here (Brownell, 1980 B). On the other hand, both ammonia forms drastically increased since the very first transport hours, with values that for HD treatment far exceeding the safe limits reported for marine fish first-feeding larvae (Brownell, 1980 A). Total ammonia nitrogen (TAN) over 7.5 mg L^{-1} is reported to cause an egg mortality of circa 75% when grey mullet eggs are shipped at densities over 15,000 eggs L^{-1} in large cubitainers even at a lower temperature of 22°C (Koven and Rosenfeld, unpublished).

As expected, also NO_2 accumulated with time, but with a less drastic trend than ammonia and was lower than the lethal values reported for Sparids larvae (Brownell, 1980 A), flathead grey mullets fry (0.07-9.1 mg L^{-1}, Hotos and Vlahos, 1998) and red drum (100 mg L^{-1}, Holt and Arnold, 1983).

The two tested densities do not seem to deteriorate water quality to a lethal level in terms of pH, NO_2 and NO_3 especially for short and medium transport times. Nitrate concentration was always far from the lethal values reported for eggs and first-feeding larvae (Simmons et al., 2012; Camargo et al., 2005; Brownell, 1980 A). However, 24 hours transport induces an extreme accumulation of ammonia which should be avoided as it affects long term survival of larvae. Exposure to ammonia above 0.55 mg L^{-1} reduces dramatically survival of red drum larvae 4 days after hatching (Holt and Arnold, 1983) without affecting hatching success. The use of water conditioners is an option that could be tested to control water quality. However, in some species, pH buffers such as Trizma® have been shown to be inefficient and even detrimental for densities as low as 1,000 eggs L^{-1} (Stuart et al., 2018). In larval fish transport promising results have been achieved with the use of probiotics in water. A mixture of Bacillus and Lactobacillus has been suggested as a beneficial additive to keep transport water quality at acceptable levels and improve survival after transport thanks to the displacement of harmful bacteria, reduction of dangerous nitrogen forms and improvement of the innate immune system (Gamoori et al., 2022; Vanderzwalmen et al., 2018; Zink et al., 2011). In freshwater fish eggs (*C. garipinus*) the use of probiotics in water improves hatching rates, and long-term larval survival in a small-scale lab experiment (Ariole and Okpokwasilii, 2012). However, so far, no such studies are available for marine fish eggs transport which would be interesting to explore especially when high densities are used and embryo development is advanced evaluating both biotic (bacterial load) and abiotic (dissolved oxygen, pH and nitrogen forms) parameters.

Variations in water quality, hatching and survival may have been also linked to the natural occurring bacterial load on the eggs. Eggs disinfection is a compulsory prophylactic practice when foreign eggs are introduced into a receiving facility to reduce the chances of bacterial and viral diseases to healthy animals (WOAH, 2019), whereas prior to transport it helps to keep suitable oxygen levels breaking down microbial background respiration and potential pathogenic agents (De Swaef et al., 2016). Different disinfection protocols are

known for several marine species (red drum, Douillet and Hold, 1994; plaice, Salvasen and Vadstein, 1995; halibut, Grotmol and Totland, 2000; sea bream, Ben-Atia et al., 2007; sea bream, red porgy and common dentex, Can et al. 2010) however, there is not an unambiguous method applicable to all as egg sensitivity to germicidals are species-specific, and stage dependent. Even if treatments may not show any observable negative effect, they can still modify post-hatching performance (Douillet and Hold 1994; Salvasen and Vadstein, 1995; Ben-Atia et al., 2007). Moreover, many chemicals have specific disposal measure and need careful manipulation (formalin, glutharaldehyde) or the use of expansive equipment (ozone generators) which are usually unavailable in remote places such as Sardinian lagoons. Due to the potential unknown additive effects of disinfectants on *M. cephalus* eggs performance this practice was avoided and carried out as in Chattopadhyay et al. (2021) and Koven and Rosenfeld (unpublished).

Manipulation of temperature to keep metabolism low, reduce activity, and hence avoid water contamination is a common procedure in fish transport (Berka, 1986). At the egg stage, temperature reduction slows down embryo development and its oxygen consumption (Walsh et al., 1989), hatching times and bacterial respiration. However, temperature cold shock at specific developmental embryo stage may affect eggs survival and larval viability as seen in salmonids (Wagner et al., 2006) and several marine species (Kazuyuki et al., 1988). The temperature chosen in the present study falls in the middle of larval thermal tolerance zone for grey mullet (Walsh et al, 1989), however, optimal incubation range seems to vary with strains. Sylvester and Nash (1975) had best eggs survival and larval performance between 22.7-23.3 °C from wild Hawaii grey mullets, whereas Ramos-Júdez et al. (2022) suggest best results with an incubation temperature of 21 °C for specimens from the Ebro River (Spain) while Liao et al. (1972) indicate a good egg survival at 23-24.5 °C for Taiwanese grey mullet eggs. Koven and Rosenfeld (unpublished) suggest that longer transport times and egg density can be successful if temperature is kept at 22 °C in Israeli specimens. However, in this latter case, larval survival is reported no further than 0 dah, giving partial information on later larval performance.

5. Conclusions

Grey mullet eggs can be shipped at a density of 1,500 eggs L^{-1} up to 16 hours at 24 °C with no detrimental effects on hatching rate and larval viability at 4 dah without the use of water conditioners. However, increasing the stocking density by three folds is accompanied by a decrease in hatching rate between 10-14% at 24 °C which should be considered for seeding purposes. Grey mullet eggs seem to be more sensitive when manipulated at blastophore closure (circa 20 hpf) and as early hatched larvae showing a significant lower survival at 4 dah than eggs manipulated at 28 hpf. Water quality parameters do not deteriorate over the safe limits for marine species in both densities except for ammonia forms which sharply increased and were negatively correlated with larval viability at 4 dah at the highest density tested.

The authors acknowledge that these results are based on a simulated transport and, therefore, a real transport validation would be needed to pinpoint the optimal density and temperature range for shipping grey mullet eggs together with the potential application of water additives and specific, sustainable disinfection protocols.

6. References

Abraham, M., Shiranee, P., Kishore Chandra, P., Kailasam, M. & Charles, V.K., 1999. Embryonic and larval development of the striped mullet *Mugil cephalus* (L). Indian Journal of Fisheries 46 (2), 123–131.

Aizen J., Meiri I., Tzchori I., Levavi-Sivan B., Rosenfeld H., 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. General and Comparative Endocrinology, 142: 212-221. https:// 10.1016/j.ygcen.2005.01.002.

Berka R.,1986. The transport of live fish: A review. EIFAC Technical Paper 48, 52 pp.

Besbes R., Besbes Benseddik A., Kokokiris L., Changeaux T., Hamza A., Kammoun F., Missaoui H., 2020. Thicklip (*Chelon labrosus*) and flathead (*Mugil cephalus*) grey mullets fry production in Tunisian aquaculture. Aquaculture Reports, 17, 100380.

Bobe J., 2015. Egg quality in fish: present and future challenges. Animal Frontiers, 5(1), 66-72. https://doi.org/10.2527/af.2015-0010.

Brownell C.L., 1980, A. Water quality requirements for first feeding in marine fish larvae. I. Ammonia, Nitrite and Nitrate. Journal of Experimental Biology and Ecology, 44, 269-283. https://doi.org/10.1016/0022-0981(80)90158-6.

Brownell C.L., 1980, B. Water quality requirements for first feeding in marine fish larvae. ll. pH, oxygen, and carbon dioxide. Journal of Experimental Biology and Ecology, 44, 285-298. https://doi.org/10.1016/0022-0981(80)90159-8.

Camargo J.A., Alonso A. & Salamanca A., 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere, 58, 1255-1267. https://doi.org/10.1016/j.chemosphere.2004.10.044.

Chattopadhyaya D.N., Chakrabortya A., Raya P.K., Mandala R.N. & Dasa A., 2021. Transportation of fertilized eggs and yolk-sac larvae of hilsa shad, *Tenualosa ilisha* (Hamilton, 1822) in different transportation systems. Aquaculture, 532,736042. https://doi.org/10.1016/j.aquaculture.2020.736042

Crosetti, D., 2016. Current state of grey mullet fisheries and culture. In: Crosetti, D., Blaber, S. (Eds.), Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, USA, pp. 398–450.

El-Baki El-Greisy Z.A., Elsaid Elgamal A.E. & Mohamed Ahmed N.A., 2016. Effect of prolonged ammonia toxicity on fertilized eggs, hatchability and size of newly hatched larvae of Nile tilapia, *Orechromis niloticus*. Egyptian Journal of Aquatic Research, 42, 215-222. https://doi.org/10.1016/j.ejar.2016.04.001.

El-Gharabawy M.M. & Assem S.S., 2006. Spawning induction in the Mediterranean grey mullet *Mugil cephalus* and larval development stages. African Journal of Biotechnology, 5(19), 1836–1845. https://doi.org/ 10.5897/AJB06.036.

Garcia L.M.B. & Toledo J.D., 1988. Critical factors influencing survival and hatching of milkfish (*Chanos chanos Forsskal*) eggs during simulated transport. Aquaculture, 72, 85-93. https://doi.org/10.1016/0044-8486(88)90149-4.

Garcia L.M.B., Hilomen & Garcia G.V., Emata A.C., 2000. Survival of captive milkfish *Chanos chanos Forsskal* broodstock subjected to handling and transport. Aquaculture Research, 31, 575-583. https://doi.org/10.1046/j.1365-2109.2000.00475.x.

Giménez G., Estévez A., Lahnsteiner F., Zecevic B., Bell J.G., Henderson R.J., Piñera J.A. & Sanchez-Prado J.A. (2006). Egg quality criteria in common dentex (*Dentex dentex*). Aquaculture, 260, 232-243. https://doi.org/10.1016/j.aquaculture.2006.06.028.

Hilomen-Garcia G., 1998. Sensitivity of fertilized milkfish (*Chanos chanos Forsskal*) eggs to mechanical shock and simulated transport. Aquaculture, 159, 239-247. https://doi.org/10.1016/S0044-8486(97)00235-4.

Holt G.J. & Arnold C.R., 1983. Effect of ammonia and nitrite on growth and survival of red drum eggs and larvae. Effects of ammonia and nitrite on growth and survival of red drum eggs and larvae, Transactions of the American Fisheries Society, 112:2B, 314-318. https://doi.org/10.1577/1548-8659(1983)112<314:EOAANO>2.0.CO;2.

Hotos G.N. & Vlahos N., 1998. Salinity tolerance of *Mugil cephalus* and *Chelon labrosus* (Pisces: Mugilidae) fry in experimental conditions. Aquaculture, 167, 329-338. https://doi.org/10.1016/S0044-8486(98)00314-7.

Jensen J.O.T. & Alderice D.F., 1983. Changes in mechanical shock sensitivity of coho salmon (Oncorhynchus kisutch) eggs during incubation. Aquaculture, 32(3-4), 303-312. https://doi.org/10.1016/0044-8486(83)90227-2.

Kazuyuki S., Hisashi K. & Shogoro K., 1988. Changes in low temperature tolerance of the eggs of certain marine fish during embryonic development. Comparative Biochemistry and Physiology, 91A, 183-187. https://doi.org/10.1016/0300-9629(88)91614-3.

Kjørsvik, E., Mangor-Jensen, A., & Holmefjord, I., 1990. Egg quality in fishes. Advances in Marine Biology, 26, 71-113. https://doi.org/10.1016/S0065-2881(08)60199-6.

Kuo C.M., Shehadeh Z.H. & Milisen K.K., 1973. A preliminary report on the development, growth and survival of laboratory reared larvae of the grey mullet, *Mugil cephalus* L. Journal of Fish Biology, 5, 459-470. https://doi.org/10.1111/j.1095-8649.1973.tb04475.x.

Loi B., Papadakis I.E., Leggieri F., Giménez Papiol G. & Vallainc D., 2020. Ontogeny of the digestive system and eye of reared flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758), and evaluation of lipid deposition in the liver according to the feeding protocol. Aquaculture 526, 735386. https://doi.org/10.1016/j.aquaculture.2020.735386.

Mylonas C.C., Sigelaki I., Divanach P., Manaños E., Carrillo M., Afonso-Polyviou A., 2003. Multiple spawning and egg quality of individual European sea bass (*Dicentrarchus labrax*) females after repeated injections of GnRHa. Aquaculture, 221, 605-620. https:// 10.1016/S0044-8486(03)00120-0.

Oz I., Gajbhiye D.S., Columbus-Shenkar Y.Y., David L., Golan M., 2022. Non-uniform metamorphosis underlies different development trajectories in hatchery-reared flathead grey mullet (*Mugil cephalus*). Frontiers in Marine Sciences, 9, 967984. https:// 10.3389/fmars.2022.967984.

Parazo M.M., Garcia L.M.B., Ayson F.G., Fermin A.C., Almendras J.M.E., Reyes D.M., Avila E.M. & Toledo, J.D., 1998. Sea bass hatchery operations (2nd ed.). Tigbauan, Iloilo, Philppines: Aquaculture Dept., Southeast Asian Fisheries Development Center.

Ramos-Júdez S., Giménez I., Gumbau-Pous J., Arnold-Cruañes L.S., Estévez A. & Duncan N., 2022. Recombinant Fsh and Lh therapy for spawning induction of previtellogenic and early spermatogenic arrested teleost, the flathead grey mullet (*Mugil cephalus*). Scientific Reports, 12, 6563. https://doi.org/10.1038/s41598-022-10371-0.

Ramos-Júdez S., Chauvigń F., González-López W.A., Rosenfeld H., Cerdà J., Giménez I. & Duncan N., 2021. Providing recombinant gonadotropin-based therapies that induce oogenesis from previtellogenic oocytes to produce viable larvae in a teleost, the flathead grey mullet (*Mugil cephalus*). Aquaculture, 536, 736418.

Reading B.J., Andersen L. K., Ryu Y.-W., Mushirobira Y., Todo T. & Hiramatsu N., 2018. Oogenesis and egg quality in finfish: yolk formation and other factors influencing female fertility. Fishes, 3, 45. https://doi.org/10.3390/fishes3040045.

Rossi G. 2004. Elementi di ragionamento statistico: per psicologia e scienze dell'educazione. Versione 11. https://www.germanorossi.it/abcstat/ABCSTAT11.pdf (accessed 28 April 2023).

Sampaio F.D., & Freire C.A., 2016. An overview of stress physiology of fish transport: changes in water quality as a function of transport duration. Fish and Fisheries, 17(4), 1055-1072. https://doi.org/10.1111/faf.12158.

Shields R.J., Brown N.P. & Bromage N.R., 1997. Blastomere morphology as a predictive measure of fish egg viability. Aquaculture, 155 (1-4), 1-12. https://doi.org/10.1016/S0044-8486(97)00105-1.

Stuart K., Losordo M., Olin P. & Drawbridge M., 2018. Optimizing procedures for shipping marine fish eggs using white seabass, Atractoscion nobilis, as a model. Aquaculture, 493, 229-232. https://doi.org/10.1016/j.aquaculture.2018.04.050.

Sylvester J.R. & Nash C.E., 1975. Thermal tolerance of eggs and larvae of Hawaiian striped mullet, *Mugil cephalus* L. Transactions of the American Fisheries Society, 104(1), 144-147. https://doi.org/10.1577/1548-8659(1975)104%3C144:TTOEAL%3E2.0.CO;2.

Tamaru C.S., Fitzgerald W.J. J. & Sato V., 1993. Hatchery Manual for the artificial propagation of striped mullet (*Mugil cephalus* L.). Guam Aquaculture Development and Training Center Technical Report n.15, pp. 167.

Thieme P., Vallainc D. & Moritz T., 2021. Postcranial skeletal development of *Mugil cephalus* L (Teleostei: Mugilidae): morphological and life-history implications for Mugiliformes. Zoological Journal of the Linnaean Society, 192, 1071–1089. https://doi.org/10.1093/zoolinnean/zlaa123.

Thompson J.M., 1963. Synopsis of Biological Data on the Grey Mullet *Mugil cephalus* Linnaeus 1758. Melbourne: CSIRO, 1, 1–75. https://doi.org/10.25919/2pmz-2721.

Toledo J.D., Doi M. & Duray M., 1996. Viability of milkfish eggs and larvae after simulated and actual transport. In: MacKinlay, D., Eldridge, M. (Eds.), The Fish Egg: Its Biology and Culture Symposium Proceedings. International Congress on the Biology of Fishes, 14-18 July 1996, San Francisco State University. Physiology Section American Fisheries Society, Bethesda, Maryland, pp. 51–58.

Valdebenito I.I., Gallegos P.C. & Effer B.R., 2015. Gamete quality in fish: evaluation parameters and determining factors. Zygote, 23(2), 177-97. https://doi.org 10.1017/S0967199413000506.

Vallainc D., Concu D., Giménez Papiol G., Loi B., Leggieri F., Brundu G., Chindris A., Sanna G., Fois N., Antognarelli F. & Rossini P., 2021. Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons. Aquaculture Reports, 21, 100844. https://doi.org/10.1016/j.aqrep.2021.100844.

Walsh W.A., Swanson C., Lee C.S., Banno J.E. & Eda H., 1988. Oxygen consumption by eggs and larvae of striped mullet, *Mugil cephalus*, in relation to development, salinity and temperature. Journal of Fish Biology, 35, 347-358. https://doi.org/10.1111/j.1095-8649.1989.tb02987.x.

Chapter V: Different weaning strategies effects on liver, gut and bone performance of larval flat head grey mullet (*Mugil cephalus*)

This chapter is based on the publication currently being written : D. Concu, R. Quirós-Pozo, B. Loi, L. Rabaina, M. Scolamacchia, A. Chindris, G. Brundu, J. Roo-Filgueira, S. Carboni, D. Vallainc. Different weaning strategies effects on liver, gut and bone performance of larval Grey Mullet (M. cephalus)

1. Introduction

The grey mullet (*Mugil cephalus*) is a cosmopolitan, omnivorous, and detritivorous fish species that in recent years is playing an increasingly important role in the diversification of Mediterranean aquaculture due to its low trophic level, rapid growth, and good adaptability to captivity (Solovyev e Gisbert, 2022). Due to heavy fishing pressure during the spawning period and aquaculture relying on the capture of wild juveniles, we are witnessing a gradual decline in populations (Withfield et al., 2012); in fact from their ovaries is produced the 'bottarga di muggine', a luxury product recognized internationally (Crosetti and Blaber, 2016). For this reason it is necessary to implement the breeding protocols of this species passing from the improvement of the larval rearing to have a more programmable production of fry in order to reduce the pressure of fishing on natural populations.

The production of juveniles of marine species in commercial hatcheries depends mainly on feeding with live prey (Rosenlund et al., 1997). During the weaning process, live food is gradually replaced by artificial diets. This is considered a very critical moment in the larval rearing phase; a prolonged period of using live foods is costly and may cause nutritional deficiency since it does not contain an adequate nutrition content for growth and development of fish larvae (Callan et al., 2003; Ma et al., 2015), furthermore, live food is a potential vector for infectious protozoan parasites, viruses and bacteria (Bonaldo et al., 2011; Makridis et al., 2000). A good weaning protocol must ensure high rates of growth and survival, with less dispersion of sizes and incidence of skeletal anomalies (Gisbert et al., 2018). The gradual weaning of larvae from live food in a co-

feeding regime with the use of MD (Micro Diet) is designed to promote digestive maturation in the early stages of life (Engrola at 2007,2009) and to improve growth and survival rates. An early co-feeding period appears to prepare the gut to accept and process inert diets, allowing earlier weaning and resulting in better growth performance than feeding strategies based on late weaning at the end of the larval stage (Conceicao et al., 2010). The replacement of the diet consisting solely of live prey in a short time is crucial for the reduction of production costs to ensure a constant production of high-quality juveniles. The purchase and production of artemia for larval feeding plays a very important role on the final costs of production of fry (Callan et al 2003).

Bone malformations of fish species reared in aquaculture play a very important economic role in terms of losses. These reduce growth performance, feed conversion, survival and increase stress, resulting in disease and related onset (Balbebona et al. 1993; Andrades et al. 1996; Divanach et al. 1996; Hilomen-Garcia 1997; Koumoundourous et al. 1997b; Boglione et al. 2001, 2003; Cahu et al. 2003; Matsuoka 2003; Lall and Lewis-McCrea 2007; Le Vay et al. 2007; Castro et al. 2008). It is currently difficult to estimate the real economic losses due to the incidence of skeletal deformities in hatcheries, due to the reluctance of farmers to provide reliable data. It has been estimated that annual losses due to the incidence of skeletal anomalies are around 50,000,000 Euro for European aquaculture (Boglione et al 2013). One of the main challenges to make this activity more productive for aquaculture, in economic and image terms, is to improve rearing techniques to reduce the impact of skeletal anomalies in the production cycle.

The ability to uptake, digest and absorb exogenous food by fish larvae depends on the development and health of the digestive tract (Przybyl et al., 2006). Specifically, both intestine and liver play a central role in digestion and absorption of nutrients, in addition are considered to be good indicators of metabolic, health and nutritional status of fish (Caballero et al., 2003; Sirri et al., 2017). The histological monitoring of these organs is considered necessary to detect diet-related morphological changes (Przybyl et al., 2006) and highlight the effects of malnourishment during the rearing process (Papadakis et al., 2009).

The deposition of lipid reserves in the liver is acknowledged as a good marker for the nutritional and physiological status of the fish (Caballero et al., 1999; Zambonino-Infante et al., 2008; Loi et al., 2020). At hepatic level, fat storage may reflect a physiological disorder due to inappropriate feeding conditions or a nutritionally unbalanced diet (Margulies, 1993; Yúfera et al., 1993, 1996; Diaz et al., 1998; Green and McCormick, 1999; Crespo et al., 2001; Gisbert et al., 2004b; Gisbert et al., 2008; Lazo et al., 2011). Concurrently, histopathological changes in the intestine due to sub-optimal feeding or starvation include, among others, lower and less frequent intestinal folds and reduced enterocyte height (Gisbert and Doroshov, 2003; Gisbert et al., 2004b; Ostaszewska et al., 2006; Przybyl et al., 2006; Kamaszewski and Ostaszewska, 2014). In particular, the reduced nutrient absorption surface area may directly compromise the growth and survival of larvae (Zambonino-Infante et al., 2008).

In view of this, the aim of the study is to reduce the use of artemia in favour of MD, maximising survival and growth in the larvae, reducing the incidence of bone deformities and reducing management costs.

2. Materials and methods

2.1 Ethics statement

The present study followed the European Union Directive (2010/63/EU) art 1 comma 5, letters (d) and (f), on the protection of animal welfare for scientific purposes and the national guidelines for the humane treatment of animals, complying with the relevant Italian legislation. The entire rearing has been carried out in the establishment of the IMC authorized by the Region of Sardinia with authorization number IT038OR501 and under a veterinary supervision.

2.2 Reproduction, Eggs incubation and Hatching

Broodstock was collected during natural spawning season in the lagoon of Porto Pino (38°57'51''N, 8°25'56''E)(2020), transferred in one 3500 L volume tank supplied by flow-trough raw seawater and induced to spawn with LH-RH anologue (ENANTONE, Takeda Italia S.p.a C59H84N16O12 amino acid sequence H-Pyr-Trp-Ser160 Tyr-D-Leu-Leu-Arg-Pro-NHEt) at a dose of 200 µg kg^{-1} for females and 100 µg kg^{-1} for males, as described by Vallainc et al., (2021). Fertilized eggs were transported to the facilities of the International Marine Centre (Oristano, Sardinia, Italy) where they were incubated as described by Loi et al. (2020). Fertilized eggs were incubated in flow-through (water flow of 4 L min^{-1}), at 1000 eggs L^{-1}. 30 hours after fertilization, eggs were transferred into an indoor recirculating aquaculture system until hatching. Larvae were then reared at a density of 130 individuals L^{-1}. Green Water was administered from 2 dph to 22 dph and consisted in the use of Nano Star Green (AlgaSpring, Almere, The Netherlands) to maintain a concentration of 400000 cells mL^{-1}. First feeding started at 2 dph and consisted of rotifers (Brachionus sp.) enriched with Nano Star Red (AlgaSpring, Almere, The Netherlands). The rotifer concentration was adjusted daily to 10 individuals ml-1 until 14 dph. The water was renewed every 7 days at a rate of 30% of total volume. Water temperature was maintained at 22.1 ± 0.7 °C using a chiller (Teco TK6000H), salinity was maintained at 36.8 ± 0.4 ppt and oxygen saturation was measured daily (Hach Lange HQ 40 d, Colorado, USA) and was 83 ± 14% throughout the trial. Ammonia and nitrite were kept < 0.25 mg L^{-1}, and nitrate < 25 mg L^{-1}.

2.3 Feeding trial

Prior to the beginning of the feeding trial, larvae were counted volumetrically and distributed manually at a density of 1090 larvae/tanks (3270 larvae $treatment^{-1}$) in the larval system consisting of 9 tanks (300 L^{-1}) divided into 3 Recirculation Aquaculture Systems (RAS). Larvae were then acclimated for 7 days and fed with5 rotifers ml^{-1} with the addition of green water (*Tetraselmis suecica* and *Isocrisis galbana*, 1:1) at a concentration of 400.000 cell mL^{-1}. Water exchange was set at 25% of the rearing volume per hour and

photoperiod was set at 12 h light: 12 h darkness. The feeding trial lasted for 14 days from 22 dph to 36 dph. Three different co-feeding protocols were tested in a triplicated randomized design according with the protocols illustrated in Figure 1. Artemia metanauplii and rotifers administered during the experiment were enriched using Nano Star Red (AlgaSpring, Almere, The Netherlands) according to manufacturer instructions. The same amount of formulated diets were administered 15 times day^{-1} ad libitum to the three treatments. Gemma Micro 0.1 (Skretting, Vervins, France) was administered from 22 dph to 35 dph and from 29 dph it was administered in a ratio of 1:1 with Gemma Wean 0.2 (Skretting, Vervins, France). Unconsumed food was weighed daily.

The average amount of food consumed in each tank was 0.56 g day^{-1} from 22 to 29 DPH, and 0.88 g day from 29 to 35 dph.

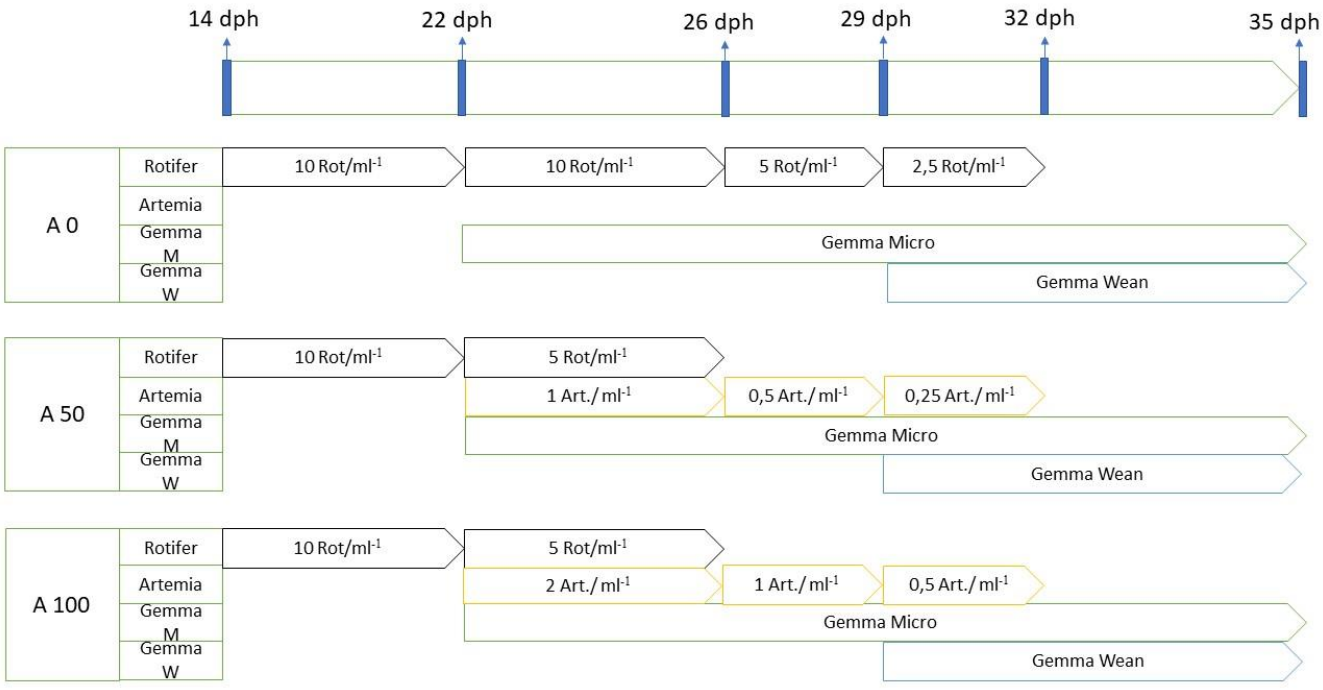

Fig. 1 – Dietary protocol of A0, A50 and A100 treatments expressed as daily concentration in 300 L tanks.

The bottom of the tanks and the surface of the water were siphoned and cleaned daily to remove excess feed and mortality.

Temperature and oxygen values were recorded daily using a probe (Hach Lange HQ 40 d, Colorado, USA), with average values of 23.0±0.16°C and 8.14±0.10 mg L^{-1} respectively.

pH, salinity, ammonia, nitrite, and nitrates were measured every 48h by using a ph-meter (Halo HI11312, Hanna Instruments, Italy), refractometer (ATC, Italy) and with a commercial kit (Prodac laboret test kit, Prodac S.L.). The average values throughout the trial for the above mentioned parameters were 7.80±0.03 (pH), 38.22±0.17 ppt (Salinity), 0.09±0.10 mg L^{-1}, 0.37±0.37 mg L^{-1}, and 8.80±5.69 mg L^{-1} (ammonia, nitrite, and nitrates).

2.4 Growth performances

Samples (n=20 fish $tank^{-1}$) were taken at the start, intermediate point (n=20 fish $tank^{-1}$) and at the end of the experiment (n=30 fish $tank^{-1}$) when larvae were 22, 29 and 36 dph respectively. Individuals were randomly collected and lethally anesthetised with clove oil.

Each fish was photographed to determine total length (TL, mm), myotome height (MH, mm), and eye diameter (ED, mm). Fish at the start of the trial were photographed and measured under a stereo microscope (Leica MZ8, Germany) connected to a digital camera (Leica DMC2900, Germany) coupled with an image analysis software (Leica Application Suite 4.5), while at the other sampling points samples were photographed by a Canon G15 camera and measured with the ImageJ software (USA). Once obtained all the measures, Condition index (CI) of fish was calculated as CI = [myotome height (mm) / total length (mm)] x100, as a measure of larval condition.

2.5 Liver and intestine histology

Five individuals were preserved for histology in 10% buffered formalin. Samples were dehydrated (70–96% ethanol), embedded in methacrylate resin (Technovit 7100®, Kulzer, Germany), sectioned at 4 µm with a manual rotary microtome (Leica RM2125, Germany), and finally stained with Methylene Blue (Alfa Aesar, Thermo Fisher GmbH, Germany)/Azure II (Sigma-Aldrich, Germany)/Basic Fuchsin (Sigma-Aldrich, Germany) (Bennett et al., 1976). All the sections were examined under a microscope (Leica DM2000, Germany) connected to a digital camera (Leica DMC2900, Germany).

For each fish, six microphotographs of histological sections of liver were taken at 100× magnification (Fig. 2 A) for the estimation of the area covered with lipid vacuoles in the liver (ACLV%), according to Papadakis et al. (2009). Pictures were converted to grey scale in order to emphasise the contrast between lipid vacuoles (in white) and the hepatic tissue (in dark grey). Blood vessels were manually eliminated from the analysis. Thereafter, the boundaries of all lipid vacuoles were marked off manually and their total area was automatically estimated by the ImageJ software (Fig. 2 B).

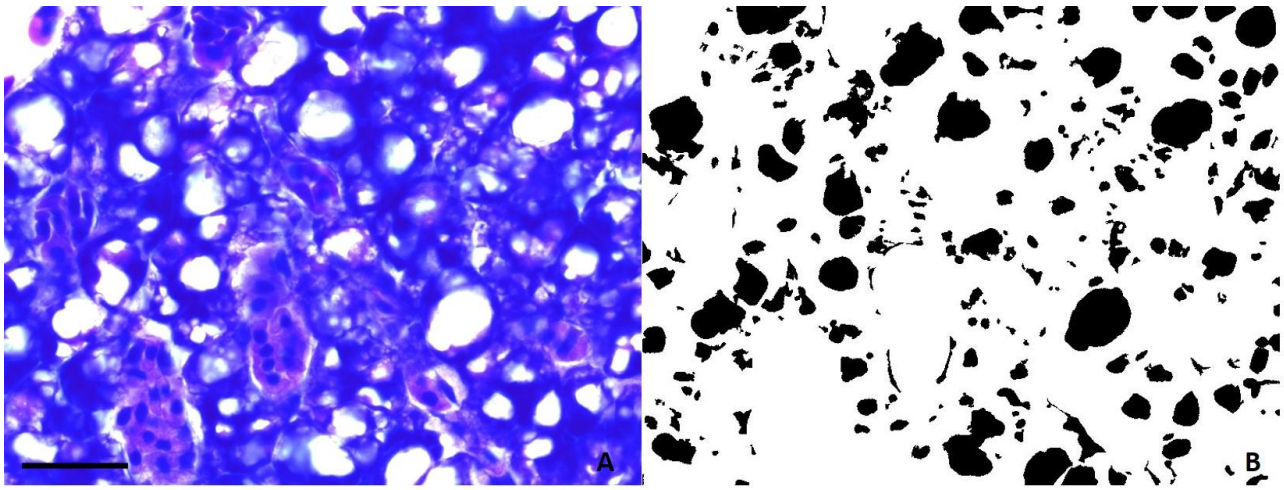

Fig. 2 – Histological microphotographs of flathead grey mullet's liver. (A) before image analysis; lipid vacuoles in white. (B) result of the image analysis; lipid vacuoles in black. Methylene blue/azure II/basic fuchsin, bar 20 µm.

In the same sample set, the possible reduction of the absorptive epithelial surface was evaluated in the distal intestine by the analysis of intestinal folds and enterocytes. For each sample, histological sections were microphotographed at 40× magnification and, intestinal fold height (Fh) was measured from the apex to the base, excluding the underlying connective layer (10 measurements per fish, 5 fish/tank) (Panettieri et al., 2020) (Fig. 3 A). Enterocyte height (Eh) was measured from the base to the top of enterocyte (10 measurements per fish, 5 fish/tank) (Przybyl et al., 2006; Peng et al., 2013) (Fig. 3 B).

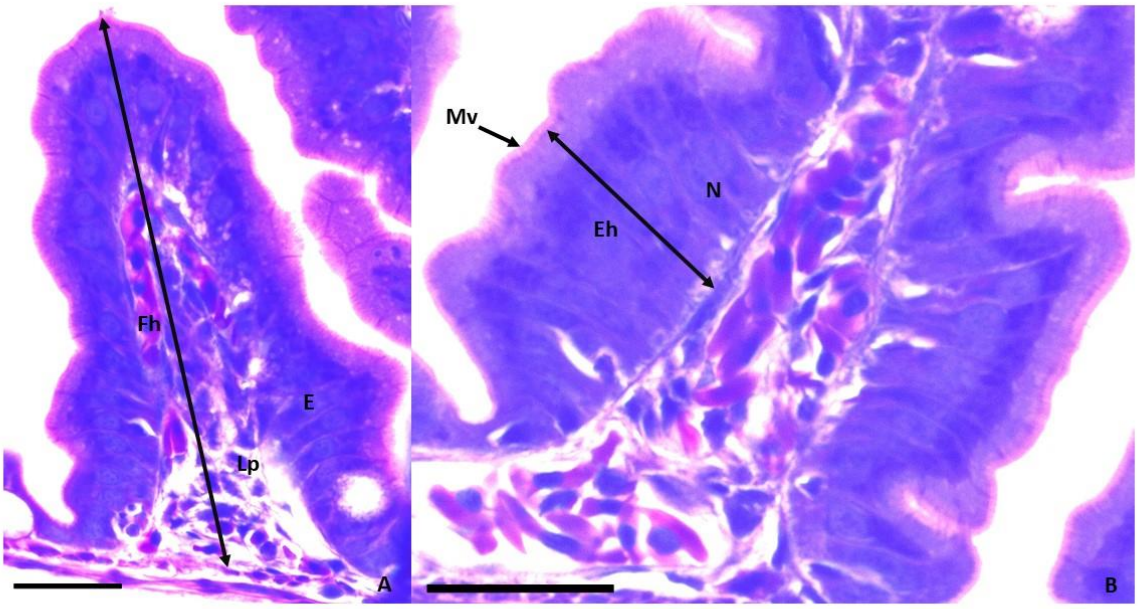

Fig. 3 – Histological microphotographs of flathead grey mullet's distal intestine. (A) general view of an intestinal fold. (B) enlarged view of the fold. Fh: fold height, LP: lamina propria, E: enterocyte, Mv: microvillus, Eh: enterocyte height, N: nucleus of the enterocyte (methylene blue/azure II/basic fuchsin, bar 20 µm).

2.6 Skeletal deformities analysis

To identify and quantify larval skeletal anomalies, a minimum of 200 larvae per treatment were randomly sampled at 36 dph and preserved in 10% neutral-buffered formalin.

After fixation, larvae were transferred in an ascending alcohol series (larvae between 4 mm and 12 mm were placed in 96% ethanol for complete dehydration and afterwards stained in Alcian Blue solution for cartilage staining for 1,5 h depending on de size), and then into 0,5% KOH, following the protocol of Schnell et al. (2016). After hydration the larvae were transferred in trypsin solution for several hours (from 2 to 24 hours depending on the size). Subsequently, the samples were transferred in alizarine solution (bone staining) from 2 to 24 hours (depending on the size). Larvae were then transferred into glycerin and KOH 0.5% (3:1), for storage and examination. The specimens were examined using a Stereoscope (Leica MZ8), through a Leica DMC2009 and measured with LAS 4.5 software (Leica,Germany).

Region	A	Cephalic vertebrae (carrying epipleural ribs) (1st–2nd vertebra)
	B	Pre-Hemal vertebrae (carrying epipleural and pleural ribs and with open hemal arch, without hemal spine) (3rd–10th vertebra)
	C	Hemal vertebrae (with hemal arch closed by a hemal spine) (11th–21st vertebra)
	D	Caudal vertebrae (with hemal and neural arches closed by modified spines) (22nd–24th vertebra)
Types	**1**	**Kyphosis**
	2	**Lordosis**
	3	**Partial vertebral fusion**
	3*	**Total vertebral body fusion**
	4	**Vertebral anomaly (shape anomaly, ossification ridges, marked reduction in length or elongation, intervertebral bony plate)**
	5	Anomalous neural arch and/or spine
	6	Anomalous haemal arch and/or spine
	7	Anomalous rib
	12	**Swim bladder anomaly**
	13	Presence of calculi in the urinary ducts
	14	**Anomalous maxillary and/or pre-maxillary**
	15	**Anomalous dentary**
	16	Other cephalic anomalies (glossohyal, neurocranium, ..)
	17L/R	**Anomalous left/right opercular plate**
	17*L/R	Anomalous, absent, fused branchiostegal ray
	21	Anomalous epipleural ribs
	22	Anomalous dorsal ribs
	23	Anomalous pleural ribs
	26	**Supernumerary vertebra**
	29	Anomalous postcleithrum
	S	**Scoliosis**
	Cl L/R	Anomalous left/right cleithrum
	Cor	L/R Anomalous left/right coracoid

Tab. 1 – List of anomalies considered. Bolt font are indicated anomalies that have been considered severe.

Deformities have been converted into binary data (presence/absence) and prevalence and severity of each deformity type was assessed according to the method described by Boglione et al. (2014). The number of individuals showing at least one deformity was recorded and expressed as a percentage of the total number of individuals observed.

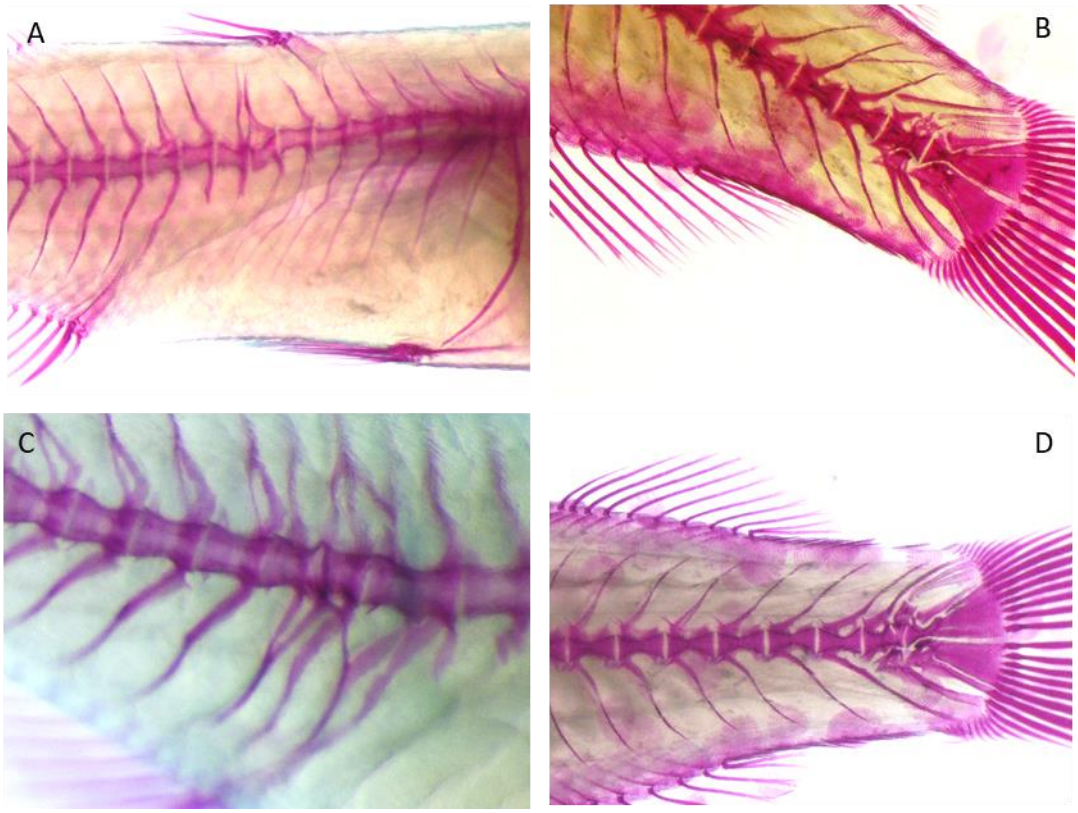

Fig. 4 - Examples of some skeletal anomalies observed in *M.cephalus*: A: Lordosis of the haemal region; B Total fusion of vertebrae in the caudal region; C: Fusion of neural and haemal arches in the haemal region; D: Partial fusion vertebrae caudal region

2.7 Data analysis

Data of, total length, myotome height, Condition Index, eye diameter, as well as liver lipid deposition, intestinal fold height and enterocyte height were checked for the normality of distribution and the homogeneity of variances. When these assumptions were not respected, a GLM were run instead of a two-way ANOVA. Comparisons were considered statistically significant when $P < 0.05$. All data expressed in

percentage were previously transformed prior to analysis using the arcsine of the square root transformation.

The analyses were performed using SigmaPlot 11 and Statistica 6.1 StatSoft, Inc. (2004) statistical packages. Data in this paper are presented as mean ± standard deviation.

All data related to skeletal deformities were tested with the Kolmogorov-Smirnoff test for normality of distribution and with the Levene test (Sokal & Rohlf, 2012) for homogeneity of variances. When these assumptions were met, one-way variance analysis (ANOVA) was performed, followed by the Tukey post-hoc test and the Student t test for coupled samples. Kruskal-Wallis test was applied when heterogeneity of variances and/or normality of distribution were not satisfied.

3. Results

3.1 Survival and growth performances

Larval survival was significantly different between treatments ($p=0.02$). A0 treatment had the highest survival (64,79±7,39%), followed by A50 treatment (42,52±8,30%), whilst A100 had the lowest survival (32,46±12,82%) (Fig. 5).

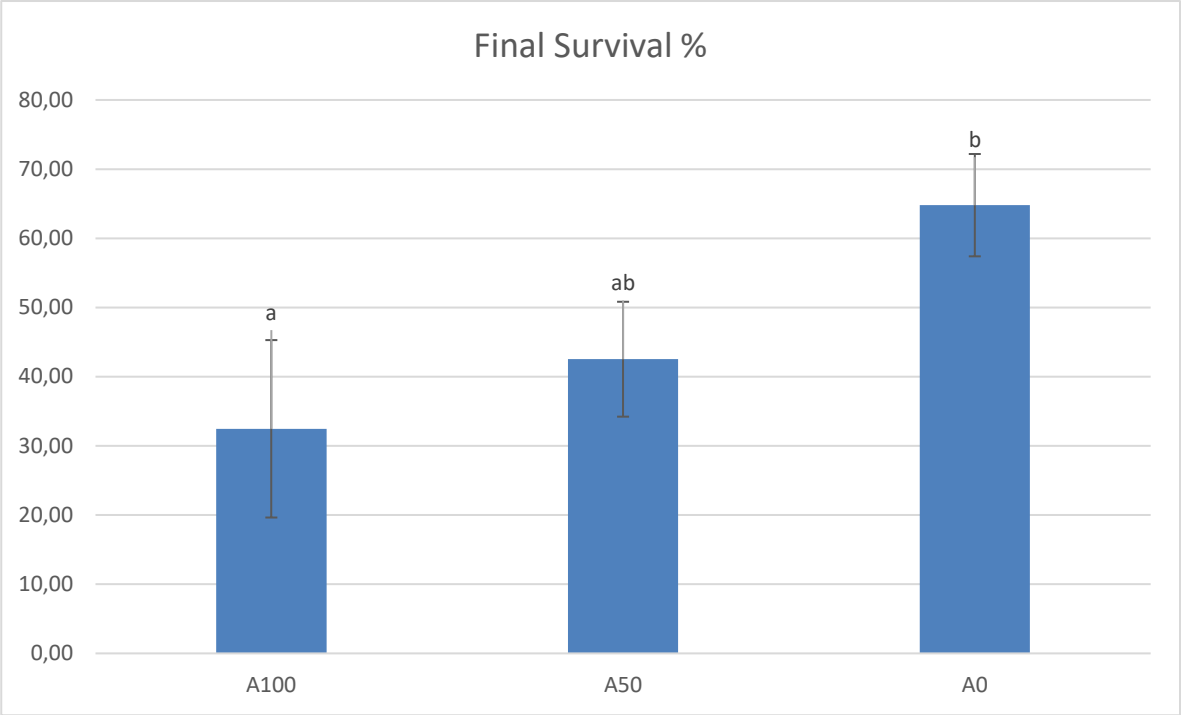

Fig. 5 – Final survival of the three different treatments expressed in average percentages ± sd.

As described by Quirós-Pozo et al., 2023, the onset of mortality in A100 and A50 began around the time the rotifer was removed (26 DPH) from the diet of these two treatments (Fig 6).

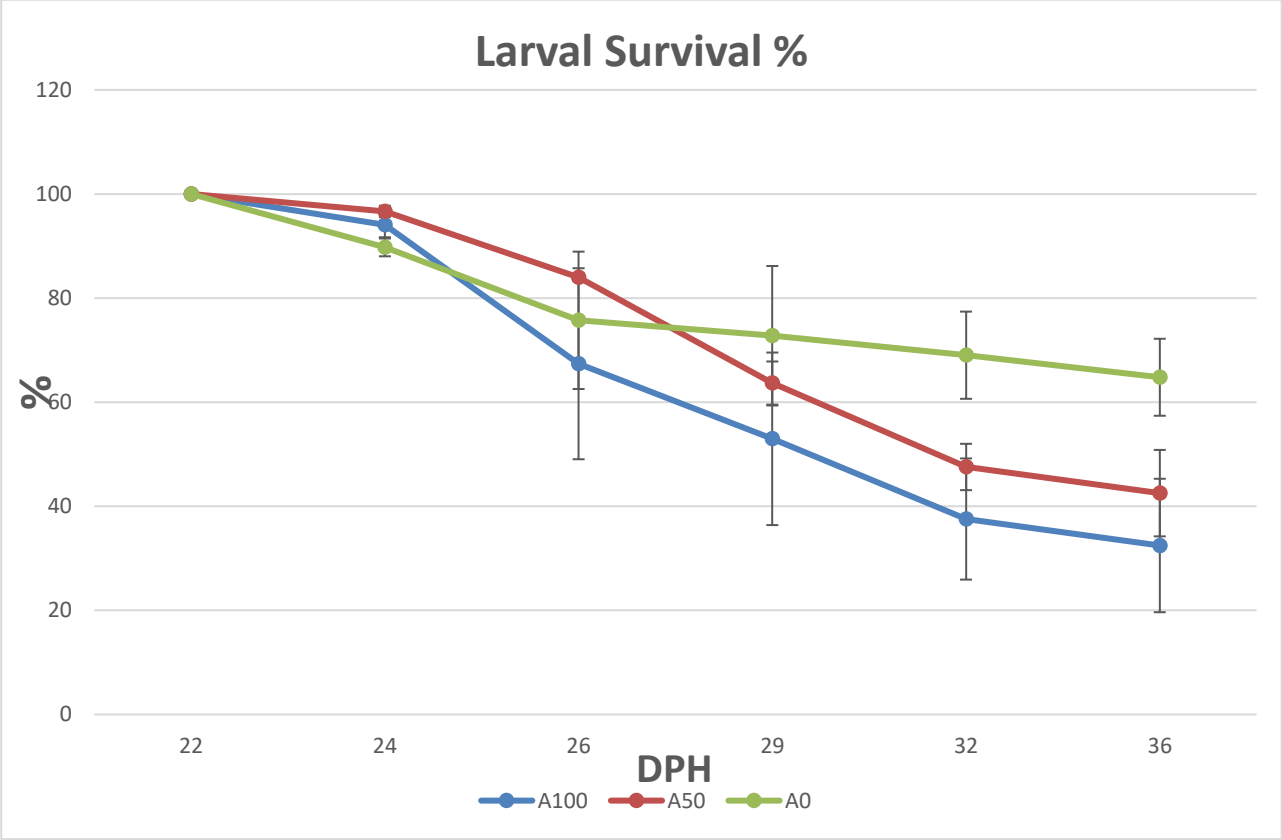

Fig 6: Larval survival over timein the three different treatments A100, A50 and A0 expressed as an average percentage ± sd.

Mean total length significantly increased over time, independently from the treatment. During the trial, TL grew from 5.54±0.12, 5.54±0.11, 5.73±0.11 mm up to 15.52±0.41, 13.92±0.48, 12.27±0.46 mm in A100, A50 and A0 respectively. At the final sampling point, TL in fish fed with Artemia was significantly higher than total length in fish fed rotifers only. Furthermore, fish fed with 100% Artemia were significantly longer than fish fed with 50% Artemia (Fig. 7).

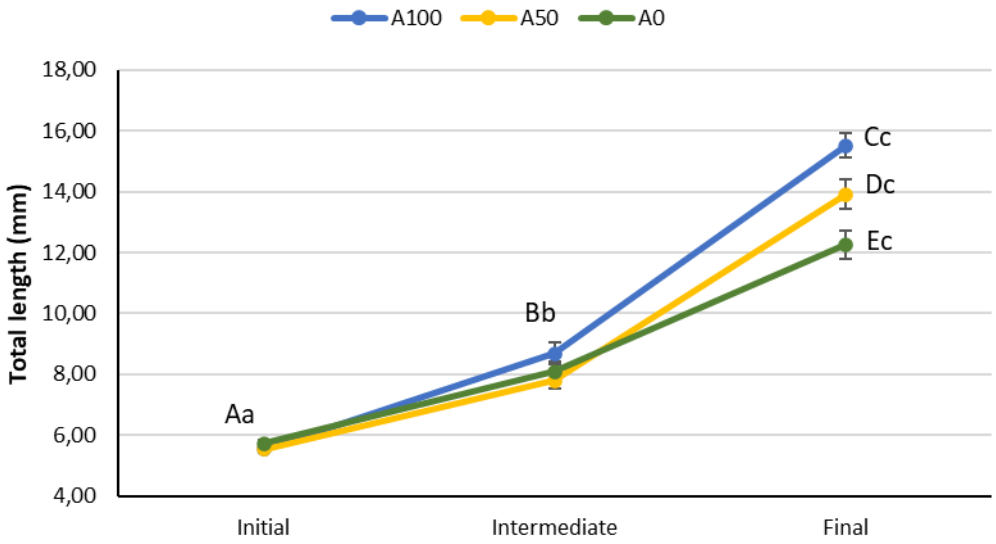

Fig. 7 – Total length of fish from the three treatments, at each sampling point. Different superscript letters indicate significant differences (GLM). Data are expressed as mean ± sd (n=3). Uppercase letters: "Treatment" factor; lowercase: "Time" factor.

As well as total length, the height of myotome grew over time, with significant differences between treatments at the final sampling point, with the greatest myotome height measured in fish from A100 (2.56±0.07 mm). On the contrary, fish fed rotifers only were characterized by the lowest myotome height (1.89±0.08 mm), while fish from A50 were at an intermediate point (Fig. 8).

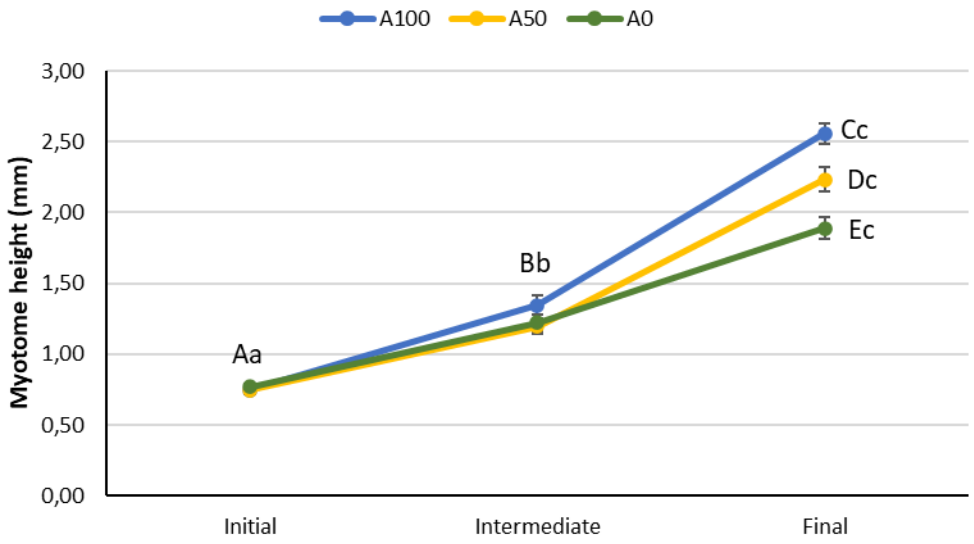

Fig. 8 – Myotome height of fish from the three treatments, at each sampling point. Different superscript letters indicate significant differences (GLM). Uppercase letters: "Treatment" factor; lowercase: "Time" factor.

Accordingly, the Condition Index reached maximum values at the end of the trial, growing significantly at every sampling point. The highest Index was obtained at the end of the experiment by fish from A100 (16.39±0.14), although it was statistically similar to the value calculated for A50 (15.82±0.19). The Condition Index of fish from A0 was lower (15.11±0.16) compared to the others, however it was not significantly different from the index calculated for A50 (Fig. 9).

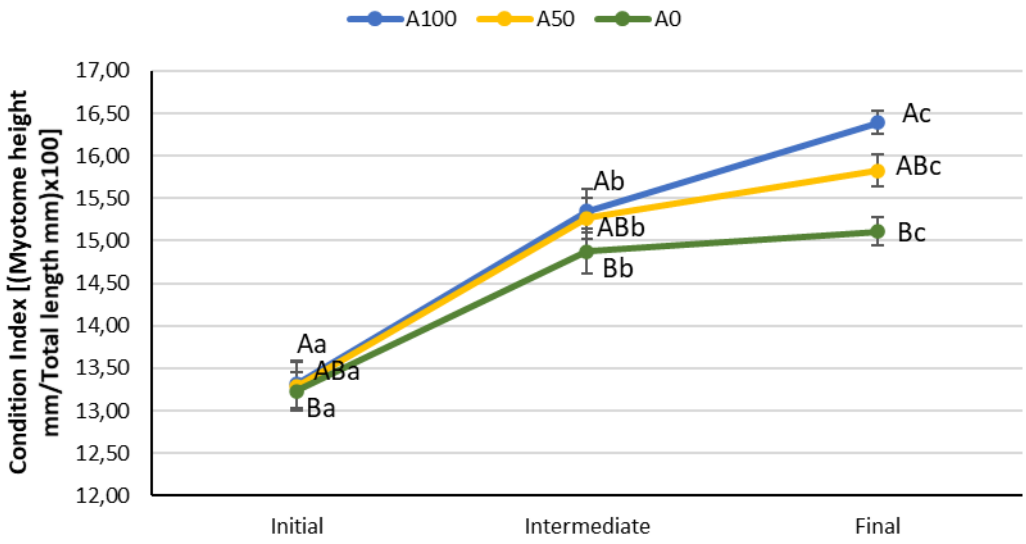

Fig. 9 – Condition Index of fish from the three treatments, at each sampling point. Different superscript letters indicate significant differences (GLM). Uppercase letters: "Treatment" factor; lowercase: "Time" factor.

The enlargement of the eye followed the trend of the other body measurements, being greater at the end of the trial. The largest diameter was measured in fish from A100 (1.29±0.03 mm), which were statistically similar to fish from A50 (1.22±0.03 mm). Significant differences were revealed by GLM between A100 and A0 (1.14±0.04 mm) (Fig. 10).

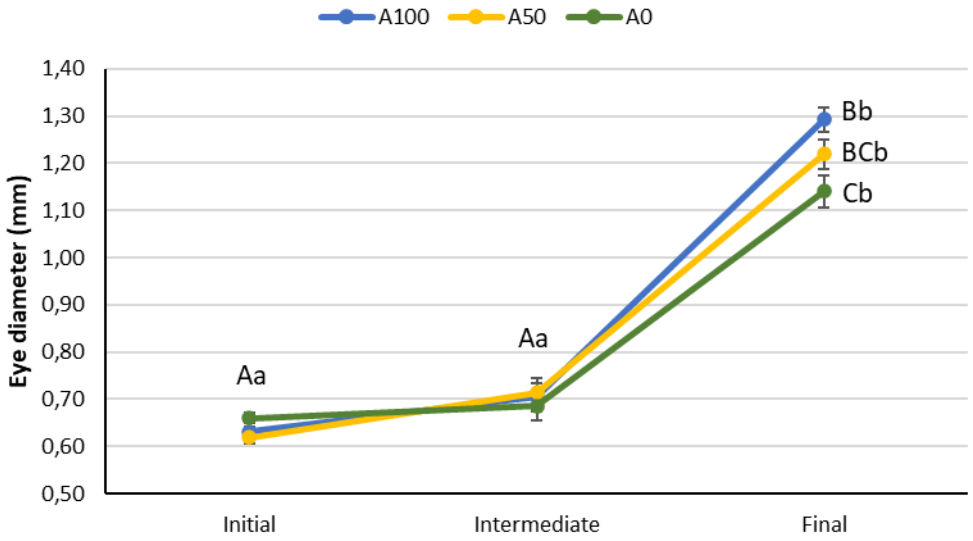

Fig. 10 – Eye diameter of fish from the three treatments, at each sampling point. Different superscript letters indicate significant differences (GLM). Uppercase letters: "Treatment" factor; lowercase: "Time" factor.

3.2 Liver and intestine analysis

Histological analysis of the liver revealed that the area covered by lipid vacuoles grew significantly in the last week of the experiment, reaching the final values of 17.11±0.98, 14.58±0.70 and 12.34±0.69 % in A100, A50 and A0, respectively. However, no difference between treatments was detected by the GLM (Fig. 11).

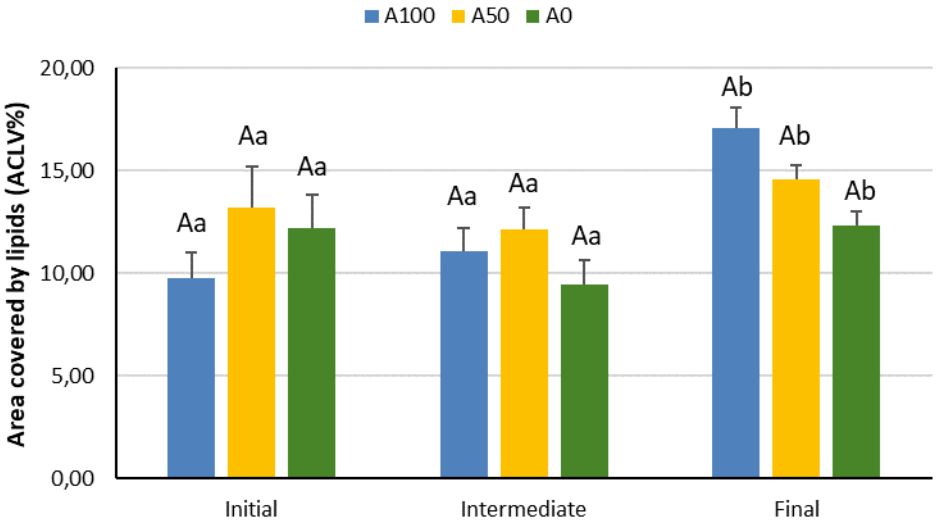

Fig. 11 – Liver area covered by lipid vacuoles in fish from the three treatments, at each sampling point. Different superscript letters indicate significant differences (GLM). Uppercase letters: "Treatment" factor; lowercase: "Time" factor.

Histology of the distal intestine evidenced a regular morphology of mucosal folds, with finger-shaped villi formed by a central, distinct lamina propria surrounded by a mono-stratified epithelial layer of well-differentiated enterocytes. These cells, provided with small nuclei, were covered by microvilli (mean height of ~1.2 µm) in the apical part, forming the brush border membrane.

The intestinal fold height was greater in fish that received 100% Artemia (172.62±10.05 µm); no statistical differences were observed with the A50 treatment, although in those fish the intestinal folds were shorter (150.66±6.67 µm). On the contrary, intestinal folds measured in fish from A100 were significantly higher than those in A0 (139.79±6.17 µm) (Fig. 12).

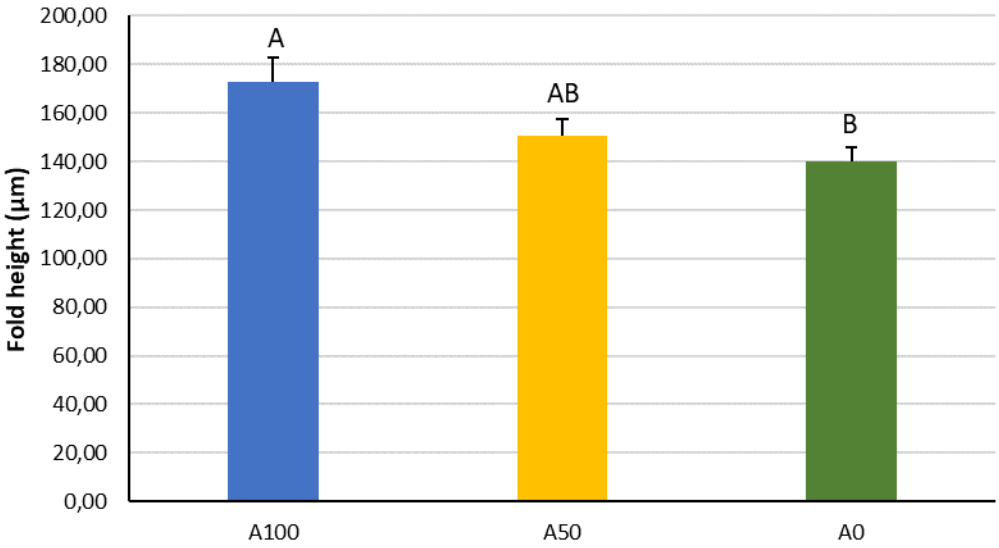

Fig. 12 – Fold height of distal intestine in fish from the three treatments, at the final sampling point. Different superscript letters indicate significant differences (One-way ANOVA).

Enterocytes reached the mean value of 26.35±0.63 µm in intestinal folds of fish from A100. Their height was significantly greater than the height of enterocytes measured in fish from the other treatments (24.12±0.51 and 22.55±0.61 µm for A50 and A0, respectively) (Fig. 13).

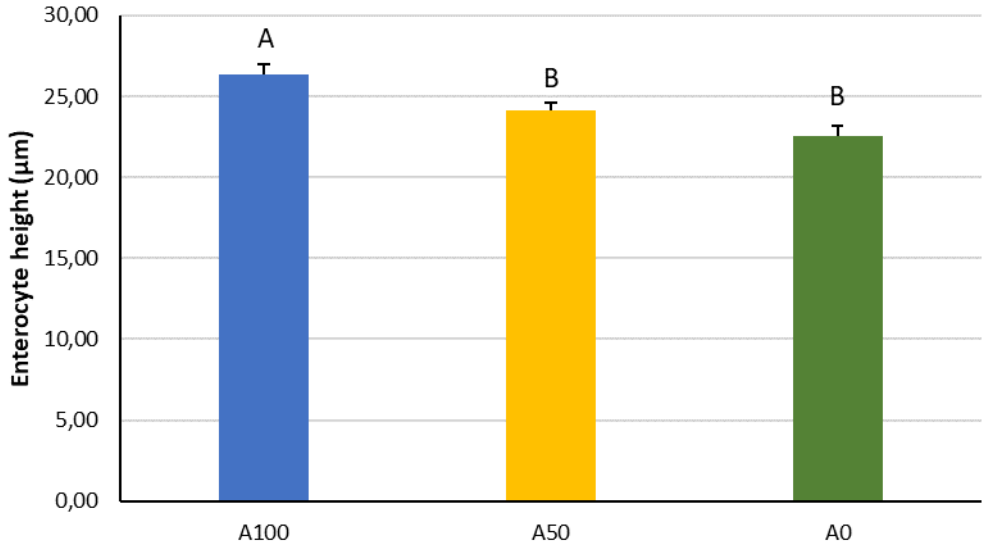

Fig. 13 – Enterocyte height of distal intestine in fish from the three treatments, at the final sampling point. Different superscript letters indicate significant differences (One-way ANOVA).

3.3 Skeletal anomalies

Considering all the areas of study shown in Table 1, deformity analyses shows that A100 presented a significantly higher (p = 0.03938) incidence of total anomalies (number of total anomalies/number of total individuals) than A50 (Fig. 14). There are no significant differences between the treatments at 4 regions considered (Cephalic vertebrae, Pre-hemal vertebrae, Hemal vertebrae, Caudal vertebrae) taken into account.

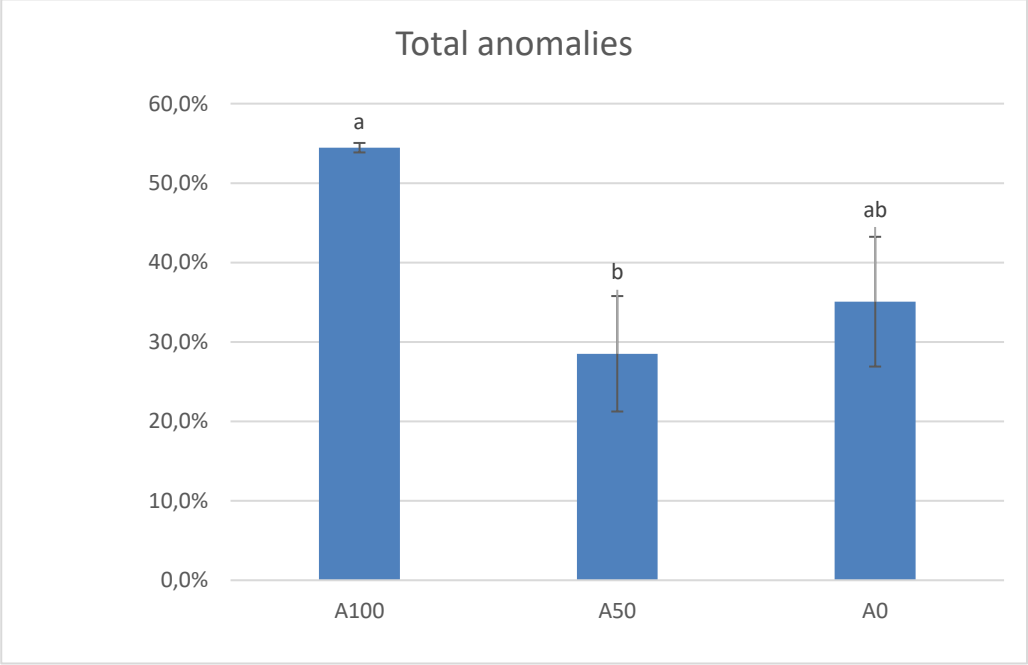

Fig. 14 – Total anomalies expressed as a percentage of incidence ± sd, considering all areas under study.

Co-feeding protocols A0 and A50 show a high rate of high-quality larvae, where only 23.23% and 22.55% of larvae had at least one skeletal anomalies. While the A100 protocol exhibited 42.08% of larvae with at least one anomaly.

Amongst skeletal anomalies some are considered severe because of their impact on the animals chances of survival and it final market value, those considered severe are shown in Tab 2. A100 treatment showed a higher incidence of severe anomalies (number of severe anomalies/total anomalies) (50.00%±4.14%)

compared to A50 and A0 (23.40%±2.77% and 29.86%±7.39%), with a significant difference of p=0.0415. (Fig. 15).

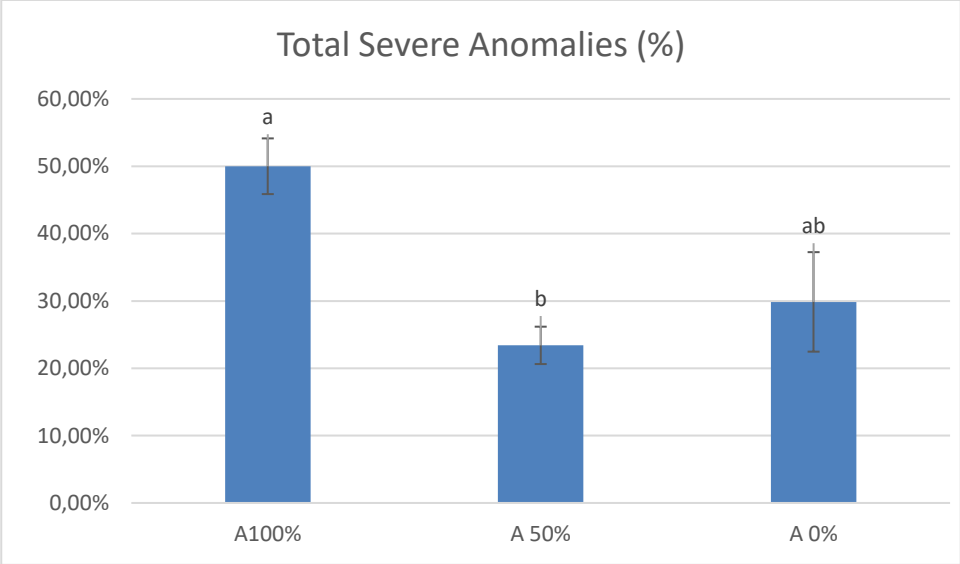

Fig. 15 – Total percentage of the incidence of severe anomalies ± sd.

A100 was found to have a higher incidence of lordosis and kyphosis (p =0.01802 and p =0.01401 compared to A50 and A0, where the higher incidence of lordosis is significantly higher in the pre-hemal region (p=0.0470). Data show higher incidence of scoliosis in A100 compared to A0 (p=0.0429).

	A100	**A50**	**A0**
Types	***Total***	***Total***	***Total***
Anomalous maxillary and/or pre-maxillary	15,34±4,91[a]	6,38±3,15[a]	3,79±2,11[a]
Anomalous dentary	0,00	0,00	0,00
Anomalous left/right opercular plate	0,00	0,00	0,00
Kyphosis	11,88±2,16[a]	2,12±0,60[b]	2,84±1,83[b]
Lordosis	6,93±0,81[a]	1,70±0,95[b]	2,84±1,07[b]
Partial vertebrae fusion	1,98±0,17[a]	1,27±0,63[a]	3,31±0,85[a]
Total vertebrae fusion	0,49±0,29[a]	0,85±0,39[a]	0,00[a]
Vertebral anomaly	15,34±3,07[a]	13,19±3,56[a]	20,85±6,55[a]
Scoliosis	2,97±0,93[a]	0,85±0,84[ab]	0,00[b]
Swim bladder	0,49±0,29[a]	0,42±0,02[a]	0,94±0,80[a]
Supernumerary vertebra	0,00	0,00	0,00

Table 2. Data expressed as mean percentage ± sd. Values of the means in the same column with different superscripts indicate the presence of significant differences (Tukey post-hoc test p≤0.05).

4. Discussion

The weaning of marine fish larvae is a very critical and species-specific process. It is generally implemented using a combination of live prey such as *Brachionus sp*. and *Artemia sp*. (Cornow et al. 2006).

The results obtained in this early weaning experiment using a co-feeding regime show substantial differences in survival between treatments, where A0 showed the best survival compared to A100 but not significantly different to A50 (Fig. 5). As described by Quiros-Pozo et al., 2023, mortality remained fairly contained for the three treatments until 26 DPH (rotiferous end of the A100 and A50 treatments). Subsequently, the A50 and A100 treatments had a steady increase in mortality, unlike A0 (Fig. 6). This as already explained by Quiros-Pozo et al., 2023, could be justified by the too premature removal of the rotifer with the inability of the smaller larvae to prey on artemia, in fact this species as described by Oz et al. 2022 shows a high variability of sizes as early as 12 DPH as their non-cannibalistic nature means that late developing larvae are not eliminated through predation and thus are well represented in the population even at later stages of development. This is evidenced by the A0 treatment showing high growth variability, where in this case mortality of the smaller larvae that failed to feed on artemia did not occur. Several authors have suggested that the optimal time for the start of weaning is species-specific and mainly related to the development of the digestive system and the larval ability to ingest, digest and absorb nutrients from MD (Cahu and Zambonino-Infante, 2001); in fact, a correct combination and timing of live food administration with an early administration of adequate micro-feeding will improve larval nutrition and thus enhance the negative effects of weaning, thus improving survival and growth in later stages (Rosenlund et al. , 1997; Canavate and Fernandez-Diaz, 1999; Cahu and Zambonino Infante, 2001; Kolkovski, 2001; Cornow et al. 2006). Curnow et al. (2006) reported that the inclusion of Artemia for 9 days in the weaning period increased larval survival

rates (over 15%) compared to the experimental group that was weaned with only rotifers for 12 days, although this trend was not significant.

A100 expressed the best growth performance evaluated in terms of TL (Fig. 7), condition index (Fig. 9), eye diameter (Fig. 10), and myotome height (Fig. 8) in relation to A0, but not significantly different in relation to A50 when considering condition index (Fig. 9) and eye diameter (Fig. 10). A factor that may have influenced the higher growth in the A100 and A50 protocols is the lower larval density produced by the higher mortality in these treatments, which may have favoured less competition for food. Yanes-Roca et al. 2018 reports similar results to those obtained in our study in pikeperch (*Sander lucioperca*) larvae where larvae fed only with rotifers had higher survivals than those fed with Artemia sp. and *Brachionus sp.*, but with lower weight and length. Imentai et al. (2020) observed higher growth indices (including TL, eye diameter and myotome height) in pikeperch larvae fed with a combination of rotifers and Artemia from 8 to 17 dph compared to larvae fed only with rotifers.

The overall size of the eye is one of the factors that determine visual sensitivity (Job and Bellwood, 2000), along with ontogenetic changes in the retinal area (Fuiman and Delbos, 1998; Higgs and Fuiman, 1998). A relatively larger eye diameter in fish larvae may support fish in catching food, since in the early stages they depend primarily on vision to detect and capture prey (Blaxter, 1986; Olla et al., 1995; Privileggi et al., 1997). Increased eye size implies a larger retinal image and thus better visual acuity (Papadakis et al., 2018; Svanbäck and Johansson, 2019). In this work, a larger eye size measured in A100 suggests that the fish were more capable of catching prey than those in the other treatments, and this may have led to greater growth in the 100% artemia-fed fish.

The development of the myotome and, more specifically, the relationship between myotome height and body length, is considered a sensitive indicator for assessing inadequate feeding conditions, especially in the early larval stages of several species (Yúfera et al., 1993; Puvanendran and Brown, 1999; Dou et al., 2002; Busch et al., 2011). Generally, the myotome develops rapidly after the first feeding in well-fed larvae, but collapses when starved. In this work, the larvae were not intentionally starved and checking the intestinal

contents at the different sampling points of the histological samples did not reveal empty stomachs (data not shown). The lower myotome height and relative Condition Index assessed in A0 may be a consequence of the poor feeding of the larvae fed only with rotifers. Busch and colleagues (2011) observed reduced muscle growth in the myotome area and a significantly lower ratio of myotome height to body length in cod larvae fed with rotifers compared to larvae fed with other zooplankton.

Analyses of the axial skeleton showed that the percentage of individuals with 24 vertebrae was 100%, as also described by Crosetti et al., 2016 and Thieme et al. 2020).

Considering all study areas reported in Table 1, the analysis of deformities shows that A100 presented a significantly higher incidence ($p = 0.03938$) of total anomalies than A50 (Fig. 14). The average percentage of animals presenting at least one anomality in the three different rearing protocols is 42.08±5.15% for A100, 22.55±9.02% for A50 and 23.24±9.80% for A0, where A100 is significantly different from the other two treatments ($p=0.024$). Boglione et al. (2003) reported cases of individuals with at least one skeletal malformation ranging from 88 to 100% in farmed sharpsnout seabream (*Diplodus puntazzo*) compared to 19-21% in wild individuals, and from 44 to 80% in juvenile common pandora (*Pagellus erythrynus*) farmed compared to 12-24% in wild animals. Furthermore, Boglione et al. (2005) reported 83 and 100 per cent deformed individuals in red porgy (*Pagrus pagrus*) and common dentex (Dentex dentex), respectively. However, it must be borne in mind that these works also consider abnormalities in the pectoral fin, anal fin, caudal fin, first dorsal fin, second dorsal fin and pelvic fin, which were not analysed here. In bass reared in aquaculture, a high number of deformities were also found in the axial skeleton and fins, with incidence frequencies of up to 75% in the vertebrae and 45% in the fins. In contrast, for the common dentex, only 6% of individuals were found to have deformities, and among these the caudal fin region was no more affected than other regions of the skeleton (Koumoundouros et al., 1999,).

The A100 treatment showed a higher incidence of severe anomalies (taken over total anomalies) (50.00%±4.14%) than A50 and A0 (23.40%±2.77% and 29.86%±7.39%), with a significant difference of $p=0.415$ (Fig. 15). The A100 was found to have a higher incidence of lordosis and kyphosis ($p=0.01802$ and

p=0.01401 compared to the A50 and A0, where the highest incidence of lordosis is significantly higher in the pre-hemal region (p=0.0470) (Tab. 2). Generally, lordosis of the pre-hemal region is due to swim bladder insufflation problems as described by Boglione et al. 2013, although in this case the percentage of animals with swim bladder insufflation problems in all three treatments was very small (Tab. 2). The data show a higher incidence of scoliosis in A100 than in A0 (p=0.0429).

Dental and opercular anomalies were found in few individuals in all treatments; thus, they do not appear to be dependent on any of the rearing conditions applied. This, suggests that in the mullet, these anomalies may be rare.

The degree of lipid vacuolisation in the liver is recognised as a valid indicator of the nutritional and physiological condition of fish (Caballero et al., 1999; Zambonino-Infante et al., 2008; Papadakis et al, 2009; Loi et al, 2020), highlighting inappropriate larval feeding conditions or a nutritionally unbalanced diet in the rearing environment (Margulies, 1993; Yúfera et al., 1993, 1996; Diaz et al., 1998; Green and McCormick, 1999; Crespo et al., 2001; Gisbert et al., 2004b; Gisbert et al., 2008; Lazo et al., 2011). In this study, fish fed with artemia, regardless of its concentration, had the highest percentage of lipids covering the liver area. This is strongly linked to the higher amount of lipids contained in artemia compared to rotifers (Imentai et al., 2020), as confirmed by biochemical analyses reported by Quirós-Pozo et al., 2023. The hepatic vacuolisation observed in fish larvae fed Artemia nauplii appears to be strongly linked to adequate nutrient uptake and lipid metabolism, as observed in previous research (Segner et al., 1994; Sarasquete al., 1995; Gisbert et al., 2004a). Furthermore, it is known that the degree of lipid accumulation in the liver is related to the type of lipids (neutral/polar), considering the role of phospholipids in lipid transport (Gisbert et al., 2005).

The gut contributes to the final digestion of food and absorption of nutrients, and its histological study can reveal pathological changes due to improper diet (Sirri et al., 2017). Alterations in the number and size of intestinal villi and/or shortening of the height of enterocytes are symptoms of starvation or inadequate nutrition (Gisbert and Doroshov, 2003; Gisbert et al., 2004b; Ostaszewska et al., 2006; Przybyl et al., 2006; Kamaszewski and Ostaszewska, 2014) and may impair growth performance or even larval survival

(Zambonino-Infante et al., 2008). In our study, in the distal gut of all samples examined, we observed several finger-like mucosal folds with regular lamina propria and well-differentiated and nucleated enterocytic cells, covered by an intact brush border membrane in the apical part. However, significant differences were observed in the size of the intestinal villi and the height of the enterocytes, which were longer in the A100 fish than in those reared without artemia. Fish fed with 50% artemia were in the middle. Similar results were obtained by Imentai et al. (2020), who observed significantly lower enterocyte height (albeit in the anterior villi of the intestine) in groups of pikeperch larvae fed exclusively with rotifers. It is plausible that fish fed 100% artemia had a greater surface area for nutrient absorption and consequently the larvae were well nourished and grew more and faster.

5. Conclusions

In summary, considering our results as a whole, we can suggest that the density of Artemia sp. in the larval co-feeding of *M. cephalus* could be reduced to at least 50% of the standard protocol, 2 Artemia sp. ml^{-1} day^{-1} being the optimal concentration in such co-feeding protocols. Although the treatment without Artemia A0 was very successful in terms of larval survival and incidence of total and severe skeletal anomalies, growth rates were low compared to the other two treatments.

6. References

Andrades J.A., Becerra J., Fernandez-Llebrez P. 1996. Skeletal deformities in larval, juvenile and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). Aquaculture 141: 1–11.Divanach et al. 1996

Balbelona M.C., Morinigo M.A., Andrades J.A., Santamaria J.A., Becerra J., 1993. Microbiological study of gilthead sea bream (*S. aurata* L.) affected by lordosis (a skeletal deformity). Bull Eur Ass Fish Pathol 13: 33.

Bennett H.S., Wyrick A.D., Lee S.W., McNeil J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technology 51: 71-97.

Blaxter J.H.S., 1986. Development of sense organs and behaviour of teleost larvae with special reference to feeding and predator avoidance. T Am Fish Soc. 115, 98-114.

Cahu, C., Zambonino-Infante, J., & Takeuchi, T. (2003). Nutritional components affecting skeletal development in fish larvae. Aquaculture, 227, 254–258. https://doi.org/10.1016/S0044-8486(03)00507-6Matsuoka 2003;

Boglione, C., Gagliardi, F., Scardi, M., Cataudella S., 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758), Aquaculture, Volume 192, Issue 1, 2001, Pages 1-22, ISSN 0044-8486, https://doi.org/10.1016/S0044-8486(00)00446-4.

Boglione, C., Costa, C., Di Dato, P., Ferzini, G., Scardi, M., Cataudella, S., 2003. Skeletal quality assessment of reared and wild sharpsnout sea bream and pandora juveniles, Aquaculture, Volume 227, Issues 1–4, 2003, Pages 373- 394, ISSN 0044-8486, https://doi.org/10.1016/S0044-8486(03)00515-5.

Boglione, C., Costa, C., Giganti, M., Cecchetti, M., Di Dato, P., Scardi, M., Cataudella, S., 2006. Biological monitoring of wild thicklip grey mullet (*Chelon labrosus*), golden grey mullet (*Liza aurata*), thinlip mullet (*Liza ramada*) and flathead mullet (*Mugil cephalus*) (Pisces: Mugilidae) from different Adriatic sites: meristic

counts and skeletal anomalies, Ecological Indicators, Volume 6,Issue4, , Pages 712-732, ISSN 1470-160X, https://doi.org/10.1016/j.ecolind.2005.08.032.

Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagné, S., & Witten, P. E., 2013. Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. Reviews in Aquaculture, 5(1), S99–S120. https://doi.org/10.1111/raq.12015

Boglione, C., Gisbert, E., Gavaia, P., Witten, P., Moren, M., Fontagné, S., & Koumoundouros, G., 2013. Skeletal anomalies in reared European fish larvae and juveniles. Part 2: Main typologies, occurrences and causative factors. Reviews in Aquaculture, 5(1), S121–S167. https://doi.org/10.1111/raq.12016

Bonaldo, A., Parma, L., Badiani, A., Serratore, P., & Gatta, P. P., 2011. Very early weaning of common sole (*Solea solea* L.) larvae by means of different feeding regimes and three commercial microdiets: Influence on performances, metamorphosis development and tank hygiene.Aquaculture, 321, 237–244. https://doi.org/10.1016/j.aquaculture.2011.09.007

Busch K., Peruzzi S., Tonning F., Falk-Petersen I.B., 2011. Effect of prey type and size on the growth, survival and pigmentation of cod (*Gadus morhua* L.) larvae. Aquaculture Nutrition 17: e595-e603.

Caballero, M.J., Izquierdo, M.S., Kjrrsvik, E., Montero, D., Socorro, J., Fernández, A.J., Rosenlund, G., 2003. Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225, 325-340.

Callan, C., Jordaan, A., & Kling, L. J., 2003. Reducing Artemia use in the culture of Atlantic cod (*Gadus morhua*). Aquaculture, 219, 585–595. https://doi.org/10.1016/S0044-8486(03)00011-5 Ma et al., 2015

Crespo S., Marín de Mateo M., Santamaría C.A., Sala R., Grau A., Pastor E., 2001. Histopathological observations during larval rearing of common dentex *Dentex dentex* L. (Sparidae). Aquaculture 192: 121-132.

Crosetti D., Blaber S.J., 2015. Biology, ecology and culture of grey mullets (Mugilidae). Boca Raton: CRC Press.

Curnow, J., King, J., Bosmans, J., & Kolkovski, S., 2006. The effect of reduced Artemia and rotifers use facilitated by a new microdiet in the rearing of barramundi Lates calcarifer (BLOCH)larvae.Aquaculture,257,204–213. https://doi.org/10.1016/j.aquaculture.2006.02.073Quiros-Pozo et al., 2023

Decreto Legislativo 4 marzo 2014, n. 26 (art. 2 comma 1). Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici. (14G00036) (GU n.61 del 14-3-2014). https://www.gazzettaufficiale.it/eli/id/2014/03/14/14G00036/sg.

Diaz J.P., Mani-Ponset E., Guyot E., Connes R., 1998. Hepatic cholestasis during the postembryonic development of fish larvae. J. Exp. Zool. 280: 277-287.

Dou S., Masuda R., Tanaka M., Tsukamoto K., 2002. Feeding resumption, morphological changes and mortality during starvation in Japanese flounder larvae. Journal of Fish Biology 60: 1363-1380.

Fuiman L.A., Delbos, B.C., 1998. Developmental changes in visual sensitivity of red drum, *Sciaenops ocellatus*. Copeia 1998: 936–943.

Gisbert E., Doroshov S.I., 2003. Histology of the developing digestive system and the effect of food deprivation in larval green sturgeon (*Acipenser medirostris*). Aquat. Living Resour. 16: 77–89.

Gisbert E., Piedrahita R.H., Conklin D.E., 2004a. Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. Aquaculture 232: 455-470.

Gisbert E., Piedrahita R.H., Conklin D.E., 2004b. Effects of delayed first feeding on the nutritional condition and mortality of California halibut larvae. J. Fish Biol. 64: 116-132.

Gisbert E., Villeneuve L., Zambonino-Infante J.L., Quazuguel P., Cahu C.L., 2005. Dietary phospholipids are more efficient than neutral lipids for long-chain polyunsaturated fatty acid supply in European sea bass *Dicentrarchus labrax* larval development. Lipids 40: 609-618.

Gisbert E., Ortiz-Delgado J. B., Sarasquete C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histology and Histopathology 23: 1525–1539.

Gisbert, E., Solovyev, M., Bonpunt, E., & Mauduit, C. (2018). Weaning in Siberian Sturgeon Larvae. In P. Williot, G. Nonnotte, & M. Chebanov (Eds.), The Siberian Sturgeon (*Acipenser baerii*, Brandt, 1869) Volume 2 - Farming (pp. 59–72). Springer. https://doi.org/10.1007/978-3- 319-61676-6Engrola at 2007,2009

Green B.S., McCormick M.I., 1999. Influence of larval feeding history on the body condition of *Amphiprion melanopus*. J. Fish Biol. 55: 1273-1289.

Hilomen-Garcia G.V., 1997. Morphological abnormalities in hatchery-bred milkfish (*Chanos chanos Forsskal*) fry and juveniles. Aquaculture 152: 155–166.Koumoundourous et al. 1997b;

Higgs D. M., Fuiman L. A., 1998. Associations between sensory development and ecology in three species of clupeoid fish. Copeia 1998: 133–144.

Imentai A., Rašković B., Steinbach C., Rahimnejad S., Yanes-Roca C., Policar T., 2020. Effects of first feeding regime on growth performance, survival rate and development of digestive system in pikeperch (*Sander lucioperca*) larvae. Aquaculture 529: 735636.

Job S.D., Bellwood D.R., 2000. Light sensitivity in larval fishes: Implications for vertical zonation in the pelagic zone. Limnol. Oceanogr. 45: 362–371.

Kamaszewski M., Ostaszewska T., 2014. The effect of feeding on morphological changes in intestine of pike-perch (*Sander lucioperca* L.). Aquac. Int. 22: 245–258.

Koumoundouros G., Gagliardi F., Divanach P., Boglione C., Cataudella S., 1997. Normal and abnormal osteological development of caudal fin in *Sparus aurata* L fry. Aquaculture 149: 215–226.

Lazo J. P., Darias M. J., Gisbert, E., 2011. Ontogeny of the digestive tract. In Holt G. J. (Ed.), Larval fish nutrition. Wiley-Blackwell, pp. 5–46.

Le Vay, L., Carvalho, G.R., Quinitio, E.T., Lebata, J.H., Ut, V.N., Fushimi, H., 2008. Quality of hatchery-reared juveniles for marine fisheries stock enhancement, Aquaculture, Volume 268, Issues 1–4, 2007,Pages169-180,ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2007.04.041.Castro et al. 2008

Loi B., Papadakis I.E., Leggieri F., Giménez Papiol G., Vallainc D., 2020. Ontogeny of the digestive system and eye of reared flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758), and evaluation of lipid deposition in the liver according to the feeding protocol. Aquaculture 526: 735386.

Margulies D., 1993. Assessment of the nutritional condition of larval and early juvenile tuna and Spanish mackerel (Pisces: Scombridae) in the Panamá Bight. Mar. Biol. 115: 317-330.

Olla B.L., Davis M.W., Ryer C.H., Sogard S.M., 1995. Behavioural responses of larval and juvenile walleye pollock (*Theragra chalcogramma*): possible mechanisms controlling distribution and recruitment. ICES. Marine Science Symposia 201: 3-15.

Ostaszewska T., Korwin-Kossakowski M., Wolnicki J., 2006. Morphological changes of digestive structures in starved tench *Tinca tinca* (L.) juveniles. Aquac. Int. 14: 113–126.

Oz I., Gajbhiye D.S., Columbus-Shenkar Y.Y., David L. and Golan M., 2022. Non-uniform metamorphosis underlies different development trajectories in hatchery-reared flathead grey mullet (*Mugil cephalus*). Front. Mar. Sci. 9:967984. doi: 10.3389/fmars.2022.967984

Panettieri V., Chatzifotis S., Messina C.M., Olivotto I., Manuguerra S., Randazzo B., Ariano A., Bovera F., Santulli A., Severino L., Piccolo G., 2020. Honey bee pollen in meagre (*Argyrosomus regius*) juvenile diets: effects on growth, diet digestibility, intestinal traits, and biochemical markers related to health and stress. Animals 10: 231.

Papadakis I.E., Zaiss M.M., Kyriakou Y., Georgiou G., Divanach P., Mylonas C.C., 2009. Histological evaluation of the elimination of Artemia nauplii from larval rearing protocols on the digestive system ontogeny of shi drum (*Umbrina cirrosa* L). Aquaculture 286: 45–52.

Papadakis I.E., Kentouri M., Divanach P., Mylonas C.C., 2018. Ontogeny of the eye of meagre (*Argyrosomus regius*) from hatching to juvenile and implications to commercial larval rearing, Aquaculture 484: 32-43.

Peng M., Xu W., Ai Q., Mai K., Liufu Z., Zhang K., 2013. Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L.). Aquaculture 392–395: 51-58.

Privileggi N., Ota D., Ferrero E.A., 1997. Embryonic and larval development of the grass goby, *Zosterisessor ophiocephalus* (Teleostei, Gobiidae). Ital. J. Zool. 64: 201-207.

Przybyl A., Ostaszewska T., Mazurkiewicz J., Wegner A., 2006. The effect of experimental starters on morphological changes in the intestine and liver of common carp (*Cyprinus carpio* L.) larvae reared under controlled conditions. Arch. Polish Fish. 14: 67–83.

Puvanendran V., Brown J. A., 1999. Foraging, growth and survival of Atlantic cod larvae reared in different prey concentrations. Aquaculture 175: 77–92.

Rosenlund, G., Stoss, J., & Talbot, C., 1997. Co-feeding marine fish larvae with inert and live diets. Aquaculture, 155, 183–191. https://doi.org/10.1016/S0044-8486(97)00116-6 Canavate and Fernandez-Diaz, 1999;

Santosh P. Lall, Leah M. Lewis-McCrea, 2007. Role of nutrients in skeletal metabolism and pathology in fish — An overview, Aquaculture, Volume 267, Issues 1–4, 2007, Pages 3-19, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2007.02.053.

Sarasquete C., Polo A., Yúfera M., 1995. Histology and histochemistry of the development of the digestive system of larval gilthead seabream, Sparus aurata L. Aquaculture 130: 79-92.

Segner H., Storch V., Reinecke M., Kloas W., Hanke W., 1994. The development of functional digestive and metabolic organs in turbot, Scophthalmus maximus. Mar. Biol. 119: 471-486.

Sirri R., Sarli G., Bianco C., Bonaldo A., Gatta P.P., Fontanillas R., De Vico G., Carella F., Brachelente C., Parma L., Mandrioli L., 2018. Retrospective study of pathology-based investigative techniques for the assessment of diet-induced changes in liver and intestine of flatfish. Italian Journal of Animal Science 17: 518-529.

Svanbäck R., Johansson F., 2019 Predation selects for smaller eye size in a vertebrate: effects of environmental conditions and sex. Proc. R. Soc. B 286: 20182625.

Solovyev M., Gisbert E., 2021. Feeding regimes affected the circadian rhythms of pancreatic digestive enzymes and somatic growth in flathead grey mullet (*Mugil cephalus*) fry. Comp Biochem Physiol A Mol Integr Physiol. 2022 Feb;264:111116. doi: 10.1016/j.cbpa.2021.111116. Epub 2021 Nov 14. PMID: 34785378.

Thieme P., Vallainc, D., Moritz, T. 2021. Postcranial skeletal development of *Mugil cephalus* (Teleostei: Mugiliformes): morphological and life-history implications for Mugiliformes, Zoological Journal of the Linnean Society, Volume 192, Issue 4, August 2021, Pages 1071–1089, https://doi.org/10.1093/zoolinnean/zlaa123

Vallainc, D., Concu, D., Gimenez Papiol, G., Loi, B., Leggieri, F., Brundu, G., Chindris, A., Sanna, G., Fois, N., Antognarelli, F., Rossini, P., 2021. Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons. Aquaculture Reports, Volume 21, 2021, 100844, ISSN 2352-5134. https://doi.org/10.1016/j.aqrep.2021.100844.

Whitfield A., Panfili J., Durand J-D., 2012. A global review of the cosmopolitan flathead mullet *Mugil cephalus* Linnaeus 1758 (Teleostei: Mugilidae), with emphasis on the biology, genetics, ecology and fisheries aspects of this apparent species complex. Reviews in Fish Biology and Fisheries 22: 641–681.

Yanes-Roca C., Mraz J., Born-Torrijos A., Holzer A.S., Imentai A., Policar T., 2018. Introduction of rotifers (*Brachionus plicatilis*) during pikeperch first feeding. Aquaculture 497: 260-268.

Yúfera M., C. Sarasquete M.C., Fernández-Diaz C., 1996. Testing protein-walled microcapsules for the rearing of first-feeding gilthead seabream, *Sparus aurata* L. Mar. Fresh Water Res. 47: 211-216.

Yúfera M., Pascual E., Polo A., Sarasquete M.C., 1993. Effect of starvation on the feeding ability of gilthead seabream (*Sparus aurata* L.) larvae at first feeding. J. Exp. Mar. Biol. Ecol. 169: 259- 272.

Zambonino-Infante J.L., Gisbert E., Sarasquete C., Navarro I., Gutiérrez J., Cahu C.L., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino J., Bureau D., Kapoor B.G. (Eds.), Feeding and Digestive Functions of Fish. Science Publishers, Inc., Enfield (NH), Jersey, Plymouth, pp. 281–348.

Chapter VI

1. General Discussions

In this study, aspects concerning the spawning induction of wild grey mullets during their natural reproductive period (Chapters II and III), their larval rearing (Chapters II and II), the management of fertilised eggs (Chapter IV) and finally the weaning of larvae (Chapter V) were examined. This is with the intention of improving the production processes of this species in order to produce a protocol that is easy to transfer to farms and has low management costs. At present, throughout the Mediterranean basin, despite the high interest in breeding this species due to its robustness in different farming environments, good market price, the quality of its meat and the great potential for added value products (roes), only one commercial scale hatchery is presently placing juveniles on the market (Dagon, Ma'agan Michael, https://www.dagon-aquaculture.com- Israel). Nonetheless, no detailed information on the farming practices or animal management is available and, as such, this operation remains a “black-box” for the scientific and farming communities. On the other hand, the vast majority of farmed product in Europe begins its production cycle with the capture of wild juveniles. This practice poses serious questions around the future sustainability of wild stocks as well as biosecurity and welfare implications of the farmed animals.

In the Mediterranean, the breeding season of *M. cephalus* begins in June and lasts until December (Brusle et al. 1982) with variations depending on the geographical area; in the Sardinian coasts, reproduction begins in August and ends in October, when the adults migrate from the lagoons in large shoals towards the open sea. *M. cephalus* females do not reproduce spontaneously in captivity, only a small proportion of them complete vitellogenesis, but they do not complete oocyte maturation through the hydration and ovulation stages (Shehadeh et al., 1973). The oocytes, as also verified by observations made during this thesis on animals kept in captivity at the IMC laboratory, stop developing around 300 µm corresponding to a primary stage vitellogenesis. This can be attributed to the disruption of one or more stages of the hypothalamic-pituitary-gonadal axis that prevents the gonads from completing maturation. The lack of gonadotropin detected in the

circulatory system of captive *M. cephalus* females could result from insufficient gonadotropin release by the pituitary gland, inadequate secretion of the gonadotropin-releasing hormone by the hypothalamus, or both (Monbrison et al., 2003). Therefore, at present, to achieve reproduction in this species, animals must be subjected to hormonal treatments that may favour the complete maturation of the gametes or interact with the last part of the maturation process thereby inducing reproduction under captive conditions.

Generally, as discussed in Chapter I and II, induction of *M. cephalus* reproduction is carried out using acute hormone treatments that consist of several phases, a priming dose and a resolving dose that is administered 24 hours after the first injection. Prior to our investigation, different induction protocols were tested: Lee and Tamaru (1988) employed CPE (20-70 mg kg-1) or hCG (10,000 IU kg^{-1}) and LHRHa (200 µg kg^{-1}) to induce spawning in mullets. Crosetti and colleagues (Crosetti, 2001; Crosetti and Cordisco, 2001) used pituitary gland extract (PG) 7 + LHRH 100/200 µg + DOM 10 mg + leuproline acetate 500/700 µg + leuproline acetate 600/700 µg kg-1 body weight. Vazirzadeh and Ezhdehakoshpour (2014) used CPE (20 mg kg^{-1})+ metaclopramide (20 mg kg^{-1}) + GnRHa (200 µg kg^{-1}). Mousa et al. (2018) administered carp pituitary extract (CPE) (20 mg kg^{-1} body weight) or human chorionic gonadotropin (hCG) (10,000 IU kg-1 body weight) + LHRHa (200 µg kg^{-1}). Besbes et al. (2020) treated fish with a priming dose and a resolving dose of LHRHa (200 µg kg^{-1} body weight). The same author also used a combination of hCG (5000 to 10000 IU kg^{-1}) as a priming injection, followed by resolving injections of hCG (5000 to 10000 IU kg^{-1}) + LHRHa (100 to 200 µg kg^{-1} body weight). Therefore, a protocol involving a single hormone treatment with low doses of LHRH was missing and inspired the work conducted in the first two experimental chapters of this thesis.

In fact, in Chapter II (for *M. cephalus*) and Chapter III (for *C. ramada*), a new protocol was developed to induce the final stages of gametogenesis and induce natural spawning with a single administration of a slow-releasing gonadotropin-releasing hormone (GnRHa) analogue preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a., C59H84N16O12, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt), under the dorsal fin at a low dosage (10 µg/Kg for males and 200 µg/Kg for females). These advances reduced the stress to the animals during the hormone treatment phases, but also made the protocol more economically sustainable and easy to use in a commercial environment for operators wishing to repeat the

procedure at scale. By following this method, the costs due to the management and maintenance of the animals (personnel costs, facilities employed) are significantly reduced or eliminated altogether. Although capturing sexually mature adult individuals is a rather simple practice, it is still necessary to overcome problems associated with: i) identifying locations where animals are synchronised in their gonadal development and suitable infrastructures are present; ii) before hormone treatment can even take place, the stress of capturing, transporting and acclimatising the animals, often leads to failure (Mathew et al, 1998). Furthermore, by breeding wild individuals, the potential advantages of genetic selection or even domestication are somewhat negated. In addition, vertical transmission of pathogens and potential contamination of the egg incubation and/or larval rearing units will always be a risk factor for this practice. To overcome this latter risk, effective egg disinfection protocols are urgently needed before this practice could be realistically taken up at commercial scale. With regard to the induction of reproduction of *M. cephalus* (Chapter II), 19 females were treated in phase 1; 7 of them released eggs naturally and only 3 of these were fertilised (43 %); roughly 70 % of the animals developed visible bacterial infections on the skin, most likely due to handling. All females that in phase 1 had oocytes with a diameter of more than 570 µm (corresponding to phase 3 of vitellogenesis), also had visible external signs of gonadal maturation such: swollen abdomen and a red, protruding papilla. As a result of this, in phase 2, it was decided not to perform ovarian biopsies, thus relying only on these external maturation features. This ensured that the stress placed on the females was reduced with a consequent improvement in the welfare of the individuals leading to improved breeding performances. None of the animals in phase 2 developed visible bacterial infections. In order to improve egg fertilisation rate, it was decisive to increase the female-to-male ratio (1/4 from the 1/3) and increase the dose of hormone used in phase 1 from 10 µg/Kg to 100 µg/Kg; in fact, in phase 2, 100% of the batches obtained were fertilised, even though the number of females used was lower. In phase 1 for the three batches the fertilisation rates were 84±6%, 88±7% and 92±6% with respective hatching rates of 84±11%, 87±8% and 72±12%. In phase 2, the fertilisation rates of the two batches obtained were 93±3% and 58±8% with respective hatching rates of 80±5% and 55±8%. The low fertilisation and hatching rates observed in phase 2 in the batch of eggs obtained from Porto Pino, could be due to lower gamete quality or influenced

by the high incubation density of the eggs tested (1000 eggs L^{-1}). Indeed, Nash and Shehadeh (1980) suggest 200 eggs L-1 as the optimal incubation density for embryos of this species. Gamete quality, both in the wild and in captive breeding, is influenced by several factors and is sometimes highly variable (Migaud et al., 2013; Bobe and Labbe, 2010; FAO, 2020). Furthermore, highly variable fertilisation rates have also been reported by Azien et al. (2005) who, by inducing a total of 12 *M. cephalus* females to spawn, obtained fertilisation rates above 90% in 4 trials and values below 40% in the remaining 8 trials. Low hatching rates were also obtained by Abrham et al. (1999), who recorded a value of 41.6% when inducing *M. cephalus* females to lay eggs. The fecundity of females observed in this study was similar to that reported by Kuo et al. (1973), 600 eggs g-1 body weight, Nash et al. (1974), 800 eggs g-1 body weight and Meseda and Samira (2006), 1395 eggs g-1 body weight.

On the other hand, this breeding induction procedure based on the capture of wild broodstock also has weaknesses such as, for example, the fact that juvenile production will be limited by the length of the natural spawning season of the species, a clear bottleneck when profit margins are often based on scale of production and the ability to produce all year-round. In this thesis we explored the potential offered by diversification toward *C. ramada* and *C. labrosus* as a mean to extend the length of production over multiple months (August-October *M. cephalus*; November- January *C. ramada* and, finally, January-March *C. labrosus*). There are also issues linked with the lack of control/standardisation over gametes quality, often ensured by provisioning of bespoke broodstock diets in captivity. The quality of the eggs is generally due to several factors such as stress but above all the nutrition provided; in fact, diets deficient in fatty acids and lipids do not meet the nutritional requirements of the spawners, creating dysfunctions in the quality of the gametes with reproductive failure, and on the quality and survival of the larvae. Moreover, wild broodstock may be subject to various health problems that could affect the survival of the larvae themselves; an example would be the vertical transmission of certain viruses such as Betenodavirus, which can cause 40% to 100% mortality of the larvae in some cases (Zorriehzahra et al., 2019).

Recent advances in the field have begun to resolve some of these issues, and a protocol was recently developed by IRTA (Institute of agrifood research and Technology) (Ramos et al. 2021), which uses

recombinant rFSH and rLH hormones to promote gonadal maturation of wild *M. cephalus* in captivity, with weekly treatments lasting 13 weeks. This is surely a significant step toward the establishment of a year-round production of juveniles and the establishment of captive, domesticated and bio-secure bloodstock populations, ultimately opening up concrete possibilities for the establishment of selective breeding programmes.

Larval survival is considered one of the most difficult obstacles preventing the wider use of *M. cephalus* in aquaculture (Eda et al. 1990; Tamaru et al. 1992). The highest mortality occurs in the first 15-20 days after hatching (U.S. Agency for International Development, 2007). Among the precautions used in this work, it appears to have been crucial for the improvement of hatching and survival rates of larvae to transfer them from the incubation system to the larval rearing system at the eye stage at 32 h post-fertilization, in fact, previous experimental evidence from Vallainc et al. 2017 suggest to avoid transporting the larvae immediately after hatching; this is to reduce the mechanical stress that could be placed on the larvae and to improve water parameters in the larval rearing system as hatching generally increases NH3 concentration in the water (Scolamacchia et al, 2023). Indeed, in phase I of Chapter II as also described by Vallainc et al, 2017 the transfer of the larvae took place at 2 DPH, while in phase II the animals were transferred as embryonated eggs at 32 hours after fertilisation improving hatching and larval survival at the earliest days. With regard to the larval feeding of *M. cephalus* in Chapter II (phase I and II) we attempted to obtain a protocol that performed well in terms of growth and survival which would also meet the larvae feeding needs based on the ontogenic developmental stages of *M. cephalus* larvae described by Loi et al, 2020. In phase I and the 2018 production cycle of phase II, we applied a feeding protocol that included feeding artemia from day 12 DPH until day 34 DPH, and feeding MD from day 22 DPH. The results showed that between 31 and 34 DPH, the larvae were in the critical weaning phase (during which the transition from live prey to inert MD feeds takes place), and the combination of lower artemia intake during this period and poor acceptance of inert feeds contributed to the reduction and eventual disappearance of intestinal lipid reserves which appeared as lipid vacuoles, which is why it was decided in Phase II with the 2019 cycle to extend the feeding of artemia

until 42 DPH and to extend the period of overlap with MD in order to facilitate the acceptance of these by the larvae.

The application of this feeding protocol also gave more than satisfactory results for larval survival where in Chapter II phase II survival at 60 DPH was 10±2 for 2018 and 19±1 for 2019. The SGR for TL was very similar between the two Phase II cycles while substantial differences are seen for BW SGR where 2018 expressed the best results. This is undoubtedly due to the lower survival of the larvae (45±5% for 2018 and 63±3% for 2019 at 3 dph) and thus higher food availability; in fact, the larval density leads to high competition for food and space, higher energy expenditure resulting from high metabolic rates and thus decreased growth (Ellis et al., 2002; Thorarensen & Farrell, 2010).

The larval rearing density used in phase II in the 2018 and 2019 cycles was 40 larvae L^{-1}, The optimal larval rearing density reported in the literature is 20 larvae L^{-1} and dates back to Nash and Shehadeh (1980). Other authors reared larvae at a density similar to the one we tested, Eda et al. (1990), rearing larvae at a density of 43 larvae L^{-1}, but obtained a survival rate of 5.6% at 60 dph. Yousif et al. (2010), rearing larvae at a density of 30 larvae L^{-1}, reported a survival of 15% at 40 dph, and Besbes et al. (2020) obtained a survival of 10% at 60 dph at a density of 1.5 larvae L^{-1}.

Concerning the simulated egg transport of *M. cephalus* treated in Chapter IV, this experiment was conducted to simulate the transport of fertilised eggs over short, medium and long distances in 3 different densities, a low one of 500 eggs/L^{-1} and a high one of 1500 eggs/L^{-1}. Mortality and hatching rate were recorded every 8 hours up to 4 days after hatching (DPH). The hatching rate was above 30% for all conditions tested but lower than previously reported by Vallainc et al., 2021 with 87%, except for the simulated 16-hour transport which achieved very similar results. The temperature in the cited experiment was 2 °C lower than in the present study, which may have influenced the results of this test, as in a population of mullet in Hawaii where peak egg mortality was reported at 24 °C (Sylvester and Nash, 1975). Hatching occurred between 29 and 38 hours post-fertilisation (hpf), varying from a minimum of 31% (HD, 8 hours) to a maximum of 54% (LD, 16 hours), while at the opening of the transport bags at 24 hours for both densities larval hatching had already occurred.

The lowest percentage of larvae surviving up to 4 days was recorded for the 24-hour transport time at both densities (6% and 4%, respectively), while the highest percentage was recorded for the 16-hour transport time at low densities (47%). The two densities tested did not appear to deteriorate water quality in terms of pH, NO_2 and NO_3, especially for medium and short transport times. Nitrite and nitrate concentrations were always far from the lethal values reported for eggs and larvae. Both forms of ammonia increased dramatically from the first hours of transport, with values for HD far exceeding the safe limits for eggs and larvae.

As mentioned above, the same breeding induction protocol used with good results for *M. cephalus* was transferred to another species of mugilidae, *C. ramada* (Chapter III). Thus, males and females were treated respectively with a single administration of a slow-releasing gonadotropin-releasing hormone (GnRHa) analogue preparation, leuprorelin acetate (ENANTONE®, Takeda Italia S.p.a, C59H84N16O12, amino acid sequence H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt), under the dorsal fin at low dosage (100 µg/Kg for males and 200 µg/Kg for females). Previous studies on spawning induction protocols in the same species (Mousa, 2006; Mousa., 2010 and Mousa and Kahil, 2013) used pregnyl (hCG) as a priming injection at a dose of 20,000 IU kg BW^{-1} followed by a resolving dose of 40,000 IU hCG kg BW^{-1}, achieving fertilisation and hatching rates of 55±8.4 and 60±6.6%, respectively. Fahmy and El-Greisy (2014) obtained fertilisation rates between 52 and 75% by administering 3,500 IU hCG + luteinising hormone-releasing hormone (LHRH) 200 + LHRH 100 µg kg BW-1. In this case, ovarian biopsies were performed on a different pool of animals from that used for breeding in order to verify the maturation status of the population present at the 'lavorieri'. As in the case of *M. cephalus*, animals in the advanced vitellogenic stage (3) with an oocyte diameter of 657.1±37.4 had a swollen abdomen and a red, protruding papilla, so the selection of animals was based on these external characteristics. This agrees with the results obtained by Mousa and Kahil (2013) who identify 600 µm as the minimum oocyte diameter to promote final maturation, ovulation and egg laying. The average fertilisation and hatching rates observed in our study were higher than those observed in previous research by 92±3 and 91.4±0.4 % respectively, probably due to the minimal stress of the animals during handling procedures and/or due to the hormone used. These factors may have resulted in better quality eggs than in previous studies where the administration of multiple doses of different hormones may have influenced the latency

time between hormone treatment and final oocyte maturation (FOM), affecting hatching rates and other egg quality parameters as described by Kucharczyk et al. (2019b; 2020) in other species. Egg hatching occurred after 36 h of incubation, the larval survival rate recorded in this study at 19 DPH (13.8±1.5%) is promising considering the larval survival data reported in other recently domesticated marine species (Roo et al., 2019; Vallés and Estévez, 2013; Abellán et al., 2000).

In Chapter V, a co-feeding larval weaning experiment was conducted on *M. cephalus*; starting from our basic protocol in which artemia was administered at a concentration of 2 artemia/ml^{-1} (protocol A100), the administration was reduced to 1 artemia/ml^{-1} (protocol A50) until complete elimination from the diet in A50. Final survival was significantly different between treatments (p=0.02). Treatment A0 had the highest survival (64.79±7.39%), followed by treatment A50 (42.52±8.30%), while A100 had the lowest survival (32.46±12.82%). This result as explained above could be due to the premature removal of the rotifer in the A50 and A100 protocols, in fact the mortality of these two treatments increased from 26 DPH. A100 expressed the best performance in terms of TL, condition index, eye diameter, and myotome height in relation to A0, but not significantly different in relation to A50 when considering condition index and eye diameter. One factor that may have influenced the higher growth in the A100 and A50 protocols is the lower larval density in these treatments resulting in less competition for food, Yanes-Roca et al. 2018 had similar results for pikeperch (*Sander lucioperca*) larvae. The lower myotome height and relative Condition Index assessed in A0 may be a consequence of poor feeding in larvae fed only with rotifers. Busch and colleagues (2011) observed reduced muscle growth in the myotome area and a significantly lower ratio of myotome height to body length in cod larvae fed with rotifers compared to larvae fed with other zooplankton. The A100 treatment showed a higher incidence of severe anomalies (considered over total anomalies) (50.00%±4.14%) than the A50 and A0 (23.40%±2.77% and 29.86%±7.39%), with a significant difference of p=0.415. A100 was found to have a higher incidence of lordosis and kyphosis (p =0.01802 and p =0.01401 compared to A50 and A0, with the highest incidence of lordosis being significantly higher in the pre-hemal region (p=0.0470). Larvae fed artemia A100 and A50 had the highest percentage of lipids covering the liver area. This is strongly related to the higher amount of lipids contained in artemia compared to rotifers (Imentai

et al., 2020), as confirmed by biochemical analyses reported by Quirós-Pozo et al., 2023. In the distal intestine of all samples examined, we observed several finger-like mucosal folds with regular lamina propria and well-differentiated and nucleated enterocytic cells, covered by an intact brush border membrane in the apical part. However, significant differences were observed in the size of the intestinal villi and the height of the enterocytes, which were longer in the A100 fish than in those reared without artemia. Fish fed with 50% artemia were in the middle. Similar results were obtained by Imentai et al. (2020), who observed significantly lower enterocyte height (albeit in the anterior villi of the intestine) in groups of pikeperch larvae fed exclusively with rotifers. It is plausible that fish fed 100% artemia had a greater surface area for nutrient absorption and consequently the larvae were well nourished and grew more and faster. Another interesting result from this trial was the successful early weaning to 32 DPH for all three protocols tested, whether or not artemia was included in the diet, which reflects on an economic benefit in managing the larval cycle and the cost of live food production.

2. Conclusions

- A single dose of 200 µg kg^{-1} body weight of the slow-release GnRHa preparation ENANTONE® (Takeda Italia S.p.a.) was successful in inducing spawning in *M. cephalus* females with an average oocyte diameter greater than 570 µm.
- The rearing density of 40 individuals L^{-1} resulted in an average survival rate comparable or even better than those obtained by other authors, supporting the possibility of producing *M. cephalus* fry in captivity from sexually mature adults captured in Sardinian lagoons.
- The same breeding induction protocol used for *M. cephalus* has also been validated for *C. ramada*, which means that there are several production opportunities over a longer period of the year.
- From the co-feeding results, the A50 protocol showed the best results in terms of growth, survival, liver vacuolisation and incidence of total and severe bone deformities compared to the A100 and A0

protocols. It is therefore possible to reduce artemia feeding by up to 50% compared to the previous protocol we used.

- The same protocol made it possible to wean the larvae at 32 DPH, thus reducing the time of the transition to micro-diets.
- Mullet eggs can be shipped at a density of 1,500 eggs L^{-1} for up to 16 hours at 24 °C without detrimental effects on the hatching rate and larval viability at 4 dah (days after hatching) without the use of water conditioners. However, the three-fold increase in rearing density is accompanied by a decrease in hatching rate of between 10-14% at 24 °C, which should be taken into account for sowing purposes.
- Mullet eggs appear to be more sensitive when handled at blastopore closure (around 20 hpf) and as early hatched larvae, showing significantly lower survival at 4 dph than eggs handled at 28 hpf (Hours after fertilisation).

3. References

Abellan, M. T., Adell, A., Honrubia, M. A., Mengod, G., and Artigas, F., 2000. GABAB-RI receptors in serotonergic neurons: effects of baclofen on 5-HT output in rat brain. Neuroreport 11, 941–945. https://doi.org/10.1097/00001756-200004070-00009.

Aizen J., Meiri I., Tzchori I., Levavi-Sivan B., Rosenfeld H., 2005 Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen Comp Endocrinol 142:212-221

Abraham M., Shiranee P., Kishore Chandra P., Kailasam M., Charles V.K., 1999. Embryonic and larval development of the striped mullet *Mugil cephalus*. Indian J Fish 46(2):123-131

Crosetti D. (2001). Messa a punto di una nuova tecnica di riproduzione controllata nei Mugilidi. Progetto di ricerca MiPA, Direzione Generale della Pesca e dell'Acquacoltura, 4° Piano Triennale Pesca Marittima ed Acquacoltura, L.41/82, 110p. Final report.

Eda H., Murashinge R., Ooseki Y., Hagiwara A., Eastham B., Bass P., Tamaru C.S., Lee C.S., 1990. Factors affecting intensive larval rearing of striped mullet, *Mugil cephalus*. Aquaculture 91:281–294.

Fahmy, A. and El-Greisy, Z., 2014. Induced spawning of *Liza ramada* using three different protocols of hormones with respect to their effects on egg quality. African Journal of Biotechnology, 13, 40, 4028-4039. https://doi.org/10.5897/AJB2013.13068.

Imentai A., Rašković B., Steinbach C., Rahimnejad S., Yanes-Roca C., Policar T., 2020. Effects of first feeding regime on growth performance, survival rate and development of digestive system in pikeperch (*Sander lucioperca*) larvae. Aquaculture 529: 735636.

Kucharczyk, D., Nowosad, J., Kucharczyk, D.J., Kupren, K., Targonska, ´ K., Wyszomirska, E., Kujawa, R., 2019b. Out-of-season artificial reproduction of common dace (*Leuciscus leuciscus* L.) under controlled conditions. Anim. Reprod. Sci. 202, 21–25. https://doi.org/10.1016/j.anireprosci.2019.01.003.

Kucharczyk, D., Nowosad, J., Wyszomirska, E., Cejko, B.I., Arciuch-Rutkowska, M., Juchno, D., Boron, ´ A., 2020. Comparison of artificial spawning effectiveness of hCG, CPH and GnRHa in combination with dopamine inhibitors in a wild strain of ide *Leuciscus idus* (L.) in hatchery conditions. Anim. Reprod. Sci. 221, 106543 https://doi.org/10.1016/j.anireprosci.2020.106543.

Lee C.S., Tamaru C.S., Miyamoto G.T., Kelley C.D., 1987. Induced spawning of grey mullet (*Mugil cephalus*) by LHRH-a. Aquaculture 62:327–336.

Lee C.S., Tamaru C.S., 1988. Advances and future prospects of controlled maturation and spawning of grey mullet (*Mugil cephalus*) in captivity. Aquaculture 74: 63–74.

Loi B., Papadakis I.E., Leggieri F., Papiol G.G., Vallainc D. (2020) Ontogeny of the digestive system and eye of reared flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758), and evaluation of lipid deposition in the liver according to the feeding protocol. Aquaculture

Meseda M.E.G. and Samira S.A., 2006. Spawning induction in the Mediterranean grey mullet *Mugil cephalus* and larval developmental stages. African J. Biotechnology 5: 1836–1845.

Mousa, M.A. and Khalil, N.A., 2013. Environmental and hormonal control of induced spawning in thin-lipped grey mullet, *L. ramada* (Risso). Egyptian Journal of Histology, 36, 2, 449-458. https://doi.org/10.1097/01.EHX.0000428962.36676.05.

Mousa M.A., Kora M.F., Khalil N.A., 2018. Evaluation of the effectiveness and cost of different hormones in stimulating the spawning of thin lipped grey mullet, *Liza ramada*. Egypt. J. Histol. 41, 275–283.

Nash C.E., Kuo C.M. and Mc Connell S.C., 1974. Operational procedures for rearing larvae of the grey mullet (*Mugil cephalus* L.). Aquaculture 3: 15–24.

Nash C.E., Shehadeh Z.H., 1980. Review of breeding and propagation techniques for grey mullet, *Mugil cephalus* L. ICLARM Studies and Reviews 3, International Center for Living Aquatic Resources Management, Manila, Philippines. 87pp.

Quirós-Pozo R., Concu D., Robaina L., Vallainc D., Loi B., Roo J., 2023. Effects of Different Co-Feeding Protocols on the Early Weaning of Flathead Grey Mullet (*Mugil cephalus*) Larvae. Animals (Basel). 2023 May 18;13(10):1685. doi: 10.3390/ani13101685. PMID: 37238115; PMCID: PMC10215270.

Roo, J., Hernández-Cruz, C.M., Mesa-Rodriguez, A., Fernández-Palacios, H., Izquierdo, M.S., 2019. Effect of increasing n-3 HUFA content in enriched Artemia on growth, survival and skeleton anomalies occurrence of greater amberjack *Seriola dumerili* larvae. Aquaculture, 500, 651-659, 10.1016. https://doi.org/10.1016/j.aquaculture.2018.09.065.Vallés and Estévez, 2013;

U.S. Agency for International Development, Bureau for Global Programs, Filed Support and Research, Center for Economic Growth and Agricultural Development (2007) Final report.

Scolamacchia, S., Concu, D., Loi, B., Chindris, A., Deiana, M., Frongia, C., Carboni, S., Vallainc, D., 2023. Simulated transport of flathead grey mullet *Mugil cephalus* eggs: Effects of density and duration, Aquaculture, Volume 576, 2023, 739856, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2023.739856.

Sylvester J.R. & Nash C.E. 1975. Thermal tolerance of eggs and larvae of Hawaiian striped mullet, *Mugil cephalus* L. Transactions of the American Fisheries Society, 104(1), 144-147. https://doi.org/10.1577/1548-8659(1975)104%3C144:TTOEAL%3E2.0.CO;2.

Thorarensen, H., Farrell A. P., 2010. The biological requirements for post-smolt Atlantic salmon in closed-containment systems, Aquaculture, Volume 312, Issues 1–4, 2011, Pages 1-14, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2010.11.043.

Vallainc D., Concu D., Giménez Papiol G., Loi B., Leggieri F., Brundu G., Chindris A., Sanna G., Fois N., Antognarelli F. & Rossini P., 2021. Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in

captivity from sexually mature adults collected in Sardinian lagoons. Aquaculture Reports, 21, 100844. https://doi.org/10.1016/j.aqrep.2021.100844.

Yanes-Roca C., Mraz J., Born-Torrijos A., Holzer A.S., Imentai A., Policar T., 2018. Introduction of rotifers (*Brachionus plicatilis*) during pikeperch first feeding. Aquaculture 497: 260-268.

ANNEX I

Publications from the thesis

- Vallainc D, Concu D, Gimenez Papiol G, Loi B, Leggieri F, Brundu G, Chindris A, Sanna G, Fois N, Antognarelli F, Rossini P (2021). Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons. https://doi.org/10.1016/j.aqrep.2021.100844

- Vallainc D, Concu D, Loi B, Pitzalis A, Frongia C, Chindris A, Carboni S (2022). Spawning induction and larval rearing in the thinlip grey mullet (Chelon ramada). The use of the slow-release gonadotropin releasing hormone analog (GnRHa) preparation, leuprorelin acetate. https://doi.org/10.1016/j.anireprosci.2022.107145

- Scolamacchia M, Concu D, Loi B, Chindris A, Deiana M, Frongia C, Carboni S, Vallainc D (2023). Simulated transport of flathead grey mullet *Mugil cephalus* EGGS: Effects of density and duration. https://doi.org/10.1016/j.aquaculture.2023.739856

- D. Concu, R. Quirós-Pozo, B. Loi, L. Rabaina, M. Scolamacchia, A. Chindris, G. Brundu, J. Roo-Filgueira, S. Carboni, D. Vallainc. Different weaning strategies effects on liver, gut and bone performance of larval Grey Mullet (*M. cephalus*) (In preparation)