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Reproduction and rearing of the flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758) and of the sea urchin, *Paracentrotus lividus* (Lamark, 1816) for restocking purposes

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Abstract

Grey mullet *Mugil cephalus* and sea urchin *Paracentrotus lividus* roe are well-known and appreciated products. A decline in wild population of both species and a growing demand promote new interest in the use of cultured individuals to help replenish depleted stocks.

The research activity of this thesis was mainly devoted to concurrently defining protocols for spawning, hatching and rearing the two species for restocking purposes.

Some of the results obtained at a laboratory scale for sea urchins produced the following results: (i) an optimisation of the larval rearing protocol; (ii) the identification of an optimal larval diet; and (iii) the use of *Ulvella lens* as metamorphosis-inducing factor in competent larvae. The experimental trials from laboratory to pilot scale comprised the use of rearing protocols and diet developed in volumes from low to high(from 5 to 300 L), in order to study the effect of stocking densities on larval survival and development. Results were encouraging. There was a reduction in water exchange, a similar phytoplankton consumption, an expected lower but satisfactory survival (13% vs 80%) and similar competence (~30%).

For the spawning in captivity of *M. cephalus* we successfully induced gametes emission in three reproductive cycles with the production of more than 300,000 larvae. Three different rearing densities were compared and an inverse correlation of growth with density was observed for both larvae and juveniles. Average biomass production in the 3 cycles ranged from 0.4 to 1.4 g/L, with the production of 1,000 to 4,700 individuals. The condition index was ~ 3 for all cycles, indicating that the *M. cephalus* juveniles produced had been correctly reared. A comparison between two different rearing methods, one 100% indoor and another 50% indoor and 50% outdoor, showed quite similar growth performances (~0.24 mm/day), indicating that the release of the fry in pre-adaptation cages into a lagoon at about 3 cm could be a good practice in terms of sustainability of the process.

Key words: *Mugil cephalus*, induced spawning, rearing, restocking, *Paracentrotus lividus*

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LIST OF ABBREVIATIONS

BW: Body Weight

C1,2,3: *Mugil cephalus* reproductive cycles

C2-1: Cycle 2 *Mugil cephalus* larvae seeded in tank 1

C2-2: Cycle 2 *Mugil cephalus* larvae seeded in tank 2

C3-1: Cycle 3 *Mugil cephalus* larvae seeded in tank 1

C3-2: Cycle 3 *Mugil cephalus* larvae seeded in tank 2

Cp: *Paracentrotus lividus* Competent larva

DPF: Days Post Fertilization

DPH: Days Post Hatching

GR: Growth Rate

HD: High Density

IJ: *Mugil cephalus Indoor reared* Juveniles.

LD: Low Density

LH-RH: Luteinizing Hormone-Releasing Hormone

LJ: *Mugil cephalus* Lagoon-released Juveniles

MD: Medium Density

Mt: Metamorphosed *Paracentrotus lividus* larva

NSW: Natural Sea Water

P6: 6-arm Pluteus

RAS: Recirculating Aquaculture System

SGR%: Specific Growth Rate

System 1,2: Egg Incubation Systems

TL: Total Length

1 GENERAL INTRODUCTION

1.1 General aims of the thesis

The general objective of this thesis is to improve hatchery and rearing techniques in order to maximize the production of the flathead grey mullet *Mugil cephalus* and of the sea urchin *Paracentrotus lividus* for restocking purposes.

1.1.1 Mugil cephalus

Specific aims:

- Define and verify a new protocol to induce *M. cephalus* to spawn in captivity
- Verify the hatching rate of eggs incubated at different densities
- Define and verify a methodology for larval seeding in the rearing system
- Verify the growth rate of juveniles reared indoors at different densities
- Define and verify a protocol for realeasing hatchery-rearedjuveniles of *M. cephalus* into the wild

Laboratory activities were aimed at defining and verifying a replicable protocol for obtaining viable fertilized eggs starting from wild broodstock individuals. In order to enhance hatchery production, increasing densities of incubated eggs were tested.

Larval survival is considered one of the hardest obstacles preventing a wider use of *M. cephalus* in aquaculture (Eda et al. 1990; Tamaru et al. 1992). As the highest mortality occurs during the first 15-20 days post-hatching (U.S. Agency for International Development, 2007), technical and methodological aspects of the protocol employed in this phase were described in detail.

Few authors reported the operational procedures for seeding the larvae into the rearing tanks, or the techniques and operations necessary for rearing *M. cephalus* larvae (Kuo et al. 1974; Nash and Shehadeh, 1980) and juveniles. In order to develop a sustainable production and maximize the process, this thesis focused on identifying a good balance between survival, density and growth of the juveniles up to about 3-4 cm in total length (TL). At this size, individuals are known to tolerate natural environment temperature and

salinity fluctuations well and can be reared in pre-adaptation cages in the wild following the slow-release restocking technique (Brown & Day, 2002). Individuals were reared for 7 months, protected from predation in a lagoon pre-adaptation cage and their growth was monitored until they reached a suitable size for introduction into the wild (> 7 cm) was reached (Leber et al., 1996).

1.1.2 Paracetrotus lividus

Specific aims:

- Test a laboratory-scale developed larval rearing protocol (Brundu et al., 2016a) at pilot scale production volumes
- Test a laboratory-scale developed diet (Brundu et al., 2016a) for larval rearing at pilot scale production volumes
- Test the use of *Ulvella lens* as methamorphosis-inducing factor at pilot scale, previously only tested at laboratory scale (Hannon et al., 2015; Brundu et al., 2016b)
- Evaluate the effects of stocking densities on larval survival and development at pilot scale volumes.

The aim of the research was to evaluate the applicability of rearing methods and diets previously identified at laboratory scale (variable method and mixture of *Dunaliella tertiolecta* and *Chaetoceros gracilis*) in a pilot production system.

It is well known that experiments carried out at laboratory scale often show?higher survival rates in comparison to those obtained at a commercial scale. For this reason it is often difficult to scale up production (Fenaux et al, 1985; Pedreotti and Fenaux, 1993; George et al, 2004; Kelly et al., 2000; Liu et al., 2007).

Mortality and development of the larvae reared at pilot scale (300 L tanks) and at laboratory scale (5 L) were evaluated. Individuals were reared following the “variable method protocol” (Brundu et al., 2016a).

Once larvae reached competence, the macroalga *Ulvella lens* was tested as a metamorphosis-inducing factor and post-larvae survival rate was determined in volumes 30 times larger (600 L vs 20 L) than those tested in Brundu et al. (2016b).

Little is known about the growth rates of *P. lividus* under laboratory controlled conditions; the few existing studies on the subject are related to other sea urchin species, *Loxechinus albus* (Cárcamo, 2015), *Strongylocentrotus droebachiensis* (Siikavuopio et al., 2007) and *Tripneustes gratilla* (Westbrook et al., 2015).

1.2 Risks of overexploitation of *M. cephalus* and *P. lividus*

The sea urchin *P. lividus* and the grey mullet *M. cephalus* roe are well known and appreciated worldwide as a delicatessen. In Sardinia their consumption is promoted by restaurants, official festivals and the black market. and It is now possible to have a pizza with sea urchins all year round (personal observation). The market of these products is mainly regional, but an enormous potential for expansion is evident considering the growing demand. Both species are harvested during their reproductive season and the depletion of *M. cephalus* and *P. lividus* stocks in the Mediterranean has been documented by ecological studies (Addis et al. 2009; Withfield et al., 2012).

In the province of Oristano there is the largest and most appreciated *M. cephalus bottarga* production in Sardinia (about 3 t /year). Data registered by the local fishermen's association "Cooperativa Pontis" (Cabras, OR, Sardinia) report that 2.6 t of *M. cephalus* sexually-mature females were captured at Cabras lagoon between August and September in 2013 and destined to the local production of salted and dried grey mullet roe, *bottarga*. In 2014, the season started in August and lasted until November, with a total capture of 7.6 t ripe females. In 2015, about 12 t were captured from August to October whileonly 3 t were recorded in 2016 (Cabras fisherman association, personal communication).

Regarding *P. lividus*, professional and recreational fishermen from all over Sardinia come to the coast of Oristano to harvest the edible sea uchins.

Both fisheries sustain an important food industry, nevertheless, improvements in management tools are necessary to increase the economical benefits in a sustainable way. Thus, in order to improve the knowledge on the conservation status of both resources and

to improve their management, the Sardinian Region recently funded some research projects on both species (Region Sardinia law n. 7, 08/2007)

The Internationl Marine Centre (IMC, Oristano – Italy) has been constantly involved in both aspects of the research, within its mission to recommend management measures to control the decline of key species (threatened or commercially relevant) and to support those activities which sustainably exploit marine biological resources in their productive process.

1.3 Introduction to the flathead grey mullet *Mugil cephalus*

1.3.1. Background data

In the 2015, the total world production of Mugilidae was 555,194 tonnes. 70% of this was from capture fisheries and the remaining 30% from aquaculture. 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 2015, the world-wide mullet production from aquaculture amounted to 179,942 tonnes (FAO 2016). Mullet culture is mainly practiced in the Mediterranean, the Black Sea and in South East Asia. Africa, thanks to the Egyptian production, is by far the leading producer with 157,543 tonnes produced in 2015 and 87% of the world's aquaculture production. This is followed by Indonesia (7,176 tonnes), the Republic of Korea (6,834 tonnes) and Taiwan (1,321 tonnes). In 2015, Europe contributed to the aquaculture production of mullets with 1,122 tonnes, of which 780 tonnes are reported to have been cultured in Italy (FAO 2016).

Among Mugilidae, the rising economical relevance of *Mugil cephalus* is mainly due to the expanding market of its roe. Once salted and dried, this represents a traditional luxury item in many regions of the world and is sold at quite a high price (Liao, 1981). Top-quality dried mullet egg roes from Tuscany and Sardinia are sold at 230 euro/kg retail price (2015).

In Italy, the dried, salted gonads of the female *M. cephalus* are known as "*bottarga di muggine*". They are known as fish roe in the UK, "*avgotaracho*" in Greece, "*karasumi*" in Japan, "*botarikh*" in Arabian countries and "*botarga*" in Spain. The price of grey mullet

roe is constantly rising in the international market. In Sardinia the price of *bottarga* can be about 10 times that of a whole fresh fish.

In the Mediterranean region, the production of dried mullet egg roe dates back to the Phoenicians. It was used throughout the region in the Middle Ages by the Arabs (Monfort, 2002). In Japan, mullet roe (*karasumi*) has been considered a delicacy since ancient times. The traditional use of the flathead grey mullet *M. cephalus* to produce dried egg roe in faraway regions of the world can also be explained by the very high gonadosomatic index (GSI) of the ripe females of this species (Katselis et al., 2005; McDonough et al., 2005). Ripe females at the secondary or tertiary yolk stage are captured during their spawning migrations.

In Italy *M. cephalus* is mainly reared extensively in coastal lagoons. During spring, juveniles naturally migrate towards the eutrophic feeding grounds of lagoons or are captured in the wild and released into confined areas.

In Sardinia, reproductive migration occurs in summer, from July to September. *M. cephalus* females are captured in the "*lavorieri*", the modern evolution of the ancient capture systems built of reeds (Del Rosso, 1905).

Lavorieri are fixed barrier traps made of concrete structures with plastic gates situated in the interface between the sea and the coastal lagoon. They allow small fish to enter the nursery habitat, the lagoon, but prevent adult fish from escaping to the sea. This type of fishery lies between extensive aquaculture and controlled management of resources. All biomass production is based on the natural trophic web, material and methiods.

The productivity for *M. cephalus* in Italian and Sardinian lagoons has constantly decreased overthe last decades. The main cause is the reduced migration of juveniles into coastal lagoons. This is due to detrimental environmental conditions, marinization of lagoons, increased predation by icthyophagus birds, (especially the great cormorant) and overfishing of sexually-mature adults (Withfield et al., 2012; Crosetti, 2015).

Bottarga production represents a highly valued traditional activity in Sardinia. Since 2002 the *bottarga* market has known an constant annual increase of about 5% per year. 2008 data suggest that the total production amounts to about 400 t/year, most of which is sold in the regional market. However, only about 2 % of the *bottarga* produced in Sardinia is obtained from females captured in Sardinian lagoons. In order to satisfy the growing *bottarga* demand, frozen female gonads of *M. cephalus* are imported in huge quantities from abroad (Africa, the USA, Brazil and Australia) (Coldiretti, Impresa pesca, personal

communication, 2013). In order to give more value to this Sardinian product which is already well known and appreciated at a national level, growing attention has been paid to the certification (DOP/IGP) of a local brand, under which only local *M. cephalus* females should be used for *bottarga* production. An example of a DOP-certified bottarga is represented by the bottarga produced in Missolongi (Greece) (Crosetti & Blaber, 2015)

Aquaculture could represent a good alternative to mullet gonad imports, a chance to enhance the *bottarga* market and to sustain natural stocks subjected to high-fishing pressure during the spawning season.

While the ecological aspects of mullets have been widely studied, aspects such as induced spawning, larval rearing and grow out culture methods still need to be implemented. Since mullet aquaculture in the world is still mostly based on wild fry collection (Crosetti & Blaber, 2015), aquaculture could assure a constant annual production of flathead grey mullet egg roe, independently from the annual availability of wild fry and juveniles,.

1.3.2 Biology and ecology

The flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758) is a member of the Mugilidae family belonging to the class of the Actinopterygii. The species has been recorded in coastal and estuarine waters of temperate, subtropical and tropical regions, mainly between latitudes 42ºN and 42ºS (Harrison, 2002; Nelson, 2006; González-Castro et al., 2008; Durand et al., 2012a; Whitfield et al., 2012). It is common at a temperature range of 8-26°C and a depth range of 0-120 m, but more usually found at 0-10 m.

Taxonomically, *M. cephalus* differs from the other mullet species in pyloric caeca number (2) and by the presence of an adipose eyelid (Turan et al. 2005). However, questions regarding its taxonomic status have been raised in many genetic studies, suggesting that *Mugil cephalus* is a complex species (Crosetti et al., 1994, Rossi et al., 1998a, Rocha-Olivares et al., 2000, Fraga et al., 2007, González-Castro, 2007, González-Castro et al., 2008; Heras et al., 2009; Jamandre et al., 2009; Durand, 2015).

In the Mediterranean, the *M. cephalus* lineage (called Mediterranean lineage) was demonstrated to represent a single population cluster (Crosetti et al., 1994, Rossi et al. 1998a, Blel et al. 2010, Durand 2015, Crosetti & Blaber, 2015) and a low genetic diversity was detected between the east coast of Italy and the Azov Sea populations (Livi et al. 2011).

It is a cathadromous euryhaline species (Riedel, 2004) which spends part of its life cycle in coastal lagoons, lakes and/or rivers and migrates back to the sea to spawn (Crosetti & Blaber, 2015). Larvae are usually planktonic feeders in the offshore marine environment (Brownell 1979), in the surf zone (Inoue et al. 2005) and when they first enter estuaries (Gisbert et al. 1996). In coastal and estuarine nursery habitats, fry and juveniles initially feed on small invertebrates and later feed mainly on benthic organisms and plant material by browsing and sifting the bottom sediments and detritus (Blaber & Whitfield, 1977). Larger juvenile and adult mullets mainly feed on detritus and benthic microalgae (especially diatoms), together with foraminifera, filamentous algae, protists, meiofauna and small invertebrates (Thomson, 1963; Blaber, 1976; Lawson & Jimoh, 2010; Whitfield et al., 2012; Cardona, 2015).

A few studies involving the growth assessment of *M. cephalus* highlighted the very variable growth rates which appear dependent on environmental factors. Significant differences in growth rate were found between individuals at different salinities such as those of brackish and marine waters (Chubb et al., 1981; Ibáñez et al., 1999; Crosetti & Blaber, 2015).

1.3.3 Reproduction and life cycle

Mullets are oviparous gonochoristic fish; individuals are either males or females with no sexual dimorphism. Flathead grey mullet are sexually undifferentiated for the first 12 months. Differentiation begins at 13 months of age, and fish are fully differentiated at 15 -19 months old. Sexual maturation in male flathead grey mullet first occurs at 1 year of age and 100% of males are mature at 2 years of age. Female flathead grey mullets first mature at 2 years of age and 100% maturity occurs at the age of 4 years(McDonough et al., 2005).

Reproduction is under neuroendocrine control, the functions of the hypothalamus and pituitary glands and gonads are influenced by environmental factors, mainly by photoperiod, water salinity and temperature. Spawning occurs once a year, oocytes develop in synchrony (Shehadeh et al., 1973a) and ovarian maturation is group synchronous (Kuo, 1995).

In the Mediterranean Sea, mature *M. cephalus* adults undertake a reproductive migration from estuaries or coastal lagoons to the sea from May to October, with a peak between August and September (Nash & Shehadeh, 1980).

Mugil cephalus has a high fecundity from ~1.5 to ~ 5.0 millions eggs/kg body weight (BW) (Grant & Spain, 1975), though values of up to 7.2 million eggs/kg BW were reported (Nikolski, 1954).

Fertilized eggs are spherical and transparent, the presence of one large oil globule makes them extremely buoyant. On hatching, the oil globule is located in the yolk sac of the pre-larvae. Newly hatched larvae of *M. cephalus* vary in length between 2.2 and 3.5 mm. The pre-larval stage is characterized by a nutritive contribution which is provided exclusively by the yolk sac and ends when the larva becomes capable of feeding itself(Balon, 1985). The larval period lasts until the number of fin rays reaches their adult complement. Then the juvenile stage begins, when the fish have a total length of 33-45 mm. This happens at 45-60 days post hatching. The juvenile stage ends when the fish reach sexual maturity (Miller & Kendal, 2009).

The fish generally enter the estuaries once they have reached a length of between 10 and 15 mm (Blaber, 1977). At this point their diet changes and their growth increases rapidly in highly productive nursery habitats. Salinity is an important driver influencing the abundance of *M. cephalus* in estuaries (Nordlie, 2015). Mesohaline and polyhaline habitats are generally preferred (McDonough and Wenner, 2003).

1.3.4 The culture of Mugil cephalus

Thanks to some peculiarity of their ecological features, grey mullets have been estensively cultured in many places in the world since the ancient times. Their euryhalinity enables them to be cultured in an ample range of salinities and in polyculture (Crosetti & Blaber, 2015). They are consumers of the lower trophic levels, feeding on the natural food web present in the environment where they live. Their ability to convert a large variety of food into high quality flesh, from microorganisms and decaying organic matter to algae and insect at the larval stages or small molluscs, make them ideal for extensive culture (Cardona, 2015).

Despite several successful experimental trials of induced spawning, no commercial mass propagation of *M. cephalus* is currently being carried out (Liao et al., 2015).

The world's mullet production from aquaculture has shown large fluctuations in the last decades, and depends exclusively on collection of fry from the wild. In the 1990s there was an intensification of culture that lead to a large increase in production, with a peak of

271,816 tonnes in 2007. Since 2010, mullet production has dropped to 133,000-150,000 tonnes. In 2015, the world's mullet production from aquaculture corresponded to ~ 30% (179,942 tonnes) of the total mullet production. Egypt is by far the leading producer with 87% of mullet aquaculture worldwide, followed by Indonesia (4%), The Republic of Korea (3.8%) and Taiwan (0.7%) (FAO, 2016).

Mugil cephalus makes up 5.3% of the total mullet production worldwide. In Italy the production of *M. cephalus* had a peak in 1996 (3100 tonnes) then started to decrease untilin 2015, only 780 tonnes were reported (FAO 2016). Such unpredictable fluctuations are difficult to couple with aquaculture planning and management. Ravagnan (1978) observed that four years out of five, the availability of mullet fry was not sufficient to stock the North Adriatic *valli* (confined portions of coastal lagoons). Artificial propagation in hatcheries could therefore be the solution for to obtaining a steady supply of fry (Liao et al., 2015).

Like most cultured finfish, *M. cephalus* does not spawn spontaneously in captivity. Females oocytes mature to vitellogenesis, then stop and eventually undergo atresia. Hormonal treatments act on different levels of the hypothalamic-hypophyseal-gonadal axis inducing final maturation and spawning. However, controlled conditions are required in order to achieve reproduction.

Numerous experimental reproduction trials employing different hormonal treatments have been carried out in different countries on 11 mullet species. All hormones tested need to be used at very high dosage to induce spawning compared to other fish species (Crosetti, 2015).

M. cephalus, was the subject of specific research programmes in Taiwan in the period from 1963-1973 (Liao, 1985) and at the Oceanic Institute of the Hawaii (USA) from the 1970s to the year 2000 (Kuo, 1995). Most of the experimental trials were carried out by administrating two hormone injections: a priming dose first, followed 24 hours later by a resolving dose.

Manuals and reviews dealing with this argument date back to the 1980s (Nash & Shehadeh, 1980) and to the 1990s (Tamaru, 1993). An update would be necessary as they didn't succeed in applying their findings on a commercial scale.

Lee et al. (1997) compared the cost-effectiveness of the different hormone treatments to induce spawning in *M. cephalus* females and identified the most reliable one to be CPH (at 20-40 mg/Kg BW) followed by LHRH-a (100 mg/kg BW). Crosetti (2001) and

Crosetti & Cordisco (2001) suggest treating females with superactive, slow-release analogues of LH-RH (leuprorelin acetate) as a resolving dose (600-700 µg/kg BW).

Males can successfully be induced to spermatogenesis and spermination on demand by treating them with 17s-methyltestosterone which is injected or administered orally (Lee et al., 1986). Other protocols do not include hormone treatments on males as they can be captured during their natural spawning period. In this period, the milt flows out when gentle pressure is applied to the abdomen (Das et al., 2008).

The artificial propagation of mullets has never reached a commercial scale. The main bottlenecks are the high hormone dosages required to induce spawning, a long culture schedule and the low price of hatchery-produced fry (Crosetti, 2015). Saleh (2008) underlines the elevated mortality rate at the larval stage and the high cost of fry production, which is similar to that of more pricy species. However, the recent rise of mullet roe prices associated with better marketing strategies has renewed the interest in the use of hatchery fry for farming, restocking and restoration needs. In Taiwan, thanks to promotion by the government and related organizations, flathead, grey mullet aquaculture has become profitable (Liao et al., 2015).

Today the grey mullet is considered a potentially interesting species for the development of aquaculture, based on its potential importance both biologically and economically. Initiatives for species diversification in aquaculture and for overcoming the well-known bottlenecks in the production of this species are flourishing with the aim of enhancing European aquaculture production with new and emerging fish species. In 2013, *M. cephalus* was included in the project DIVERSIFY (2013-2018; http://www.diversifyfish.eu), funded under the 7th Framework Programme of the European Commission (7FP-KBBE).

In particular, theimportance of mullets for feeding populations in developing countries is easily understood when we consider the wide range of conditions in which it can be grown and its high nutritional value (U.S. Agency for International Development, 2007).

Although reliable techniques for inducing mullets and *M. cephalus* to spawn have been developed, further refinement is still necessary in order to obtain good quality mullet seed in sufficient quantities to stock grow-out systems. Moreover, the added value of processed products from the flathead grey mullet is more than their fishery value. Hence the importance of the management and development of the flathead grey mullet aquaculture industry (Crosetti & Blaber, 2015).

1.3.5 Restocking

In the 1980s, some US states began new stock enhancement programs, following advances in marine fish culture and fish tagging technologies. In the 1990s and beyond, numerous additional stocking programs around the world were carried out (International Symposium on Stock Enhancement and Sea Ranching (ISSESR, 1990) (www.SeaRanching.org).

The restocking of threatened species of economic relevance is now considered an efficient fisheries management tool (Welcomme & Bartley, 1998; Sass & Allen, 2014). Emphasis is placed on a responsible approach to restocking programs by improving planning, fishery management, genetic considerations, pilot experiments aimed at enhancing the survival of released fishes, and the use of adaptive management (Blankenship and Leber 1995, Walters & Martell, 2004; Lorenzen et al., 2010; Sass & Allen, 2014; Crosetti & Blaber, 2015).

Restocking directives and protocols represent helpful tools to establish how best to release hatchery-reared fisheshave been published (Hutchison et al., 2012).

The flathead grey mullet *Mugil cephalus* has a unique role in the modern development of marine fishery enhancements. *M. cephalus* was the test species chosen for one of the first systematic series of empirical studies to evaluate the effectiveness of aquaculture-based marine fishery enhancements. Beginning in 1988, the Oceanic Institute (OI), located on Oahu, Hawaii (USA), conducted several years of experimental pilot releases with grey mullets and collaborated with the Hawaii Division of Aquatic Resources (DAR). The goal was to transfer mullet stock-enhancement technology to the state for implementation in a recreational mullet fishery in Hilo, Hawaii (Nishimoto et al., 2007).

The first batch of hatchery-produced mullets was tagged and released in 1989 (K. Leber, pers. comm.). A collaborative project by the Division of Aquatic Resources and the Oceanic Institute followed in 1990–2000. The Hilo project verified the potential of stock enhancement as an effective tool to replenish diminishing stocks for mullets, and led to the implementation of several management measures to improve mullet fishery management based on the results of this project (Nishimoto et al., 2007).

The survival of mullets (*M. cephalus)* was enhanced when mullet fingerlings were stocked in freshwater streams rather than bay environments (Leber et al., 1996).

Hutchison *et al.* (2006) concluded that the 50-65 mm fingerlings were the most cost-effective size? to stock in impoundments with high densities of predators (Leber & Arce, 1996), based on prevailing hatchery prices at the time of their experiments.

Grey mullets were also used in the Hawaii studies in the early 1990s to demonstrate the effectiveness of using pilot-release experiments to optimize release strategies. Such pilot releases are a fundamental aspect of a 'Responsible Approach' to marine enhancements (Blankenship & Leber, 1995; Lorenzen et al., 2010). The study compared survival rates among different sizes (total length) of *M. cephalus* juveniles released in Kaneohe Bay (Hawaii, USA), in spring and summer. The best performances in terms of survival were recorded when 70-110 mm (TL) fishe were released in summer (Leber et al., 1996).

The size and the season of the release are fundamental factors in the success of restocking activities, but there are also other aspects to consider in order to enhance the survival rates of released fish. A low survival rate is commonly recorded in marine finfish restocking trials. This has been also attributed to behavioural deficits (*domestication effect)* which could contribute to making them incompatible with life in the wild (Olla et al., 1994; Stickney, 1994). Several research activities have focused on the main causes of high post-release mortality and on how to reassess the techniques of fish rearing for restocking purposes (Brown & Day, 2002).

The domestication effects of captive fishes concern feeding behaviour (Furuta, 1998; Olla et al., 1994), predation escaping ability (Malavasi et al., 2004; Stunz & Minello, 2001; Yamamoto & Reinhardt, 2003), orientation and other ecological aspects (Petersson & Jaervi, 1999).

Brown & Day (2002) suggest that soft release techniques, which foresee fish being kept in confined structures placed in the natural environment for a period of time prior to being effectively released into the wild, contribute to accustoming them to the prevailing environmental conditions. The same authors also suggest the possibility of releasing juveniles immediately from the lab into the wild, which they call a "hard" release technique.

The stock enhancement of *M. cephalus* was mostly carried out in confined coastal lagoons, though this could be considered capture-based aquaculture, according to Ottolenghi et al. (2004).

1.4 Introduction to the sea urchin *Paracentrotus lividus*

1.4.1 Background data

The gonads of sea urchins are a prized delicacy in Asian and Mediterranean countries, as well as in some countries in the Western Hemisphere like Barbados and Chile (Ding et al., 2007).

In the Mediterranean, *P. lividus* is the most consumed and harvested sea urchin species (Guillou & Michel, 1993; Brundu et al. 2015).

The high demand for its gonads, their high prices and its depth range (normally between 0 and 10 m), make *P. lividus* very attractive for small scale fisheries. They can make a high profit with a very low investment (Le Gall, 1987).

However, their slow growth rate and the high fishing pressure occurring during the reproductive period has produced an alarming decline in wild populations over the last decades (Le Gall, 1990; San Martin, 1995; Pais et al., 2007; Addis et al., 2009).

In many regions, sea urchin harvesting has been added to higher-trophic-level fisheries (Anderson et al., 2011). This is the case of some regions in Southern Italy where the edible sea urchin *Paracentrotus lividus* (Lamarck, 1816) is subject to high fishing pressure (Tortonese, 1965; Guidetti and Dulcic, 2007; Pais et al., 2007). The most striking effect of sea urchin fishing is the fast decrease of the resource in terms of total density and abundance (Andrew et al., 2002; Bertocci et al., 2014).

In Sardinia, commercial fishery of the sea urchin is limited by law to catches of sizes larger than 50 mm test diameter (TD), and restricted in time (from November to April). However, despite regional decrees concerning fishing periods, minimum size and catch quotas per day per fisherman, the harvesting of *P. lividus* is intensively practiced, and removal by recreational fisherman occurs throughout the year because of the long tourist season (Pais *et al.*, 2007). The systematic removal of the larger sea urchins may decrease the number of fertile individuals that release gametes into the surrounding environment, therefore leading to a population collapse, as reported for some overfished areas (Pais *et al.*, 2007; Addis et al., 2009).

Together with a better management of the resource, restocking programs could represent a suitable tool for the enhancement of wild populations. Indeed, the very slow growth rate of *P. lividus* suggests that rearing it until it reaches a suitable size for harvesting is not economically profitable. However, improvements in *P. lividus* rearing techniques are still necessary in order to maximize the juvenile production for restocking purposes (Andrew et al., 2002).

A mass production of *P. lividus* is still limited by the high mortality rate of larvae and post-larvae, the transition from planktonic to benthic stages and the low growth rate of juveniles (Carboni et al., 2012; Daume et al., 2000).

At present, sea urchin restocking programs based on hatchery-reared individuals which are released into the wild have not produced the desired results in Japan, Chile or the USA (Saito, 1992). In the French Mediterranean, a pilot scale experiment was conducted to assess the feasibility of such an operation and to evaluate the impact on both the population structure and genetic diversity of wild comunities (Couvray et al., 2015).

1.4.2 Biology and Ecology

Paracentrotus lividus (Lamarck, 1816) is a species of the Class Echinoidea. Its distribution area extends from Scotland to the Canary Islands, in the northeastern Atlantic and it is also distributed throughout the Mediterranean Sea (Boudouresque and Verlaque, 2007).

P. lividus is particularly common at seawater temperatures ranging from 10 to 25 °C (Boudouresque and Verlaque, 2007). It is a subtidal species, mostly distributed at a depth ranging from 0 to 30 m (Relini and Tunesi, 2009). It colonizes rocky bottoms, preferably ones with a good cover of marcroalgae, seagrass meadows of *Posidonia oceanica* and *Zoostera marina* (Fernandez, 1996; Tortonese, 1965).

P. lividus is considered an opportunistic generalist species. It feeds mostly on photophilous macroalgae, but is able to consume a wide variety of trophic resources, from porifers to hydrozoans and copepods (Régis, 1978; Pastor, 1971; Tortonese, 1965).

Furthermore, at high densities, *P. lividus* adults were reported to feed on conspecific juveniles (Pastor, 1971). Individuals do not distribute themselves randomly. Habitat heterogeneity, wave exposition, bottom slope, light, food presence, predation and larval transport can all strongly influence population densities (Guidetti & Dulcic, 2007).

The growth rate of *P. lividus* is influenced by seawater temperature (Fernandez, 1996). The ideal temperature for good growth performance ranges between 18-22 °C (Le Gall et al., 1990). However, food availability represents one of the main factors determining *P. lividus* growth. It has, in fact, been demonstrated that larger-size individuals and higher growth rates are associated with an abundance of food (Turon et al., 1995).

The feeding preferences of *P. lividus* varied on the basis of diversity and the abundance of vegetable species, and according to the size of the individuals (Verlaque & Nedelec, 1983a).

1.4.3 Reproduction and life cycle

Paracentrotus lividus is a separate-sexes species, without sexual dimorphism, although hermaphroditism has occasionally been observed (Boudoresque & Varleque, 2001).

The reproductive organ consists of five gonads radially disposed in the coelomic cavity; *P. lividus* becomes sexually mature when its diameter is between 20 to 25 mm (Grosjean, 2001).

Somatic and germinal cells (oogonia or spermatogonia) make up the *P. lividus* gonads; the former represent a reservoir of nutrients available for the gametogenesis of the latter (Walker et al., 2007).

The annual gametogenic cycle of *P. lividus* can be divided into six stages based on the proportions of the somatic and germinal cells (Byrne, 1990).

The reproductive cycle is highly variable (Guettaf et al., 2000) and significant differences in gonad growth and stages of maturity have been observed observed even between organisms collected in close sites (Spirlet et al., 1998).

In the Mediterranean Sea, spawning peaks occur from spring to summer (Sellem and Guillou, 2007) and in autumn (Pedrotti, 1993), but in some cases mature individuals were reported all year round (Soualili and Guillou, 2009).

Reproduction is mediated by external factors, such as water temperature or mechanical disturbance. Gametes are contemporarily released by both sexes in the water column and fertilization is external (Spirlet, 1999; Spirlet et al., 1998). At 48 hours post-fertilization the Echinoid larva (echinopluteus) is capable of swimming and eating (Falugi and Angelini, 2000). At 6 days post-fertilization the larva develops the first pair of additional

arms (6-arm pluteus): the formation of the second additional pair occurs at about 12 days post-fertilization (8-arm pluteus). Larvae are considered competent when the rudiment is equal to or larger than the stomach. At this stage the larva start seeking a substrate to settle and metamorphose (Carboni et al., 2012).

The post-larva is endotrophic. The mouth and anus develop within seven days from what? when the post-larva becomes an exotrophic juvenile complete with a developed and functional digestive tract (Grosjean, 2001).

The growth of juveniles is influenced by temperature, food availability, hydrodynamics and density (Gago et al., 2003; Grosjean et al., 1996; Fernandez, 1996; Turon et al., 1995). Several studies have aimed at determining the age and somatic growth of *P. lividus*,. The latter resulted in about 1 cm (test diameter) per year (Boudouresque & Verlaque, 2007).

1.4.4 Echinoculture of Paracentrotus lividus

The rearing cycle of *P. lividus* consists of six steps: reproduction, larval rearing, metamorphosis,the growth of post-larvae and juveniles, the growth of subadults, and the growth and conditioning of adults.

Spawning can be induced without killing the breeders, by injecting Potassium chloride (KCl) or Acetylcholine (Ach) into the peristomial membrane. Gametes can also be obtained by dissecting the individuals. Female gametes are kept in natural seawater while sperm is collected dry and mixed with female gametes to promote fertilization (Falugi & Angelini, 2000).

When at least 80% of the eggs show the elevation of the fertilization membrane within 80 seconds, fertilization is considered successful (Falugi & Angelini, 2000).

Larval rearing begins at 40 h post-fertilization. The stocking density of Echinopluteus varies from 0.25 to 4 larvae/mL, with an optimum density at 1-2 larvae/mL (Fenaux et al., 1985; Pedrotti and Fenaux, 1993; Pedrotti and Lemée, 1999; Kelly et al., 2000; Càrcamo et al., 2005; Liu et al., 2007; Azad et al., 2011; Privitera et al., 2011).

Larvae are reared in static filtered seawater with continuous light. Though new methodologies characterized by low mechanical disturbance and maintenance of a constant, daily restored microalgal concentration were tested at laboratory scale with encouraging results (Brundu et al., 2016a). Most of the rearing protocols foresee a 50%

water exchange performed every three days and a fixed daily amount of food(Càrcamo et al., 2005; Gibbs et al., 2009; Azad et al., 2011; Privitera et al., 2011; Swanson et al., 2012; Carboni et al., 2014).

Larval rearing lasts for 18-25 days post-fertilization, when larvae reach the competence for settlement and shift from a planktonic to a benthic animal (Falugi and Angelini, 2000). At this stage, the presence of specific macroalgae stimulates the settlement (Carboni, 2013) which occurs about one hour (Burke, 1987) from the exposition of the individuals to the metamorphosis-inducing factor.

When at least 75% of the larvae have metamorphosed within 72 hours, the larval culture is transferred to settlement tanks containing filtered natural sea water (NSW) and the metamorphosis-inducing factor (Carboni, 2013; Carboni et al., 2012; Liu et al., 2007; Kelly et al., 2000). Mortality rates higher than 90% were recorded by several authors during the post-metamorphic period (Buitrago et al., 2005; Rahim et al., 2004; Grosjean et al., 1998; Shimabukuro, 1991).

The juvenile stage starts after the opening of the mouth and anus, which become functional, as doesthe digestive system. At this point, individuals are exotrophic and start to graze the biofilm used as metamorphosis-inducing factor (Carboni, 2013). Juveniles can be reared in flow-through or recirculating aquaculture systems (RAS) (Carboni et al., 2013). When individuals have a test diameter of 3-4 mm, macroalgae such as *Enteromorpha* spp., *Ulva* spp., *Saccharina* spp. and *Laminaria* spp. can be added to the diet. The stocking density for growing *P. lividus* juveniles is usually 400 individuals/m^2 (Carboni, 2013). Subadults (individuals with a test diameter >10 mm) are reared apart from smaller individuals (Grosjean, 2001). The conditioning process starts when the sea urchins reach 40 mm test diameter and consists in stocking the animals at 16°C and feeding them ad libitum with a diet rich in proteins for about three months (Grosjean, 2001; Klinger et al, 1998).

1.4.5 Enhancement of natural stocks

Generally, the enhancement of wild sea urchin stocks is divided into three categories: reseeding, habitat enhancement and transplantation to the wild (Andrew et al., 2002).

Reseeding is defined as the release of hatchery-reared juveniles into a natural environment (Andrew et al., 2002). Successful reseeding rests on the assumption that the

population receiving the out-planted animals is recruitment limited. Processes acting before, during and after settlement, including successful fertilization, food limitation, larval and post-larval predation, can limit recruitment of the species (Doherty, 1999). In addition, to ensure successful reseeding, the operator must check that the population does not exceed the carrying capacity of the environment. This is mainly for density-dependent processes and is related to feed limitations (Lawrence, 2001, Andrew et al., 2002). Different reseeding programmes were carried out overthe last decades using various sea urchin species such as *Strongylocentrotus intermedius*, *S. nudus*, *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina* and *Tripneustes gratilla*. *T. gratilla* has been reseeded and grown-out in the Philippines since 1993 (Juinio-Meñez et al., 1998). Recently, reseeding programmes of *A. crassispina* and *S. intermedius* were carried out in South Korea (National Fisheries Research and Development Institute, 2000). However, the full expression of sea urchin reseeding was reached in Japan many years ago, increasing sharply from the 1980s up to 1994 (Andrew et al., 2002). In Japan, larvae and post-larvae are reared in hatchery tanks up to a seed size of about 5 mm; at this size, they are placed into small mesh cages for intermediate culture in tanks or suspended in the sea. Juveniles are fed with kelp or knotweed and they are released when the test-diameter is equal to or larger than 15 mm (Tegner, 1989). Individuals with a test diameter of >15 mm proved to be less vulnerable to predation than individuals of <15 mm (Miyamoto et al., 1985).

Often, the effectiveness of the reseeding programmes was not evaluated (Kitada, 1999; Saito, 1992) due to the difficulties in telling reseeded and wild individuals apart (Andrew et al., 2002), Many methods of tagging sea urchins externally were developed. Some examples are stainless steel, colour-coded labels, monofilament wire, passive integrated transponder tags (PIT), aluminium tags and chemical tagging.t Unfortunately, many of these increase the risk of mortality or affect juvenile growth (Carboni, 2013; Andrew et al., 2002).

Omi (1987) reported the results of the seeding experiment of reared *S. intermedius* spread uniformly in Hokkaido. The survival rate of the juveniles released ranged from 23% to 39%, and about 92% of these achieved 40 mm in test diameter after about 21 months.

The success of *S. franciscanus* reseeding at small-scale was evaluated in California. Ebert et al. (1992) recorded a strongly size-dependent mortality rate, with values ranging from 0% to 0.3% for 5 mm individuals, from 0% to 6.8% for 10 mm ones and from 1% to 22%

for 15 mm ones. In another reseeding trial, Dixon et al. (1997) reported a catastrophic mortality (more than 99%) in a first site and 81% in a second site, after one year. Good results were obtained by Sakai et al. (2004) with *S. intermedius*; they found that 62-80% of their total landings were reseeded sea urchins and therefore, suggested that the reseeding of hatchery-raised *S. intermedius* is effective for rebuilding stocks.

A negative point of the reseeding activity is that it may influence the genetic diversity and structure of wild populations (Lorenzen et al., 2013; Juinio-Meñez et al., 2008). Factors which increase the risk of lowering the level of genetic diversity are using an inadequate number of progenitors and promoting inbreeding (Lorenzen et al., 2010). Aiming to evaluate the genetic impact on wild populations, a study of Couvray et al. (2015) reported the results of a stock enhancement pilot project carried out in France. Survived sea urchins were identified by genetic parentage assignment tests and were collectd at two sites, representing 3% and 12% of the total recaptured urchins The genetic diversity within- and among-populations did not seem to be affected by reseeding. Couvray et al. (2015) concluded that reseeding could be considered a potential and efficient management tool to restock or sustain overexploited populations.

Although different studies focused on sea urchin reseeding and positive results were previously obtained, it is still unknown if the mortality of hatchery-reared individuals is higher than the wild ones and, consequently, if additional methods of acclimatization should be used before release. Effectively, many studies have shown that vertebrate organisms grown in captivity are generally less resistant to environmental variations than wild ones (McGinnity et al., 2009). In addition, their antipredator behaviour is different, since they are more vulnerable to predators (Meager et al., 2011). However, previous studies focusing on the effects of sea urchin reseeding (*P. lividus* and *S. franciscanus*) did not include the acclimatizing stage of the animals before their release (Couvray et al., 2015; Sakai et al., 2004; Andrew et al., 2002; Juinio-Meñez et al., 1998; Dixon et al., 1997; Ebert et al., 1992; Tegner, 1989). Habitat enhancement aims at expanding the areas suitable for sea urchin growth and promoting algae colonisation to ensure the presence of live feed (Morikawa, 1999). For example, artificial reefs (stones or cement blocks) were introduced in Japan (Taki and Higashida, 1964; Kawamura, 1973) and South Korea (Andrew et al., 2002) to increase the suitable habitats for shallow-water species, such as sea urchins, abalones, top shells and algae.

2 MATERIALS AND METHODS

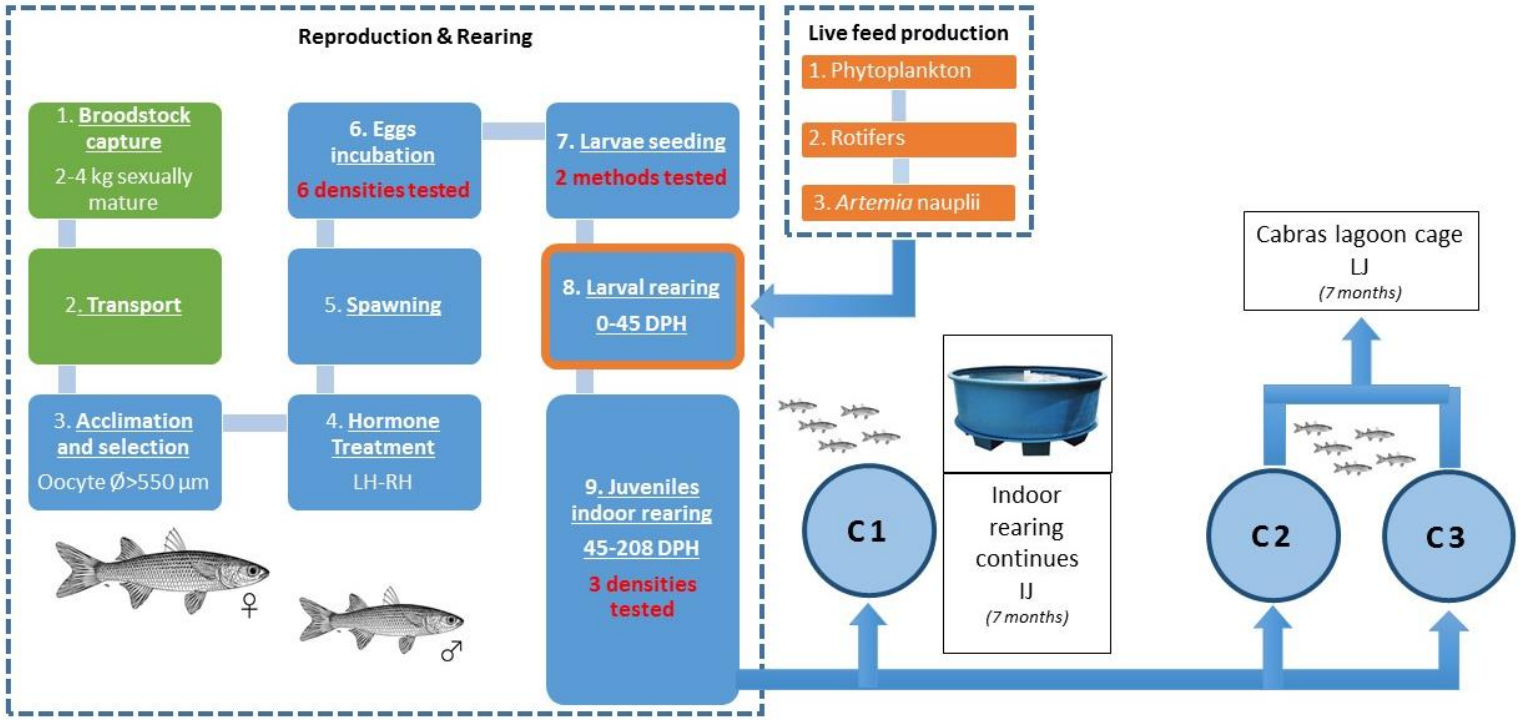
2.1 *Mugil cephalus*

The reproduction and rearing trials of the flathead grey mullet were carried out in indoor facilities at the International Marine Centre - IMC laboratories (Oristano, Sardinia, Italy).

Grey mullet broodstock were collected during their natural spawning season (August-September) at the *lavorieri* (a systemof trapping mullets during their natural migration towards the sea, cfr. Fig. 1bis) in the lagoons of Cabras (39°54'31" N; 8°29'34" E) and Mistras (39°54'08" N; 8°27'59" E) (Figure 1).

Figure 1: Cabras and Mistras lagoons lavorieri

The hatchery was equipped with a spawning and egg collection system, two egg incubation systems, a larval rearing system and a live-feed (phyto- and zooplankton) culture system.



Schematic diagram of the production process and research activities on *Mugil cephalus*

1: Broodstock capture at the lavorieri of the Cabras and Mistras lagoons. **2**: Transport in fish transport bags. **3**: Acclimation and biopsy; three females selected for the spawning induction. **4**: Leuprorelin acetate injections (female dosage: 200 µg/kg body weight; male dosage: 10 µg/kg body weight). **5**: Spawning; each female produced a reproductive cycle (C1, C2, C3). **6**: Egg incubation at 6 densities in two hatching systems, determination of the hatching rate. C1 egg density: 200/L in system 1 and 150/L in system 2; C2 egg density: 400/L in system 1 and 300/L in system 2; C3 egg density: 1000/L in system 1 and 600/L in system 2. **7**: Seeding of the larvae in the larval rearing system at two densities (20/L in C1; 40/L in C2 and C3) according to two methods: immediately after hatching (C1 and 50% of C2) and at two days post hatching (50% of C2 and 100% of C3). **8**: Larval rearing (from day 0 to 45 days post hatching); growth rate and survival determination. **9**: Juvenile rearing (from 45 to 208 days post-hatching); individuals were reared at 3 densities: C1: 1 individual/L; C2: 7.5/L; C3 15/L; growth rate and survival determination, statistical comparison between C2 and C3 juveniles, condition index determination. At 208 days post-hatching C1 juveniles were kept indoors while C2 and C3 individuals were released together at Cabras lagoon into a pre-adaptation cage. Growth rate and condition index were determined.

2.1.1 Hatchery

The seawater used in the hatchery system was pumped from the Gulf of Oristano, 30 m from the beach of Torregrande(39°54'21" N; 8°29'47" E). Before being used in the hatchery, the seawater was micro filtered (0.5 µm, sand filter) and UV sterilized (80 W).

Broodstock were induced to spawn in an indoor recirculating aquaculture system (RAS) consisting of two 2,500 L fiberglass tanks painted in black. The system was equipped with a mechanical filter (cartridge, 10 µm), UV lamp, protein skimmer, a 200 L SUMP filled with mature bio-balls as a biological filter and a water chiller (TECO, 91 KW).

As ripe fertile eggs tend to float, a pipe was cut in half and placed horizontally on the water surface in the tank, makeing the eggs converge into a 500-µm net-mesh egg collector (300 L) connected to the broodstock tank recirculation system and equipped with aeration (Figure 2).

Figure 2: Broodstock tanks and egg collector

Fertilized eggs were transferred to and incubated in two different systems. System 1 was an RAS made of 3 circular, truncated, cone-shaped tanks with a 250 L volume, supplied

with a 200 L SUMP/bio-filter equipped with active bio-balls, two mechanical filters (cartridge, 50 and 10 µm), a UV lamp (80 W), a protein skimmer (3 x 53 W) and a water chiller (TECO, 440 W).

System 2 was an RAS made of 3 circular, truncated, cone-shaped tanks with a 300 L volume, a 300 L SUMP/bio-filter equipped with active bio-balls, and a mechanical filter (cartridge, 10 µm). All tanks from the 2 systems were equipped with a 500 mesh-size banjo filter to avoid egg loss (Figure 3).

Figure 3: Incubation systems (system 1 on the left, system 2 on the right)

Larvae were reared in an indoor RAS (system 3) which consists of 4 circular fiberglass tanks with a 2000 L volume; the sides and bottom of the tanks were black and white, respectively. The system was equipped with a 10 µm cartridge filter, UV lamp, protein skimmer, a 200 L SUMP filled with mature bio-balls (bio-filter) and a water chiller (Figure 4).

Figure 4: Larval rearing system (system 3)

2.1.2 Live food

2.1.2.1 Phytoplankton

The microalgae species used were *Nannochloropsis oculata* and *Isochrysis galbana*. Both species were successfully used in several larvae-rearing trials to meet flathead grey mullet nutritional requirements (Eda et al., 1990; Gautier and Hussenot, 2005; Harel et al., 1998).

Phytoplankton cultures were permanently kept in Erlenmeyer flasks containing pre-filtered (1 µm filter paper) marine seawater at 30 ± 1 ppt enriched with Guillard F/2 medium (Guillard et al., 1962-1975). Cultures were maintained at 23 ± 1 °C and exposed to a 16 h L/8 h D photoperiod; pre-filtered (1 µm cartridge filter) air was gently supplied.

Massive phytoplankton production started from this culture as *inoculum*, that was previously cultivated to reach the desired concentration and then inoculated into two photo bioreactors (215 L volume) and into two phytoplankton bags (300 L volume).

Photo bioreactors worked continuously; a peristaltic pump furnished 30 L of seawater enriched with Guillard F/2 medium each 24 h. A pipe carried the phytoplankton from the photo bioreactor to the rotifer tanks.

Phytoplankton bags filled with seawater enriched with Guillard F/2 medium were inoculated and kept for 6 days with constant illumination and aeration at 30 ± 1 ppt and 23 ± 1 °C before being used in rotifer cultures or in larval rearing tanks.

2.1.2.2 Rotifers

The rotifer *Brachionus plicatilis* (S-type, 110 to 230 µm lorica length) was cultured in seawater with 30 ± 1 ppt salinity and a temperature of 23 ± 1 °C, with constant light and aeration. Starting with a density of 60 ± 10 individuals/ml, rotifers were grown in two tanks (450 L volume) filled on day 1 with 200 L of *N. oculata* and *I. galbana* 2:1 (minimum density of 10 x 10^6 cells/ml). Each day, up until day 4, 50 L of phytoplankton were added to the rotifer culture. Approximately 60% of the phytoplankton used in the rotifer production was provided by the FBR peristaltic pumps.

On day 4, when the rotifer density was about 300/ml, the culture was filtered with a 50 µm mesh-size filter, an aliquot was administered to the larvae and the culture regrown to harvest density. No formulated feed or rotifer enrichers were supplied to rotifer cultures (Figure 5).

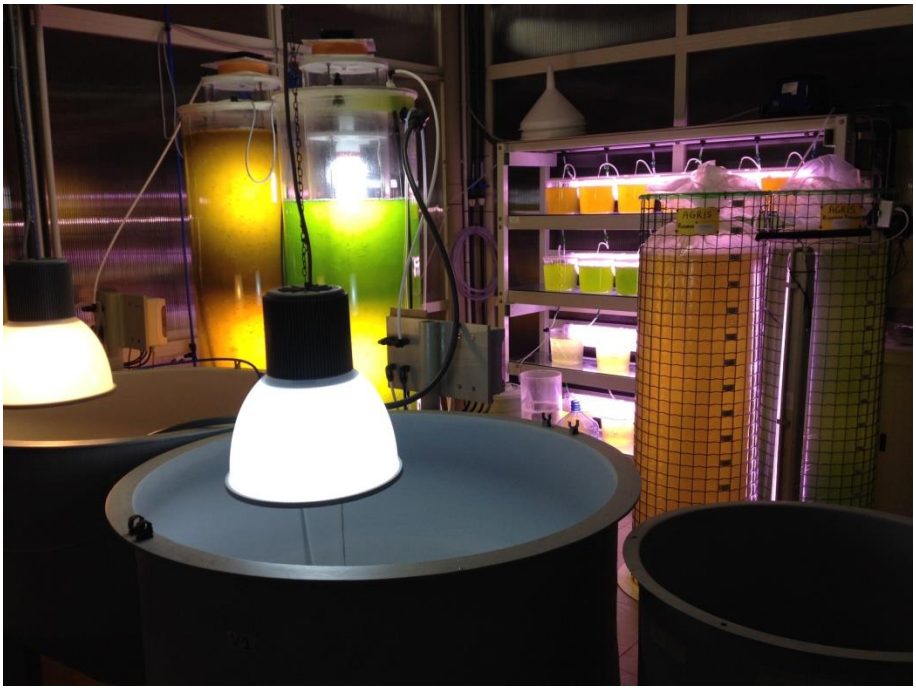


Figure 5: Live feed production room. Photobioreactors, phytoplankton bags and rotifer tanks.

2.1.2.3 *Artemia* nauplii

Artemia cysts were hatched daily into 150 L truncated cone tanks at 25 ppt salinity and 23 °C. *Artemia* nauplii were obtained by supplying the cysts with light and strong aeration.

2.1.3 Reproduction and hatching

Fish were captured by hand, trapped against the gates of the lavorieri and gently blocked for a first selection. Females (2.8±1.4 kg BW) with a bulging abdomen and red papilla and fluent ripe males (1.2±0.4 kg BW) were preferred (Figure 6).

Selected adults were transferred in fish transport bags (120 x 50 cm) filled 1/3 with seawater and 2/3 with oxygen. Bags were inserted into 45 L containers which were thermally insulated and refrigerated. These were kept in the dark, and ice was added when the temperature in the bags exceeded 24.5 °C. The fish were then transported to the IMC facilities for acclimation. Salinity, temperature and DO were recorded for each fish transport bag.

Figure 6: Broodstock capture at Cabras and Mistras lagoon.

The fish were transferred into fish acclimation tanks (45 L) filled with the seawater contained in the transport bags and then kept in the dark. A flow rate of 1 L/minute of seawater was introduced from the broodstock system, while an equivalent water flow was siphoned out until the salinity and temperature of the acclimation tanks coincided with that of the broodstock system.

At the laboratory, each single broodstock was checked to see that fin margins were intact, no scales were lost, and that there were no pathologies or parasites.

Individuals were anesthetized with a solution of 0.08% clove oil and weighed.

The state of ovarian maturity for each female (F) was assessed prior to each spawning attempt with a polyethylene cannula (0.86 mm inner diameter, 1.52 mm outer diameter).

The 550 µm diameter of the oocyte was established as the minimum egg diameter for successful spawning induction, according to Nash & Shehadeh (1980). Males (M) were checked for the presence of milt by applying gentle abdominal pressure. Selected fish were then transferred to spawning tanks.

Hormonal spawning induction was promoted by a singler intramuscular injection practiced below the dorsal fin of the slow release luteinizing-releasing hormone (LHRH) agonist, Leuprorelin acetate (Enantone). Females received a 200 µg/kg body-weight (BW) dose, while males were treatened with 10 µg/kg BW of the same hormone (Figure 7).

Figure 7: Fish acclimation tanks (1). Ovarian biopsy (2). Cannula with oocytes (3). Hormone dosage calculation (4). Hormone injection (5).

Three reproduction cycles were carried out. In cycle 1, reproduction was obtained by dry-stripping the individuals, while in cycles 2 and 3 brooders reproduced naturally in the brooder tanks. The sex ratio adopted were 1F:3M in cycle 1 and 1F:4M in cycles 2 and 3.

Salinity and temperature for the spawning induction was 36.8±0.6 ppt and 22.5±1.2 °C respectively. Dissolved oxygen was kept at saturation (8.9±0.2 mg/L) by supplying

brooder tanks with strong aeration. Water flow was set at ~ 8000 L/h. Both tanks were supplied with a 500 µm mesh banjo filter to avoid egg loss. Natural photoperiod was used.

Fertilization rate was determined at 40 minutes from gametes emission by checking, the presence of the first cleavage under a stereoscope, (3 replicates of about 40 embryos were observed).

2.1.3.1 Dry stripping

After the administration of the hormone, if the female appeared extremely hydrated but did not spawn, egg release was obtained by applying very gentle pressure to the abdomen. Sex ratio was 1 female:3 males. Males were captured and stripped into the egg collectors. The total sperm volume was about 10 ml. Eggs were collected in 5 L sterilized beakers, counted volumetrically and checked for viability. A sterile feather was used for guiding the solution of eggs and sperm in order to promote fertilization. After 2-3 minutes, 2 L of water from the hatching system was added to the solution and stirred for 5 minutes. Gentle aeration was provided for the embryos.

About 40 minutes after fertilization, eggs were collected to determine fertilization rate by checking the presence of the first cleavage under a stereoscope.

2.1.3.2 Natural spawning

When females appeared hydrated after the hormonal treatment, males swam next to their cloaca. Spawning and fertilization occurred spontaneously. Immediately after emission eggs were checked under a stereoscope for viability.

RAS was turned off for 40 minutes after spawning, in order to promote fertilization. Strong aeration was supplied to the broodstock tank. Eggs were made to converge towards an egg collector, supplied with strong aeration in small vessels, counted volumetrically and checked for fertilization rate.

2.1.3.3 Egg incubation

Fertilized eggs were incubated at different densities in the 2 hatching systems.

In both systems, seawater recirculated with a flow rate of 3.5 L/min, while salinity and temperature were as close as possible to those employed in the broodstock tanks. DO was

kept over 7 mg/L by strong aeration produced by circular air stones placed on the bottom of the tanks.

A 20% water exchange was performed every 6 hours and sedimented eggs were removed from the bottom of the incubation tanks every 4 hours.

The physical and chemical parameters of the seawater were monitored.

Six *M. cephalus* egg incubation densities (three in system 1 and three in system 2) were evaluated to determine hatching rate during three reproductive cycles.

Table 1: Egg densities tested for hatching % during the three reproductive cycles

	Cycle 1	**Cycle 2**	**Cycle 3**
System 1	200/L	400/L	1000/L
System 2	150/L	300/L	600/L

Each density was determined volumetrically. At hatching (36-48 hours), the RAS (hatching systems 1 and 2) and their aeration systems were turned off and the water column was gently stirred.When larvae were randomly distributed, a 5 L volume sample was collected from each tank; the percentage of unhatched eggs was determined by counting them into 3 subsamples of 100 ml (3 replicates).

A stereoscope equipped with a digital image analyser was used for the documentation of embryo development.

2.1.4 Larval and juveniles rearing

Newly hatched larvae were stocked at two densities (20/L in cycle 1 and 40/L in cycles 2 and 3) in the larval rearing thanks. Survival rates were determined at 2, 20 and 45 days post-hatching (DPH).

Two methods of larval transfer were adopted in the 3 cycles:

- Seeding the larvae immediately after hatching:

This method was used for seeding Cycle 1 larvae (C1) and 50% of Cycle 2 larvae (C2-1).

Immediately after hatching, larvae were transferred in the larval rearing system and kept for 2 days in static conditions. Larvae were transferred manually by collecting 5 L volume aliquots from the hatching systems.

- Seeding the larvae at 2 DPH:

  This method was used for seeding both 50% of Cycle 2 larvae (C2-2) and Cycle 3 larvae (C3-1 and C3-2).

  Larvae were kept in the hatching system for 2 days, in darkness and static conditions; 20% water was exchanged daily and dead larvae were removed. Larvae were transferred into 5 L beckers in the larval rearing tanks.

Larvae were reared in the larval rearing system. Seawater was 36 ± 1 ppt salinity with a temperature of 23 ± 2 °C; a natural photoperiod (14/10 L/D) was adopted. Recirculation flow and banjo-filter mesh nets were changed during larval development and juvenile growth.

From 3 DPH until 16 DPH the RAS was turned on at night, producing a 2.5 L/min flow rate (100% exchange/day). From 16 until 50 DPH, a 5 L/min flow rate was supplied constantly except for 2 hours after feeding (300% water recirculation daily). From 50 DPH until individuals reached 2.5 cm TL, the flow rate was constantly increased to reach a maximum of 1,000L/h (when fish total length was >3 cm). A surface skimmer was used from 2 DPH until 45 DPH (except for the larvae of the first reproductive cycle which were reared with the use of the device from 12 DPH). No aeration was supplied to rearing tanks until individuals were younger than 45 DPH. Faeces and unconsumed feed were siphoned out daily from 50 DPH up until when *M. cephalus* juveniles reached 3 cm TL.

A 30% water exchange was applied every 10-14 days from 50 DPH (Table 2).

Larval rearing tanks were equipped with a 100 µm mesh-size banjo filter from 1 to 40 DPH, a 250 µm mesh-size banjo filter from 41 to 50 DPH, a 500 µm mesh-size banjo filter from 51 to 120DPH and a 1 mm mesh-size banjo filter from 120 DPH (Table 3).

Table 2: Operation program and operation frequencies during the first 208 days post-hatching (DPH)

Operation type	DPH	frequency
Banjo filter cleaning	1-120	2 times/day
Siphoning off	50-365	daily
Water exchange	50-365	every 10-14 days
Surface skimmer cleaning	2-45	4-5 times day

Table 3: Banjo filters adopted during larval (0-45 DPH) and juvenile (> 45 DPH) rearing

Banjo filters	
Mesh	**Days**
100 µm	1-40
250 µm	40-50
500 µm	50-120
1 mm	120-365

M. cephalus larvae were reared by applying the green water technique which consists in rearing the larvae in a microalgae medium. The first feed the was L- type rotifer *Brachionus plycatilis,* followed by *Artemia salina* nauplii.

2.1.4.1 Feeding program

Since 2 DPH, phytoplankton (1:1 ratio of *N. oculata* and *I. galbana*) was given to the larvae at the density of 300,000 cell/ml; larvae were fed the rotifer *Brachionus plycatilis*; 4.5 rotifers/ml from 3 DPH to 35 DPH. *Artemia* nauplii at the density of 2/ml were given from 12 to 45 DPH.

A concentration of microalgae, rotifers and *Artemia* nauplii was restored daily in the larval tanks up to 35 DPH.

Larval samples were collected every 24 hours for the first 20 DPH and the larval development was documented with a stereoscope until *M. cephalus* juveniles reached 3.5 cm TL.

The formulated feed adopted in these trials was supplied from Skretting S.r.l: from 20 to 50 DPH, larvae were fed a 50 µm Gemma micro 50 in addition to *Artemia* nauplii. Juveniles were fed with 100-150 µm, Gemma Wean 0.1 in addition to *Artemia* until 55 DPH. From 50 DPH to 200 DPH, the feed employed was Perla Larva. Juveniles were fed from day 195 with the pellet Larva Plus (Table 4).

The formulated feed was administered three times per day and was composed of:

- Gemma Micro 50: 55% crude protein, 15% crude oils and fats, 13.5% crude hash, 5% crude fiber and 2% crude phosphorus
- Gemma Wean0.1: 60% crude protein, 15% crude oils and fats, 13.5% crude hash, 0.5% crude fiber and 1.5% crude phosphorus
- Perla Larva: 62% crude protein, 11% crude oils and fats, 10% crude hash, 0.8% crude fiber and 1.1% crude phosphorus
- Larva Plus: 62% crude protein, 11% crude oils and fats, 9% crude hash, 0.8% crude fiber and 1.2% crude phosphorus.

Table 4: Larval and juvenile feeding program

Feed	Density	Days
Phytoplancton *Isochrisys galbana* *Nannochloropsys oculata*	300, 000 cells/ml	2-35
Rotifers *B. plicatilis* L-type	4-5/ml	2-35
Artemia *Artemia* nauplii	3-4/ml	12-45
Gemma micro 50	*Ad libitum*	20-30
GEMMA Wean 0.1	*Ad libitum*	28-55
Perla Larva	*Ad libitum*	50-200
Larva plus	*Ad libitum*	195-365

2.1.4.2 Survival rates and densities

As most mortality occurs during the fist 30 days, larval survival was determined at 2, 20 and 45 DPH (at the end of live food administration).

At 2 and 20 DPH, larvae were counted volumetrically (3 replicates of 5 LT randomly sampled from each larval rearing tank).

At 45 DPH, image analysis was applied to high resolution pictures of the larval rearing tanks. Individuals were counted with the software "Image J". Three pictures for each tank were taken at 1 minute intervals and the maximum number of individuals detected were reported as the more accurate count. At the end of the indoor rearing phase (~200 DPH), the same technique was employed for cycles 2 and 3 for determining the number of fish, while cycle 1 individuals were counted by capturing and transferring them into 1 tank of the broodstock system (2.5 m^3). The initial rearing density of juveniles was determined at 45 DPH (Anderson, 1958).

Survival results always refer to the initial number of larvae introduced into the larval system.

2.1.4.3 Growth

Larvae and juveniles were monitored for somatic growth, total length (TL) and body weight (BW). Larvae were measured at hatching and during the first 45 DPH witha binocular equipped with a Leica digital camera and image analysis software.

In order to avoid mortality due to manipulation, individuals were sampled for measurement at hatching, at 45 DPH, 140 DPH and 200 DPH.

From 45 DPH to 200 DPH individuals were considered as juveniles (Anderson, 1958).

A total of 291 individuals were measured and weighed (e.g. ~ 100 individuals for each reproductive cycle).

Individuals were anesthetized with 0.08% clove oil and measured with an electronic calliper. An electronic scale was used for determining the BW of the individuals.

Biometrical data, TL (mm) and BW (g), were used to determine the growth rate (GR) (Hopkings, 1992) and the specific growth rate (SGR%) (Ricker, 1979):

1. $GR = (BW_t - BW_i) / t$

where BWt is the body weight at time t and BWi is the initial body weight; t is the time (days) between BWt and BWi.

2. $GR = (LT_t - LT_i) / t$

where LT_t is the total length at time t and LT_i is the initial total length; t is the time (days) between LT_t and LT_i.

1. $SGR\% = [Log(LT_t) - Log(LT_i)] / t \times 100$
2. $SGR\% = [Log(BW_t) - Log(BW_i)] / t \times 100$

Data on length and weight were also used also to determine the condition index, following the equation described by Bagenal (1978):

$$BW = a\ TL^b$$

where **a** and **b** are the parameters of the allometric relation. a and b were estimated by the linear regression of length (TL) and weight (BW) data logarithmically transformed.

The relation obtained is described by the equation: $Log_{10}\ BW = a' + b\ Log_{10}\ TL$

where **a'** is the log_{10} of the coefficient **a**, which represents the intercept of the straight line with the ordinate axis and b is the slope of the regression line.

Regression coefficient **b** was used as a condition index (Safran, 1992). When growth is isometric, i.e., when length and growth increase in the same proportion, the value of the slope is equal to 3. A condition index < 3 indicates that individuals are slim and length increases more than weight. When b is > 3, individuals in the sample are robust. Condition index b and the coefficient a were determined at the end of the indoor rearing phase for each cycle (Crosetti & Blaber, 2016).

2.1.4.4 Juveniles growth at 3 densities

Three *M. cephalus* juvenile rearing densities were evaluated for growth rate and condition index.

Juvenile rearing densities were: 1 individual/L (LOW), 7.5/L (MEDIUM) and 15/L (HIGH). Each density was tested in 1 rearing tank.

The total length (TL) and the body weight (BW) were measured at 2/3 of the rearing period (127-147 DPH) and at the end (188-208 DPH) of the rearing period. The reason was to check for any significant difference in growth performance in the last two months before the scheduled release in the wild.

Individuals were reared simultaneously in the larval rearing system, fed *ad libitum* with the same formulated feed, therefore density was the only factor which changed among the different rearing cycles.

2.1.5 Juveniles release

On April 6, 2015 ~13,000 individuals from cycle 2 and 3 (201 and 188 DPH respectively) were transferred into fish transport tanks equipped with oxygen to the lagoons of Cabras and Mistras. "C2" and "C3" juveniles, were grouped as "LJ" (lagoon juveniles) and released in two lagoons.

C1 individuals (208 DPH) were kept indoor and transferred to the broodstock rearing system at the density of 140/m^3. We will refer to C1 individuals as "IJ" (indoor juveniles).

At Mistras lagoon, part of the LJ, a total of ~8,000 fish were acclimated and released at the lagoon mouth. Acclimation was performed by introducing 1 L/minute of seawater from the site of release, while an equivalent water volume was siphoned out from the transport tank until water salinity and temperature coincided with that of the release site.

At Cabras lagoon, 5,074 juveniles (LJ) were released after acclimation into a concrete rectangular facility placed inside the laggon a few meters from the lagoon coastline. The cage was supplied with 6 couples of gates equipped with 3 mm mesh size nets (pre-adaptation cage). During the rearing period, the gates were frequently washed from fouling to guarantee an adeguate water flow through the cage; the presence of double gates allowed operators to clean the nets without opening the cage. The cage had a surface of 54 m^2 and a medium depth of 1 m (Figure 8).

Individuals were reared for 7 months into the lagoon cage, sampled for measurements at 154 (T1) and 216 days (T2).

Physical water parameters (temperature, salinity and dissolved oxygen) were monitored every 10/14 days. Individuals from cycle 1 (IJ) were kept at the IMC laboratories, reared in controlled conditions and measured at the same time intervals of LJ. The feeding of IJ was the same as in the previous period whilst LJ individuals were fed twice a day at about 1% of initial body weight.

Condition index was calculated for LJ and IJ at the end of the rearing period.

Figure 8: Cabras lagoon facility

2.2 *Paracentrotus lividus*

Reproduction and rearing of the sea urchin *Paracentrotus lividus* were carried on in indoor at the International Marine Centre - IMC laboratories (Oristano, Sardinia, Italy).

The sea urchin hatchery was made of a larval rearing system and a juvenile rearing system. The larval system consisted of 6 circular, truncated cone shaped 300 LT tanks, equipped with 100 µm mesh-size banjo-filter to avoid larval loss, a 300 LT SUMP/bio-filter equipped with active bio-balls and a mechanical filter (cartridge, 1 and 10 µm) (Figure 9).

Figure 9: Larval rearing system of *Paracentrotus lividus*

The juvenile rearing system consisted of 2 rectangular tanks with 600 L volume (length: 385; width: 18; height: 85 cm), supplied with a recirculating system (flow rate of 5 L/min) and equipped with a biological and a mechanical filter (cartridge, 10 µm) (Figure 10).

Figure 10: Juvenile rearing system of *Paracentrotus lividus*

2.2.1 Reproduction and embryos

Adults *Paracentrotus lividus* (test diameter > 40 mm) were collected during their natural spawning season; 5 males and 5 females were induced to spawn (Evans & Marshall, 2005).

Gametes were obtained after animal dissection; male gonads were collected and kept dry at 4°C, while female gonads were kept in a beaker containing filtered (0.47 µm) NSW. Four drops of diluted sperm (5:100) were added to the eggs and the solution was gently stirred in order to facilitate fertilization. The number of fertilized eggs was determined as a result of 5 replicates, using a tubular plankton chamber and a Leica MZ8 Stereo Microscope. The fertilization rate was verified by the presence of the fertilization membrane.

Embryos were kept in 5 L white, cylindrical, plastic tanks under static conditions, in filtered (0.47 µm) NSW at 36.5 ± 0.2 ppt salinity, continuous light and a temperature of 19.0 ± 2.0 °C. Density was at 20 larvae/ml until they reached the echinopluteus stage, about 40 h after fertilization.

2.2.2 Larval rearing

Echinoplutei were transferred into the larval rearing tanks, containing filtered (1 µm) NSW at 36.5 ± 0.2 ppt salinity, with aeration and in continuous light (OSRAM Natura type lamps). The temperature was maintained at 19.0 ± 2.0 °C.

Larvae were reared in static conditions and according to the variable method previously tested (Brundu et al., 2016a). Larvae were fed a mixture of *Dunaliella tertiolecta* and *Chaetoceros gracilis* in equal quantities (50:50 ratio). The first feed supply (2 DPF) was a fixed quantity of 8,000 cells/mL (4,000 cells/mL of each species), while the subsequent administrations of feed were established after measuring the residual phytoplankton concentration in the larval tanks.

The amount of feed and seawater to exchange were established every 3 days and adjusted according to larval consumption. Feed was not given when cells were abundant in the rearing tanks (more than 8,000 and 16,000 before and after 10 DPF, respectively). For details see Brundu et al. (2016a).

Cultures of *D. tertiolecta* and *C. gracilis* were maintained in batch lines at 25 °C, exposed to a 16 h L/8 h D photoperiod and supplied with gentle aeration. The 30 ppt salinity

seawater was pre-filtered (1 µm filter paper), enriched with modified Guillard f/2 and autoclaved at 121 °C for 30 min. The phytoplankton was supplied to the larvae during the exponential growth phase.

2.2.3 Experimental design

The effects of two stocking densities, Low (1.5 larvae/mL) and High (4 larvae/mL), on the development and survival of larvae.

Three replicates were carried out for each density treatment.

Larval development was evaluated by observating larval structures (number of arms, presence and size of the rudiment) under the microscope, and development stages were defined according to previous studies (Brundu et al., 2006a; Brundu et al., 2006b). A minimum of 10 randomly sampled larvae were observed every 3 days post-fertilization (DPF). A development stage was considered achieved when at least 75% of the sampled larvae were determined to be at that stage. Competence (Cp) for settlement was considered achieved when the rudiment was equal to or larger than the stomach.

Larval survival was assessed volumetrically and the mean value of each measurement was then used to calculate the number of larvae in the tanks. Survival at each measurement was finally expressed as a percentage of the initial number of larvae stocked.

2.2.4 Metamorphosis and juvenile rearing

Metamorphosis tests were carried out when larvae reached competence stage. A stock of 50 larvae was transferred into a 50 mL volume beaker containing filtered NSW and a 50x50 mm layer colonized by the macroalgae *Ulvella lens* as a metamorphosis-inducing factor (Brundu et al., 2016b). The number of metamorphosed individuals was counted at 24, 48 and 72 h post-exposure to the settlement cue. Metamorphosis (Mt) was considered achieved when at least 75% of the larvae had metamorphosed.

At this stage,the larval culture was considered ready to settle and was transferred into the juvenile rearing system containing NSW and layers colonized by *U. lens*. The animals were kept in a static system for a month at 36.5 ± 1.0 ppt salinity without aeration, and a 50% seawater exchange was performed twice a week. The temperature was maintained

at 19.0 ± 2.0 °C by air-conditioning during the trial period and a 14 h light photoperiod at 22 µmol photons/m2/s was applied using fluorescent lamps. After one month, the tanks were connected to the recirculating system, and provided with biological and mechanical filters.

Sea urchins were fed *ad libitum* every two days with fresh fronds of *Ulva* spp. Fronds were harvested in the wild, carefully washed with fresh water to remove epiphytes and frozen at -20 °C. Prior to administering, the *Ulva* spp. was thawed.

The number of juveniles was recorded after 18 months post-settlement and the survival rate was calculated as a percentage of the initially stocked competent larvae. A sample of 100 juveniles was measured for test diameter and weighed. To simplify these operations, a solution of KCl 1% was used to induce paralysis and detachment of juveniles from the tanks, as tested by Hagen (2003) for *S. droebachiensis*.

2.2.5 Laboratory – Pilot scale comparison

Results recorded at pilot scale were compared to those obtained at laboratory scale in terms of:

- Total seawater exchange (% of the initial rearing volume)
- Phytoplankton consumption (thousand cells/mL)
- Larval development at P6
- Larval development at Cp
- Larval survival at Cp and Mt
- Metamorphosis rate

2.3 Statistical analysis

Grey mullet

Juvenile growth was statistically compared in function of the factor “density”. Statistical comparisons were performed to verify significant differences between MD and HD. The Shapiro test was used to verify wether TL and BW data were normally distributed. The

Barlet test was applied to verify if data variance was homogeneus. The Welch Two Sample t-test was applied to underline significant differences in normally distributed, non-homogeneus data. The Wilcoxon test was used to verify significant differences in data which was not normally distributed. Biometric data were analyzed by R 3.3.0 (2016).

Sea urchins

The effects of stocking density on larval development and survival were assessed by using one-way analysis of variance (ANOVA). Shapiro Wilk's W test was used to verify the normality of the data distribution and Levene's test was used to verify the homogeneity of variances. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$). Data were analyzed by Statistica 6.1 StatSoft, Inc. (2004).

3 RESULTS

3.1 *Mugil cephalus*

3.1.1 Reproduction

23 adults were captured from 30 August to 6 October 2015. 13 of these were successfully used in three reproductive cycles (Table 6).

In Cycle 1 (C1), one *M. cephalus* female (4.4 kg BW) and three males (0.8, 1.4, 2.1 kg BW) were captured in September at the Cabras *lavorieri*. The water salinity was 25 ppt and the temperature was 22 °C. The oocyte diameter determined by ovarian biopsy was 562 ± 6 µm. The female was treated with LHRH and dry stripped after 36 hours it. The female spawned a total of 3.1 ± 0.4 million eggs. Ripe unfertilized eggs were spherical and transparent, had a diameter of 769 ± 18 µm and contained a single oil drop (diameter 307 ± 6 µm) at the animal pole. Males were captured and stripped into the egg collector. The total volume of sperm was estimated at about 10 ml.The fertilization rate, determined at 40 minutes from emission, was ~ 84%.

In Cycle 2 (C2), one *M. cephalus* female (2.0 kg BW) and three males (0.9, 1.7 and 1.2 kg BW) were captured on 15 September at Cabras lagoon, with an oocyte diameter of 599 ± 5 µm. Water salinity was 25 ppt and the temperature 22 °C. Another male (1.7 kg BW) was captured at the Mistras lagoon at 23°C and 42 ppt salinity. The male was injected

with the hormone and added to the broodstock tank, resulting in a sex ratio of 4:1 male to female. 12 h after the hormone treatment, the female was hydrated and the males were swimming next to her cloaca. Spawning and fertilization occurred spontaneously 36 h after treatment. The female released 1.6 ± 0.3 million eggs. The egg diameter at spawning was 771 ± 20 µm, the oil drop had a diameter of 321 ± 14 µm. The fertilization rate was 88 %.

In Cycle 3 (C3), on 28 September, one 1.1 kg ripe male was collected at Cabras lagoon and transported to the laboratory. Water salinity was 34 ppt and the temperature 23 °C. A 2.2 kg BW female and 3 ripe males (0.9, 1.2 and 0.8 kg BW) were captured at Mistras lagoon. Water salinity was 43 ppt and the temperature 22 °C. A 4:1 male to female sex ratio was adopted in the broodstock system. The female oocyte diameter was 593 ± 6 µm. Spawning occurred naturally 39 h after treatment. Male emission occurred immediately after spawning and an elevated sperm concentration was observed in the spawning tank. The female spawned 1.8 ± 0.3 million eggs with a diameter of 774 ± 16 µm, the oil drop diameter was 309 ± 8 µm. The fertilization rate was 90%.

Figure 11: treated *Mugil cephalus* female with bulging abdomen

Table 5: Seawater parameters during induced spawning

Temperature	22.5±1.2°C
Salinity	36.8±0.6 ppt
Dissolved oxygen	8.9±0.2 mg/L
pH	8.4±0.1
Nitrite	0.15±0.16 mg/L
Nitrate	0.2±0.2 mg/L
Ammonia	0.04±0.04 mg/L

Table 6: Induced spawning trials

Cycle 1 (Cabras, 07/09/2015)					
Female					
BW kg	**Oocyte diameter**	**Injected**	**Spawn**	**Fertilization**	**Number of eggs**
4.4	562±6 µm	Yes	Dry stripping	yes	3.1 M
Males					
BW kg	**Ripe**	**Injected**	**Emission**	**Sperm volume**	
0.8	yes	no	Stripping	7.3 ml	
1.4				27 ml	
2.1				0.6 ml	
Cycle 2 (Cabras, 14/09/2015)					
Females					
BW kg	**Oocyte diameter**	**Injected**	**Spawn**	**Fertilization**	**Number of Egg**
1.0	599 ± 5 µm	yes	Yes	yes	1.6 M
Males					
BW kg	**Ripe**	**Injected**		**Emission**	
0.9	yes	No		Natural	
1.7	no	Yes			
1.2	no	Yes			
Cycle 3 (Cabras and Mistras) 28/09/2015					
Female					
Lagoon	**BW kg**	**Oocyte diameter**	**Injected**	**Spawn**	**Fertilization**	**Number of Eggs**
Mistras	2.2 kg	593 ± 6 µm	yes	natural emission	yes	1.8 M
Males					
Lagoon	**BW Kg**	**Ripe**	**Injected**	**Emission**	
Mistras	0.8	yes	yes	natural	
Mistras	1.0				
Mistras	1.2				
Cabras	0.9				

3.1.2 Embryos

In C1, a total of 315,000 eggs were incubated in the hatching systems with a density of 200 in system 1 (180,000 total eggs) and 150 eggs/L in system 2 (135,000 total eggs). Hatching started about 36 h after fertilization and lasted for about 12 h. The hatching rate was ~70%. Newly hatched larvae measured 2.2 ± 0.2 mm total length (TL).

In C2, eggs were transferred to the hatching systems at a density of 300 and 400 eggs/L in systems 1 and 2, respectively. A total of 630,000 eggs were incubated in the two hatching systems (270,000 in system 1 and 360,000 in system 2). Hatching started about 36 h after fertilization and lasted for 4 h, resulting in a hatching rate of ~ 80%. Larvae measured 2.2 ± 0.4 mm TL.

In C3, fertilized eggs were distributed in the two hatching systems at a density of 1,000/L in system 1 and 600/L in system 2. A total of 1.4 million eggs were incubated in the two incubation systems (900,000 in system 1 and 540,000 in system 2). Hatching rate was ~ 90% in both incubation conditions. Newly hatched larvae measured 2.1 ± 0.4 mm (Table 7 and Figure 12).

Table 7: Seawater parameters during egg incubation

Temperature	22.7±0.4°C
Salinity	36 ±0.5 ppt
Dissolved oxygen	8.8±0.4 mg/L
pH	8.5±0.1
Nitrite	0.06±0.1 mg/L
Nitrate	0.2±0.3 mg/L
Ammonia	0.06±0.04 mg/L

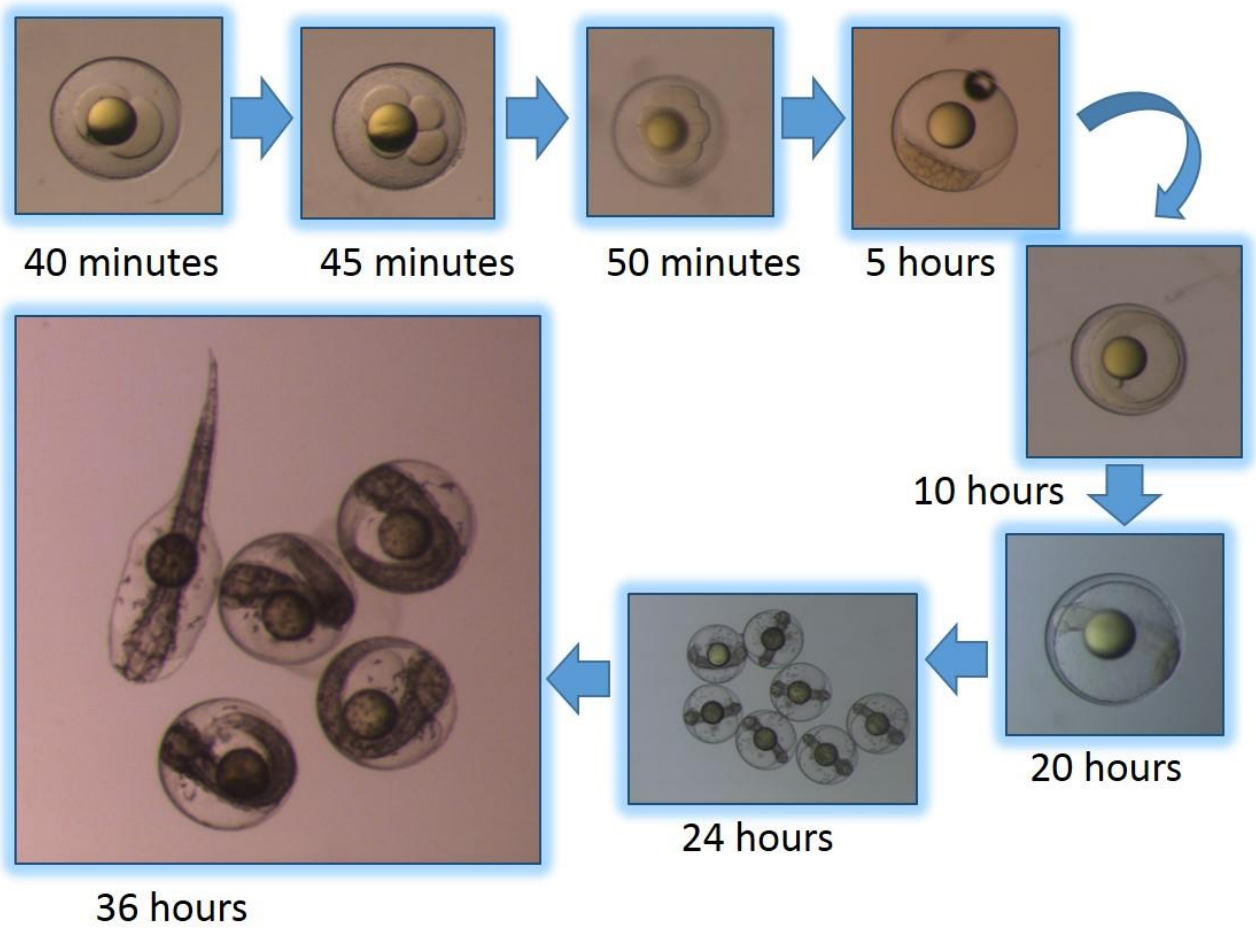


Figure 12: Embryo development from first cleavage (40 minutes) to hatching (36-48 hours) (22.7±0.4°C)

3.1.3 Larvae

In C1, 40,000 larvae were stocked int a larval rearing tank at the density of 20 larvae/L. Immediately after transportation to the larval system, a high mortality was observed. At 2 days post-hatching (DPH), survival was about 40% and about 20 % at day 20.

In C2, 160,000 larvae were stocked at a density of 40 larvae/L in two larval rearing tanks (80,000 larvae/2000 L each). At day 2, the survival rate of the larvae stocked in the rearing tank (C2-1) immediately after hatching was 0 %.In contrast, the survival rate of the larvae seeded at day 2, C2-2 larvae, was about 75 %. Larvae were transferred manually (5 L beaker) into the larval rearing system. Survival at day 20 was about 60%.

In C3, 160,000 individuals were stocked in 2 larval rearing tanks (80,000 each) at adensity of 40 larvae/L. Survival was about 80 % at 2 DPH and 70% at day 20.

C1 and C2-1 were transfered immediately after hatching. Survival was 40% for C1 and almost 0% for C2-1. Survival increased up to 75% for C2-2 and 80% for both C3-1 and C3-2 when larvae were transferred at 2 DPH.

From now, we will compare data from the three cycles, using one of the two C3 tanks and we will refer to it as C3 for the follow-up of the experimental trials (Figure 13).

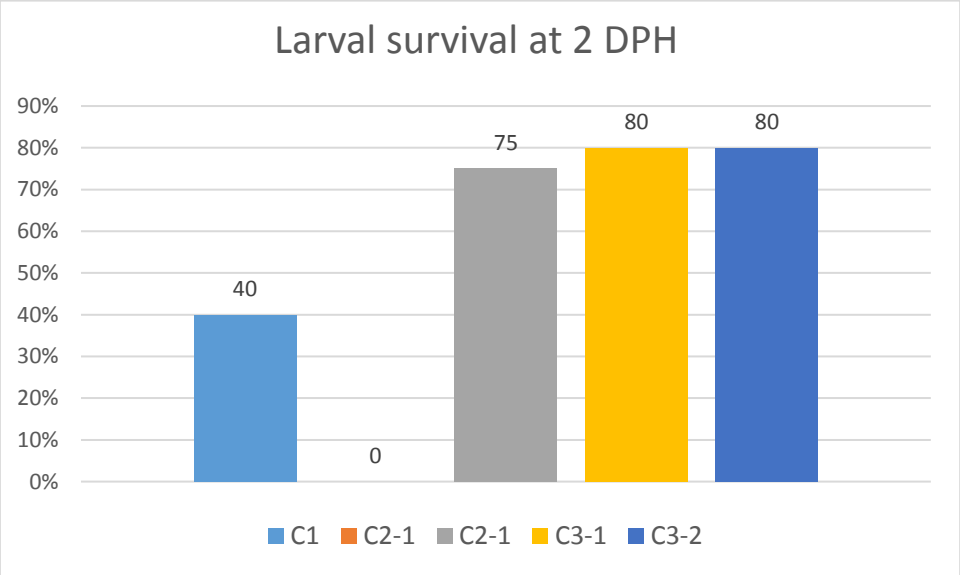


Figure 13: Larval survival (%) at two DPH

At hatching, the mouth of the larvae was not yet developed and the digestive tube was not completely developed. The mouth development started at the end of the 2nd day post-hatching.

Between days 3 and 4 the mouth opened, the upper and lower jaws were well developed and the larvae were feeding actively. The yolk diminished to ¼ of the original size and the oil globule was also reduced.

At 12 DPH, the mouth size increased and larve were being fed *Artemia* nauplii (Fugure 14).

Individuals were considered to have switched from larval to juvenile stage at 45 DPH.

The growth rate (GR) in terms of total length (TL) was determined when the three cycles reached ~ 45 days (from 39 to 46) from hatching. At 46 DPH, C1 larvae measured 29 ± 2 mm TL, on the same date (39 DPH) C2 larvae were 5 ± 1 mm TL and C3 (42 DPH) were 6 ±1 mm TL (Table 8).

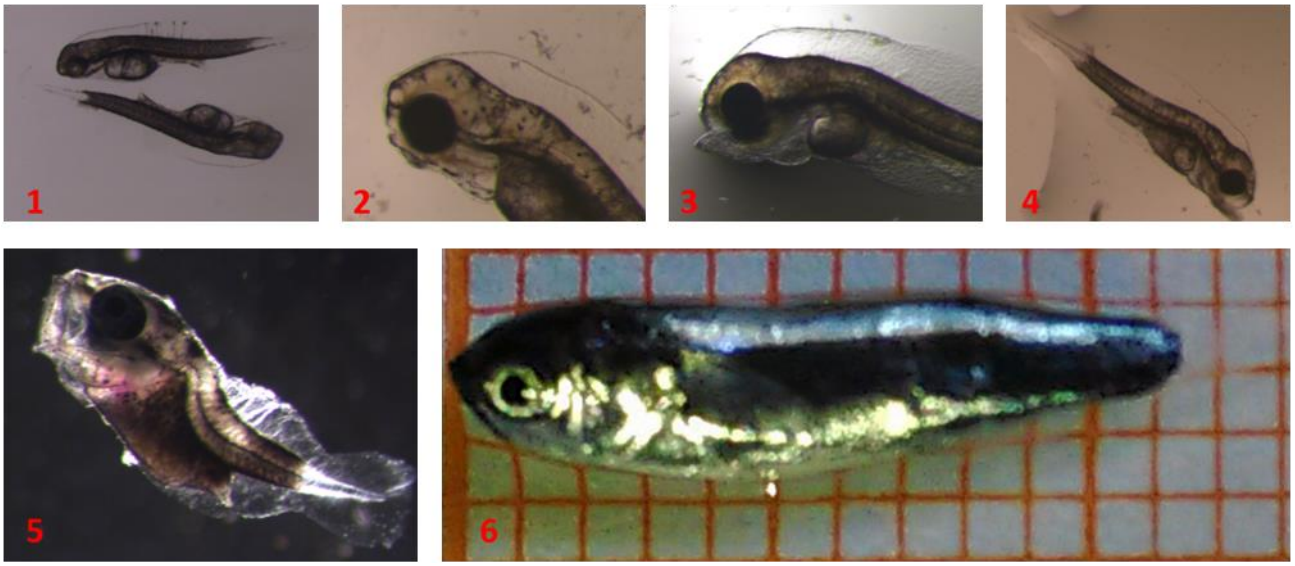


Figure 14: Larval development from hatching to ~ 40 DPH. 1= Newly hatched larvae; 2= larva at 2 DPH; 3= Mouth forming (3DPH); 4= The mouth is formed (4 DPH); 5= larvae are able to feed on Artemia nauplii (12 DPH); 6= Larva at 40 DPH.

Table 8: Total length (TL) at the end of the larval stage. DPH = days post-hatching

Cycle	TL (mm)	S.D. (mm)	DPH
C1	29	2	46
C2	5	1	39
C3	6	1	42

In C1, the growth rate of the larvae (from 0 to 46 DPH) was 0.6 ± 0.04, in C2 0.1 ± 0.01 and in C3 it was 0.1 ± 0.03 mm/day (Figure 15).

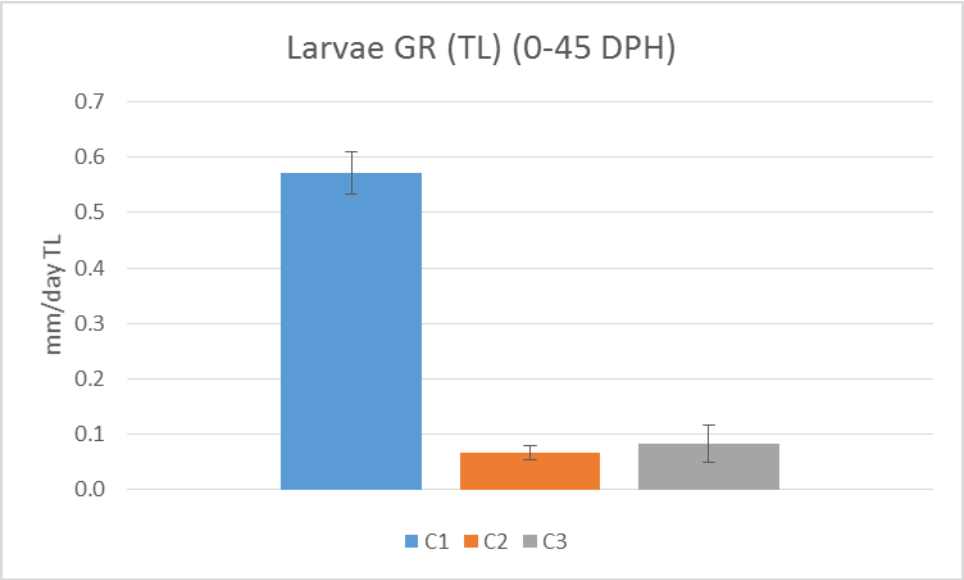


Figure 15: Larval growth rate (GR, mm/day) at the end of the larval rearing phase. TL = total length

The specific growth rate (SGR) was calculated referring to the total length at the end of the first rearing phase: it measured 2.4 %/day for C1, 0.9 %/day for C2 and 1.1 %/day for C3 (Figure 16).

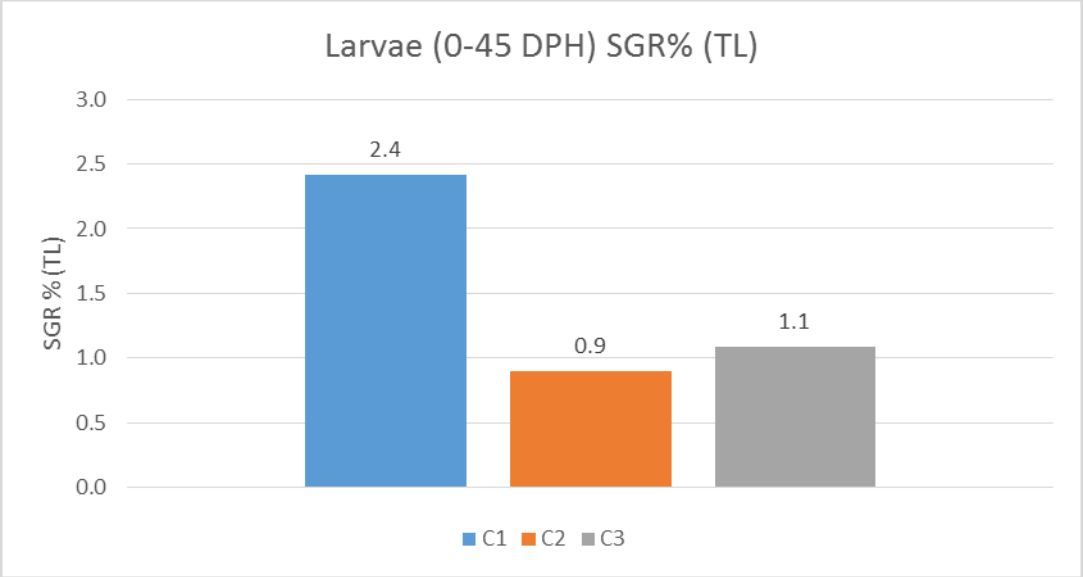


Figure 16: Specific larval growth rate (SGR, %) referred to total length (TL) at the end of the larval rearing phase

Larval survival at the end of the rearing phase was 36% in C3, 18% in C2 and 2% in C1, thus affecting the densities inside the 3 different tanks (Figure 17). The number of individuals that were used for the following phase of juvenile growth (at about 45 days)

were ~1,000 in C1, ~15,000 in C2 and ~30,000 in C3, producing densities of 0.5 ind./L for cycle 1 (LOW), 7.5 ind./L for cycle 2 (MEDIUM), 15 ind./L for cycle 3 (HIGH).

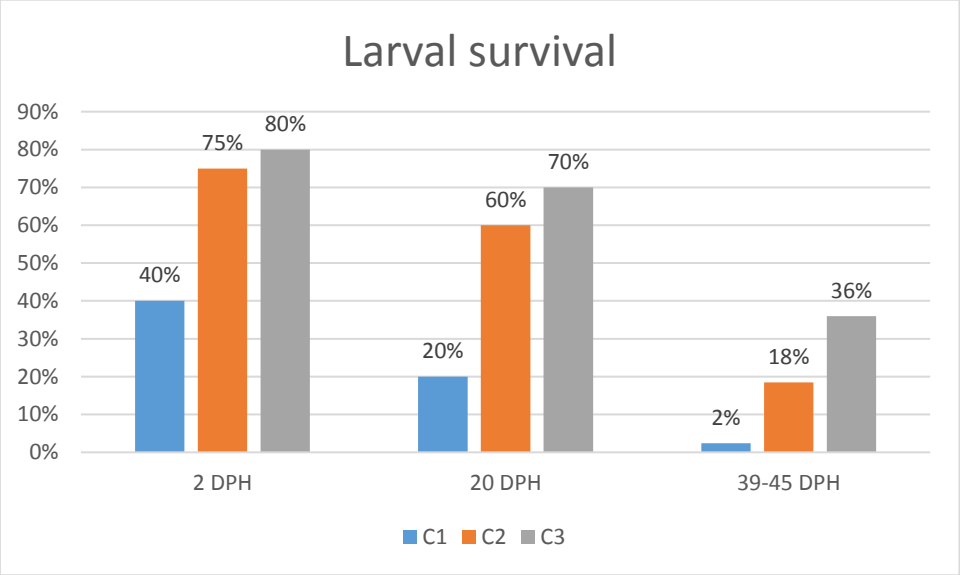


Figure 17: Larval survival (%) at 2, 20 and 39-45 days post-hatching (DPH).

In Table 9 are reported data of water parameters during the first 45 days.

Table 9: Parameters registered during the first 45 days of larval rearing

Temp (°C)	Sal (ppt)	DO (mg/L)	pH	Nitrite (mg/L)	Nitrate (mg/L)	Ammonia (mg/L)
22.2±0.5	35.8±0.9	8.8±0.2	8.4±0.4	0.04±0.1	6.7±1.4	0.001±0.003

3.1.4 Juveniles, growth and survival

Juvenile GR for the total rearing period of ~ 150 days (from ~ 45 to ~ 200 DPH) was 0.19 ± 0.03 mm/day in C1, 0.20 ± 0.03 in C2 and 0.18 ± 0.04 mm/day in C3 (Figure 18).

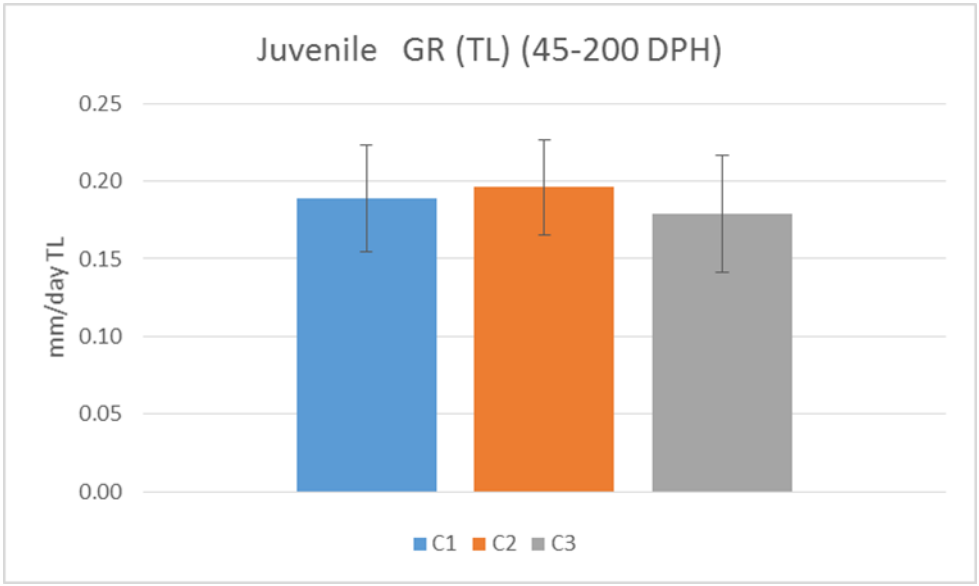


Figure 18: Growth rate (GR, mm/day) of the 3 cycles from 45 DPH to 200 DPH

At 127-147 DPH, TL ranged from 25±4 mm of C2 to 50±3 of C1. BW was 1.5±0.2 g in C1 and 0.2±0.1 g in C2 and C3.

At 188-208 DPH, TL ranged from 59 ± 6 mm of C1 to 31 ± 5 mm of C3, BW ranged from 2.4± 0.6 g of C1 to 0.4 ± 0.2 g of C3 (Table 10).

Table 10: Total length (TL) and body weight (BW) of the juveniles at ~ 140 DPH and at ~ 200DPH

TL	**(127-147 DPH)**	**(188-208 DPH)**
Cycle	**TL±D.S. (mm)**	**TL±D.S. (mm)**
C1	50±3	59±6
C2	25±4	36±5
C3	26±4	31±5
BW	**(140-201 DPH)**	**(188-208 DPH)**
Cycle	BW±D.S. (g)	BW±D.S. (g)
C1	1.5±0.2	2.4±0.6
C2	0.2±0.1	0.6±0.2
C3	0.2±0.1	0.4±0.2

From 45 to 140 DPH, GR was 0.2 ± 0.03 mm/day in C1 and C2, 0.2 ± 0.04 in C3 (Figure 19)

C1 and C2 juvenile GR in the last two months (140-200 DPH) was 0.2 ± 0.1 mm/day, whilst in C3 it was 0.1± 0.1 mm/day (Figure 20).

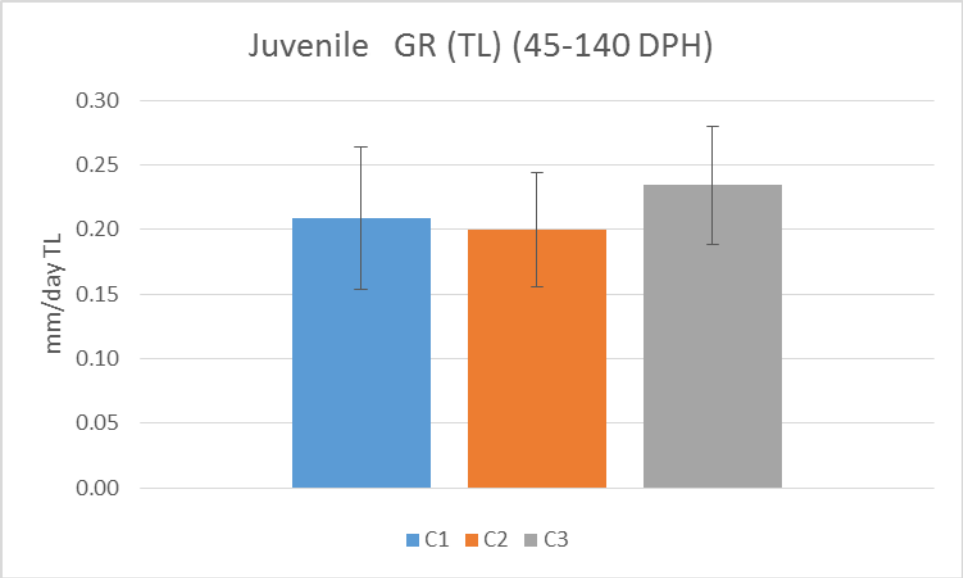


Figure 19: Juvenile growth rate (GR, mm/day) from ~45 to ~140 DPH

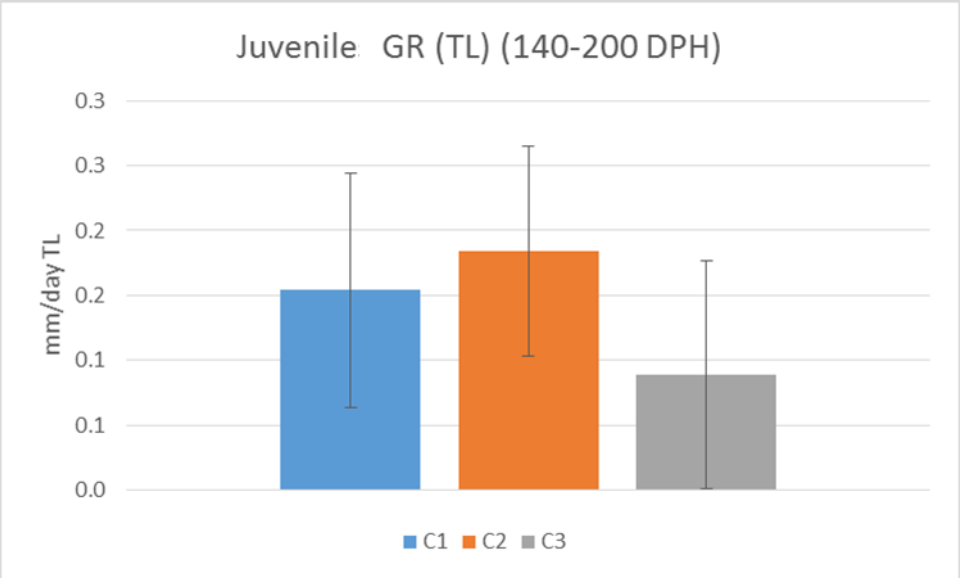


Figure 20: Juvenile growth rate (GR, mm/day) in the last two months (140-200 DPH)

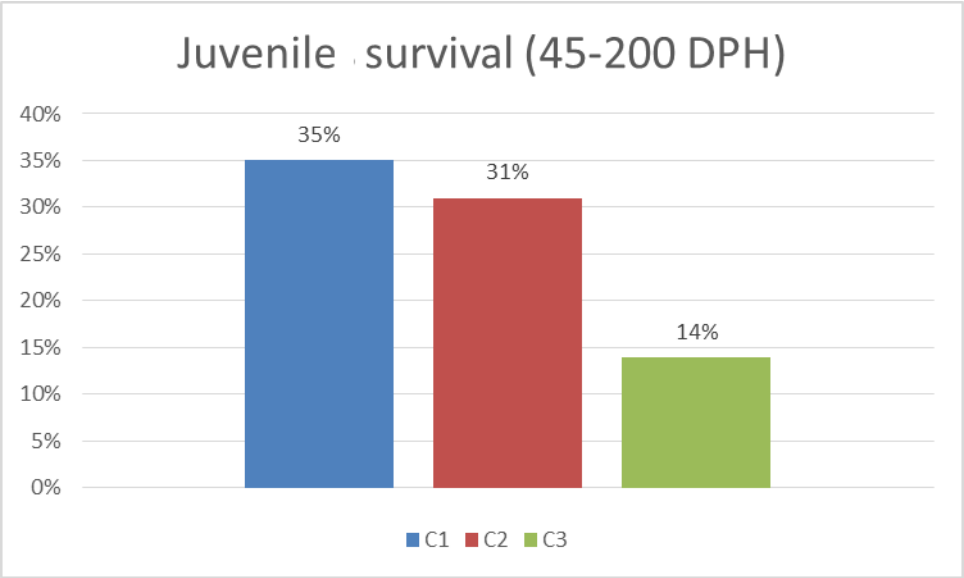


Figure 21: Juvenile survival (%) at the end of the indoor rearing phase (45-200 DPH)

The condition index (**b**) calculated for each cycle at the end of the indoor rearing phase ranged from 2.9 of C1 to 3.3 of C3 (Figures 22, 23 and 24).

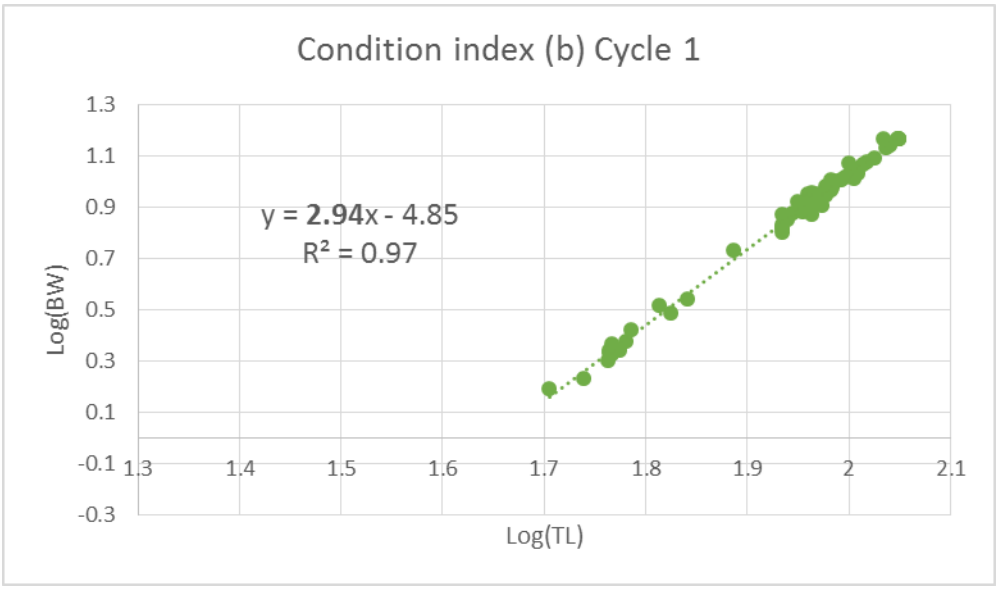


Figure 22: Condition index (b) of C1 juveniles

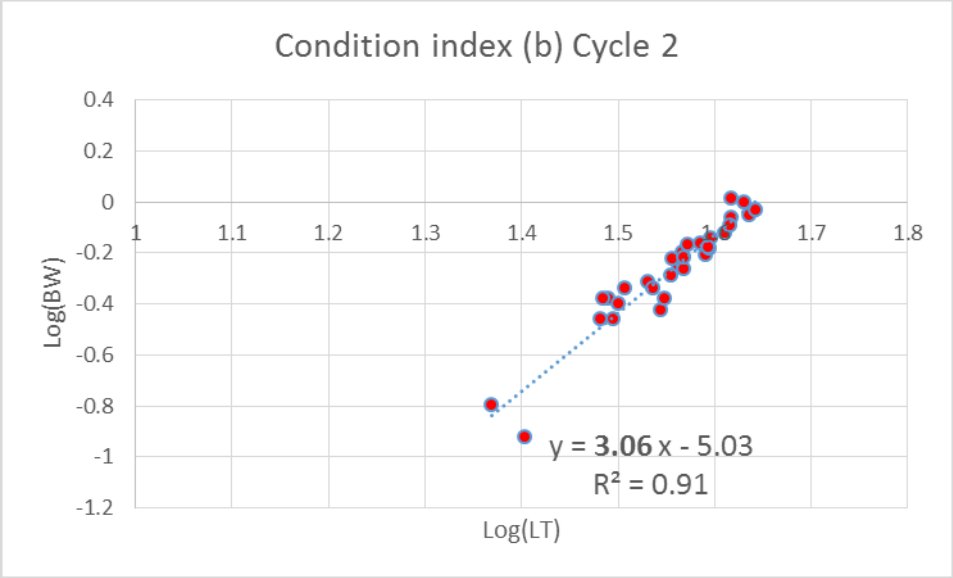


Figure 23: Condition index (b) of C2 juveniles

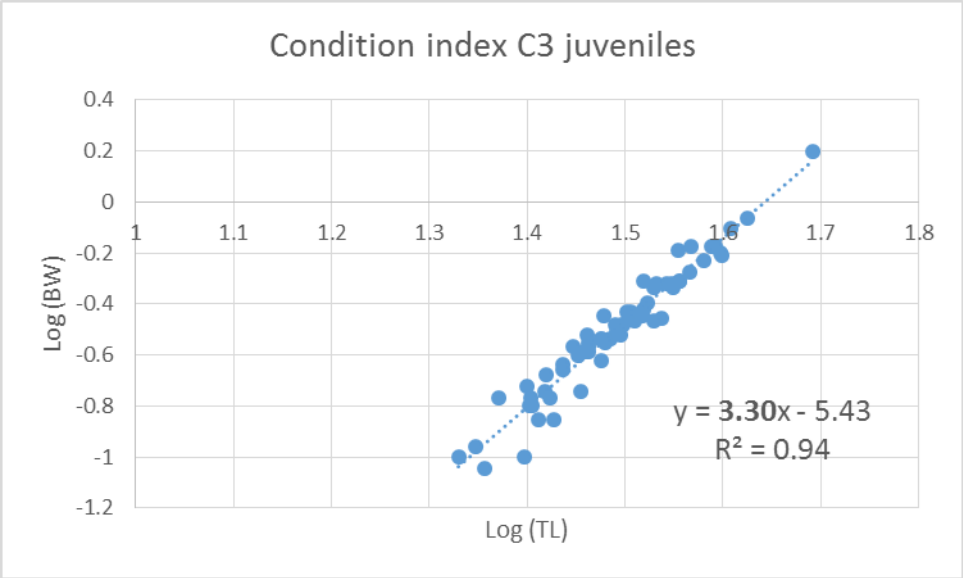


Figure 24: Condition index (b) of C3 juveniles

TL data resulted normally distributed (*p*-value = 0.50) and their variance was not homogeneus (p-value = 1.5e-05). Welch's Two Sample t-test did not show significant differences between MD and HD (p-value = 0.4471).

BW data were not normally distributed (p-value = 6e-08) and the Wilcoxon test showed significant differences between the juveniles reared at MD and HD densities (p-value = 0.019) (Figure 25).

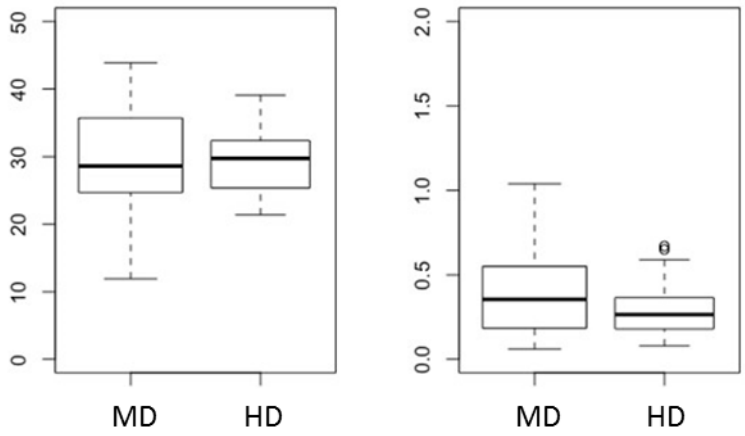


Figure 25: Box plot results of the t-test applied to the total length of juveniles at the end of the indoor rearing phase (left). Box plot results of the Wilcoxon test applied to the body weight of juveniles at the end of the indoor rearing phase (right). MD=medium density; HD=high density

3.1.5 Indoor rearing, survival and growth

For the entire period of the indoor rearing (200 DPH), larval + juvenile survival was 1% for C1, 6% for C2 and 5% for C3. In the period of 45-200 DPH, survival was 35%, 31% and 14% for C1, C2 and C3 respectively (Figure 26, Table 11).

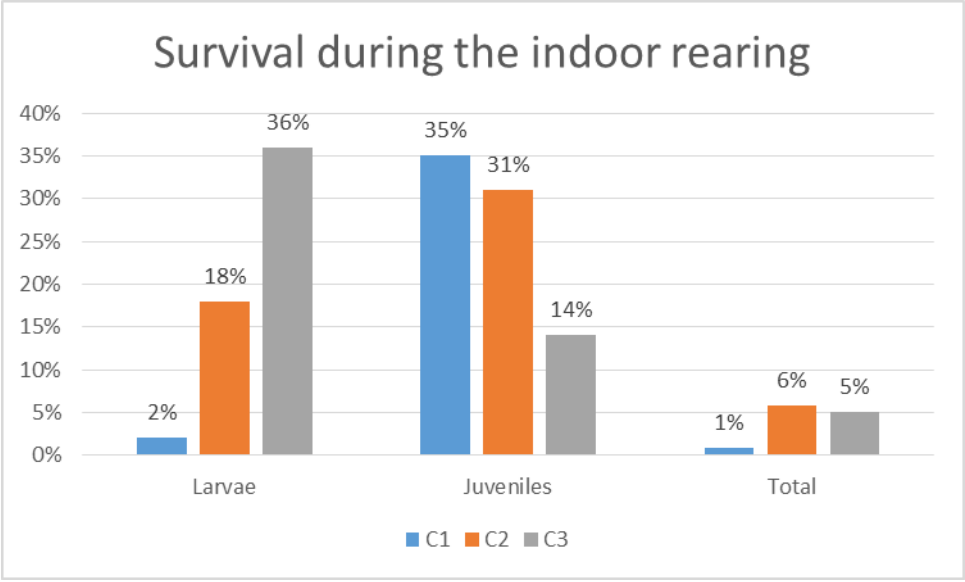


Figure 26: Larval (0-45 DPH), juvenile (45-200 DPH) and average final survival rates (200 DPH) of the indoor rearing phase (%)

Table 111: Number of individuals and survival rates obtained in the three cycles during the different stages of the indoor study

	Initial density	N° of larvae seeded (0-2 DPH)	Larval survival 45 DPH	N° of individuals 45 DPH	Juvenile survival 45-200 DPH	N° of individuals 200 DPH	Final survival 200 DPH
C1	20 /L	40,000	2%	1,000	35%	350	1%
C2	40/L	80,000	18%	15,000	31%	4,700	6%
C3	40/L	80,000	36%	30,000	14%	4,100	5%

Considering the mean growth rate (±SD) at about 200 DPH, the C1 growth rate (208 DPH) was the highest (0.3 ± 0.03 mm/day), whilse C2 and C3 showed the same value of 0.2 ± 0.02 mm/day (Figure 27).

The mean growth rate (±SD) in terms of BW at about 200 DPH was 0.011 ± 0.003 g/day in C1, 0.003 ± 0.001 g/day in C2, and 0.002 ± 0.001 g/day in C3 (Figure 28).

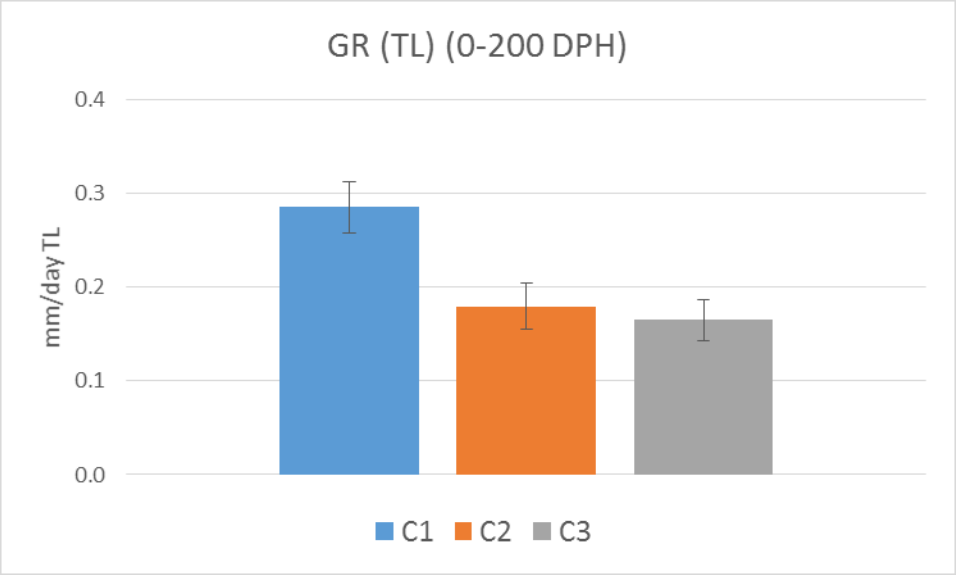


Figure 27: Total length growth rate (GR TL, mm/day) calculated for the whole period from hatching to 200 DPH

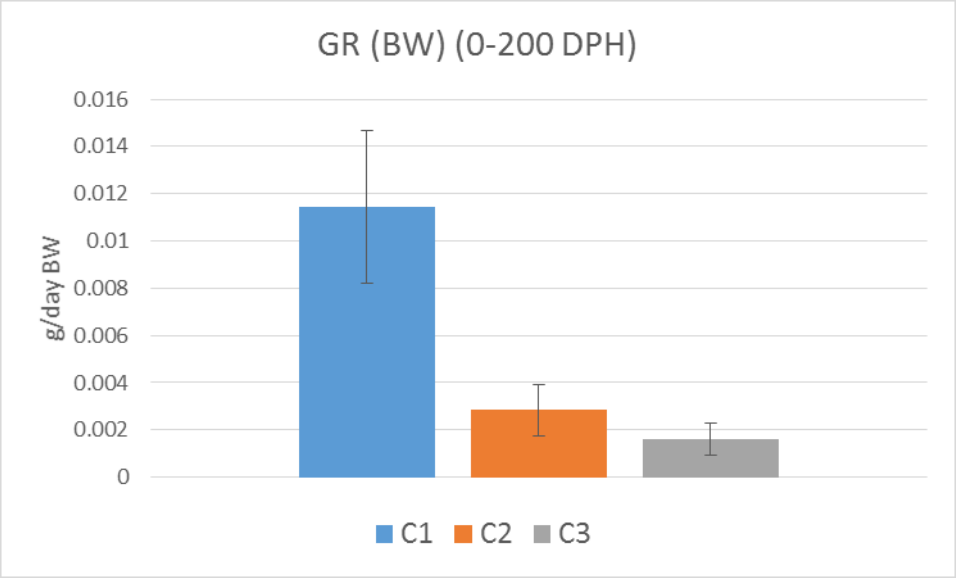
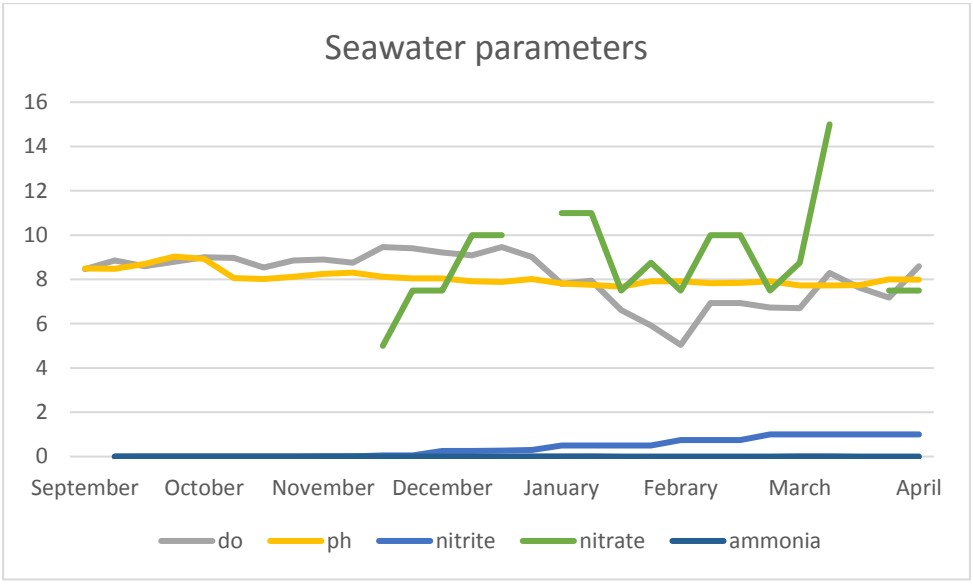


Figure 28: Body weight growth rate (GR BW, g/day) calculated for the whole period from hatching to 200 DPH

The average water parameters registered during the entire indoor rearing phase are reported in Table 12. The monthly variations of parameters from September to April are reported in Figure 29.

Table 122: Seawater parameters registered during the complete indoor rearing phase

Temp	sal	DO	pH	nitrite	nitrate	ammonia
20.3± 1.9°C	36.6±1.0 ppt	8.1±1.2mg/L	8.1±0.3	0.4±0.4 mg/L	8.9±2.2 mg/L	0.00-0.01 mg/L



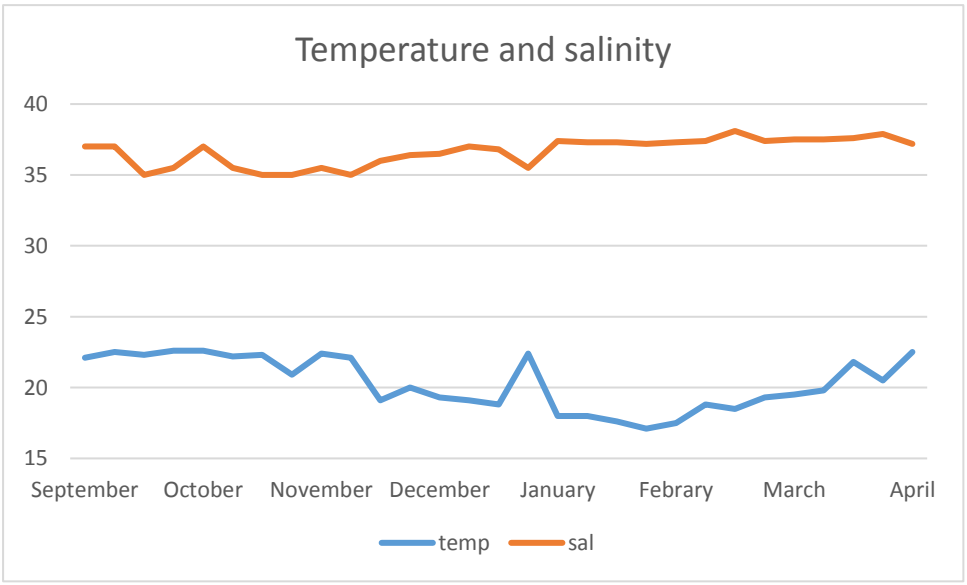


Figure 29: Water parameters recorded during the indoor rearing phase

3.1.6 Juvenile growth: indoor vs. released individuals

At the end of the rearing phase, the growth performance was evaluated of *M. cephalus* juveniles reared indoors (IJ) and released into pre-adaptation cages in the lagoon (LJ).

Growth rates (TL) at 154 days (T1) were 0.2 ± 0.04 mm/day in IJ and 0.3 ± 0.11 mm/day in LJ (Figure 32); whereas at 216 days (T2) they were 0.14 ±0.12 mm/day in IJ and 0.08 ± 0.23 mm/day in LJ (Figure 30).

Considering the whole period (Total), GRs were 0.19 ± 0.04 mm/day and 0.23 ± 0.07 mm/day for IJ and LJ respectively (Figure 34).

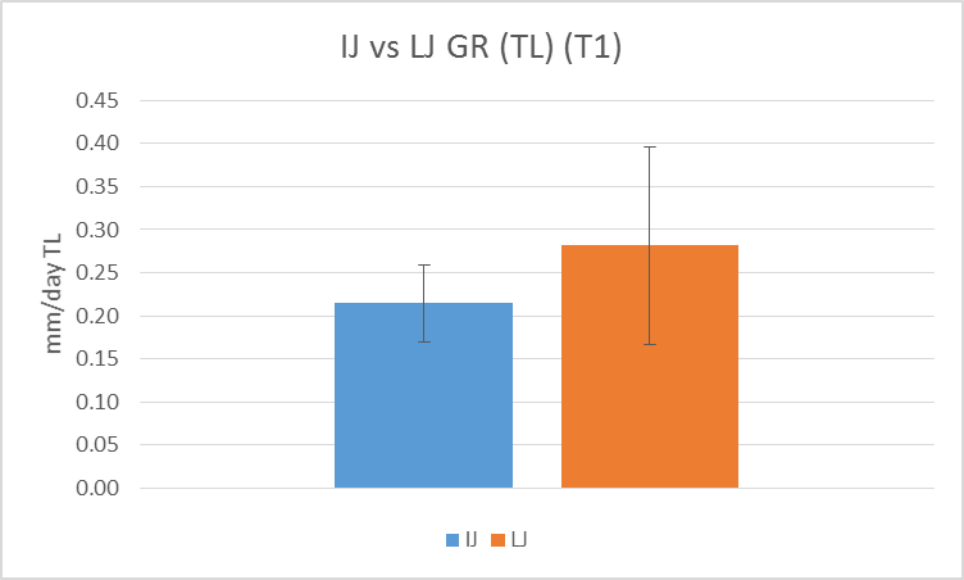


Figure 30: Total length (TL) growth rate (GR, mm/day) of indoor-reared juveniles (blue bars, IJ) and lagoon-released juveniles (red bars, LJ) for T1

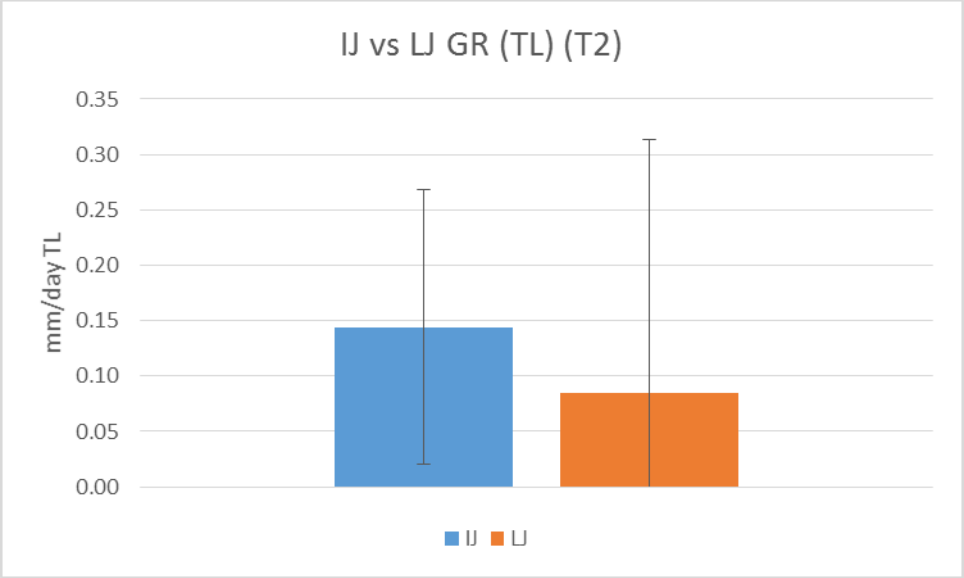


Figure 31: Total length (TL) growth rate (GR, mm/day) of indoor-reared juveniles (blue bars, IJ) and lagoon-released juveniles (red bars, LJ) for T2

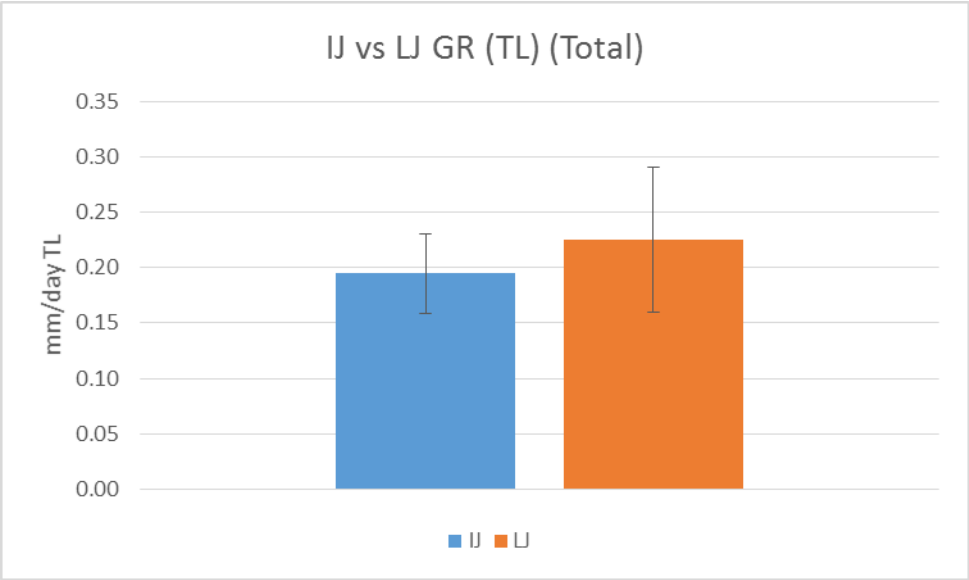


Figure 32: Total length (TL) growth rate (GR, mm/day) of indoor-reared juveniles (blue bars, IJ) and lagoon-released juveniles (red bars, LJ) for the whole period (T1+T2)

The specific growth rate (TL) was recorded for the same periods, and the highest value of 0.24% per day was reported in the first 5 months for the juveniles released into the lagoon (LJ at T1). The SRG value for the whole period (~ 7 months) was 0.18 % per day and 0.11% per day for LJ and IJ respectively (Figure 35).

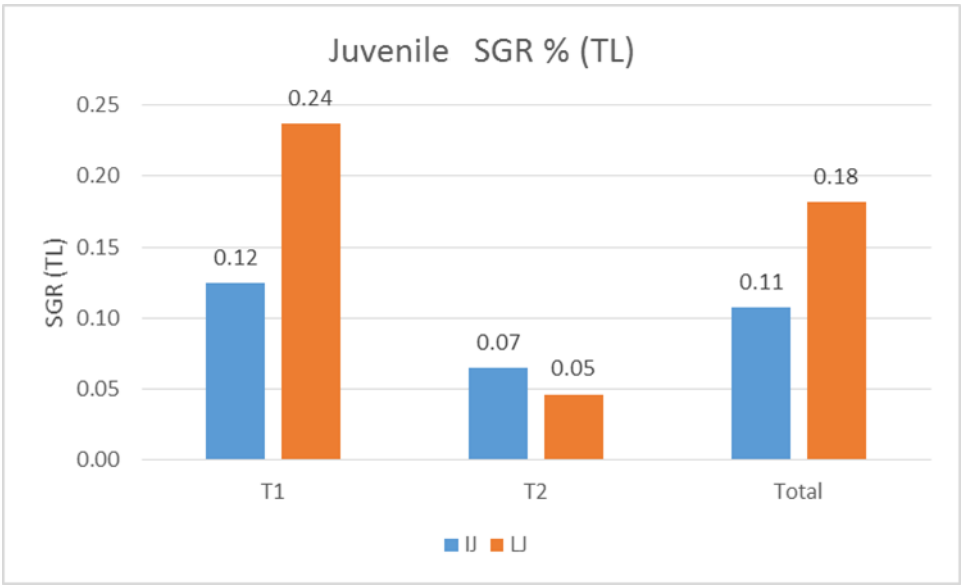


Figure 33: Total length specific growth rate (SGR, %) of indoor-reared juveniles (blue bars, IJ) and lagoon-released juveniles (red bars, LJ) for T1, T2 and for the whole period (T1+T2)

The same comparison of growth rates in terms of body weight (BW) was carried out by measuring the fish after ~ 5 months (T1), at the end of the following two months (T2) and at the end of the whole period of ~ 7 months (Total).

GR recorded at T1 were 0.04 ± 0.01 g/day in IJ and 0.03 ± 0.02 g/day in LJ (Figure 34).

At T2, GR was 0.04 ±0.04 g/day in IJ and 0.01 ± 0.05 g/day in LJ (Figure 35).

The GR values for the whole period (Total) were 0.05 ± 0.01 g/day and 0.02 ± 0.01 g/day for IJ and LJ respectively (Figure 36).

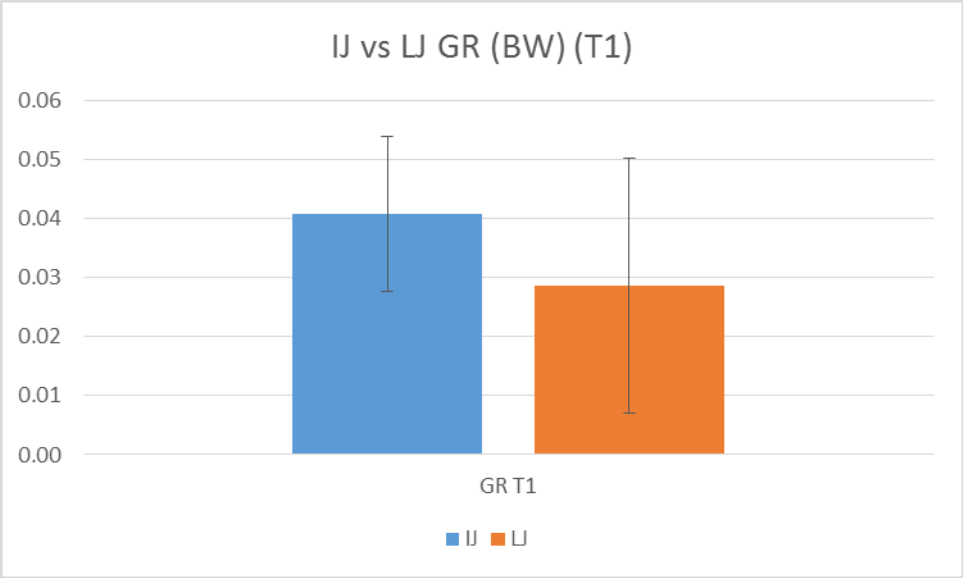


Figure 34: Body weight growth rate (GR BW, g/day) of IJ and LJ for T1. IJ = indoor-reared juveniles, blue bars; LJ = lagoon-released juveniles, red bars

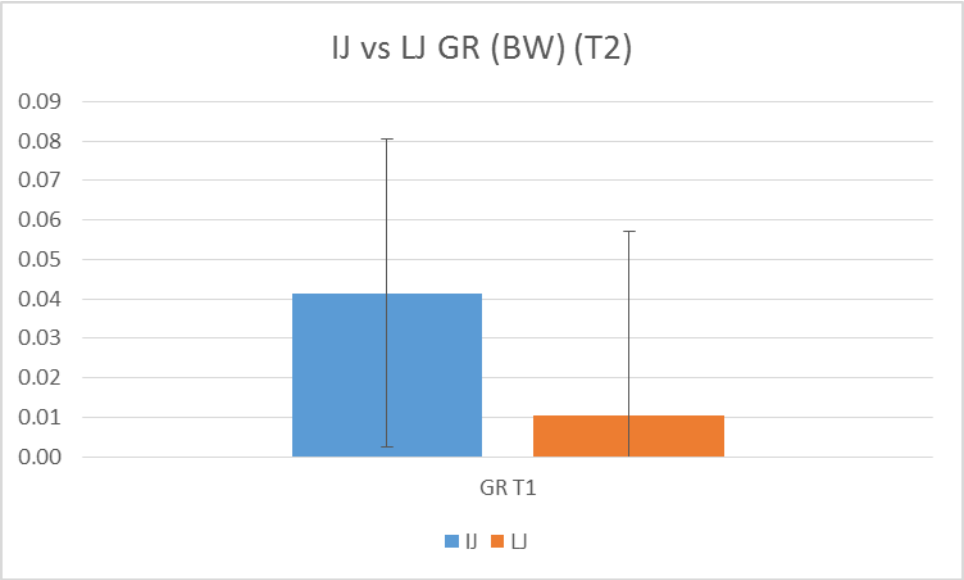


Figure 35: Body weight growth rates (GR BW, g/day) of IJ and LJ for T2. IJ = indoor-reared juveniles, blue bars; LJ = lagoon-released juveniles, red bars

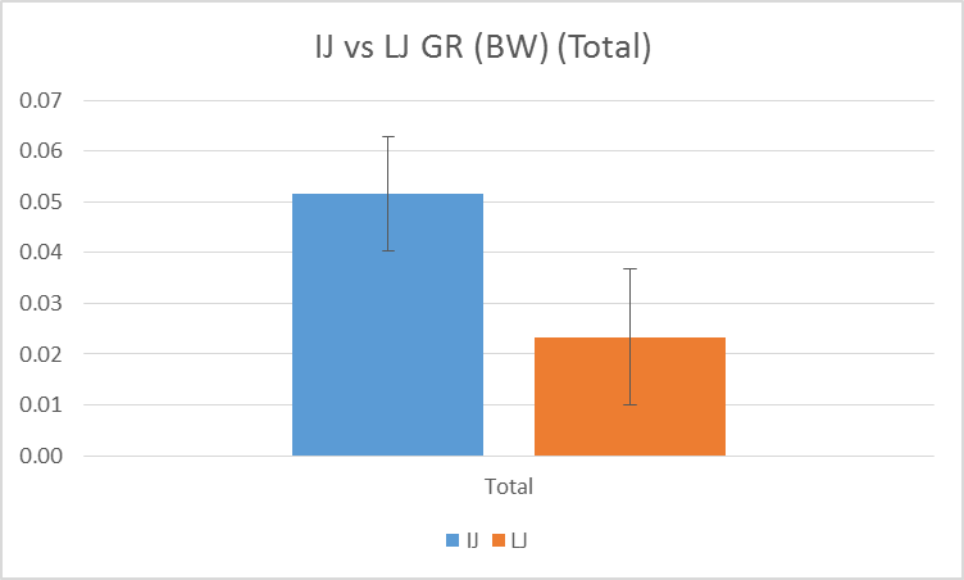


Figure 36: Body weight growth rate (GR BW, g/day) of IJ and LJ for the whole period (Total). IJ = indoor-reared juveniles, blue bars; LJ = lagoon-released juveniles, red bars

The SGR% of IJ was 0.37 % in T1 and 0.18 % in T2. LJ SGR% was 0.68 in T1 and 0.08% in T2.

The average SGR value for IJ and LJ at the end was 0.31 % per day and 0.51% per day, respectively (Figure 37).

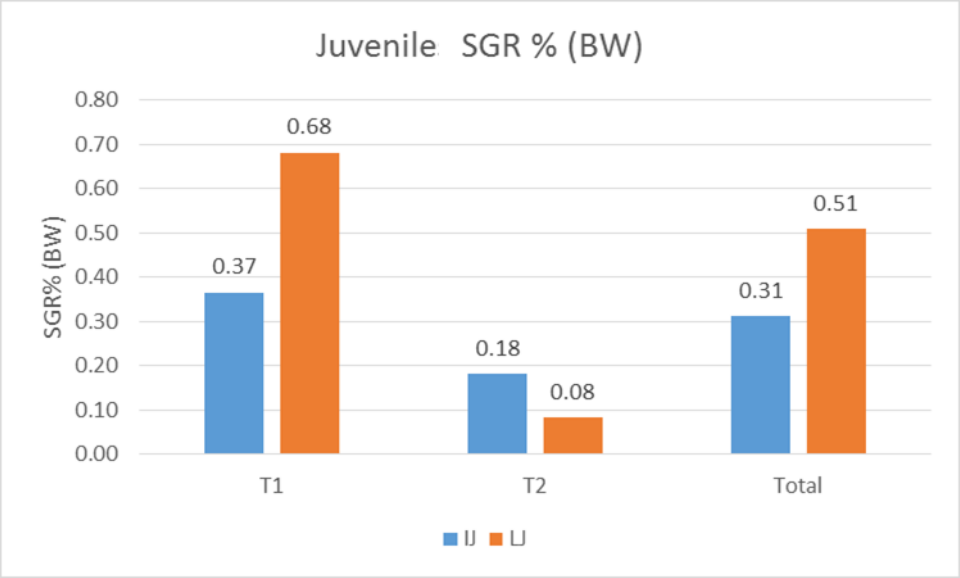


Figure 37: Specific body weight growth rate (SGR BW, %) of IJ and LJ at T1, T2 and for the whole period (Total). IJ = indoor-reared juveniles, blue bars; LJ = lagoon-released juveniles, red bars

The total length and body weight for IJ and LJ at the beginning of the experiment (6 April), after ~5 months (7 September) and at the end, after ~7 months (8 November) are reported in Table 13. The temperature and salinity registered during the juvenile rearing in the lagoon cage are listed in Table 14.

The average length of juveniles kept indoors was 59 mm at the beginning of the second part of the experiment, while the individuals released in the lagoon were 33 mm on average in TL. The initial difference in body weight was even more evident, being IJ 2.4 g and LJ 0.5 g. After 5 months, IJ reached ~ 9 cm and after 7 months it reached ~ 10 cm . The TL of LJ in the same periods attained ~ 8 cm. In both cases, most of the growth occurred in the first 5 months. Body weight pattern was slightly different, with a constant increase for the IJ of 0.04 g/day and a better performance in T1 (0.03 g/day) compared to T2, where the growth rate values decreased to 0.01 g/day.

Table 133: Total length (TL, mm) and body weight (BW, g) of mullets on April 6, September 7 and November 8, 2015 for indoor-reared (IJ) and lagoon-released juveniles (LJ)

TL (mm)	06-apr	D.S:	07-sep	D.S.	08-nov	D.S.
IJ	59	6	92	7	101	8
LJ	33	6	76	18	82	14
BW (g)	06-apr		07-set		08-nov	
IJ	2.4	0.7	8.7	2.0	11.2	2.4
LJ	0.5	0.3	5.0	3.3	5. 7	2.9

Table 144: Temperature and salinity registered during juvenile rearing in the lagoon cage

	Temp (°C)	S.D.
Apr-Jul	21.3	3.2
Jul-Oct	26.1	1.5
	Sal (ppt)	S.D.
Apr-Jul	31.0	6.6
Jul-Oct	34.7	3.3

In the figure below (Figure 38, 39), we have synthesized the increasing size (TL, mm) and weight (BW, g) of the reared mullets throughout the duration of the whole experimental trials (~ 14 months).

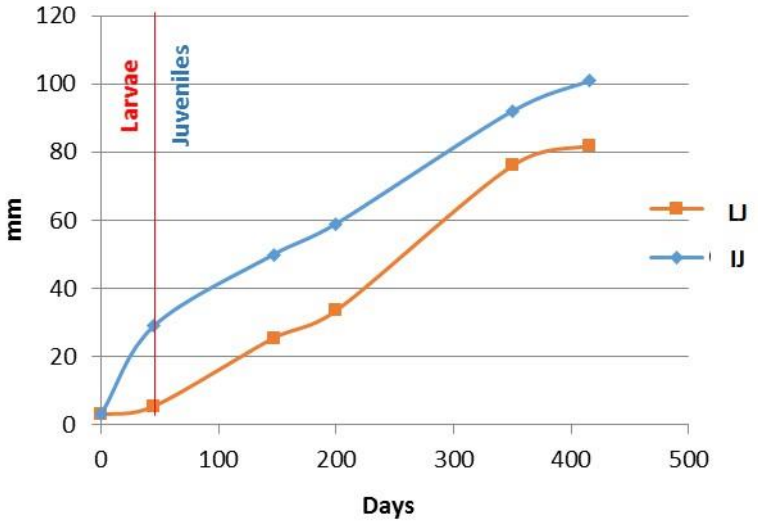


Figure 38: TL of juveniles reared 100% indoors (IJ) and 50% indoors and 50% outdoors (LJ)

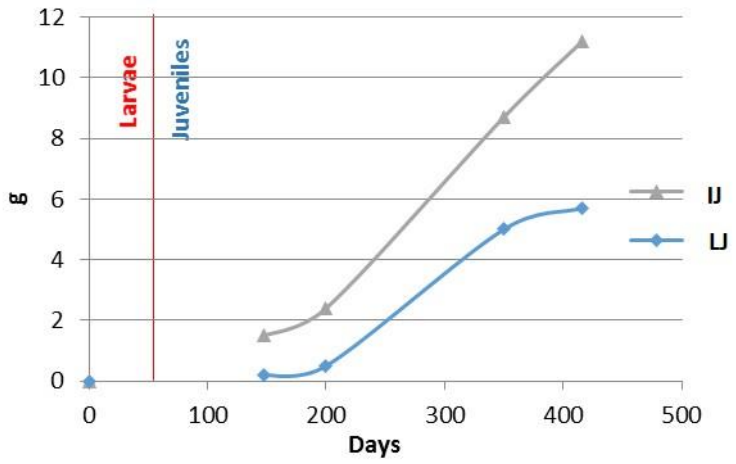


Figure 39: BW of juveniles reared 100% indoors (IJ) and 50% indoors and 50% outdoors (LJ)

In order to check the performances of both rearing environments, the condition index of both IJ and LJ were calculated at the end of the 7 months (Figures 40 and 41).

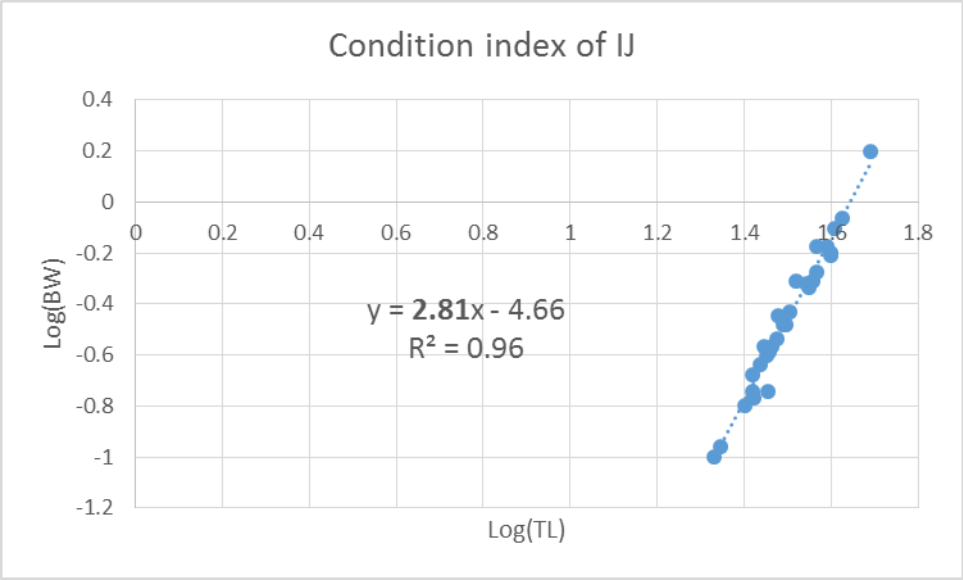


Figure 40: Condition index (b) of IJ at the end of the indoor experiment of 216 days

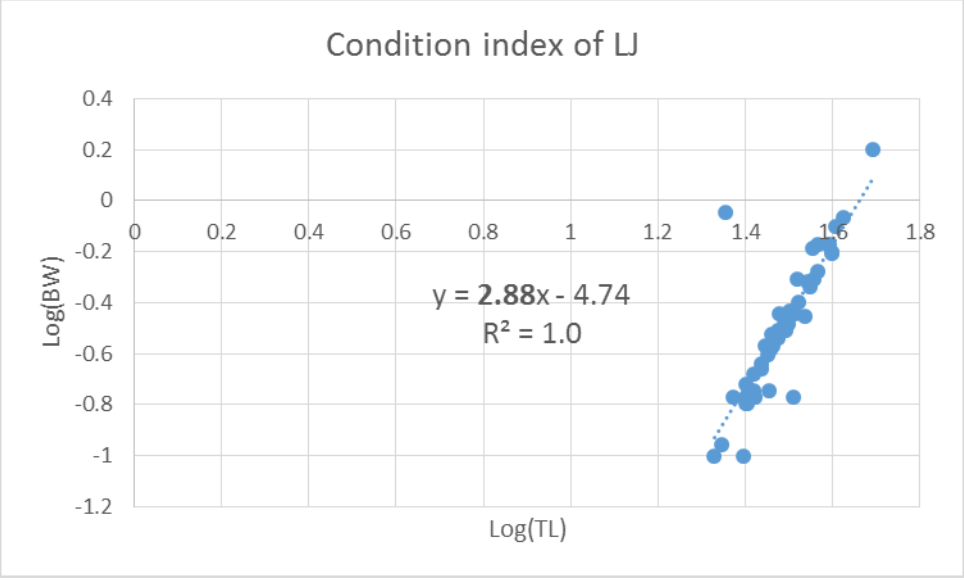


Figure 41: Condition index (b) of LJ at the end of outdoor experiment (216 days from the release) in lagoon cages

3.2 *Paracentrotus lividus*

3.2.1 Larval rearing

Larvae reared at low density (LD= 1.5 ind/ml) developed to the P6 stage at 13 DPF (~ 90 % of the larvae) whereas the larvae at high density (HD= 4 ind./ml) achieved this stage at 16 DPF (~88 %) ($p < 0.05$) (Figure 42).

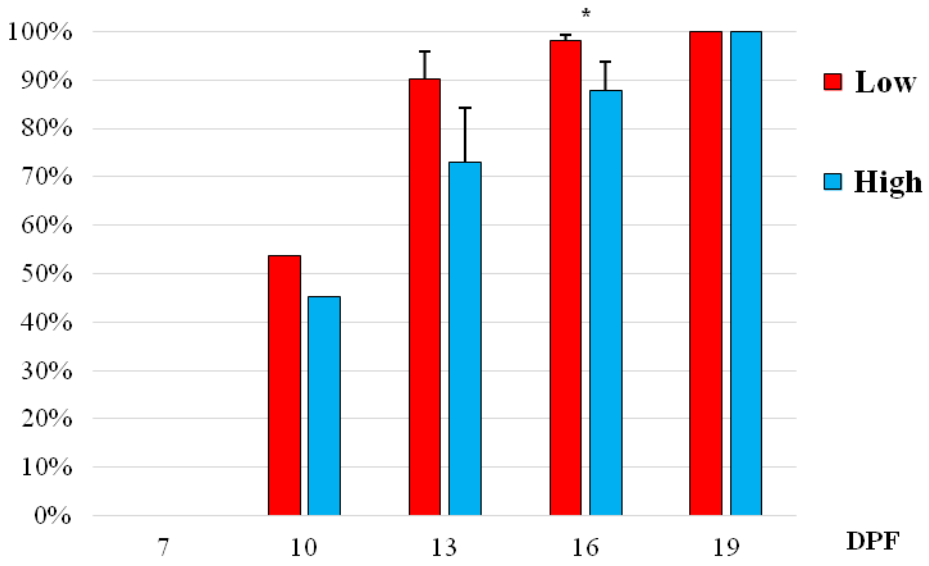


Figure 42: Larval development of Paracentrotus lividus at 6-arm stage (P6) with Low and High density. Values are expressed as the mean ± SE (n = 3). Asterisks indicate a significant difference between density treatments.

Competent larvae were recorded from16 DPF both for Low (14 ± 11%) and High (43 ± 23%) densities. The competence stage was achieved at 22 DPF by both densities tested (LD=86 ± 15%; HD=85 ± 10%). No significant differences were observed between the two densities tested (Figure 43).

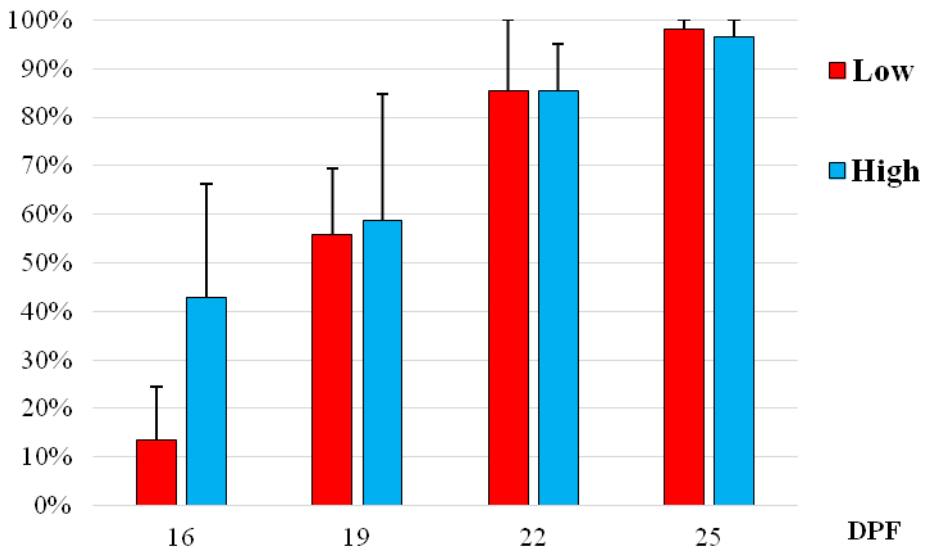


Figure 43: Larval development of *Paracentrotus lividus* at comptence stage (Cp) with Low and High density. Values are expressed as the mean ± SE (n = 3)

Larval survival at LD was 63%, 30% and 12% at 16 DPF, 22 DPF and 31 DPF, respectively. Larval survival at HD was 25%, 5% and 3% at 16 DPF, 22 DPF and 31 DPF, respectively. Significant differences resulted between the two densities at 22 and 28 DPF ($p < 0.05$) (Figure 44).

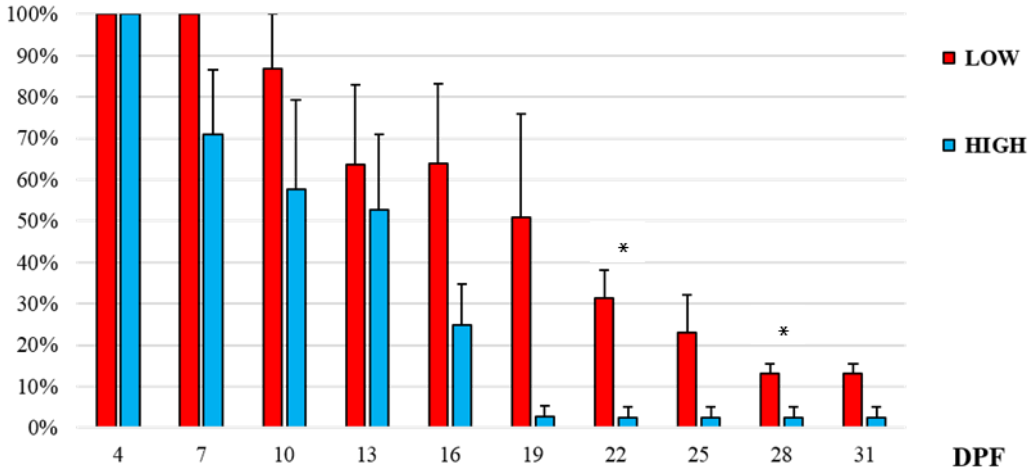


Figure 44: Larval survival of *Paracentrotus lividus* up to the end of the rearing cycle, with Low and High densities. Values are expressed as mean ± SE (n = 3). Asterisks indicate significant differences between density treatments (p < 0.05).

After achieving the competence stage, a delay was observed before larvae metamorphosis. Larvae were ready to methamorphose at 28 DPF and 26 DPF for Low and High densities, respectively.

3.2.2 Metamorphosis and juvenile rearing

Competent larvae were stocked for metamorphosis resulting in a metamorphosis rate of 31%. After 18 months of laboratory rearing, a total of 833 metamorphosed individuals survived, corresponding to ~1% of the total post-larvae obtained. The mean test diameter was 14.9 ± 0.3 mm (mean ± SE). The test diameter growth was heterogeneous and ranged from 9 mm to 22 mm, with a size-class of 16 – 18 mm being the most representative (Figure 45).

The mean wet weight was 1.8 ± 0.1 g (mean ± SE). Wet weight ranged between 0.5 and 4 g, and the most representative size-class resulted 0-1 g with a total biomass of 176 g (Figure 46).

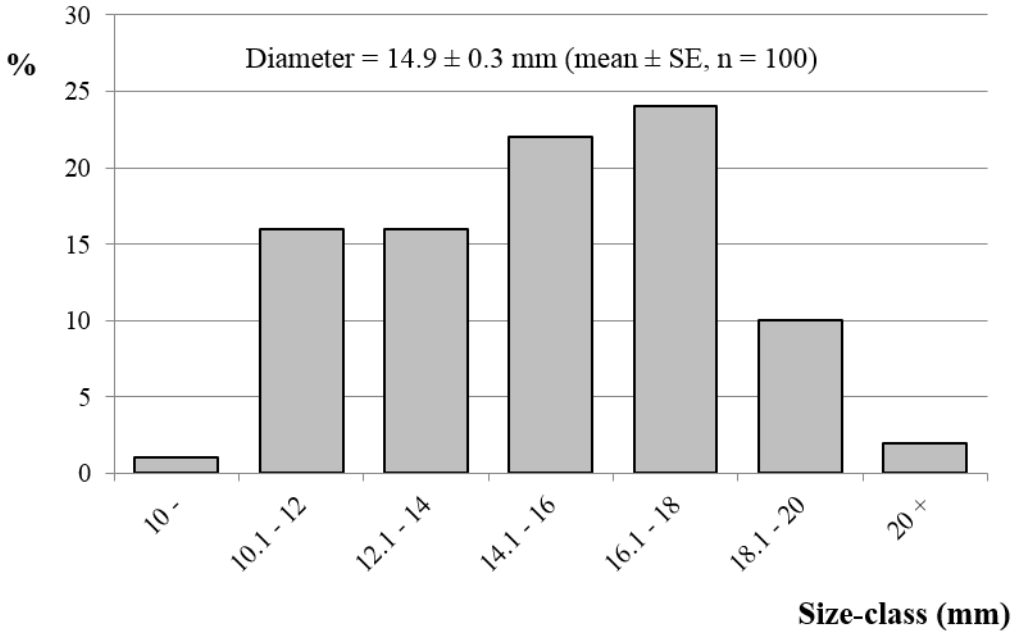


Figure 45: Size-class distributions (diameter in 2 mm intervals) of *Paracentrotus lividus* post-larvae 18 months after metamorphosis

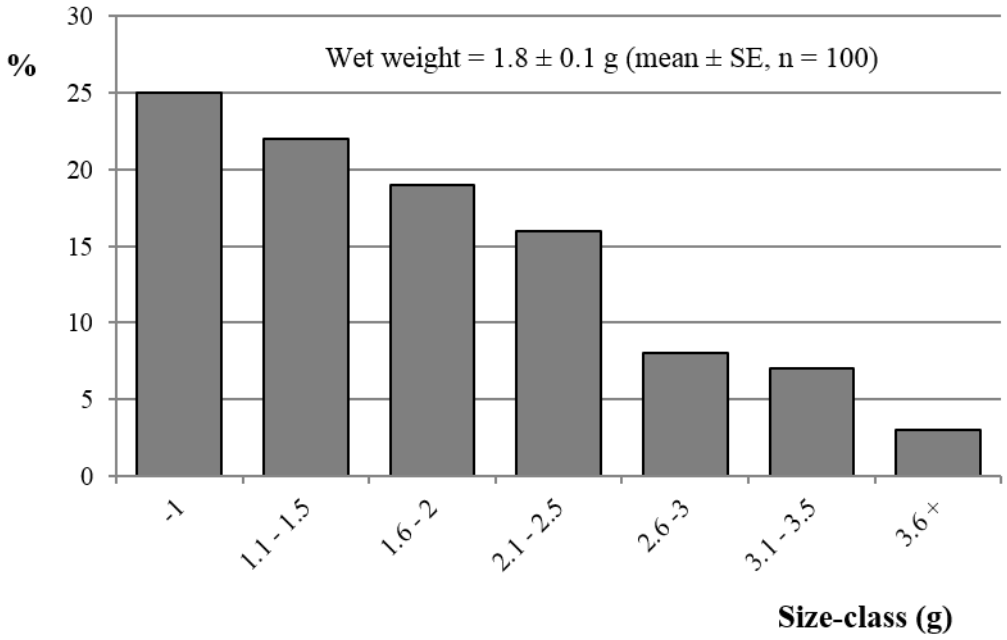


Figure 46: Size-class distributions (wet weight in 0.5 g intervals) of *Paracentrotus lividus* post-larvae 18 months after metamorphosis

3.2.3 Laboratory – Pilot scale comparison

Similar results in the phytoplankton consumption and larval metamorphosis percentage (*U. lens* as metamorphosis-inducing factor) were obtained between laboratory scale (preliminary experiment #2, mixture of *D. tertiolecta* and *C. gracilis* diet) and pilot scale (Low density treatment). A quite low volume (8 ± 4%) of NSW was exchanged during the whole preliminary laboratory experiment, while no water exchanges were done at pilot scale (Table 16).

Different results in larval development and survival were obtained between scales. At pilot scale, larval development was slow, 13 and 22 DPF to achieve P6 and Cp stage, respectively, in comparison to laboratory scale, only 10 DPF for P6 stage and 13 DPF for Cp stage (Table 16).

Larval survival at Cp and Mt recorded lower values at pilot scale (32 ± 18% and 13 ± 7%, respectively) in comparison with laboratory scale (85 ± 10% and 78 ± 10%, respectively) (Table 15).

Table 155: Comparison between results obtained at Laboratory scale (preliminary experiment #2, mixture of *D. tertiolecta* and *C. gracilis* diet) and at Pilot scale. P6 = 6-arms pluteus; Cp = competence; Mt = metamorphosis.

	Laboratory scale 1.5 larvae/ml	**Pilot scale 1.5 larvae/ml**	**Pilot scale 4 larvae/ml**
Total seawater exchange	8 ± 4%	0%	0%
Phytoplankton consumption (thousand cells/mL)	51.2 ± 5.5	61 ± 6.9	91 ± 13.2
Development at P6	10 DPF	13 DPF	16 DPF
Development at Cp	13 DPF	22 DPF	22 DPF
Larval survival at Cp	85 ± 10%	32 ± 18%	5 ± 3%
Larval survival at Mt	78 ± 10%	13 ± 7%	3 ± 1%
Metamorhposis rate (*Ulvella lens*)	34%	31%	28%

4 DISCUSSION AND CONCLUSION

Our research activity was mainly devoted to concurrently defining protocols for induced spawning, hatching and rearing in echinoculture and grey mullet culture. Some of the outcomes of the echinoculture trials were used to implement the mullet culture systems and *vice-versa*. In the following sections, major outcomes of the two studies are reported.

4.1 Grey mullet

Induced spawning in captivity in *M. cephalus* was successfully performed in three reproductive cycles with the LHRH analogue Leuprorelin acetate (Enantone) at a concentration of 200 µg/kg body weight. This concentration was three times lower than those reported in Crosetti (2001) and Crosetti & Cordisco (2001). Other authors reported the successful spawning of *M. cephalus* by adopting LHRH analogues at the same dosage used in the present thesis, but the hormone was administered in addition to the carp pituitary gland extract (PG) at 20-70 mg/kg BW female or HCG from 1000 to 10 000 IU/kg BW female (Meseda & Samira, 2006; Yousif et al. 2010).

Probably the capture, transport, acclimation and treatment protocols used in this thesis succeded in limiting the manipulation stress in the grey mullet broodstock, promoting the action of the hormone at low dosages. Indeed, it is known that reduced stress is fundamental for successful spawning induction in numerous fish species (Haddy & Pankhurst, 2000; Schreck et al., 2001; Ibarra-Castro & Alvarez-Lajonchere, 2009).

Adopting lower dosages to induce spawning could represent a step towards a more economically sustainable production process of the grey mullet. This could reduce the costs and therefore promote the commercial-scale production of this species (Crosetti, 2015). Indeed, the identification of the optimum dose of hormones to induce spawning is desirable for obtaining the best breeding performance in fish (Rahdari et al., 2013).

Another result of our study was that the average oocyte diameter (at the tertiary yolk globule stage) for successful spawning induction resulted between 550 and 600 µm. This concurs with the results obtained by Lee et al. (1991), although their experimental trials were carried out in Hawaii (USA) using a different lineages of *M. cephalus* (Durand, 2015), and is in partial contrast with Meseda & Samira (2006) in Egypt. The latter

obtained viable grey mullet eggs by treating females with an average oocyte diameter of 500 µm.

During the experimental trials, the *M. cephalus* females spawned from 1.2 to 3 million eggs. This corresponds to an average of 0.9 milion eggs/kg body weight and confirms the high fecundity of the species (Liao 1981).

The number of spawned eggs per female was similar to those reported by Nash et al. (1974) and Liao et al. (1972) in Hawaii, whilst Kuo et al. (1973a) obtained about 450 eggs/ kg BW.

Taking the results obtained in the spawning trials into account, with an average fertilization rate of ~87% and an average hatching rate of ~80%, a potential production of ~ 600,000 larvae could be obtained for each spawned female.

During the reproductive season it is possible to induce more than one female to spawn.We therefore calculated that the potential production considerably exceeds the maximum number of larvae which could be reared at the IMC hatchery: about 40 larvae/L in a total volume of 8,000 L (320,000 larvae).

A maximum of 900,000 eggs can be incubated at the IMC facilities (at the highest density tested during experimentation, 1,000 egg/L). A high potential is therefore represented by the excess of embryos which could be transported and incubated elsewhere, greatly enhancing the production. This could also define a more ample process involving fishermen's associations and lagoon facilities elsewhere in Sardinia.

Another interesting result was that embryos can be incubated at much higher densities (up to 1,000 egg/L) than those reported in the literature (140 egg/L) by Habram et al. (1999), with good hatching rates (from 70 to 90%).

As far as larval seeding methods are concerned, the experimental trials suggest that transportation of the larvae should not be done immediately after hatching. This would avoid the high mortality observed during the first 2-4 DPH in C1 (~60%) and in C2-1 (100%). Seeding at 2 DPH, as used in the following cycles, resulted in larval survival ranging from 75% to 80%.

The mortality observed in C1 was also attributed to the late use of the surface skimmer (from 12 DPH) A thick biofilm was present on the water surface, probably determining a low exchange of gas/oxygen between the air and water. Indeed, allowing larvae to gulp

air at the water surface is crucial for a proper inflation of the swim bladder (FAO, Fisheries and Aquaculture Department, 1996).

4.1.1 The influence of density on larval and juvenile growth

The influence of density on larval GR was observed during the first 45 DPH by comparing the data of the three cycles with increasing density. The growth rate of the C1 larvae, 0.6 mm/day, was 6 times faster than the value obtained in C2 and C3. This is confirmed, and made even more evident, when we consider the specific growth rates (SGR, %) obtained in the same period in C1: 2.4% versus an average of 1% observed in C2 and C3.

The growth rates observed in our study are similar to those recorded by Liao (1975) (0.6 mm/day) for 45 DPH *M. cephalus* larvae reared at 25°C at a density of 20 individuals/L.

Tamaru et al. (1994) reported a GR of about 0.25 mm/day in larvae reared at 25°C, 15 DPH.

At 23°C, Lee & Kelly (1991) and Kuo et al. (1973a) obtained a GR of 0.3 mm/day. Yousif et al. (2010) reported a GR of ~0.4 mm/day when larvae were reared at the initial density of 30 individuals/L and 23°C.

Taking into account the juvenile rearing phase (45-200 DPH), according to Eda et al. (1990), density seems to influence survival more than it influences growth. The survival rates of the three cycles suggest that high density could have determined the low survival rate recorded in HD (C3). It was 14%, compared to those observed in LD (C1) and MD (C2), which were 35 and 31 % respectively. However, growth rate decreases and becomes more homogeneous in all the three cycles during the juvenile rearing phase (45-200 DPH). GR observed was 0.19 mm/day for C1, 0.20 mm/day for C2 and 0.18 mm/day for C3.

FLittle data on size-at-age for reared *M. cephalus* are reported in the literature (Crosetti & Blaber, 2015) and no data concerns growth performance in RAS systems. De Silva and Silva (1979) estimated a rate of 0.24 mm/day for 1-2 g BW for reared *M. cephalus* juveniles at 20°C. Eda et al. (1990) and Yousif et al. (2010) obtained a GR of about 0.4 mm/day by rearing grey mullet juveniles (60 DPH) at 24.5°C and with an initial larval

density of 30 larvae/L. Since there is a large variability in growth rates in this species, the present thesis could provide significant information on the growth of *M. cephalus*.

4.1.2 Biomass production

Considering the biomass produced during our experimental trials, the best performance was obtained in a medium density. Indeed, in this cycle, the average biomass at the end of the indoor rearing phase (200 DPH) was about 2.8 kg, compared to the 1.6 kg and 0.8 kg obtained respectively in the other two cycles. This biomass corresponds to a density of 1.4 g/L in MD, 0.8 g/L in HD and 0.4 g/L in LD. MD performed better even in terms of number of individuals produced (4,700). At higher densities, juvenile mortality determined a slightly lower survival rate of about 4,100 individuals.

We state that the survival rate of reared juveniles is more important than the individual growth since they are used for restocking purposes and growth occurs in the natural environment (in pre-adaptation cages).

4.1.3 Condition index

The condition index identified was not influenced by density and resulted in a value of 3 in all cycles.

A condition index close to 3 indicates an isometric growth, a good proportion between length and weight in the fish (Crosetti & Blaber, 2015). Values ranged from 2.94 to 3.30, the highest being recorded at the highest density tested. This indicates that *M. cephalus* juveniles were suitably reared, by providing them adequate diets and culture conditions during their development.

Studies carried out in Spain reported condition index values from 2.96 (Lake Albufera, 2000) to 3.36 (Mar Menor lagoon, 2002-2004) for wild individuals (Fishbase). In Croatia values ranged from 3.12 (River Neretva estuary, middle Adriatic, 2000-2004) to 3.18 River Mirna estuary, northern Adriatic, 2000-2004), in France from 3.14 to 3.58 (Gulf of Lion), and in Greece from 3.16 (Korinthiakos Gulf, 2008-2009) to 3.18 (Rihios estuary, NW Aegean, 1997-99) (Fishbase). Currently no data on condition indexes are reported for *M. cephalus* reared indoors.

4.1.4 Juvenile growth in lagoons

Examining the growth rate of the individuals released in the pre-adaptation cages (LJ), we identified a value 3-times higher in spring/summer (April - September) compared to autumn (September - November).

This is also confirmed by Leber (1996) who suggests that the best performances in terms of growth and survival of released juveniles occurred in summer, and by Garbin et al. (2013) who demonstrated the fastest growth from June to August. Probably this is due to the higher average temperature normally registered in spring and summer and what it implies in terms of food availability in the lagoon. A juvenile growth of 0.5 mm/day was described for wild, one-year-old Australian specimens (Chubb et al., 1981). Anderson (1958) observed a growth rate of 0.41 mm/day in juveniles. Ibáñez et al. (2015) reports a GR of about 0.48 mm/day for the same period in brackish water and a GR of 0.43 mm/day in sea water for one-year-old juveniles.

Growth rates recorded in IJ did not vary between T1 (0.21 mm/day) and T2 (0.15 mm/day), thus showing an evident ”indoor rearing effect”. These individuals received a constant amount of feed in controlled conditions (temperature, salinity and water flow), and we hypothesize that they had a more constant energy consumption than those in the lagoon (where mullets are subjected to physiological and hydrodynamic stress). However, fish growth decreases with increasing fish length, so a reduction in growth performances in subsequent time periods is expected (Hamre et al., 2014).

Taking into account the whole rearing period for IJ (100 % indoor) and for LJ (~50% indoor and ~50% outdoor), growth performances were similar. This indicates that releasing the individuals at about 3 cm TL could be a good practice in terms of economical sustainability of the production process. In addition, the larger mullets produced at the end of the whole rearing period IJ (~ 11 cm) could be utilised for the strong release method as described by Hutchison et al. (2012).

4.2 Sea urchins

The shift from laboratory-scale experiments to pilot-scale volume was tested in the present thesis. The variable method protocols were applied on a pilot scale and the effect of stocking density on larval survival and larval development was tested. The main outcome was that the variable method protocols can be successfully applied on a pilot scale.

The sustainability of the method at pilot scale was identified in a seawater exchange equal to 0%, with a similar phytoplankton consumption between densities. This result has the potential to reduce manual labour in the production process, diminishing both costs and the use of resources.

It is well known that the laboratory scale results in higher survival rates, but these techniques are not always applicable on a larger scale (Carboni et al., 2012; Liu et al., 2007; George et al. 2004; Kelly et al., 2000). Our results clearly show slower larval development and lower survival at pilot scale compared to the laboratory scale.

Larval development and survival were significantly affected by density. Development was faster at a low density. Larval survival at metamorphosis recorded at pilot scale was only 13%, while at laboratory scale it was significantly higher: almost 80% of individuals survived up to this stage of larval development. Conversely, the use of larger volumes did not seem to have an influence on the metamorphosis rate.

Our results on larval survival and development concur with previous studies by other authors who worked with large volumes and stocked larvae at low density (1.5/mL). Our results are similar to to those obtained by Paredes et al. (2015), who had a survival rate of ~40%. However, our results differ from the results reported by Liu et al. (2007), who obtained a survival rate of 68-76% and the achievement of competence at 18 DPF.

Carboni et al. (2012), focused on the application of the *P. lividus* echinoculture at a commercial scale and investigated the effects of different phytoplankton diets at large volumes (80 L). They reported survival rates (from 5 to 14%) similar to those obtained in this study (~5%).

A total of 144,000 competent larvae were obtained at low density and 58,500 compentent larvae at high density. This results in a survival rate at metamorphosis of 60,000 post-larvae at LD and 36,000 post-larvae at HD. Our results showed that low density performs better than high density in terms of the number of individuals per cycle.

In conclusion, the variable method peformed in our work has the potential to reduce manual labour, water volumes and phytoplankton requirements in a sea urchin hatchery.

5 FURTHER STUDIES

5.1 Mugil cephalus larvae growth and survival

The feeding program adopted during the larval rearing phase in our study was characterized by low rotifer densities and green water concentrations compared to other authors (Eda et al., 1990; Lee & Kelley 1991; Tamaru et al., 1994; Tamaru et al., 2005). Moreover, Tamaru et al. (1993b) suggests feeding rotifers with commercially available enrichment media to improve growth and survival in grey mullet larvae.

However, temperature is known to have a significant influence on *M. cephalus* larval growth. Many authors (Crosetti & Blaber, 2016) report larval growth data at higher temperatures than those adopted in this thesis.

Therefore, more trials of larval rearing would be required to better evaluate the effects of temperature on growth and survival of larvae:

- fed with higher rotifer densities and green water concentrations
- using enrichment media to culture rotifers
- reared at different temperatures

5.2 Can “stressed phytoplankton” positively influence larval survival and growth?

The main factor limiting the commercial rearing of the grey mullet is the high mortality which occurs during early larval development when the fish are feeding on rotifers (Brachionus spp.). Although little is known of the nutritional requirements of mullet larvae, preliminary studies have shown that enriching the rotifers with the n-3 highly unsaturated fatty acids (HLTFA), eicosapentaenoic acid (EPA, 20511-3) and docosahexaenoic acid (DHA, 22:6n-3) improved

survival, growth and stress resistance (Eda et al. 1990, Tamaru et al. 1992; U.S. Agency for International Development, 2007) ".

This demand for high lipids can be satisfied by employing enrichment in the zooplankton culture medium (U.S. Agency for International Development, 2007) but such products are expensive and add to hatchery costs.

Microalgae lipid content can be increased without additional costs by cultivating them under "stress conditions" (Converti et al. 2009). For example, *N. oculata* can double its lipid content by increasing the culture temperature from 20 to 25 degrees or by reducing the nitrogen content in the culture medium (Converti et al. 2009).

In order to enhance larval survival and growth and concurrently define an economically sustainable protocol for rearing *M. cephalus* larvae, a preliminary experiment was carried out at the IMC. Survival and growth in larvae fed with rotifers cultured with stressed phytoplankton was compared to those cultured with standard phytoplankton.

Stressed cultures of *N. oculata* and *I. galbana* were obtained by modifying nitrogenous content in the culture medium (Converti et al. 2009).

Rotifers were cultured separately both in stressed and standard microalgae and administered to the larvae with the respective green water (stressed and standard microalgae cultures).

The comparison was made after rearing the individuals from 0 to 21 DPH in 12 tanks of 15 L volume each (experimental larval rearing system).

Larval growth in terms of total length resulted significantly higher ($p<0.05$) in the larvae fed with the stressed diet than in those reared with the standard one. This encouraging result was invalidated by the high mortality observed during the experiment. The high mortality observed was attributed to the inadequate set up of the experimental larval rearing system used in this experiment. A thick biofilm was, in fact, present on the water surface of the rearing tanks and probably determined a low exchange of gas/oxygen between the air and the water. This would also have prevented a proper swim bladder inflation (FAO, Fisheries and Aquaculture Department, 1996).

New trials of the same experiment performed with a re-designed experimental larval rearing system are necessary in order to better evaluate the effects of the "stressed diet" on *M. cephalus* larvae survival and growth.

5.3 Mugil cephalus juveniles grow out

The juvenile grow out protocol presented in this thesis was tested in the Cabras lagoon. It would be very interesting to evaluate the growth and survival of reared *M. cephalus* juveniles released in different lagoons in pre-adaptation cages. This research activity would be aimed at better identifying the physical parameters and biological aspects which influence the growth of grey mullet juveniles.

5.4 Survival of released fish

In this study, individuals were released into the wild after 7 months (pre-adaptation period in a lagoon cage). Their TL was at about 8 cm , when their survival is supposed to be comparable with wild individuals (Leber et al., 1996).

The survival of released individuals is fundamental in order to evaluate the success of restocking programs. In order to test the survival of the released individuals, juveniles could be tagged with PIT tags before their release into the wild.

A significant quantity of information would be available thanks to the recording of the survival rate, movements and habitat preferences of the released fish, and by comparing them to the ecological and physical data of the release site.

5.5 Sea urchin restocking and tagging

The high mortality observed during the sea urchin laval rearing phase was probably due to the inadequate set up of the rearing system. New rearing trials will be necessary in order to design a better performing system and to identify the best set up (banjo-filter surface, mesh size and water flow).

Further studies are necessary to evaluate the survival of laboratory-reared sea urchins in the wild. Advances in sea urchin tagging technologies are still necessary in order to define a reliable rate of survival. Tests aimed at evaluating different tagging methods could represent a first step in order to start defining a Mediterranean restocking program for this species.

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