Exploring the biological and socio-economic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry

A compilation of 9 Articles from the EU DIVERSIFY project published in the Aquaculture Europe magazine of the European Aquaculture Society 2014 to 2018

Co-funded by the 7th Framework Programme of the European Union

7FP-KBBE-2013-GA 602131, DIVERSIFY
www.diversifyfish.eu

Table of contents

DIVERSIFY
New species for EU aquaculture

Co-funded by the Seventh Framework Programme of the European Union

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DIVERSIFY:

Enhancing the European aquaculture production by removing production bottlenecks of emerging species, producing new products and accessing new markets

DR. CONSTANTINOS (DINOS) C. MYLONAS,
RESEARCH DIRECTOR, INSTITUTE OF MARINE BIOLOGY, BIODIVERSITY AND AQUACULTURE, HELLENIC CENTER FOR MARINE RESEARCH, GREECE MYLONAS@HCMR.GR

DR. ROCIO ROBLES,
TECHNICAL DIRECTOR, ANDALUSIAN AQUACULTURE TECHNOLOGY CENTRE, SPAIN
R.ROBLES@CTAQUA.ES

On 29-30 January 2014, the European Commission project DIVERSIFY (FP7-KBBE-2013, GA 603121) had its kickoff meeting at the Hellenic Center for Marine Research (HCMR) in Iraklion, Crete, Greece. The project is coordinated by Dr. Constantinos C Mylonas of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC), one of the three institutes of the HCMR. DIVERSIFY has a total budget of 11,8 million € for its 5 year duration and it is one of the largest research project in the area of aquaculture funded by the European Commission. DIVERSIFY's consortium (Table 1) includes twenty research and academic institutions, three Large Enterprises, nine Small and Medium Enterprises (SME), five Professional Associations and one consumer NGO.

The project DIVERSIFY (www.diversifyfish.eu) has identified a number of new/emerging finfish species, with a great potential for the expansion of the EU aquaculture industry. Although the emphasis is on Mediterranean cage-culture, fish species suitable for cold-water, pond/extensive and fresh water aquaculture have been included as well. These new/emerging species are fast growing and/or large finfishes marketed at a large size and can be processed into a range of products to provide the consumer with both a greater diversity of fish species and new value-added products. The fish species to be studied include **meagre** (*Argyrosomus regius*) and **greater amberjack** (*Seriola dumerili*) for warm-water marine cage culture, **wreckfish** (*Polyprion americanus*) for warm- and cool-water marine cage culture, **Atlantic halibut** (*Hippoglossus hippoglossus*) for marine cold-water culture, **grey mullet** (*Mugil cephalus*) a euryhaline herbivore for pond/extensive culture, and **pikeperch** (*Sander lucioperca*) for freshwater intensive culture using recirculating systems.

These species were selected based both on their biological and economical potential, to cover the entire European geographic area and to stimulate different aquaculture types. In collaboration with the participating SMEs and/or Large Enterprises, DIVERSIFY will build on recent/current national initiatives for species diversification in aquaculture, in order to overcome the documented bottlenecks in the production of these species. Research will be carried out in the scientific disciplines of Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health, Final product quality and Socioeconomics. The combination of biological, technological and socioeconomic research planned in DIVERSIFY are expected to support the diversification of the aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets. To ensure the dissemination and implementation of the new knowledge that will be developed by the project, a wide range of dissemination activities have been planned, targeted both to the aquaculture production and its associated sectors (*i.e.*, food processing and retailing), as well as the European consumers.

Meagre cages.

Seriola in cage.

Stripping eggs from meagre.

Participating organizations in DIVERSIFY

Greece: Institute of Marine Biology, Biotechnology and Aquaculture (HCMR/IMBBC); ARGOSARONIKOS FISHFARMS AE; AQUACULTURE FORKYS AE; IRIDA AE; Hellenic Research House AE; VAS. GEITONAS & Co Ltd; Federation of Greek Maricultures.

Spain: Institut de Recerca i Tecnologia Agroalimentaries (IRTA-San Carles de la Rapita); Parque Científico y Tecnológico de la Universidad de Las Palmas de Gran Canaria; Centro Tecnológico de la Acuicultura de Andalucia (CTAQUA); Universidad de la Laguna; Instituto Español de Oceanografía; Asociación Empresarial de Productores de Cultivos Marinos-APROMAR; Consellería do Medio Rural e do Mar-Xunta de Galicia; Ayuntamento de A Coruña (Museos Científico Coruñeses); CULMAREX SAU; CANEXMAR SL; ANFACO-CECOPESCA.

France: French Research Institute for the Exploitation of the Sea (IFREMER); Université de Lorraine; ASIALOR Sarl

Israel: Israel Oceanographic and Limnological Research-National Center for Mariculture; DOR AQUACULTURE Ltd

Norway: Institute of Marine Research, National Institute of Nutrition and Seafood Research; Skretting Aquaculture Research Center AS; Stirling White Halibut AS

The Netherlands: LEI-Wageningen UR (DLO/LEI); Eindhoven University of Technology

United Kingdom: The University of Aberdeen

Italy: Universitá degli Studi di Bari «Aldo Moro»; AZIENDA AGRICOLA ITTICA CALDOLI Srl

Belgium: Université de Namur; European Food Information Council

Denmark: Technical University of Denmark, Aarhus University (MAPP Center)

Germany: German Association of Fish Processors (Bundes Verband Fisch, BVFi E.V.)

Hungary: Hungarian Aquaculture Association (Mayar Akvakúltra Szövetség, MASZ)

MEAGRE

Meagre is found in the Mediterranean and Black Sea, and along the eastern Atlantic coast (Haffray et al., 2012). It has attractive attributes for the market that include **large size, good processing yield, low fat content, excellent taste and firm texture** (Monfort, 2010). The species also has attractive biological characteristics such as a **fast growth of ~1 Kg per year** (Duncan et al., 2013), a **low feed conversion ratio of 0.9-1.2** (Duncan et al., 2013; Monfort, 2010) –which is similar to the Atlantic salmon-, relatively easy larval rearing (Papadakis et al., 2013; Roo et al., 2010; Vallés & Estévez, 2011, 2013) and established induced spawning protocols for the production of viable eggs (Duncan et al., 2012, 2013; Mylonas et al., 2013a, b). Meagre was first produced in 1997 in a commercial hatchery in France and since then it has exhibited annual production increases as high as 7 fold (FAO, 2012). In 2010, European meagre aquaculture production was 2,387 t, mainly in Spain, with smaller quantities from France, Portugal, Italy, Greece, Cyprus and Croatia (FAO, 2012). Production of meagre is also carried out in Egypt, but there it is based exclusively on the collection of wild fry.

A survey of meagre producers carried out during the proposal stage of DIVERSIFY identified four principal bottlenecks to the expansion of the industry. Firstly, **variable growth rates** are reducing yield greatly (Duncan et al., 2013). A multidisciplinary approach is required in order to examine the role of genetics, nutrition --particularly dietary requirements during weaning, nursery and grow out-- feeding behaviour and grow out husbandry. Secondly, the distribution of this fish only in specific areas in the Mediterranean region has resulted in the acquisition of broodstocks from a limited number of sources (mainly a hatchery in France), resulting perhaps in a **limited genetic variation of the**

Checking PIT tag.

available broodstocks. This will have significant negative implications for the future initiation of breeding selection programs, which are necessary to move the industry to the next level of efficiency and production. Thirdly, the industry must address issues in **fish health**, emerging diseases, parasites (Koyuncu et al., 2012; Merella et al., 2009; Ternengo et al., 2010; Toksen et al., 2007) and the wide occurrence of Systemic Granulomas (Elkesh et al., 2012), which may stem from the fact that no diets have been developed for this fish. Finally, **socioeconomic factors have been identified as bottlenecks**, including the need for a more expanded market and diversification of provided products (Monfort, 2010) beyond the whole fresh fish. National initiatives for meagre domestication are underway in Spain and Greece (kranios.weebly.com), and DIVERSIFY will build on the acquired information by targeting specific issues recognized as bottlenecks for further production.

GREATER AMBERJACK

This is a cosmopolitan species (Andaloro & Pipitone, 1997; Cummings et al., 1999; Thompson et al., 1999) of great interest to the aquaculture sector due to its **excellent flesh quality**, **worldwide market availability** and high **consumer acceptability** (Nakada, 2000). Its rapid growth (*i.e.*, **short time to market size**) and large size makes this species **very suitable for product diversification and development of value added products**. In the Mediterranean (Lovatelli & Holthus, 2008), farming started with capture-based activities using wild juveniles (Crespo et al., 1994). Fish of ~90 g reached ~1 kg in a year, and 6 kg in a period of 2.5 years (Jover et al., 1999; Mazzola et al., 2000). The high growth rate of cultured greater amberjack and its feeding on fish of low commercial value made the activity profitable. Using standard dry feeds, wild caught individuals of 50-100 g exhibited great growth performance of 1.8, 4 and 7.5 kg body weight in 1, 2, and 3 years, respectively (Jover et al., 1999; Mazzola et al., 2000). Still, the Mediterranean production in 2012 was only ~2 t, while market price –mainly for capture fisheries catches-- reached values >14 € kg^{-1}. Today, **a very limited commercial activity with hatchery-produced individuals exists in Malta**, though interest exists and efforts have been made by various aquaculture companies in the Mediterranean.

The **major bottlenecks f**or the incorporation of greater amberjack in the EU aquaculture industry include lack of (a) **reliable reproduction** and (b) **production of adequate numbers of juveniles**. In captivity, reproduction has been problematic (Kozul et al., 2001), but **captive-reared broodstocks have reproduced after hormonal treatments** (Fernandez-Palacios et al. 2013; Mylonas et al., 2004), and in some cases also spontaneously (Jerez et al., 2006). Also, some knowledge has been acquired on the nutritional requirements

of reproduction (Rodríguez-Barreto et al., 2012). **DIVERSIFY will study the reproduction in captivity and in the wild, and develop efficient spawning induction methods, as well as appropriate broodstock diets.**

Larval rearing of greater amberjack was done initially using semi-intensive methods (Papandroulakis et al., 2005). Survival was limited (3%), but recently it has been improved with adaptations in feeding regime and diet quality (Anonymous, 2008). Since both the greater amberjack (Matsunari et al., 2012) and its congeners the yellowtail (*S. quinqueradiata*) (Nakada, 2000), yellowtail kingfish (*S. lalandi*) (Ma et al., 2012) and almaco jack (*S. rivoliana*) (Roo et al., 2012) have been produced in hatcheries, once the bottleneck of egg availability is surpassed, the available information on these congeners can hasten the development of larval rearing protocols for the greater amberjack.

Another area of concern for the commercial production of greater amberjack is **fish health**. Bacterial pathogens cited in the literature as potential threats include *Photobacterium damsella* (Crespo et al., 1994) and epitheliocystis (Rigos and Katharios, 2010), and *Cryptocaryon irritans* has caused severe losses in broodstock (Rigos et al., 2001). During grow out, monogenean parasites cause occasional mass mortalities in farmed fish (Grau et al., 2003; Montero et al., 2004), while *Neobenedenia* spp was identified in a major outbreak causing losses in both juveniles and broodstock. Therefore, DIVERSIFY will study the potential pathologies that will occur in the course of the project in an effort to develop **early diagnosis tools**, **veterinary solutions** and **preventive veterinary protocols** that will be available and will support the sustainable rearing of the species.

PIKEPERCH

This freshwater fish is considered to have the highest potential for inland aquaculture diversification in Europe (Wang et al., 2008). Through the EU projects LUCIOPERCA and LUCIOPERCIMPROVE, reproductive control (Kucharczyk et al., 2007) and bio-economic feasibility of pikeperch intensive rearing (Steenfeldt & Lund, 2008; Steenfeldt et al., 2010a,b) have been demonstrated. **Pikeperch demand has been strengthened** by the strong decline of wild catches from Russia, Estonia and Finland from 50,000 t in 1950 to 20,000 t currently (FAO, 2009). Over the last decade, 10 new farms have been built in Europe to produce pikeperch using RAS (Fontaine et al., 2012), producing an estimated 300-400 t (1st Workshop of the European Percid Fish Culture Group, 1 Sept 2012, Prague). Numerous more commercial operations have been designed and/or are under construction in Belgium, Czech Republic, Denmark, France, Germany, Hungary,

Italy, Poland, Portugal and the Netherlands. Year-round production of pikeperch requires constant high temperatures (24-26°C), which is only feasible in RAS to ensure relatively high growth rates (*i.e.*, **production of 1.2 kg fish in 15 -18 months** from non-selected strains). These RAS also allow high densities of 80-100 kg m^{-3} (Dalsgaard et al., 2013). Pikeperch flesh quality has a neutral taste, thus lending itself to different forms of preparation, and the filets are without bones --unlike carp, which competes on the same market segment. At present, pikeperch is sold either as whole fish at a weight of 600-3,000 g or as filets of 100-800 g to markets in Europe (mainly Western, Eastern and Northern areas)

Pikeperch bleeding.

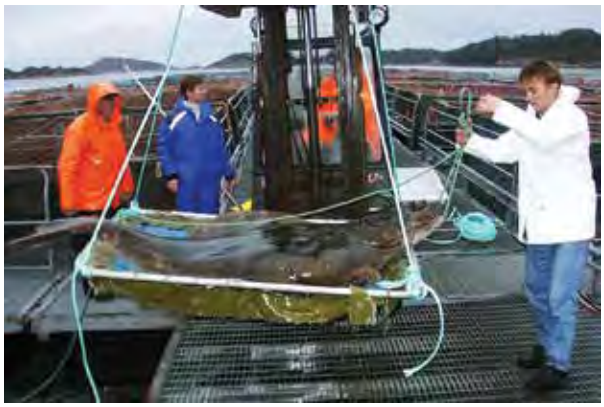
and North-America, showing strong demand. The market value is high at 8-11 € kg^{-1} at farm gate, whole fish.

Identified by a survey addressed to fish farmers in preparation for DIVERSIFY, the **major bottlenecks for further expansion of pikeperch** culture today include (a) **high sensitivity to stressors, handling and husbandry practices** that result in high and sudden mortalities, (b) **low larval survival** (typically 5-10%) and **high incidence of deformities**, and (c) **lack of knowledge of the genetic variability of the used broodstocks**. Identification of genetic relationships among different broodstocks, inbreeding phenomena and loss of heterozygosity is important in aquaculture, since it may result in subsequent reproductive and productive failure (reduced progeny survival, growth, food conversion efficiency and increased frequency of deformities). It is also important to know how the domesticated stocks differ from their wild counterparts, which could potentially be a future source of fish to include in effective breeding programs. Overcoming the above bottlenecks is very important to reduce production costs and, therefore, expand the aquaculture production of this species in the EU, and will be the objective of DIVERSIFY.

ATLANTIC HALIBUT

The **Atlantic halibut is the world's largest flatfish** and can attain a weight of over 300 Kg. It is **highly prized at markets worldwide**, but availability of wild Atlantic halibut is decreasing and the fish is classified as **endangered on the IUCN red list**. Two years ago a complete ban was imposed on Icelandic fisheries, and stocks along the Norwegian coast are declining and under strict regulation. This has led to a **higher market demand for Atlantic halibut than cannot be met by fisheries alone**. Cultured Atlantic halibut has an excellent reputation, but is rarely available outside specialty restaurants due to low annual production. The Atlantic halibut is a semi-fat fish rich in omega-3 fatty acids, with a characteristic flaky white meat with few bones. In terms of product diversification, Atlantic halibut is traditionally marketed as large fish steaks or cutlets. It can be smoked or marinated in the typical Scandinavian style. These characteristics led to the **inclusion of Atlantic halibut in DIVERSIFY, as a great candidate for fish species and product diversification in European aquaculture**.

Research and cultivation efforts of Atlantic halibut started in the 1980's, but the **total annual production of cultured Atlantic halibut is still only ~1,600 t** (Norwegian Directorate of Fisheries). In Europe, Atlantic halibut farms exist in Norway and Scotland. The desired market size is 5-10 kg and production time is currently 4-5 years. Despite a significant research effort between 1985 and 2000, the complicated life cycle of Atlantic halibut made aquaculture progress slow, and very little research funding has been allocated thereafter. However,



during this time slow but steady progress has been made by the farmers in order to improve production stability, and **interest in cage culture is growing**. The remaining **bottlenecks for increased and stable production are related to a steady supply of fry and a need to decrease the production time**. The latter may be achieved with the recent establishment of "all female" juvenile production (Babiak et al., 2012; Hendry et al., 2003). This is expected to have a major impact on production time as females grow faster and mature later –80% of slaughtered fish <5 kg are mature males (unpublished data). DIVERSIFY will address these important bottlenecks with a coordinated **research effort in reproduction, and larval nutrition and husbandry**.

WRECKFISH

Wreckfish is one of the largest Serranid species, **reaching a size of 100 Kg**. It is a deep-water fish found **almost throughout the world** and is characterized by an extended pelagic juvenile phase (Ball et al., 2000; Deudero et al., 2000; Sedberry et al., 1999). Wreckfish is one of the most interesting new species for aquaculture, due to its **fast growth** (Rodriguez-Villanueva et al., 2011; Suquet & La Pomélie, 2002), **late reproductive maturation** (Sedberry et al., 1999), **high market price and limited fisheries landings** --quotas have been reduced by 90% in 2012 in the U.S.A. (NOOA, www.fishwatch.com)-- and ease of manipulation in captivity (Papandroulakis et al., 2008; Rodriguez-Villanueva et al., 2011). Its large size lends itself to **processing and development of value added products**, and its **cosmopolitan distribution may enable** EU exports.

Wreckfish acclimatizes easily to captivity and, despite its large size, no mortalities have been reported due to handling. It accepts inert food easily, being a very voracious carnivore. In a recent study of wild-caught individuals it was shown that fish **grew from 1 kg to**

Stripping eggs from wreckfish.

5 kg in a period of 10 months (Rodriguez-Villanueva et al., 2011). The slow reproductive maturation of wreckfish, which occurs at an age of 5-10 y in captivity,

may be a problem for broodstock development and management. On the contrary, its **long juvenile stage is a great advantage from the aquaculture viewpoint**, allowing for commercialization before sexual maturity, and thus avoiding problems linked to maturation, such as reduction in growth, or loss of flesh quality and organoleptic properties.

Lack of reproduction control and of established larval rearing protocols are considered major bottlenecks preventing wreckfish aquaculture. Limited egg collection has been achieved from captive spawners using hormonal induction (Papandroulakis et al., 2008) or stripping of naturally maturing fish (Peleteiro et al., 2011). Embryonic development and the early life stages have been described (Papandroulakis et al., 2008, Peleteiro et al., 2011), indicating that the **large egg size of this fish (~2 mm in diameter) may offer significant advantages for its larval rearing**. Reproduction and larval rearing of a very close relative, the hapuku (*Polyprion oxygeneios*) has been achieved recently in New Zealand (Anderson et al., 2012). The **scarcity of broodstock is a disadvantage for this fish**, but the **clear biological and economical potential of this species justifies allocation of part of the effort of DIVERSIFY** in bringing together almost all partners involved so far in Europe in wreckfish domestication, to **overcome its documented bottlenecks --*i.e.*, reproduction and larval rearing--** in order to produce appropriate numbers of juveniles to launch commercial production.

Reading PIT tag.

Conducting an ovarian biopsy.

GREY MULLET

Farming of grey mullet has been practiced for centuries, but production of this potentially invaluable source of animal protein in Europe has been small and non-intensive (Nash & Koningsberg, 1981; Pillay, 1993). It is a **euryhaline species, found throughout the world** (Oren, 1981) and is a **rapid-growing, herbivorous species** that can be **reared over the wide geographical and temperature range of the Mediterranean basin**. As it is detritivorous in the wild, it has been stocked in fish ponds to improve sediment quality and avoid oxygen depletion (Milstein et al., 1991). Therefore, it can be an **excellent candidate for the enhancement of aquaculture in earthen ponds, coastal lagoons, "valli" and deserted Salinas** that exist throughout the EU Mediterranean countries. Hatchery produced juvenile females have been **grown to 1.9 kg in 2 years** on a fishmeal-containing pelleted feed. The development of fishmeal-free feed will reduce the cost of fish production, and will be **more sustainable and environmentally friendly**. In this way, grey mullet would be more acceptable to an increasingly aware consumer public that demands sustainability and lower environmental impact. Moreover, grey mullet aquaculture has the advantage of providing not only affordable whole fish and fillets, but also **fish roe ("bottarga" in Italian), a high value product (>100 € kg^{-1}),** whose market is expanding around the Mediterranean. Therefore, grey mullet has a great **biological and economical potential for fish species and product diversification, and development of value added products**.

A market for grey mullet is well established, though a niche one, in the Mediterranean. Even without any marketing effort by the aquaculture industry, the European market demand for grey mullet is likely to increase in the coming years, due to the demand from established and newly immigrant families originating from North Africa, Middle East and Asia. Currently, **the industry is a capture-based aquaculture, relying exclusively on capture of wild fry** (ca 1,000,000,000) that are subsequently grown out to market weight (600-1,200 g) in captivity, in lagoons or earthen ponds. The sustainability of such an activity is, of course, questionable, and the **future growth of the grey mullet aquaculture is limited by a number of bottlenecks**, which will be addressed in DIVERSIFY. Firstly, **controlling the reproductive cycle and improving egg quality** via broodstock management and nutrition is necessary not only for the production of robust larvae, but also for producing high value bottarga. Secondly, **development of a larval rearing protocol** is necessary to reduce early mortalities, size dispersion as well as increasing metamorphic synchrony, which will lead to a supply of high quality juveniles. Finally, development of a sustainable, economical, **fishmeal-free grow out feed** is needed, which would perform well under different environmental conditions of temperature, pond type, and water quality, thus broadening the geographical range of grey mullet aquaculture in Europe.

Socioeconomics

(including new product development)

Besides the technical improvement of the selected species, the socio-economic research in DIVERSIFY includes applied market development approach solutions on perception of aquaculture products, market demand, buyer preferences, new product development, value adding and market development. These outcomes will help the EU aquaculture sector and the supply industry in targeted marketing and improvement of its international competitive position.

Based on the development of the EU market and the demand characteristics, the following socioeconomic bottlenecks were identified during the preparation of DIVERSIFY:

- **Demand for seafood in the EU is increasing.** While the EU fisheries are stable or decreasing, the total EU demand for seafood is increasing. This increasing demand is currently fulfilled with imports from third countries. However, in order not to become overly dependent on seafood that is sourced in an increasingly competitive international market, it is important to introduce locally produced, sustainable and safe seafood to meet the demands of EU consumers.
- **EU consumer's negative attitude towards aquaculture fish and products**. This means that effective communication strategies have to be developed for the existing and newly developed products (new fish species and their value added products). This requires changing consumer perceptions and attitudes towards the entire aquaculture industry and range of products.
- **Demand for new aquaculture products in the EU market and subsequently in the world has to be developed**. New quality products for new markets have to be developed and targeted to potential market segments, in order to increase demand in the EU and world markets. New species have to be introduced in the market to diversify the aquaculture assortment, so that the risk of image loss of a specific species has lower market consequences for the whole sector.
- **Demand for European aquaculture products in the world markets has to be created**. Rising global consumption of aquaculture fish constitutes a great challenge and opportunity for the EU aquaculture industry. DIVERSIFY's species, cultured with sustainable methods and leading to high added-value products, can be a driver for growth of the market share of EU aquaculture in local and global markets.
- The range and **added value of the aquaculture products has to increase**. Consumers ask for more convenient products in the seafood market. In addition, the added value and cost price of the products have to be positioned in relation to other protein sources. This requires that the price elasticity of fish must be related to the price elasticity of other protein sources. In addition, additional value of European aquaculture products has to be implemented in chain revenue models that lead to a better livelihood for aquaculturists.
- The **sustainability of the aquaculture sector has to be improved further**, as sustainable fish products are requested more and more by EU and global consumer segments, industrial buyers and regulators; at the same time, investing on a sustainable image of the EU aquaculture will create a competitive advantage for the EU aquaculture industry. This requires that technological innovations have to be achieved, which are driven by market demand (consumers and retailers) and sustainability demands of NGO's.

All the above aspects underline that the image of the aquaculture sector has to be improved. New, sustainable --and high added value-- products with a longer shelf life have to be developed and SME's have to be more innovative for the introduction and market development of these new species.

Each of the species selected for DIVERSIFY has the potential to grow in the market and to be perceived as an added value product, and their **biological and economical potential is expected to stimulate the growth of the European aquaculture sector**. The economic potential of each species in relation to the socioeconomic bottlenecks, and the actions planned in DIVERSIFY to overcome them are:

Meagre is a **large fish with excellent taste**. As it is rather rare in fishery captures in the Mediterranean, it is not well known by consumers and the European market is still a niche product. Market development and consumer acceptance of relative species is done successfully in Japan, Australia and the USA. **Market development is imperative for the EU** and should focus mainly on consumer and retail awareness, and a better positioning with regard to gilthead sea bream and European sea bass. **New product** development could support market development.

The **greater amberjack** is a **large fish with high flesh quality and market value**. In addition to its economic potential in the EU market, cultured greater amberjack has a significant potential for exports, as it is distributed worldwide, and congener species are produced commercially elsewhere. This cultured fish **has proven its potential in** other markets. In Europe, there has recently been an intense interest from the aquaculture sector for this species, but production levels are miniscule. Therefore, **a consumer oriented market introduction of cultured amberjack is necessary**. Also, **market development is necessary for growth** with preservation of the added value and price, once production increases.

Pikeperch is a medium size freshwater fish, with a **good taste and a high market value**. There is already a market in Europe and North America, showing strong demand. The production capacity of this fish is expected to grow fast in the coming years. To keep up the high market value, **product development and market development is necessary** for coordinated growth. Therefore, potential markets and consumer segments have to be identified to maintain or increase the added value.

Atlantic Halibut is a **large fish with a very good reputation** in the north European market and a high market value. Demand exceeds the current production capacity. Market and product development is not necessary for the short run, but a **market development strategy** for the long run is necessary, because new entrants in the market can be expected given the added value of Atlantic halibut.

Wreckfish is a **large fish with excellent flesh**, but not available as a cultured fish. It is distributed throughout the world and products from the capture fishery are highly regarded. A very close relative is produced experimentally in New Zealand, where it is considered one of the best marine fishes. Because of this potential excellence, wreckfish could be interesting for the European market. For this species, technical bottlenecks have to be solved first. So, only **market positioning in relation to other species is necessary** for the short run, and for the long run the **market potential will be identified**.

Grey mullet is **a medium size herbivorous fish**, **cultured extensively throughout the world, but often not well regarded by consumers**. It has a niche market in the Mediterranean for its flesh and high priced roe. Due to its good taste and low cost of rearing, grey mullet could have large potential market all over Europe, especially within segments of population of North African, Middle Eastern or Asian origin. **Market and new product development** are necessary for growth in the middle-long run in the native European market and the immigrant market.

To cover these market bottlenecks, DIVERSIFY gives a central role to positioning of species, and market and product development. In the first year a competitive market and environment analysis will be done and a study on consumer preferences with regard to cultured fish will be undertaken. Both studies are the basis for the development of new product prototypes, which will be developed in this project. These prototypes will be tested on food safety, preservation and market acceptance by consumers. Communication research will find out ways to overcome the negative image of aquaculture fish. The outcomes of all these studies will be the basis for the business plans per species, which will be developed together with the partner SMEs.

The combination of biological, technological and socioeconomic research activities planned in DIVERSIFY are expected to **support the diversification of the aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets**.

"This work has been carried out with financial support from the Commission of the EuropeaCommunities, specific RTD programme of Framework Programme 7 (FP7-KBBE-2013-07 single stage, GA 603121 DIVERSIFY). It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area."

SEVENTH FRAMEWORK PROGRAMME

www.diversifyfish.eu

References

Andaloro, F., Pipitone, C., 1997. Food and feeding habits of the amberjack, *Seriola dumerili,* in the Central Mediterranean Sea during the spawning season. Cahier de Biology Marine 38: 91-96.

Anderson, S.A., Salinas, I., Walker, S.P., Gublin, Y., Pether, S., Kohn, Y.Y., Symonds, J.E., 2012. Early development of New Zealand hapuku *Polyprion oxygeneios* eggs and larvae. Journal of Fish Biology 80: 555-571.

Anonymous, 2008. Innovative Methodologies for the reproduction and larval rearing of fast growers. Final Report. OPF 2000-2006 Measure 4.6, 66 pp.

Babiak, J., Babiak, I., Harboe, T., Haugen, T., van Nes, S. and Norberg, B., 2012. Induced sex reversal using an aromatase inhibitor, Fadrozole, in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 324-325: 276-280.

Ball, A.O., Sedberry, G.R., Zatcoff, M.S., Chapman, R.W., Carlin, J.L., 2000. Population structure of wreckfish *Polyprion americanus* determined with microsatellite genetic markers. Marine Biology137: 1077-1090.

Crespo, S., Grau, A., Padrós, F., 1994. The intensive culture of 0+ amberjack in the western Mediterranean is compromised by disease problems. Aquaculture International 2: 1–4.

Cummings, N.J., Turner, S.C., McClellan, D.B., Legault, C.M., 1999. Atlantic greater amberjack abundance indices from commercial handline and recreational charter, private, and headboat fisheries through fishing year 1997. National Oceanic and Atmospheric Sciences, 77 pp.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and further perspectives. Aquacultural Engineering. 53: 2-13.

Deudero, S., Morales-Nin, B., 2000. Ocurrence of *Polyprion americanus* under floating objects in western Mediterranean oceanic waters, inference from stomach contents analysis. Journal of the Marine Biological Association of the UK. 80: 751-752.

Duncan, N.J., Estévez, A., Fernández-Palacios, H., Gairin, I., Hernández-Cruz, C.M., Roo, J., Schuchardt, D., Vallés, R., 2013. Aquaculture production of meagre (*Argyrosomus regius*): hatchery techniques, ongrowing and market. In: Allan, G., Burnell, G. (Eds.), Advances in aquaculture hatchery technology. Woodhead Publishing Limited, Cambridge, UK.

Duncan, N., Estévez, A., Porta, J., Carazo, I., Norambuena, F., Aguilera, C., Gairin, I., Bucci, F., Valles, R., Mylonas, C.C., 2012. Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatized to captivity. Fish Physiology and Biochemistry 38: 1273-1286.

Elkesh, A., Kantham, K.P.L., Shinn, A.P., Crumlish, M., Richards, R.H., 2012. Systemic nocardiosis in a Mediterranean population of cultured meagre, *Argyrosomus regius* Asso (Perciformes: Sciaenidae). Journal of Fish Diseases. 36: 141-149.

FAO, 2009. The state of the world fisheries and aquaculture 2008. FAO, OUN. Rome, 216 pp.

FAO, 2012. The State of World Fisheries and Aquaculture: 2012. Rome: Food and Agriculture Organization of the United Nations. 209 pp.

Fernández-Palacios, H., Schuchardt, D., Roo, J., Hernández-Cruz, C.M., Izquierdo, M., 2013 (in press). Multiple GnRHa injections to induce successful spawning of wild caught greater amberjack (Seriola dumerili) matured in captivity. Aquaculture Research DOI: 10.1111/are.12330

Fontaine, P., Wang, N., Teletchea, F., 2012. Domestication of new species and diversification in inland aquaculture, the example of Percid fish. Third workshop on fish culture, 3-4th July, Paris, France (in French).

Grau, A., Crespo, S., Pastor, E., Gonzalez, P., Carbonell, E., 2003. High infection by *Zeuxapta seriolae* (Monogenea: Heteraxinidae) associated with mass mortalities of amberjack *Seriola dumerili* Risso reared in sea cages in the Balearic Islands (western Mediterranean). Bulletin of the European Association of Fish Pathologists 23: 139–142.

Haffray, P., Malha, R., Sidi, M.O.T., Prista, N., Hassan, M., Castelnaud, G., Karahan-Nomm, B., Gamsiz, K., Sadek, S., Bruant, J.S., Balma, P., Bonhomme, F., 2012. Very high genetic fragmentation in a large marine fish, the meagre *Argyrosomus regius* (Sciaenidae, Perciformes): impact of reproductive migration, oceanographic barriers and ecological factors. Aquatic Living Resources 25: 173-183.

Hendry, C.I., Martin-Robichaud, D.J., Benfey, T.J., 2003. Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 219: 769-781.

Jerez, S., Samper, M., Santamaría, F.J., Villamados, J.E., Cejas, J.R., Felipe, B.C., 2006. Natural spawning of greater amberjack (*Seriola dumerili*) kept in captivity in the Canary Islands. Aquaculture 252: 199-207.

Jover, M., Garcia-Gomez, A., Tomas, A., De la Gandara, F., Pérez, L., 1999. Growth of Mediterranean yellowtail (*Seriola dumerilii*) fed extruded diets containing different levels of protein and lipid. Aquaculture 179: 25-33.

Lovatelli, A., Holthus, P.F., 2008. Capture-based aquaculture; Global overview. Food and Agriculture Organization of the United Nations, Rome, 298 pp.

Koyuncu, C. E., Castro Romero, R., Karaytug, S., 2012. *Lernanthropus indefinitus* N. Sp (Copepoda, Siphonostomatoida, Lernanthropidae) parasitic on *Argyrosomus Regius* (Asso, 1801) (Pisces, Sciaenidae). Crustaceana 85: 1409-1420.

Kozul, V., Skaramuca, B., Glamuzina, B., Glavic, N., Tutman, P., 2001. Comparative gonadogenesis and hormonal induction of spawning of cultured and wild Mediterranean amberjack (*Seriola dumerili*, Risso 1810). Scientia Marina 65: 215-220.

Kucharczyk, D., Kestemont, P., Mamcarz, A., 2007. Artificial reproduction of pikeperch. Practical manual, Polish Ministry of Science, 80 pp.

Ma, Z., Qin, J.G., Hutchinson, W., Chen, B.N., 2012. Food consumption and selectivity by larval yellowtail kingfish *Seriola lalandi* cultured at different live feed densities. Aquaculture Nutrition.

Matsunari, H., Hashimoto, H., Oda, K., Masuda, Y., Imaizumi, H., Teruya, K., Furuita, H., Yamamoto, T., Hamada, K., Mushiake, K., 2012. Effects of different algae used for enrichment of rotifers on growth, survival, and swim bladder inflation of larval amberjack *Seriola dumerili*. Aquaculture International 20: 981-992.

Mazzola, A., Favaloro, E., Sara, G., 2000. Cultivation of the Mediterranean amberjack, *Seriola dumerili* (Risso, 1810), in submerged cages in the Western Mediterranean Sea. Aquaculture 181: 257-268.

Merella, P., Cherchi, S., Garippa, G., Fioravanti, M.L., Gustinelli, A., Salati, F., 2009. Outbreak of *Sciaenacotyle panceri* (Monogenean) on cage-reared meagre *Argyrosomus regius* (Osteichthyes) from the western Mediterranean Sea. Diseases of Aquatic Organisms 86: 169-73.

Milstein, A., Alkon, A., Avnimelech, Y., Kochba, M., Hulata, G., Schroeder, G., 1991. Effects of manuring rate on ecology and fish performance in polyculture ponds. Aquaculture 96 (2): 119-138.

Monfort, M.C., 2010. Present market situation and prospects of meagre (*Argyrosomus regius*), as an emerging species in Mediterranean aquaculture, Studies and Reviews, General Fisheries Commission for the Mediterranean No. 89, FAO, Roma, pp. 28.

Montero, F.E., Crespo, S., Padrós, F., De la Gándara, F., García, A., Raga, J.A., 2004. Effects of the gill parasite *Zeuxapta seriolae* (Monogenea: Heteraxinidae) on the amberjack *Seriola dumerili* Risso (Teleostei: Carangidae). Aquaculture 232: 153–163.

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

Mylonas, C.C., Mitrizakis, N., Castaldo, C., Cerviño, C., Papadaki, M., Sigelaki, I., 2013a. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality. Aquaculture 414-415: 318-327.

Mylonas, C.C., Mitrizakis, N., Papadaki, M., Sigelaki, I., 2013b. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle. Aquaculture 414-415: 309-317.

Nakada, M., 2000. Yellowtail and related species culture. In: Stickney, R. (Ed.), Encyclopedia of Aquaculture, Wiley, pp. 1007–1036.

Nash, C.E., Koningsberg, R.M., 1981. Artificial propagation. In: Oren, O.H. (ed.), Aquaculture of Grey Mullets, Cambridge University Press, pp. 265-312.

Oren, O.H., 1981. Aquaculture of Grey Mullets, Cambridge University Press, 506 pp.

Papadakis, I., Kentouri, M., Divanach, P., Mylonas, C.C., 2013. Ontogeny of the digestive system of meagre *Argyrosomus regius* reared in a mesocosm, and quantitative changes of lipids in the liver from hatching to juveniles. Aquaculture 388-391: 76-88.

Papandroullakis, N., Mylonas, C.C., Syggelaki, E., Katharios, P., Divanach, P., 2008. First reproduction of captive-reared wreckfish (*Polyprion americanus*) using GnRHa implants. Aquaculture Europe 08, September 15-18, Krakow, Poland, European Aquaculture Society Special Publication 37, pp. 507-508.

Papandroulakis, N., Mylonas, C.C., Maingot, E., Divanach, P., 2005. First results of greater amberjack (*Seriola dumerili*) larval rearing in mesocosm. Aquaculture 250: 155–161.

Papandroulakis, N., Suquet, M., Spedicato, M.T., Machias, A., Fauvel, C., Divanach, P., 2004. Feeding rates, growth performance and gametogenesis of wreckfish (*Polyprion americanus*) kept in captivity. Aquaculture International 3: 1-13.

Peleteiro, J.B., Saavedra, C., Perez-Rial, E., Soares, E.C., Álvarez-Blázquez, B., Vila, A., 2011. Diversificación de especies en acuicultura marina. Desarrollo de técnicas de cultivo de la cherna (*Polyprion americanus*). XIII Congreso Nacional de Acuicultura, Castelldefels, Barcelona, Spain.

Pillay, T.V.R., 1993. Aquaculture. Principles and Practices. Fishing News Books, Oxford, UK, 575 pp.

Rigos, G., Katharios, P., 2010. Pathological obstacles of newly-introduced fish species in Mediterranean mariculture; a review. Reviews in Fish Biology and Fisheries 20: 47-70.

Rigos, G., Pavlides, M., Divanach, P., 2001. Host susceptibility to *Cryptocaryon sp*. infection of Mediterranean marine broodfish held under intensive culture conditions: a case report. Bulletin of the European Association of Fish Pathologists 21: 33-36.

Rodríguez-Barreto, D., Jerez, S., Cejas, J.R., Martin, M.V., Acosta, N.G., Bolaños, A., Lorenzo, A., 2012. Comparative study of lipid and fatty acid composition in different tissues of wild and cultured female broodstock of greater amberjack (*Seriola dumerili*). Aquaculture 360–361: 1–9.

Rodriguez-Villanueva, J.L., Peleteiro, J.B., Perez-Rial, E., Soares, E.C., Álvarez-Bláquez, B., Mariño, C., Linares, F., Mañanós, E., 2011. Growth of wreckfish (*Polyprion americanus*) in Galicia, Spain. Aquaculture Europe 2011 (EAS), 18-21 October, Rhodes, Greece.

Roo, F. J., Hernández-Cruz, C.M., Borrero, C., Shuchardt, D., Fernandez-Palacios, H., 2010. Effect of larval density and feeding sequence on meagre (*Argyrosomus regius*; Asso, 1801) larval rearing. Aquaculture 302: 82-88.

Roo, J., Fernández-Palacios, H., Hernández-Cruz, C.M., Mesa-Rodriguez, A., Schuchardt, D., Izquierdo, M., 2012 (in press). First results of spawning and larval rearing of longfin yellowtail *Seriola rivoliana* as a fast-growing candidate for European marine finfish aquaculture diversification. Aquaculture Research. doi: 10.1111/are.12007.

Sedberry, G.R., Andrade, C.A.P., Carlin, J.L., Chapman, R.W., Luckhurst, B.E., Manooch, C.S. III, Menezes, G., Thomsen, B., Ulrich, G.F., 1999. Wreckfish *Polyprion americanus* in the North Atlantic: fisheries, biology and management of a widely distributed and long-lived fish. American Fisheries Society Symposium. Life in slow lane: ecology and conservation of long lived marine animals, 23: 27-50.

Steenfeldt, S., Lund, I., Höglund, E., 2010a. Is batch variability in hatching time related to size heterogeneity and cannibalism in pikeperch (*Sander lucioperca*)? Aquaculture Research 42(5): 727-732.

Steenfeldt, S.J., Vestergaard, M., Overton, J.L., Lund, I., Paulsen, H., Larsen, V.J., Henriksen, N.H., 2010b. Further development of intensive pikeperch rearing in Denmark. DTU Aqua Research Report no. 228-2010, Technical University of Denmark, Denmark (in Danish).

Steenfeldt, S.J., Lund, I., 2008. Development of methods of production for intensive rearing of pikeperch juveniles. DTU Aqua Research Report no. 199-2008, Technical University of Denmark, Denmark (in Danish).

Suquet, M., La Pomèlie, Ch., 2002. Le cernier (*Polyprion americanus*): biologie, pêche, marché et potentiel aquacole. Plouzané: IFREMER, cop. 2002. 279 h. (Ressources de la mer). ISBN 2-84433-075-4.

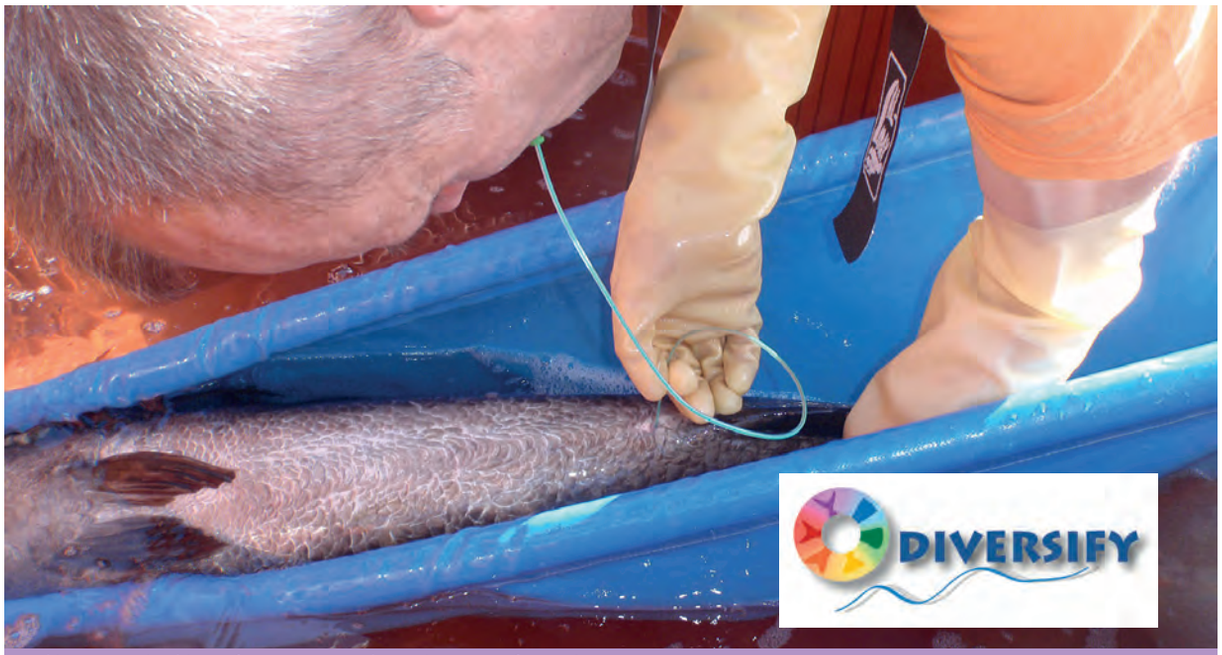
Ternengo, S., Agostini, S., Quilichini, Y., Euzet, L., Marchand, B., 2010. Intensive infestations of *Sciaenocotyle pancerii* (Monogenea, Microcotylidae) on *Argyrosomus regius* (Asso) under fish-farming conditions. Journal of Fish Diseases 33: 89–92.

Toksen, E., Buchmann, K., Bresciani, J., 2007. Occurrence of *Benedenia sciaenae* van Beneden, 1856 (Monogenea: Capsalidae) in cultured meagre (*Argyrosomus regius* Asso, 1801) (Teleost: Sciaenidae) from western Turkey. Bulletin of the European Association of Fish Pathologists 27(6): 250.

Wang, N., Milla, S., Fontaine, P., Kestemont, P., 2008. Abstracts of the Percid fish culture workshop: From research to production, January 23-24, Namur, Belgium.

Vallés, R., Estévez A. 2011. Effect of difrerent DHA concentrations on the growth and survival of megre (*Argyrosomus regius*) larvae under intensive culture. XIII National Congress of Aquaculture, Casteldefells, Spain

Vallés, R., Estévez, A., 2013. Light conditions for larval rearing of meagre (*Argyrosomus regius*). Aquaculture 376-379: 15-19.



Taking an ovarian biopsy from meagre to determine stage of maturity.

ADVANCES IN MEAGRE *(Argyrosomus regius)* RESEARCH DURING THE FIRST YEAR OF THE PROJECT "DIVERSIFY"

CONSTANTINOS MYLONAS AND ROCIO ROBLES

During the first year of the DIVERSIFY project (Dec 2013-Nov 2014), a variety of research activities have been undertaken with meagre, and a summary of the most relevant results is provided below.

REPRODUCTION

An evaluation of the genetic variation of a large number of the available captive meagre broodstocks of 13 research institutes and SMEs from 7 European countries has been carried out by Fundacion Canaria Parque Cientifico Tecnologico de la Universidad de las Palmas de Gran Canaria (FCPCT, Spain, Dr. J.M. Afonso) using 2 multiplexes of 18 microsatellite markers. The examined broodstocks, as a whole, appeared to originate from three different populations and sufficient genetic variation exists to form a base population for a breeding program (Fig. 1). However, care will be needed in selecting families within each broodstock and an increase in the number of families is recommended, in order to avoid problems and ensure improvement of desirable traits.

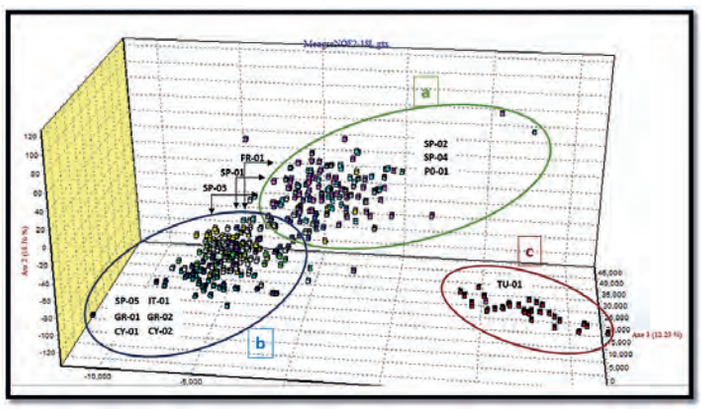


Fig 1.- Factorial Correspondence Analysis from 18 loci and 376 fish distributed in 13 Mediterranean meagre broodstocks maintained in captivity for research or aquaculture production, showed three different original populations.

continued on page 6

Updated findings of all of the species involved in the DIVERSIFY project will be presented in a session entitled "New and Emerging Finfish Species" at Aquaculture Europe 2015 in Rotterdam

aquaculture europe 15

Paired crossings with six pairs of females and males were carried out in the Institute de Recerca I Technologia Agroalimentáries (IRTA, Spain, Dr. N. Duncan). Spawning was induced with GnRHa injections (15 µg Kg^{-1} for females and 7.5 g kg^{-1} for males) every 7-10 days. Breeders that did not spawn after 2-3 induced spawning attempts were replaced. A total of 41 different pairs were induced to spawn, of which 10 pairs produced >500,000 eggs, 16 pairs produced >250,000 eggs and 19 pairs produced >100,000 eggs that hatched (Fig. 2). Poor spawning results were not caused by maturity status, repeated spawning or inductions, and different individuals had clear differences in egg production and quality.

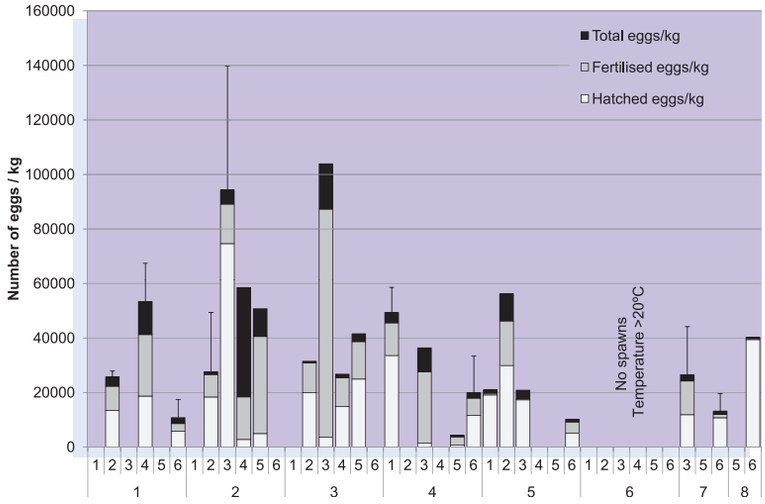


Fig 2.- Mean (± SD) daily fecundity of meagre in response to multiple (8) GnRHa injections. Total number of eggs was multiplied by percentage fertilization and hatch to determine the number of fertilized eggs and larvae produced.

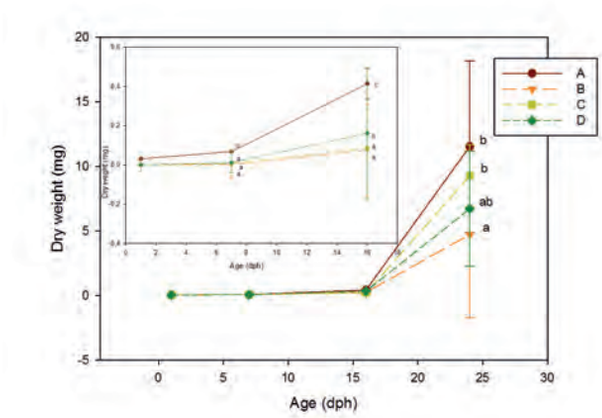
An additional experiment was also carried out at the Hellenic Center for Marine Research (HCMR, Greece, Dr. C.C. Mylonas) with four pairs of breeders to determine how many successful spawns can be produced in response to consecutive weekly injections of GnRHa. Up to 17 consecutive spawns were obtained with high quality eggs that had >80% hatching success and larval survival to 5 days post hatch. These two trials demonstrated that paired spawning of high quality eggs is possible, and the method could be used in breeding selection programs.

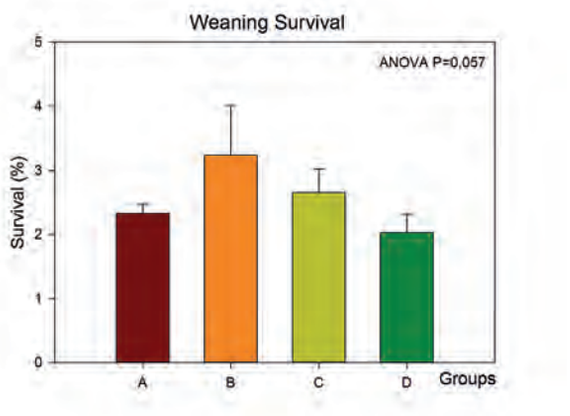
LARVAL CULTURE

A weaning assay was carried out in IRTA (Dr. A. Estevez) to advance the time for weaning in meagre. Larvae were weaned either at age 12, 15 and 20 (the usual age) days post hatch (dph) using half the amounts of enriched *Artemia* metanauplii and a commercial weaning diet (Gemma Micro, Skretting). Growth (Fig. 3), survival rate (Fig. 4), fatty acid composition as well as digestive system development (histology and enzymes) were analysed. A high incidence of cannibalism was detected from day 12 dph onwards, resulting in very low survival (2-3.3%). The experiments will be repeated in 2015 and several new approaches will be taken, including increasing the photoperiod to give more chances of the fish to eat the weaning diet or increase the initial stocking density.

NUTRITION

A trial was conducted by FCPCT (Dr. L. Robaina) to investigate the requirements of meagre larvae for n-3 HUFA in relation with vitamin E (vit E) and vitamin C (vit C). After feeding the larvae with combinations of different levels of n-3 HUFA (0.5 and 3.5%) and vit E and vit C (150 vit E + 180 vit C, 300 vit E + 180 vit C and 300 vit E + 360 vit C) from day 14 to 28 dph, results showed a clear improvement in growth when dietary n-3 HUFA levels were 3.5%, whereas the effects of vit E or vit C and the interaction between both nutrients and the n-3 HUFA levels were not significant. Regarding biochemical composition, larval contents of n-3 HUFA reflected clearly dietary levels (table 1), being significantly higher in larvae fed fish oil, and elevation of dietary n-3 HUFA and vit E + vit C tended to increase larval lipid contents. Study of larval foregut histological characteristics showed that larvae fed 0.5% HUFA presented very pigmented enterocytes with centered nucleoli and very little lipid vacuoles, while larvae fed higher levels of dietary HUFA, such as in the 3.5/150/180 combination, showed larger





Figs. 3 and 4: Growth (dry weight in mg) and survival (%) of larvae of the different groups after weaning.

Table 1.- Culture performance and morphometric parameters of meagre larvae (initial total length 4.07±0.26 mm and dry weight 0.058±0.01 mg) fed early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels from 14 dph to 28 dph.

	Diet					
	0.5/150/180	0.5/300/180	0.5/300/360	3.5/150/180	3.5/300/180	3.5/300/360
Total length (24 dah)	4.8±0.44b	5.0±0.39a	4.9±0.40ab	5.0±0.45a	5.0±0.48a	5.1±0.38a
Total length (28 dah)	5.2±0.46ab	5.2±0.43ab	5.1±0.51ab	5.3±0.44a	5.0±0.31b	5.3±0.59a
Dry weight (24 dah)	0.19±0.04c	0.21±0.02bc	0.20±0.03bc	0.21±0.02bc	0.22±0.02ab	0.24±0.03a
Dry wieght (28 dah)	0.23±0.02	0.21±0.04	0.21±0.03	0.27±0.05	0.23±0.05	0.24±0.04
Survival (%)	12.1±4.9	8.0±5.2	15.1±4.1	14.2±8.3	16.7±3.5	15.2±7.7

* Values (mean ± standard deviation) with the same leters are not significantly different; ANOVA. P_{Length} <0.01; P_{Weight} <0.05.

and more developed enterocytes containing lipid vacuoles around the nucleous, reflecting the higher lipid absorption activity. These results suggest that there is a high requirement of this species for n-3 HUFA to promote growth, and vit E and vit C to prevent fatty acid oxidation during larval stages. Thus, weaning diets for meagre larvae must be supplemented with increased n-3 HUFA, vit E and vit C in order to be improved. Selected diets were used to conduct studies on resistance to handling stress, stress bio-markers such as gene expression of HSPs (FCPCT), specific fish behaviour, evaluation of metabolic cost after sub-lethal stress, video analysis of activity, escape responses and sensory acuity (Danmarks Tekniske Universitet, Denmark, Dr. I. Lund) and digestive enzyme (protease, amylase and lipase) and gut ATPase activities (University of La Laguna, Spain, Dr. C. Rodriguez).

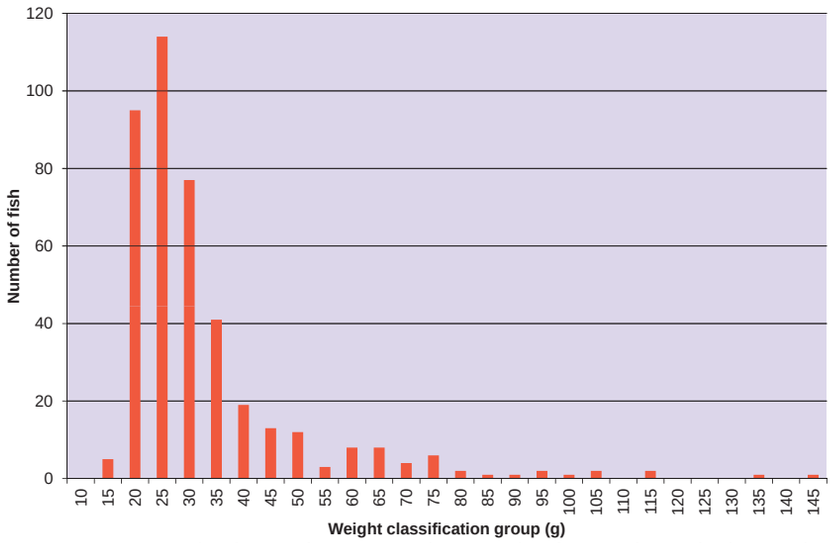


Fig 5.- Frequency distribution of meagre in each 5-g size classification. The weight shown is the upper value of the classification, for example classification 15 g contains fish from 10.1 to 15 g.

In the following years, the essential fatty acid requirements will be examined in grow out diets (Skretting Aquaculture Research Center, Norway, Dr. R. Fontanillas) for meagre by feeding six levels of docosahexaenoic, eicospentaenoic and arachidonic acids (FCPCT). During the last three months of 2014, information on the nutritional requirements of meagre have been collected and a basal diet formulation for grow out has been defined.

ONGROWING

Size variability in juvenile meagre during pre-grow out makes regular grading essential to avoid cannibalism, and grades of smaller fish may be related to poor performance when transferred to sea cages. Experiments were carried out by IRTA using meagre juveniles of a mixture of 5 known families, to simulate the commercial hatchery situation and in order to study differences in growth rate. Juveniles were separated into three size grades, and were stocked into tanks at the same initial density and fed the same commercial diet. After 4 months the distribution of all the size grades across the different tanks / grades was compared and 70% of the population was observed to be in the size range of 15-30 g (Fig 5).

The population was skewed to larger fish with 30% of the population in the range of 30-145 g and this wide dispersion of sizes made management difficult. The normally distributed 70% of the population was graded into three grades of 73 large fish (25-30 g), 89 medium fish (20-25 g) and 86 small fish (15-20 g) and growth was monitored.

A random sample of 50 fish from each group was weighed and measured (length) every 3 weeks. The large fish have grown from 27.2±1.5 g to 113±21.0 g, medium fish have grown from 22.7±12.2 g to 94.2±19.8 g and small fish have grown from 17.9±1.8 g to 71.6±31.31 g (Fig. 6). On all sample dates there have been significant differences (ANOVA, P<0.05) between the grades, and the fish in each group have grown significantly during the study (ANOVA, P<0.05). The different size grades appeared to have very similar growth potential. The trial finished on 11th December 2014 and the fish will be characterised genetically for parentage assignment (HCMR, Dr. C. Tsigenopoulos) to establish if differences in growth were a consequence of genetic origin.

The effect of cage depth on meagre grow out was studied by HCMR. The trial started in May 2014 using cages of 180- m^3 (6x6x5 m, Shallow) and 290-m3 (6x6x8 m, Deep) at the Souda Bay pilot cage farm (Dr. N. Papandroulakis) in duplicates, and juveniles

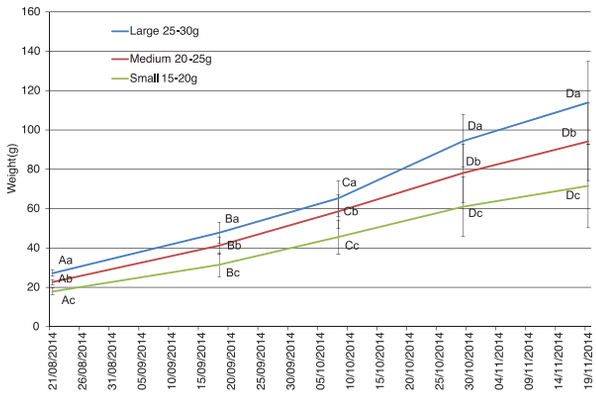


Fig 6.-Mean (± SD) wet weight of meagre classified to three grades as large (initially 25-30 g), medium (initially 20-25 g) and small (initially 15-20 g). Capital letters represent significant differences (P<0.05) between sample dates for the same size grade. Lower case letters represent significant differences (P<0.05) between size grades on the same sample date.

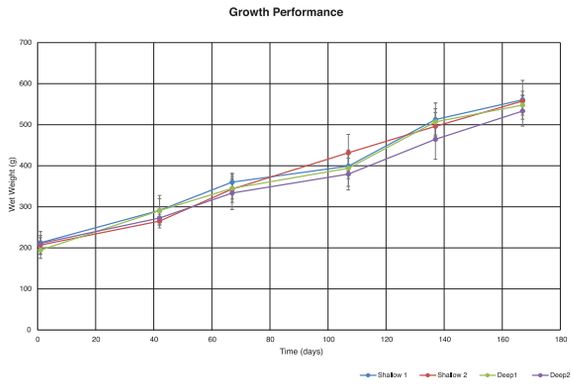


Fig 7.- Mean (±SD) growth performance of meagre at the Souda Bay pilot cage farm (HCMR).

Monitoring growth of meagre juveniles

obtained from the HCMR hatchery. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery. Juveniles of 2 g were transferred at the cage facility and they were reared under similar conditions until the beginning of the trial. Then, 4 groups were created, two of ~5,150 fish for the 180-m3 cages and two of ~8,240 fish for the 290-m3 ones. The wet weight at the beginning of the trial was 200±20 g. During the experiment, growth performance was estimated with monthly samples (Fig. 7).

Every second month, blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid, free fatty acids), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. The samples are currently being analyzed.

The vertical distribution in cages was monitored using an echo integrator. Although a technical problem has not allowed the monitor during the first month of the trial, an upgraded system (CageEye 1.3, Lindem Data Acquisition AS, Norway) was installed in June 2014 and the trial was implemented as planned without further alterations. The analysis of the data is not completed yet, but an interesting observation has been made already. The vertical distribution of meagre shown for a period of 3 days (Fig. 8), demonstrates clearly that the fish are located mostly at the lower half of the cage for a period of ~12 hours, while the rest of the period are distributed almost homogeneously in the whole available volume of the cage. This observation is independent of the cage depth and it is correlated with the light and dark period of the day. To our knowledge this is the first time that such behavior has been observed.

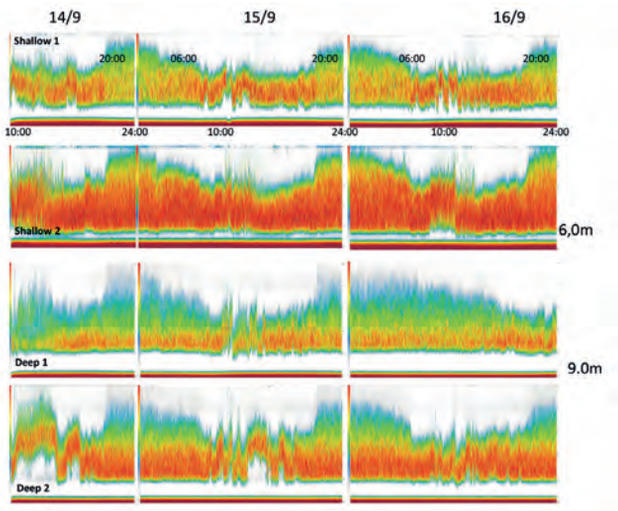


Fig 8.- Vertical distribution of meagre in the experimental cages for a period of 3 days.

HEALTH

Meagre were sampled for collecting data on specific growth rate and to collect chronological samples for the immune ontogeny study. Duplicate sets of samples were collected at each time point (Fig. 9); one set was fixed in formalin for histological analysis, and a second set was collected in RNAlater for extraction of RNA to be used in gene expression analysis. As fish became more developed and organ tissues were recognized easily, individual tissue samples were collected in formalin and RNAlater. Tissues collected included spleen, head kidney, gills and intestine. Samples for immune gene expression analysis are being stored at -80°C. The original plan was to collect animals that were of a medium size, as well as animals from the larger end of the growth spectrum to see how differential growth may lead to premature immune maturation. We eliminated this idea due to a reduction in the overall size of the population. The original population was diminished greatly due to cannibalism during the growout.

A search of the online database GenBank was performed to identify and collect existing sequences

for genes of interest from extant marine teleost species for the study of the immune system. The sequences collected were used for the preparation alignments for designing degenerate/consensus primers for amplification from cDNA of meagre tissues. Samples for the preparation of RNA and subsequent synthesis of cDNA for preparation of these gene expression asssays (table 2) has already been done during the growout period of fish being used in the earlier experiment. All of this process for isolation of gene sequences and development of the specific gene expression assays were initiated in the first quarter of 2015. ■

Co-funded by the Seventh Framework Programme of the European Union

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from the project site at "www.diversifyfish.eu".

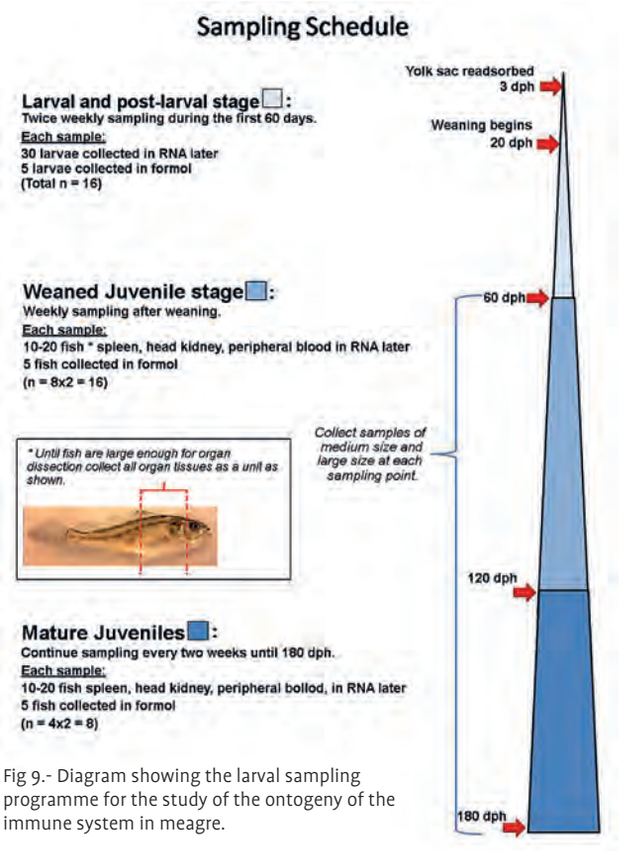


Fig 9.- Diagram showing the larval sampling programme for the study of the ontogeny of the immune system in meagre.

Table 2.- Genes targeted for characterization of the immune system of meagre. The unknown gene sequences should provide amplicon sizes approximating those shown, if there exists a high degree of conservation between species. These estimates are based upon data from existing sequences found in GenBank.

	Target Gene	Degenerate/ Consensus Primers	Amplicon size
Endogeneous Controls	EF1 (Elongation Factor)	X	230
	GAPDH (Glyceraldehyde Phosphate Dehydrogenase)	X	239
	18S	X	-
Innate Immunity	Piscidin1 ("Defensin")	X	110
	Piscidin2 ("Defensin")	-	-
	Piscidin3 ("Defensin")	-	-
	Lysozyme	X	220
	Metallothionein	X	80
	MX protein	X	570
	NOD2 (Toll Like Receptor - TLR)	X	1390
Adaptive Response	RAG1 (Recombination Activating Gene)		
	IgM		
	IgT		
	TcR (T-cell Receptor)		
	C3 (complement)	X	1202
	TNFa (Tumor Necrosis Factor)	X	250
	IFN alpha (interferon)		
	IFN gamma		
	IL-1beta (Interleukin)		
	IL-2		
	IL-4		
	IL-10		
	IL-17		
	IL-22		
Inflammatory Response	COX2 (cyclooxygenase 2)	X	1500
	MyD88 (myeloid differentiating factor)	X	130

Advances in pikeperch (*Sander lucioperca*) research during the first 18 months of the project

PASCAL FONTAINE
(UNIVERSITY OF LORRAINE)

COSTAS TSIGENOPOULOS
(HELLENIC CENTER FOR MARINE RESEARCH)

PATRICK KESTEMONT
(UNIVERSITY OF NAMUR)

IVAR LUND
(TECHNICAL UNIVERSITY OF DENMARK)

Pikeperch (*Sander lucioperca*) is a promising emerging fish species for intensive freshwater aquaculture, based on Recirculation Aquaculture Systems (RAS). During the first 18 months of the DIVERSIFY project, a number of studies have been initiated (a) to obtain knowledge in support of future breeding programs and (b) to solve the major bottlenecks identified previously by fish farmers (*e.g.*, cannibalism during larval rearing and stress sensitivity). This article presents a summary of the main results obtained so far.

GENETICS

The first genetic work was organized under the responsibility of Dr C. Tsigenopoulos (Hellenic Center for Marine Research, Greece). The primary objective was to use genetic markers (microsatellite loci) to evaluate the genetic variability of some wild pikeperch populations in comparison to the variability of captive broodstocks in commercial RAS farms around Europe. Thirteen cultured and eight wild populations with more than 950 fish in total were analyzed for a final set of 10 microsatellite genetic markers. On average, and contrary to what could be theoretically expected, the thirteen domesticated populations exhibited a slightly higher number of alleles compared to the wild ones (2.634 *versus* 2.580, not significantly different with an F-test). Likewise, unbiased expected heterozygosity estimates were slightly higher in wild population (0.573 *versus* 0.553, but again not significantly different with an F-test). Inbreeding coefficient (F_{IS}) values showed that the domesticated populations are in general not inbred and that some wild populations may also suffer from kin mating, too. In general, the mean heterozygosity estimates and the count of the number of alleles per population indicate that domesticated samples do not suffer from inbreeding. There are few domesticated populations that have some level of inbreeding, either due to their small sample size or their use as 'selected' fish.

Our studies also provide evidence that pikeperch populations in Europe are part of at least two genetically differentiated groups (Figs. 1 & 2). The first group is found in northern Europe from the Netherlands/Denmark to the West, Poland (at least) to the East, and to Finland to the North (Fig. 2). The second group comprises all remaining populations in Central Europe to as south as Tunisia (and probably Spain, Italy and northern Greece). In the second stock, the Hungarian

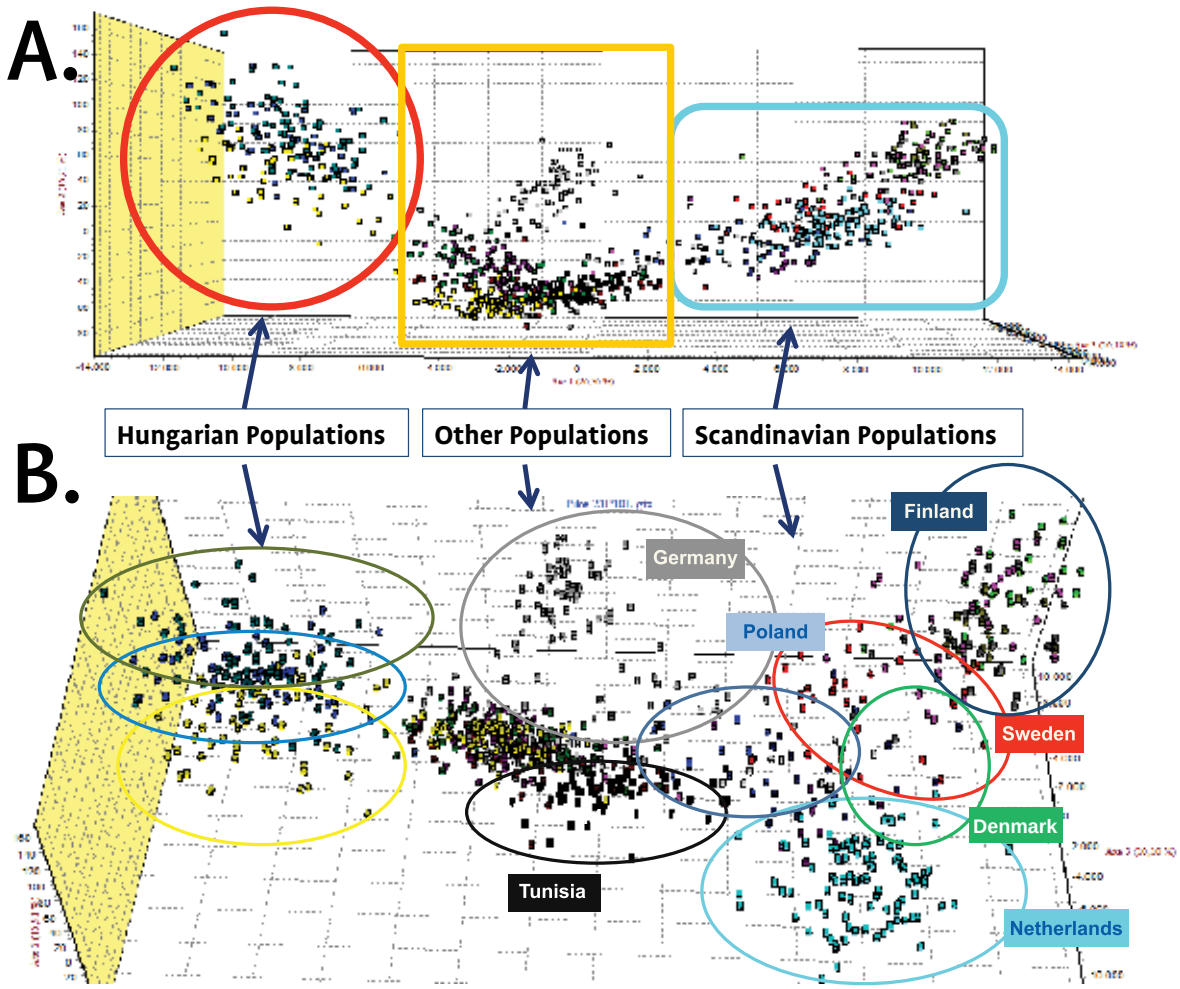


Figure 1. Factorial Correspondence Analysis (FCA) for all twenty-one populations and ten loci using the GENETIX v. 4.05 software.

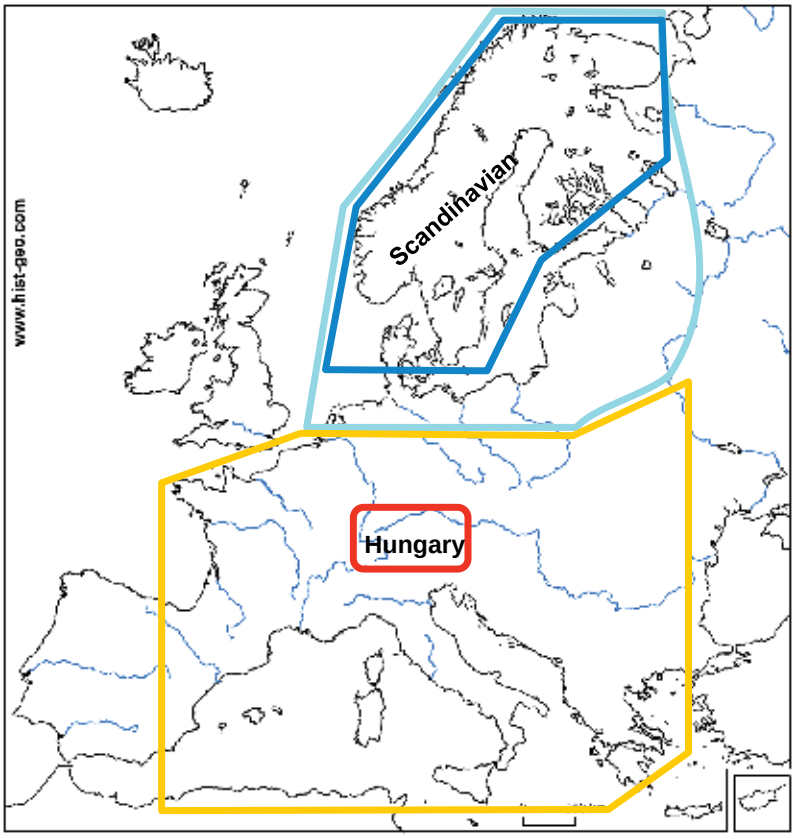


Figure 2. Map of Europe showing the major pikeperch genetic groups included in the study.


Figure 3. Pilot scale larval rearing system used for pikeperch research at the University of Lorraine (France).

populations are having a key-position being different from those found geographically close, *e.g.*, from Czech Republic and Germany. It might be another stock associated with Hungarian lakes, as opposed to all other populations that probably has dispersed through the Danube River west and southwards. Based on this grouping, it can be stated that most analyzed populations seemed to contain fish of a single origin; nevertheless, in few domesticated populations this ratio varied from 5-19%, possibly due to the mixing of fish from multiple sources.

LARVAL REARING

Three main bottlenecks have been identified as preventing the success of larval rearing: a high rate of mortality due mainly to cannibalism, a high rate of deformities and a strong growth heterogeneity characterized by important differences in size between larvae of the same age but at various developmental stages. Several trials have been completed using a pilot scale larval rearing system (RAS, eight 700 L tanks, Fig. 3). The main goal was the identification of optimal combinations of major culture factors (environment, population and nutrition) to increase larval survival and growth. This task has been managed by Pr P. Fontaine (University of Lorraine, France). In a first experiment, the effects of four environmental factors with two modalities were tested (light intensity: 5 *vs* 50 lx, water renewal rate: 50 *vs* 100% per hour, water current direction: up-flow *vs* down-flow, tank cleaning time: at morning just after the first feeding vs at the evening after the last meal). A fractional factorial experimental design (24-1) of resolution IV was used to study simultaneously the effect of these four factors and their possible interactions. Every week, from four days post hatching (dph) and after the first feeding, 60 larvae were sampled in each tank. Individual weight, morphological measurements (total length, mouth size, myotome height and eye diameter), occurrence of deformities, inflation rate of swim bladder and histological analyses (retina, intestine and musculature of jaws, in collaboration with Dr E. Gisbert, IRTA, Spain) were made at 25 and 40 dph. Results showed that light intensity, water renewal rate and cleaning period have a direct impact on growth, deformities and swim bladder inflation success. For example, larval total length at 40 dph was influenced by the interactions between (i) light intensity and water renewal rate (Fig. 4) and (ii) light intensity and cleaning period. The water current direction had no impact on these developmental parameters.

Then, in a second experiment using similar methodology, we studied the impact of four feeding factors on the growth of pikeperch larvae during the first weeks after fertilization. The four tested factors were: quantity of live preys (2,100 or 10,500 *Artemia* nauplii/larvae/day), weaning duration (three or nine days), frequency of food distribution (continuous or a meal each 1.5 hour from 8:30 to 17:30) and use of co-feeding (Prowean Larviva, BioMar) or not. Significant effects were observed in pikeperch larval growth and growth heterogeneity (CV, %) at 11 and 18 dph. At both sampling dates, the mean body weight of larvae was significantly higher when a higher quantity of *Artemia* nauplii were distributed (4.79 mg *vs* 3.07 mg at 11 dph and 20.10 mg *vs* 9.39 mg at 18 dph). Likewise, similar effects were observed on larval length (1.35 cm *vs* 1.08 cm at 11 dph). Moreover, significant effects on growth heterogeneity were caused by the quantity of live preys, the frequency of food distribution and co-feeding. A higher coefficient of variation for body weight at 11 dph was observed in response to discontinuous *vs* continuous food distribution (29.8% *vs* 28.3%) and to a lower *vs* higher quantity of live preys (37.8% *vs* 30.3%). Finally, at 18 dph, a significant interaction between co-feeding and duration of the weaning period was observed. When co-feeding was applied, the duration of the weaning period had no effect on larval size heterogeneity, whereas without co-feeding a significant increase of size heterogeneity appeared when the weaning period was longer *vs* shorter (13.9% *vs* 10.5%). In conclusion, the amount of distributed food was the main factor affecting the development of pikeperch larvae, but some effects of food distribution, co-feeding and weaning duration were also observed.

NUTRITION

Some nutritional bottlenecks remain to be solved to sustain a successful commercial production of pikeperch. Among these is the development of larval diets for optimal growth and performance. Several studies have indicated that low dietary levels of long-chain highly unsaturated fatty acids (LC HUFAs) may cause dysfunctions, such as increased stress sensitivity

and mortality and may have long term consequences on brain size, behavior and neuromuscular escape responses. The research undertaken so far in the project (managed by Dr I. Lund, Technical University of Denmark, DTU Aqua) involves a study on larval requirement of phospholipids (PL) and LC HUFAs and a pilot study on tolerance to salinities in order to observe how performance, essential fatty acid (FA) requirements and FA metabolism may be altered by changes in environmental salinity. To study the effects of PL and LC HUFAs, six different diets with increasing content of PL, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were tested (Table 1). The diets were fabricated by SPAROS (Portugal). At 30 dph, larval performance, biochemical composition as well as digestive enzymatic activity, liver proteomics, gene expression and skeleton morphogenesis were evaluated. Larvae were obtained by AquaPri A/S (Denmark) and reared on *Artemia* nauplii until 10 dph and then gradually switched to compound feeds within 5 days. The trial was performed in 18 tanks of 50 L (6x3) in which ~800 larvae were stocked and fed in surplus (approximately 25% of estimated total average wet weight) until 30 dph. Results are still being analysed, but the growth result (Fig. 5) showed that increasing levels of PL had a significant positive impact on larval size and that LC HUFAs such as DHA and EPA may increase those values.

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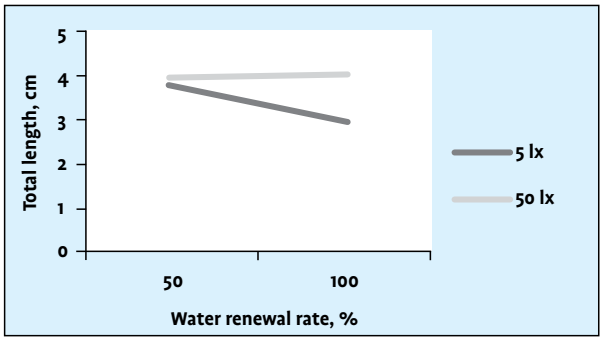


Figure 4. Influence of light intensity and water renewal rate on the total length of pikeperch larvae at 40 days post hatching (dph).

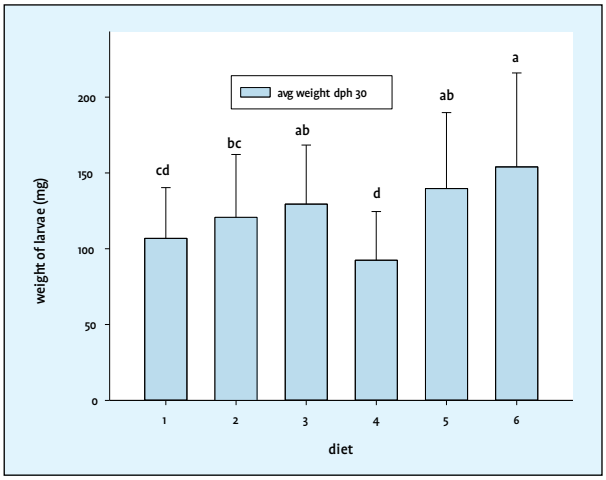


Figure 5. Size of pikeperch (*Sander lucioperca*) larvae at 30 days post hatching (dph) in response to six experimental diets. Means with statistically significant differences (ANOVA, P<0.05) are indicated by different letter superscripts.

As fed basis (% wet weight)	1	2	3	4	5	6
Crude protein	52,74	52,72	52,69	52,74	52,72	52,69
Crude fat	27,01	26,99	27,01	27,01	26,99	27,01
Fiber	0,14	0,14	0,13	0,14	0,14	0,13
Starch	4,02	3,90	3,72	4,02	3,90	3,72
Ash	8,12	8,12	8,11	8,12	8,12	8,11
Gross Energy	24,02	23,34	22,48	24,02	23,34	22,48
Fatty acids (% total fat)						
Eicosapentaenoic acid (EPA)	0,41	0,41	0,41	0,47	0,61	0,75
Docosahexaenoic acid (DHA)	0,66	0,66	0,66	1,04	2,06	3,04
Phospholipid classes (% total fat)						
Phosphatidylcholine (PC)	1,51	2,88	4,64	1,51	2,88	4,64
Phosphatidylethanol-amine (PE)	0,62	1,58	2,81	0,62	1,58	2,81
Phosphatidylinositol (PI)	0,69	2,10	3,90	0,69	2,10	3,90
Total phospholipids (TPL)	3,16	7,45	12,96	3,16	7,45	12,96

Table 1. - Dietary content of six formulated weaning diets.

Another pilot study showed that pikeperch larvae can survive abrupt salinity changes of 8-10 ppt directly after hatching without noticeable mortality, but 12 ppt might be the upper tolerance level. A subsequent study involving three salinity levels was initiated (0, 5 and 10 ppt) in a triplicate set-up on larvae fed either *Artemia* enriched with high levels of linolenic acid (ALA, 18:3n-3) or linoleic acid (LA, 18:2n-6). Larvae seemed to grow well, but the study needs to be repeated later this year, as the initial stocked numbers of larvae were too low. The capability of larvae to synthesize LC-HUFAs, as well as the fatty acid esterification pattern into different lipid classes will be studied by *in vivo* radio-tracing of 14C fatty acids and lipid classes (phosphatidylcholine-phosphatidylethanolamine, PC-PE).

GROWTH - STRESS

A first experiment was carried out in order to study the effects of husbandry practices and environmental factors on pikeperch growth, immune and physiological status (task led by Pr P. Kestemont, University of Namur, Belgium). The aim was to optimize the conditions for pikeperch grow out. This study is a screening approach of the main stressful factors identified for pikeperch juveniles reared in intensive culture conditions such as RAS. Eight factors with two modalities have been selected according to the bibliography and fish farmer observations (Table 2). This experiment (June – August 2015) is based on a multifactorial experimental design and new experimental facilities consisting of 16 independent RAS of 3 m^3 each (Fig. 6), located at the University of Lorraine (France). Pikeperch juveniles (70 g) were supplied by the company SARL Asialor, also a partner of the DIVERSIFY project. Different parameters of stress response will be analyzed, including cortisol, glucose or cerebral serotonin. Immune markers will be also investigated, including lysozyme and complement activities, concentration of immunoglobulin (Ig) and the expression of immune genes (*e.g.*, lysozyme, C3-1, TNF-α, IL-1β). Following this multifactorial experiment, a validation experiment will be done at the University of Namur facilities for further investigation, focusing on the interaction between stress intensity and resistance against pathogens. The results will enable the determination of the major aquaculture stressors in pikeperch in order (a) to reduce stress exposure during rearing by applying the optimal rearing conditions, (b) to increase disease resistance and (c) to improve growth performance.

Before the actual start of this multifactorial experiment, two preliminary experiments were planned in order to (i) standardize the analytical protocols for physiological and immune markers using pikeperch submitted to stressors and (ii) define the lethal dose of *Aeromonas hydrophila* or *A. salmonicida* that will be used for the challenge tests after stress experiments. Compared to salmonids, the first observations showed high levels of cortisolemia in pikeperch (88-122 ng/ml), confirming its high sensitivity to captive environmental conditions, and that emersion stress induced a significant increase in plasma glucose.

Factor	Modality
1 - Grading	- 2 times per month - No
2 - Initial rearing density	- 30 kg/m^3 - 15 kg/m^3
3 - Light intensity	- 100 Lx - 10 Lx
4 - Light spectrum	- White - Red
5 - Photoperiod	- L:D 24 : 0 - L:D 10 : 14
6 - Hypoxia	- 50-60 % of O_2 saturation - 90-100 % of O_2 saturation
7 - Temperature	- 21-22°C - 26-27°C
8 – Type of feed	- Semi floating - Sinking

Table 2. - Experimental conditions and modalities

Figure 6. Examples of two environmental conditions applied on pikeperch juveniles to study. Upper photo: white spectrum, 100 Lx, lower photo: red spectrum, 100 Lx

The project DIVERSIFY has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). Further information for the project is available at www.diversifyfish.eu.

Advances in greater amberjack *(Seriola dumerili)* research: the DIVERSIFY project

MYLONAS C.C., KATHARIOS, P., GRIGORAKIS, K. AND PAPANDROULAKIS, N. (HELLENIC CENTER FOR MARINE RESEARCH, HERAKLION, CRETE, GREECE)

ROCIO, R. (CTAQUA, SPAIN)

CORRIERO, A., POUSIS, C. AND ZUPA, R. (UNIVERSITY OF BARI, ITALY)

FERNÁNDEZ-PALACIOS, H., MONTERO, D., IZQUIERDO, M.S., ROO, J. AND HERNÁNDEZ-CRUZ, C.M. (FUNDACIÓN CANARIA PARQUE CIENTÍFICO TECNOLÓGICO, UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA, SPAIN)

RODRÍGUEZ, C. AND PÉREZ, J.A. (UNIVERSIDAD DE LA LAGUNA, TENERIFE, SPAIN)

JEREZ, S. (INSTITUTO ESPAÑOL DE OCEANOGRAFIA, TENERIFE, SPAIN)

SECOMBES, C. (UNIVERSITY OF ABERDEEN, SCOTLAND, U.K.)

GUERRERO, L. (IRTA, SPAIN)

The greater amberjack (*Seriola dumerili*) is a species with high potential for the EU aquaculture due to its fast growth (6 kg in 2.5 years), excellent flesh quality and global market. Its farming in the Mediterranean region started in the 1990s with wild-caught juveniles, but the production is still negligible, as several bottlenecks exist for its industrial production. These include the absence of reliable reproduction, limited availability of juveniles, lack of knowledge on the nutrient requirements and pathology of the species. The EU FP7-funded DIVERSIFY project (www.diversifyfish.eu) examines the major aspects of greater amberjack aquaculture in order to overcome these bottlenecks and develop appropriate rearing methods for commercial production. This article provides some highlights from the first 2 years of the project.

Reproduction & Genetics

The research activities on greater amberjack reproduction have focused on three aspects. Firstly, on the identification of the reproductive dysfunctions in greater amberjack reared in captivity compared to fish in the wild, in terms of sex steroid plasma levels, histological and nutritional assessment of gonad maturation, the vitellogenic process (liver vitellogenin synthesis and oocyte yolk accumulation), as well as male germ cell proliferation and apoptosis. Secondly, on the development of a spawning induction protocol and an egg collection method for wild-caught greater amberjack maintained in land-based tanks and cages in the Mediterranean Sea and in the eastern Atlantic Ocean. Finally, work has focused on the development of an optimized spawning induction protocol for a greater amberjack broodstock born in captivity (F1 generation) in the eastern Atlantic.

Sex steroid plasma levels and histological evaluation of ovaries and testes underlined that gametogenesis in some captive-reared greater amberjack broodstocks is unreliable and may be seriously impaired (**Fig. 1**). Although liver vitellogenin gene expression was lower in captive fish, the capacity of the oocytes to accumulate yolk did not seem to be affected compared to wild fish. In the males, an early decrease of germ cell proliferation was observed and spermatogenesis ceased completely during the natural spawning period in captivity. In addition, captive male germ cells appeared to be affected by

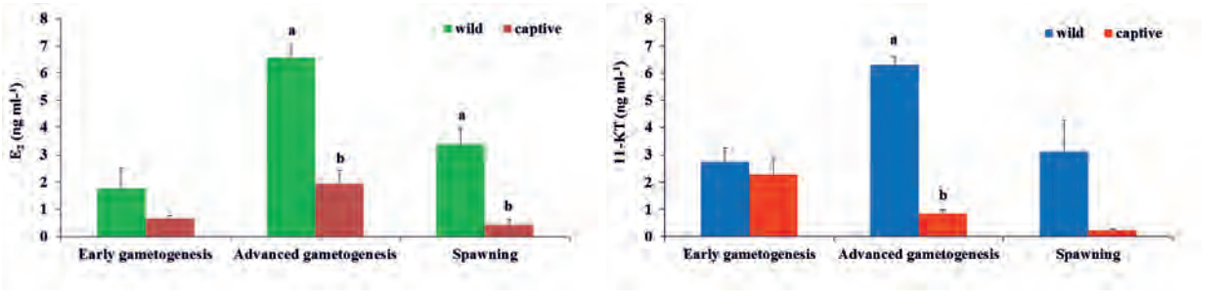


Figure 1. Plasma levels of 17 -Estradiol (E_2) in female and 11-ketotestosterone (11-) in male wild and captive greater amberjack during the reproductive season. Asterisks indicate significant differences between wild and captive fish (ANOVA, $P < 0.05$), both in males and females.

an unnaturally high rate of apoptosis. It is hypothesized that the reproductive dysfunctions observed in captive-reared greater amberjack might result from the combination of captivity-induced stress, the lack of appropriate 'natural' spawning conditions, as well as nutritional deficiencies.

Experiments to induce maturation and spawning in the Mediterranean greater amberjack broodstocks by means of gonadotropin-releasing hormone agonist (GnRHa) implants, showed difficulties in obtaining eggs from broodstocks maintained in tanks. On the contrary, encouraging results toward the development of methods for reliable egg production have been obtained from fish maintained in cages (**Fig. 2**). Good quality of sperm during the spawning period, better development of the ovaries and better response to hormonal treatment with good fertilization of eggs was obtained when fish were maintained in sea cages during the year and either allowed to spawn in the cage or transferred to land-based tanks for spawning and egg collection. Unfortunately, egg collection in sea cages was either inefficient or negligible, and more effort is needed to develop appropriate egg collection methods for sea cages. However, the alternative method to transfer the fish to tanks for spawning after GnRHa induction was very effective, leading to the collection of ~22 million eggs from a small number of broodfish (n=6).

Experiments carried out in the eastern Atlantic Ocean (Canary Islands) demonstrated that wild-caught fish adapt readily to the captive environment and are capable of undergoing complete reproductive maturation, and are spawning spontaneously without the need of any hormonal therapies. Also, for the first time F1 generation greater amberjack at the final stages of vitellogenesis and at spermiation, were induced to spawn with treatment with GnRHa implants (**Fig. 3**). The fish underwent repeated spawnings for a period of 3 months, with a total production of about 15 million eggs. A major difference observed between the Mediterranean and Canary Islands broodstocks was the timing and duration of the reproductive season. Spawning started earlier (May) and lasted much longer (September) in both broodstocks in the Canary Islands, whereas in the Mediterranean the spawning season was limited to June and early July. These differences are probably due to differences in photoperiod and water temperature fluctuations, although genetic differences between the stocks have not been ruled out yet.

Figure 2. Induction of spawning in a sea cage-reared broodstock in Greece (left), after evaluation of oocyte maturation stage (right). Some fish were transferred to land-based tanks for spawning after GnRHa treatment, but the majority of the fish were allowed to spawn in the cage (bottom).

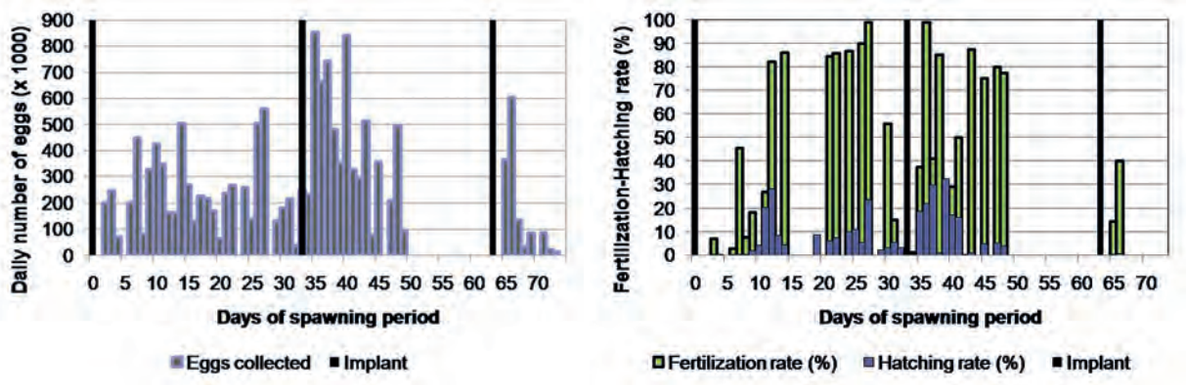


Figure 3. Induction of spawning in an F1 greater amberjack broodstock in Tenerife, Canary Islands (Spain) after three consecutive treatments with GnRHa implants.

Nutrition

The scarce knowledge on greater amberjack larval nutritional requirements leads to low larval survival and performance, and poor juvenile quality. Under this perspective, the overall objective of three feeding experiments performed by FCPCT and IEO/ULL was to determine the optimum levels of long-chain poly-unsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA), eicosapentaeonic acid (EPA) and arachidonic acid (ARA), as well as the combination of LC-PUFA and carotenoids in enrichment products for rotifers and *Artemia*. Trials were performed using different lipid and antioxidant sources, and evaluations included their effects on survival, growth, welfare, stress resistance/tolerance, bone development and tissue composition of the larvae. The results suggested that *Artemia* enriched with DHA at levels of 1.5 g 100 g $^{-1}$ dry weight and a 1.6 DHA/EPA ratio is sufficient to promote fast growth, whereas increased levels were associated with skull anomalies (**Fig. 4**). Skeletal elements such as the maxilla or mandible that develop from a cartilaginous precursor would be more sensitive to oxidative risks and, therefore, to dietary DHA elevation. Results also suggested that essential fatty acid requirements during rotifer and *Artemia* feeding are similar to those reported for larvae of other marine fish species. Requirements of greater amberjack larvae for DHA were higher than those found in other marine fish species and similar to those for other fast growing species, such as the yellowtail (*Seriola quinqueradiata*) or striped jack (*Pseudocaranx dentex*), whose larvae require 1.3–2.6 g and 1.6–2.2 g DHA 100 g^{-1}, respectively.

In addition, an enrichment protocol for rotifers was developed, containing 10 ppm of astaxanthin-based carotenoids, and a DHA-rich marine lecithin (LC-60) supplemented slightly with ARA (E1-10). Its use for just 3 h resulted in rotifers with high contents of DHA in the polar lipids, and a 2.7 DHA/EPA, resembling the composition of greater amberjack eggs. Feeding trials showed also improved larval performance (**Fig. 5**), and we expect that the data obtained will be used to design well-balanced enrichment products and weaning diets for this species.

Husbandry

The specific husbandry requirements for the rearing of greater amberjack are studied at different developmental stages to define appropriate and efficient practices. Preliminary results studying light conditions (photophase, intensity and background color) during the larval stages showed a beneficial effect of the long photophase on growth and survival of the larvae. Rearing trials comparing intensive and semi-intensive conditions are also implemented, and analysis is underway relating the ontogenetic changes of the digestive system with the expression of somatotropic axis genes. Results until now showed that intensive rearing conditions favor amylase, alkaline protease and pepsin activities in 30 days post hatching (dph) larvae, while in earlier stages (12 dph) amylase activity was also higher, in contrast to alkaline protease and lipase activities (**Fig. 6**).

Figure 4. Greater amberjack juvenile with cranial malformation from the study of the effect of dietary Artemia DHA content.

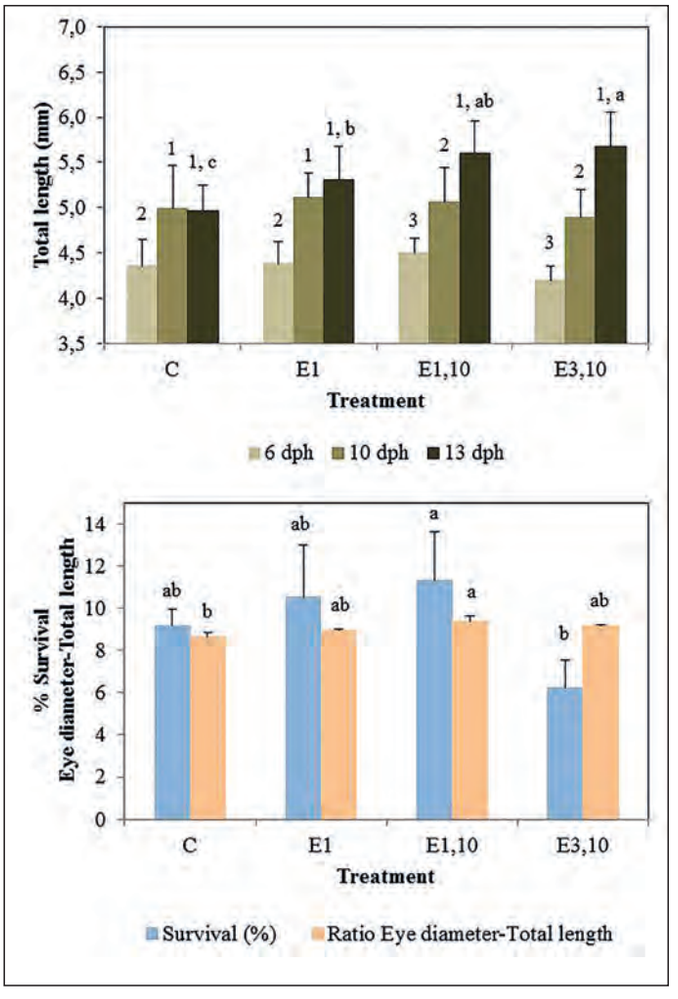


Figure 5. Effect of rotifer enrichment protocols on greater amberjack larval performance (a) Evolution of total length, (b) survival and eye diameter-total length ratio at the end of the experiment. Different numbers indicate significant differences along the feeding period, different letters indicate significant differences among dietary treatments. C, commercial enrichment; E1, LC-60 based emulsion; E3, triacylglycerol (TAG) DHA-rich emulsion; 10, 10ppm astaxanthin-based carotenoid supplementation.

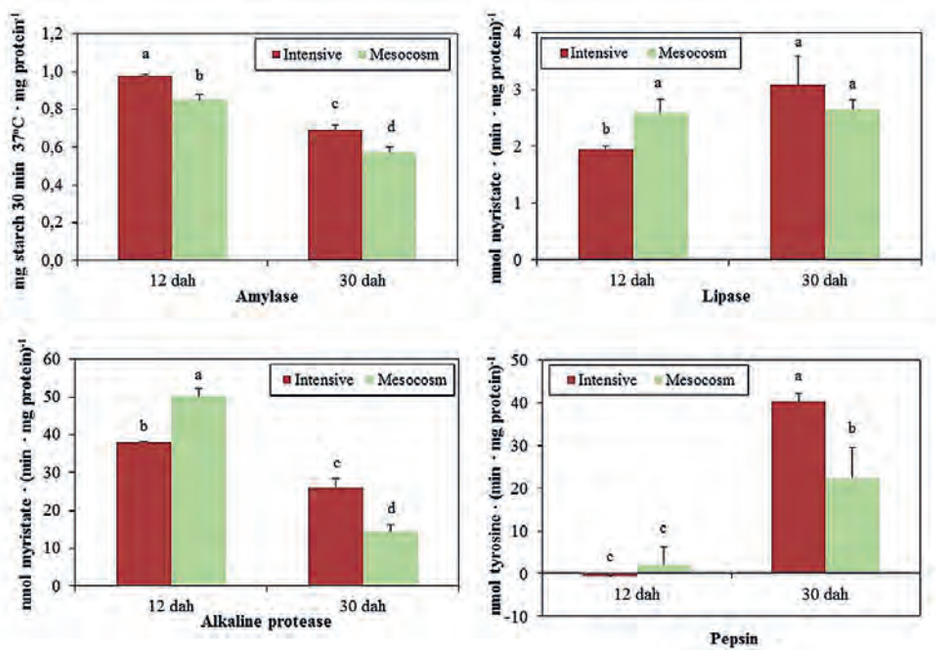


Figure 6. Comparison of digestive enzyme activities in greater amberjack larvae reared using intensive and Mesocosm protocols.

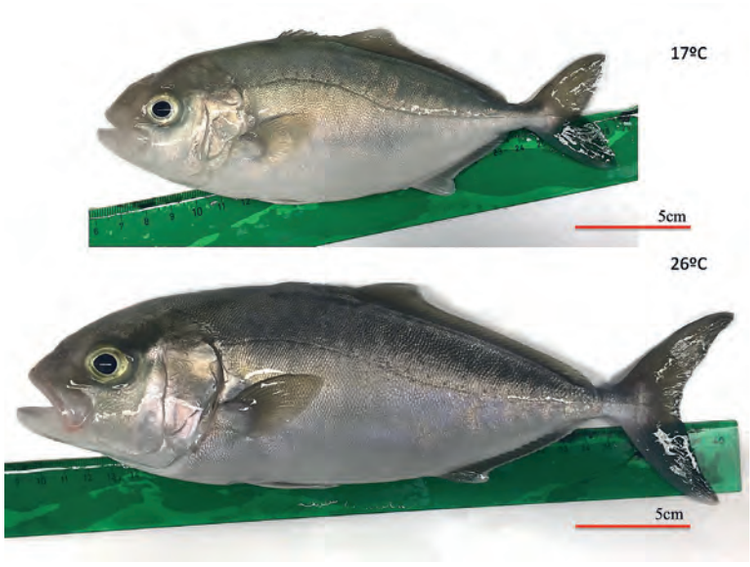


Figure 7. Greater amberjack reared at 17°C (above) and 26°C (below) showing the effect of rearing temperature on body morphology.

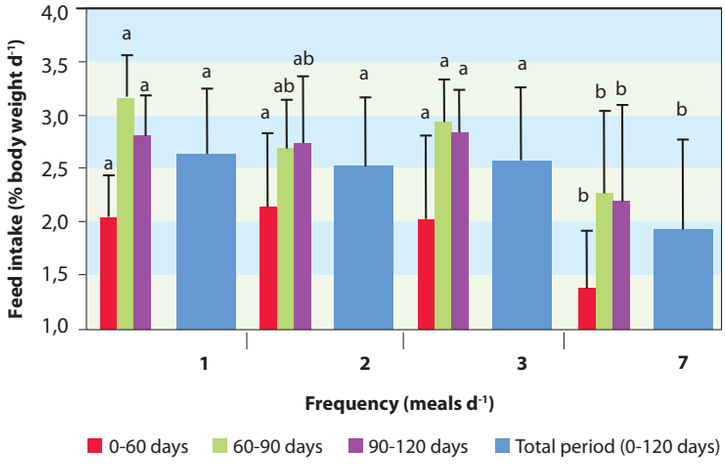


Figure 8. Feed intake (% of body weight d^{-1}) of greater amberjack juveniles at various feeding frequencies, at different times during the experiment (0-60 days, 60-90 days, 90-120 days and the overall effect). Different letters indicate significant differences (ANOVA, $P < 0.05$) for each period.

A study has been implemented examining the effect of stocking density during on-growing (initial densities of 0.2, 0.3 and 0.5 kg m^{-3} reaching 3.5, 5.7 and 7.4 kg m^{-3} after 4 months) on growth. The first results with juveniles (5 g) showed significantly lower specific growth rates (SGR) and condition index at high-density conditions. A second trial is currently under way with 200 g individuals, looking at feed intake, immune and welfare conditions. Regarding temperature tolerance, we have evaluated the performance of juveniles at 17, 22 and 26°C, with the latter being the most effective for growth and feed utilization. Furthermore, the body morphology of the individuals was affected by the temperature, with 26°C resulting in a better, more elongated body shape (**Fig. 7**).

The effects of different feeding rhythms on growth performance and welfare status were also examined. A trial was implemented with juveniles (~200 g) fed 1, 2, 3 and 7 meals day^{-1} for 4 months. Fish fed 1 meal day^{-1} showed the lowest SGR, condition index and hepatosomatic index, while those fed 7 meals day^{-1} showed the lowest feed intake (% of body weight day^{-1}) suggesting greater feed efficiency (**Fig. 8**). Although some monthly differences in mucus immunological, hematological and biochemical parameters, as well as oxidative stress enzymes in several tissues were observed, at the end of the study there were no statistical differences suggesting that greater amberjack juveniles are able to adapt to the different feeding frequencies under the particular culture conditions.

Health

Over the past 2 years, we have been studying the disease issues that impact production of greater amberjack, by monitoring several populations of cage-cultured fish for their health status. In Greece the main pathological problems have been caused by opportunistic bacteria and parasitic infections, especially during the transitional period between the hatchery and on-growing at the open sea. The most important bacterial infections were caused by *Vibrio harveyi*, which causes typical vibriosis (**Fig. 9**) and may result in high mortality, especially when water temperature rises above 20°C. The monogenean parasite *Zeuxapta seriolae* (**Fig. 10**) was the most prevalent and important parasitic pathogen. It is transmitted to cultured fish from wild populations and since its life cycle is direct (not requiring an intermediate

Figure 9. Juvenile greater amberjack infected by *Vibrio harveyi*.

host), it can propagate rapidly, reaching enormously high numbers on the host fish. The parasite is attached on the gills and feeds on blood, causing severe anaemia. Currently there are no adequate registered therapeutics for this parasite and we are investigating several experimental drugs that can be used to mitigate its impact on greater amberjack aquaculture.

Figure 10. The polyopisthocotylean monogenean parasite *Zeuxapta seriolae*.

Apart from *Zeuxapta seriolae*, we have also identified the blood fluke *Paradeontacylix* sp. to be present in greater amberjack reared in Greece. This digenean parasite resides within the blood vessels of the fish and releases its eggs into the blood stream. The eggs and the encysted metacercariae obstruct the gill capillaries, causing severe inflammation and damage of the gill tissue (**Fig. 11**). There is scarce information on the biology of this parasite and almost nothing is known about its life cycle. We are currently investigating possible alternative or intermediate hosts, and we are trying to locate the source of infection. In parallel, we are aiming to develop tools to treat this parasitic disease. The skin fluke *Neobenedenia spp.* is a monogenean parasite that is especially important for aquaculture due to his broad host range and the damage that it causes to cultured greater amberjack (**Fig. 11**). The parasite is well distributed in temperate waters around the world and it caused mortalities to one of our stocks in the Canary Islands. In Japan, infection prevalence rates of 70% have been reported for reared amberjack. Once infected, fish scratch the nets and tanks in order to remove the parasite, producing wounds that cause important injuries on the skin, leading to secondary infections, immunosuppression and, in most cases, the death of the fish.

For this reason strategies for reducing skin fluke incidence are being examined. For example, ways to promote the fish mucosal immune system, particularly skin mucus production (quality and quantity) are being studied since this is the host's first defensive barrier against this parasite. Thus, histopathological analyses for evaluating the derived-skin mucosa wounds, mor-

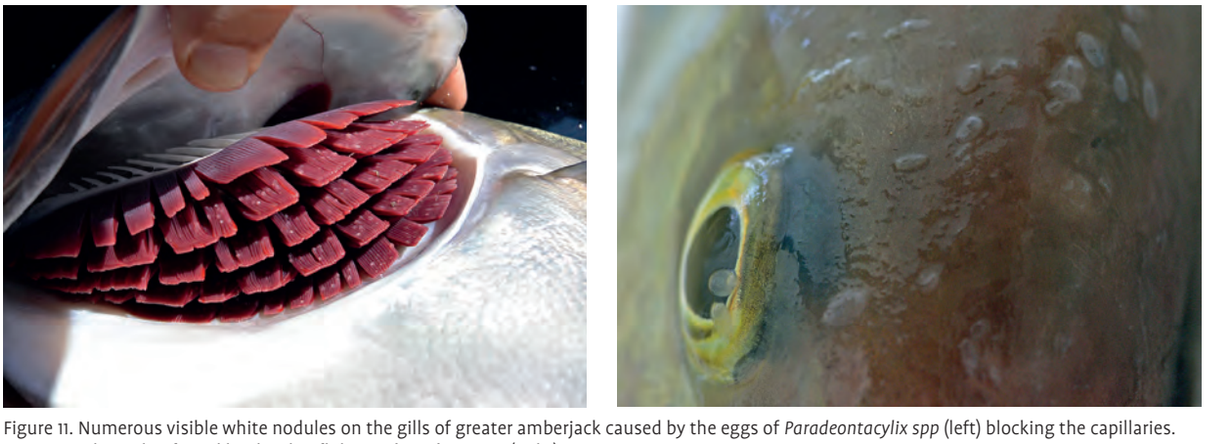


Figure 11. Numerous visible white nodules on the gills of greater amberjack caused by the eggs of *Paradeontacylix spp* (left) blocking the capillaries. Greater amberjack infected by the skin fluke ***Neobenedenia spp*** (right).

phometric studies for determining skin mucus quantity and an evaluation of the host immunological status are being conducted. The morphological and hydrodynamic differences between cranial and dorsal regions suggest that the cranial region is an easier place for the attachment of the parasite. *N. girellae* attachment induces epidermis disorganization, increase of goblet cells and massive migration of immune cells (mononuclear lymphocytic type) around the site of parasite attachment. The attachment of *N. girellae* in greater amberjack causes a dermatitis that can be the site for opportunistic pathogens to occur. The incidence of *Neobenedenia* seems to be related to fish size and temperature, with outbreaks of parasites observed in animals larger than 100 g and temperatures above 20°C. These studies are being completed with biochemical and immunohistochemical analysis and examination of the expression of immune and mucus production related genes.

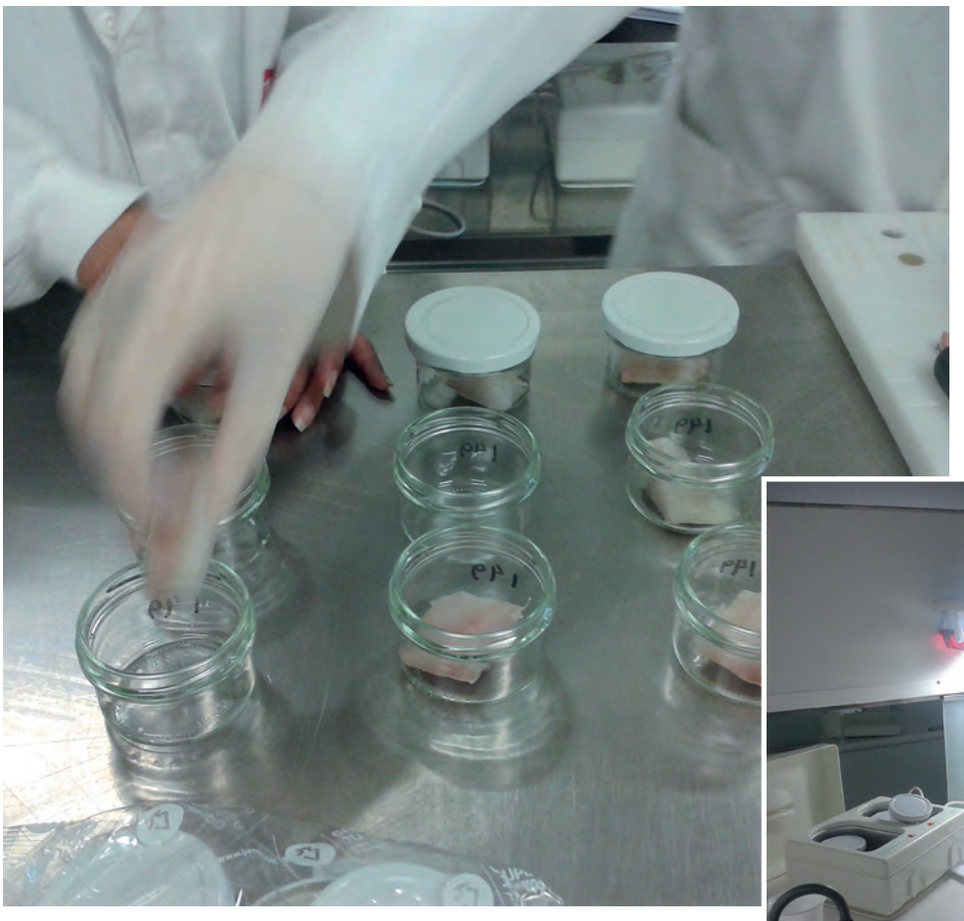
New product development

The technical characteristics and muscle composition of greater amberjack were studied in two different size groups (**Fig. 12**), in order to define both the range of these quality characteristics and the effect of fish size on them (**Table 1**).

Figure 12: Farmed greater amberjack during yield measurements and filleting.

	Group A (small fish)	Group B (big fish)
Size (fish weight in Kg)	1.19±0.19	13.00±1.62
Dressing yield (% of body weight)	92.8±0.79	94.7±0.39
Filleting yield (% of body weight)	50.5±2.89	
Visceral loss (% of body weight)	5.60±0.71	2.89±0.83
Fillet composition (%)		
Protein	22.9±1.29	20.5±0.56
Fat	3.87±0.93	12.3±0.11
Moisture	71.03±1.07	65.5±0.55
Ash	1.35±0.49	1.31±0.04

Table 1. Somatic yield and fillet composition of farmed greater amberjack



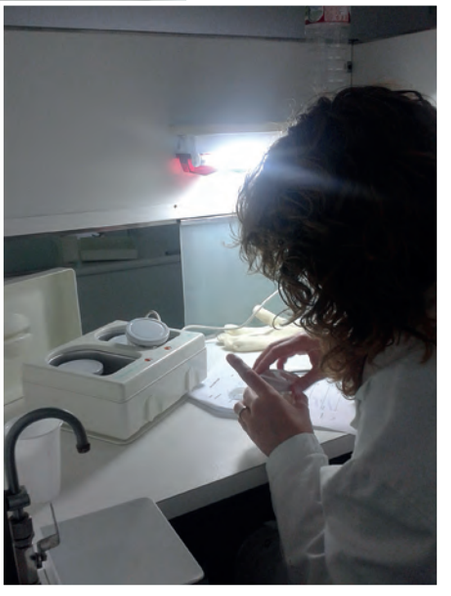


Figure 13. Sample preparation and testing of great amberjack (taste panel, IRTA – Spain).

groups was the much higher fillet fat contents in big fish. The sensory characteristics of the species' fillet have been examined analytically with descriptive sensory analysis by trained panelists (**Fig. 13**). In summary, the great amberjack fillet exhibits homogenous color, laminar structure, high juiciness and acid and butter flavors, while its texture is characterized by high teeth adherence and chewiness but of medium hardness (when compared to other fish species). Based on the technical and sensory characteristics of the species and out of a list of 43 different products of variable process, three products have been chosen to be generated as prototypes: frozen fish fillet that is seasoned or marinated (product 1), ready-made fish tartar with additional soy sauce (product 2) and fresh fish steak for grilling in the pan (product 3). One of these products will be selected for consumer acceptance in the near future within the DIVERSIFY project.

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 2 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece.

DIVERSIFY

Co-funded by the Seventh Framework Programme of the European Union

Further information may be obtained from the project site at "www.diversifyfish.eu".

Advances in Atlantic halibut (*Hippoglossus hippoglossus*) research: the DIVERSIFY project

BIRGITTA NORBERG, TORSTEIN HARBOE, AUDUN H. NERLAND AND SONAL PATEL (INSTITUTE OF MARINE RESEARCH, IMR, AUSTEVOLL, NORWAY)

KRISTIN HAMRE, (NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH, NIFES, NORWAY)

BØRRE ERSTAD, (STERLING WHITE HALIBUT, SWH, NORWAY)

MYLONAS C.C. (HELLENIC CENTER OF MARINE RESEARCH, CRETE, GREECE)

The Atlantic halibut (*Hippoglossus hippoglossus*) is the world's largest flatfish and can attain a weight of over 300 Kg (**Fig. 1**). It is highly prized at markets worldwide, having a characteristic flaky white meat with few bones and semi-fat flesh that is rich in omega-3 fatty acids. However, availability of wild Atlantic halibut has been decreasing steadily in the last decades, and the fish is classified as endangered on the IUCN red list. These characteristics led to the inclusion of Atlantic halibut in the project DIVERSIFY – (FP7-602131, www.diversifyfish.eu)- as a great candidate for fish species and product diversification in European aquaculture.

Research and cultivation efforts of Atlantic halibut started in the 1980's, but the total annual production of cultured Atlantic halibut is still only ~1600 t (Norwegian Directorate of Fisheries). In Europe, Atlantic halibut farms exist in Norway and Scotland. The desired market size is 5-10 kg and production time is currently 4-5 yrs. Despite a significant research effort between 1985 and 2000, the complicated life cycle of Atlantic halibut made aquaculture progress slow, and very little research funding has been allocated thereafter. However, during this time slow but steady progress has been made by the farmers in order to improve production stability, and interest in cage culture is growing. The remaining bottlenecks for increased and stable production are related to a steady supply of fry and a need to decrease the production time. The latter may be achieved with the recent establishment of "all female" juvenile production (Hendry et al., 2003; Babiak et al., 2012). The

Figure 1. Atlantic halibut broodstock at Institute of Marine Research, Austevoll Research Station.
Photo: Institute of Marine Research.

project DIVERSIFY addresses some other important bottlenecks in reproduction, larval nutrition and husbandry, and fish health, in order to improve the existing rearing methods and enhance the commercial production of Atlantic halibut.

REPRODUCTION & GENETICS

Even though empirical data suggest a significant difference in spawning performance between wild-caught (wild) and hatchery-produced (farmed) Atlantic halibut females, systematic documentation is lacking. The Atlantic halibut is a group-synchronous, batch spawner and in captivity wild females will release 6-12 batches of eggs during a period of 2-4 weeks in the spawning season (February to late April in southwestern Norway). Females have to be manually stripped of their eggs according to their individual ovulatory rhythms, to prevent over-ripening and deterioration of

	Domesticated Females	Farmed (F1) Females
n	3 (4•)	5
length (cm)	150.7 ± 6.2	113.4 ± 3.9
weight (kg)	48 ± 5.7	19.2 ± 2.3
number of batches - female $^{-1}$	7.3 ± 0.6	9.4 ± 1.7
spawning interval (hours)	82.2 ± 8.4	72.4 ± 22.9
batch volume (mL)	2300 ± 900	700 ± 300
total fecundity (mL - female^{-1})	16700 ± 420	6800 ± 130
relative fecundity (mL - kg^{-1})	347 ± 70	349 ± 84
average fertilization (%)	89 ± 7	61 ± 29

Table 1. Spawning performance of wild and farmed Atlantic halibut breeders at IMR, Austevoll.

the eggs (Norberg et al., 1991). Although wild females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, farmed females (F1/F2 generation) appear to suffer from a reproductive dysfunction, releasing small batches of eggs at irregular intervals. There is, however, a lack of thoroughly documented evidence describing this reproductive dysfunction in farmed females. Consequently, reproductive performance of wild Atlantic halibut and farmed females was compared in the framework of DIVERSIFY.

One group of wild breeders held in captivity for at least 4 years, and one group of farmed females were closely monitored for ovulation during the spawning seasons of 2015 and 2016 and were strip-spawned and the eggs fertilized *in vitro*. Eggs were incubated at 6°C under standard hatchery procedures (Mangor-Jensen et al., 1998) for 72 day-degrees (11 days at 6°C). For calculation of hatching percentage, eggs were collected and divided into 500 ml beakers with sterile-filtered seawater and incubated in darkness at 6°C for 72 hours. Hatched larvae and dead eggs were counted in a binocular microscope and larvae were also photographed in a dissecting microscope, in order to document any possible aberrations from normal development.

Overall, the wild females appeared to spawn fewer, larger egg batches with higher and more stable fertilization success (**Table 1**). Relative fecundity did not differ between the two groups. Careful monitoring and timing of stripping, as close to ovulation of the whole batch as possible, was necessary in order to obtain high fertilization of eggs (by avoiding over-ripening). In cases where the whole egg batch could not be strip-spawned, domesticated females generally released the remaining eggs into the tank. Farmed females, in apparent contrast, tended to keep a small "residue" of eggs, typically 100-250 ml, which were held in the ovary. These eggs had to be stripped 6-12 hours after the main batch so that the overripe residue would not have a negative impact on the viability of the next, maturing cohort. Once this was established as an additional routine in strip-spawning eggs from farmed females, the fertilization success stabilized at levels above 75-80% in most individuals, with occasional batches having up to 90-94% fertilization.

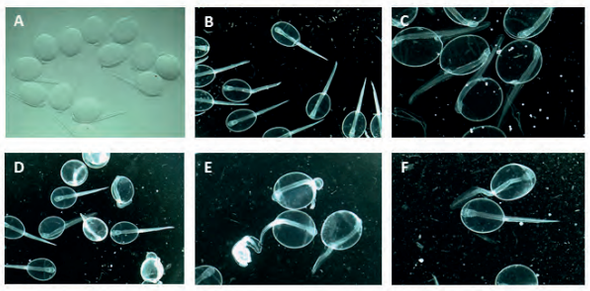


Figure 2. Newly hatched larvae from wild (A-C) and farmed (D-F) Atlantic halibut females. Note spinal deformities in embryos from farmed females. *Photo: Institute of Marine Research.*

Eggs from farmed females generally appeared heavier, and would sink to the bottom of the incubator/beaker, while eggs from domesticated females remained buoyant near the surface. Hatching success was lower in eggs from farmed females, and dead or deformed larvae were observed more frequently when eggs from farmed females hatched (**Fig. 2**). It is not clear what caused the deformities, but one possible cause may be mechanical damage of the heavy eggs, that sank and rested at the bottom of the beaker for two days. Further work is needed, however, in order to establish whether this is the cause or if there are genetic/epigenetic factors that contribute to a higher rate of deformities in larvae from those females.

Overall, wild females were predictable spawners that consistently gave eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified and stripping carried out close to ovulation. However, for commercial, as well as breeding purposes, it is not practical to rely on wild-caught females. As at both IMR and SWH, relatively few farmed females produced eggs with fertilization rates >80-85% consistently, it may be necessary to include wild-caught broodstock also in future in order to ensure a broad enough genetic material. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the very considerable workload connected with spawning and egg collection of Atlantic halibut.

One way of alleviating the reproductive dysfunctions of farmed females, might be to use reproductive hormone therapies, such as controlled-release delivery

Figure 3. Atlantic halibut broodstock treated with GnRHa implants to induce ovulation at IMR. *Photo: Constantinos C. Mylonas.*

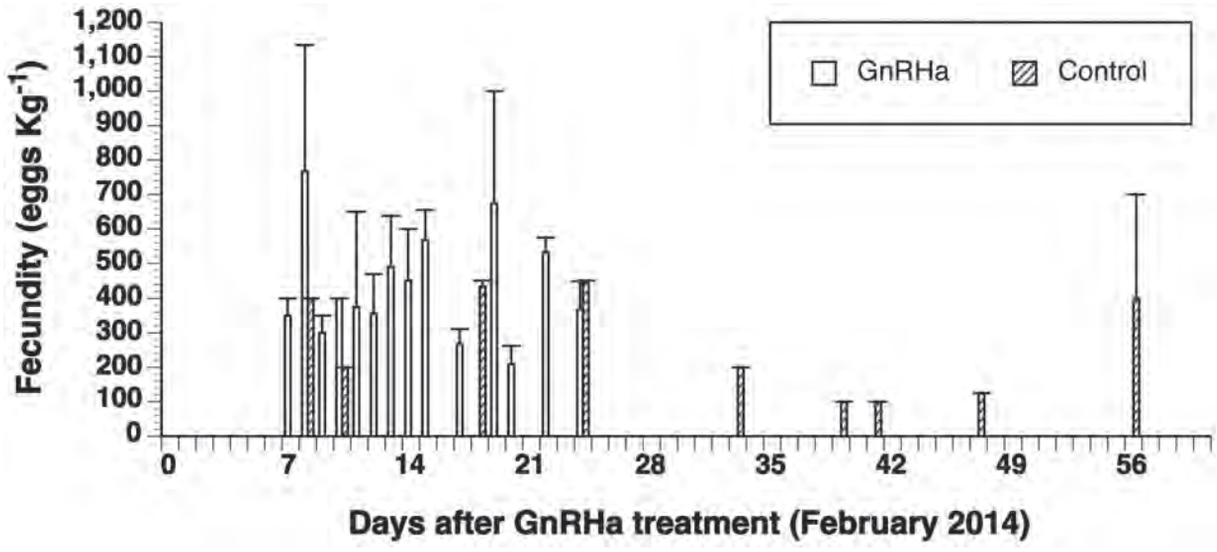


Figure 4. Mean (±SEM) daily egg production of Atlantic halibut treated with GnRHa implants (50 or 100 µg kg-1) or sham-injected as Controls at IMR, Austevoll.

systems (implants) loaded with gonadotropin releasing hormone (GnRHa) to induce oocyte maturation and ovulation. Such therapy has been highly effective in other teleosts (Mylonas et al., 2010) including flatfish, such as the coldwater batch-spawner yellowtail flounder (*Pleuronectes ferrugineus*; Larsson et al., 1997). To examine the application of GnRHa implants in improving reproductive performance of farmed Atlantic halibut (**Fig. 3**), females were selected based on ovarian biopsies and were treated either with GnRHa implants for an effective dose of 50 µg kg^{-1} or 100 µg kg-1, or were sham injected (Control group). Two commercial trials were then made based on these result, using females with outer signs of maturation: ovary visible on the exterior of fish but not enlarged near the ovipore, degree of swelling and color of ovipore, and based on documented spawning performance. All females chosen had given average to low amounts of eggs in previous seasons. Females were implanted with 75 µg GnRHa kg-1 or were sham-injected as Controls.

Although GnRHa implantation did not advance spawning time significantly in Atlantic halibut females, in two of the trials there was an apparent synchronization in spawning time between individuals, as all treated females had completed spawning 1 month before all Control fish were spent (**Fig. 4**). Spawning in Atlantic halibut normally occurs during a period of 2 to 3 months both in captive broodstock and in natural populations (Norberg et al, 1991; Haug1990). This is most likely an adaptation that will ensure production of viable offspring independent of year-to-year fluctuations in temperature and feed availability for larvae. In a commercial production, however, synchronization between individuals can be an advantage as staff efforts can be concentrated to a relatively short period. Atlantic halibut females ovulate and release their eggs (*i.e.* spawn) in captivity, but fertilization of eggs released in the broodstock tank happens only occasionally. Therefore, Atlantic halibut breeders need to be monitored closely for ovulation and stripped on a regular basis for *in vitro* fertilization to be successful, and the use of GnRHa implants offers a logistic advantage to the commercial broodstock management of the species, by shortening the spawning season.

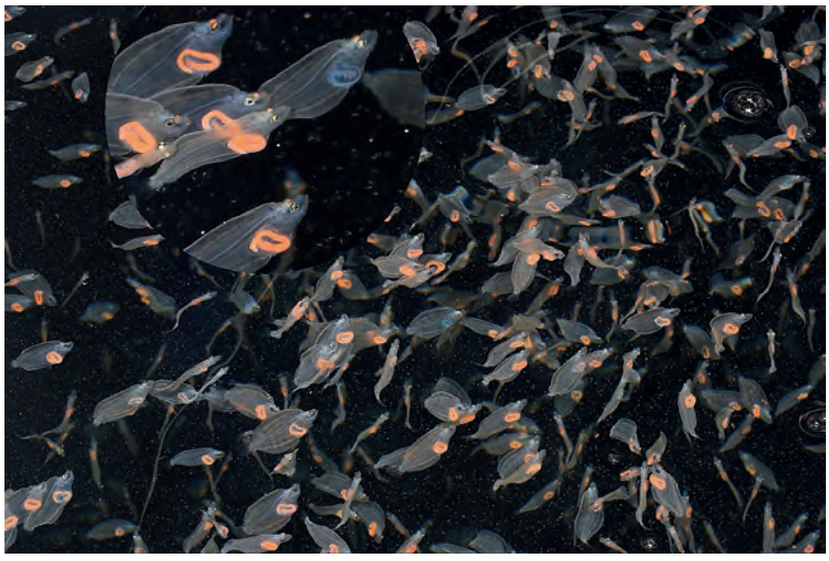


Figure 5. First-feeding Atlantic halibut larvae.

Photo: Institute of Marine Research.

On the other hand, spawning performance in terms of fecundity per female and fertilization success was not significantly affected by GnRHa treatment in Atlantic halibut females. In the first experiment, which was carried out at IMR, females implanted with GnRHa had a marked trend towards a higher fecundity than Control females. In the 2 commercial trials, however, this trend was not observed. So, at this stage the use of GnRHa

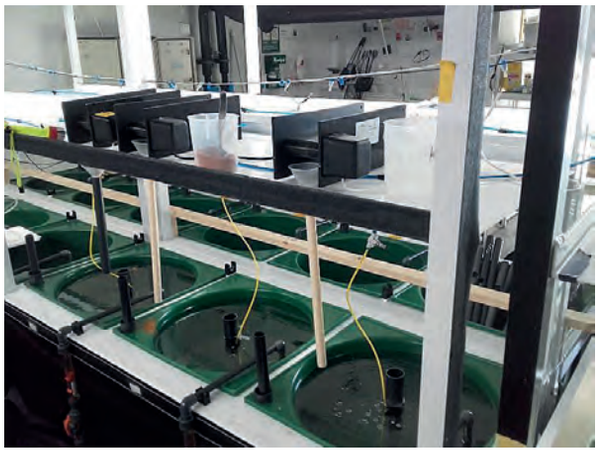


Figure 6. Tanks used for early weaning of Atlantic halibut larvae. *Photo: Institute of Marine Research.*

therapy to increase fecundity and/or fertilization success is not confirmed. Apparently, spontaneously maturing and ovulating females may produce as many eggs as GnRHa treated individuals. However, GnRHa was demonstrated to be highly effective in ensuring that all females matured and ovulated, as all treated females ovulated at least 3 to 4 egg batches, whereas in all trials some of the Control fish did not ovulate and appeared to resorb their ovaries. So, these results indicate that GnRHa implantation may be a useful tool to ensure that all females in a broodstock group reach maturation and ovulation, increasing parentage contribution to the next generation and increasing overall broodstock fecundity, without having deleterious effects on egg viability.

LARVAL REARING AND NUTRITION

Atlantic halibut larvae are approximately 12 mm in standard length (SL) at first-feeding and because of their relatively large larval size they are first-fed on *Artemia* (**Fig. 5**). The main constrains for Atlantic halibut hatcheries are (1) slow growth during the late larval stages and (2) high mortalities caused by opportunistic bacteria, and 3) slow growth after weaning. The slow growth in late larval stages may be overcome by early weaning. Most often, weaning of Atlantic halibut occurs only at 60 days post first-feeding (dpff), but attempts have been made to introduce formulated diets from 20 and 50 dpff, with varying results. The first problem arising is that the larvae refuse to eat formulated feed (Harboe, Hamre and Erstad, unpublished results). It has been observed frequently, however, that they ingest inert particles such as *Artemia* cysts and pollen from pinewood, the main similarity being that both particles have neutral buoyancy and a bright color. Previous experiments have also shown improved feed ingestion with floating compared to sinking feed particles. Furthermore, the structure of the visual system of Atlantic halibut larvae indicates that they hunt prey in the horizontal plane, favoring feed intake when particles stay in the same position in the water column for some time. Additionally, the type of feed could also affect digestive capacity of proteases, carbohydrases and lipases (Caruso et al., 2009) or even ATPase activity, which is essential to ensure the ion gradient necessary for nutrient uptake in the gut.

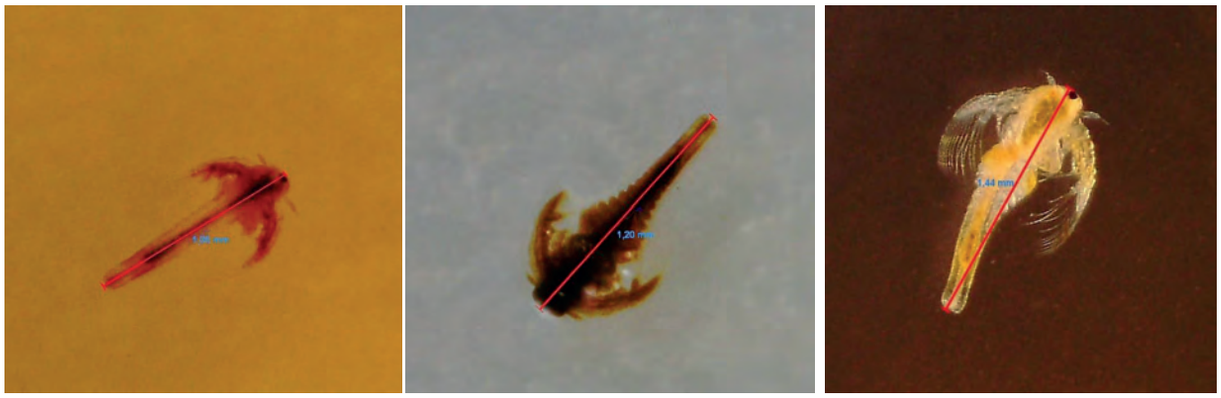


Figure 7. Artemia grown from nauplii for 2, 3 and 4 days. Length: 1.06, 1.2 and 1.4 mm, respectively. *Photo: Institute of Marine Research.*

Another strategy to alleviate the slow growth of later stage Atlantic halibut larvae is to feed them on-grown *Artemia*. On-grown *Artemia* are larger, contain more protein and phospholipids and have different micronutrient status from *Artemia* nauplii (Hamre and Harboe, unpublished results). Because of the larger size, they will probably also have a lower shell-to-soft tissue ratio. These differences may explain why Atlantic halibut fed on-grown *Artemia* have grown faster and develop into juveniles with better pigmentation and eye migration than larvae fed *Artemia* nauplii (Olsen *et al.*, 1999; Hamre and Harboe, unpublished).

In the course of DIVERSIFY, we have chosen three candidate feeds, anonymized as Feed A, Feed B and Feed C) based on their chemical content and earlier experience, and tested them on early weaning of larvae at 28 dpff and for 5 d (**Fig. 6**). In addition, experiments were performed in order to develop a production strategy for on-grown *Artemia* which was tested on the larval rearing of Atlantic halibut.

In the early weaning experiment, gut fullness was lower in the morning than in the evening, possibly because the larvae were measured before hand feeding and clay addition in the morning and after this procedure in the evening. According to the evening measurements, larvae fed *Artemia* were almost full on the first day and stayed full for the rest of the experiment. Larvae fed Feed B showed increasing fullness over the whole period and on day 5 almost 100% of the larvae were full in the evening. The fraction of larvae with food in their

gut increased more slowly on Feed A and Feed C. On the evening of day 5, 14.7±1.2 and 12.0±0.6 larvae, respectively, out of 28 had filled guts, while 0-0.3 larvae had partly filled guts on these diets.

For the production of on-grown Artemia, experiments were performed IMR and SWH. *Artemia* were hatched and either enriched directly with Larviva Multigain (Biomar, Denmark) or grown for 3-4 days on OriGreen (IMR) or Ori-One (SWH) and then enriched with Larviva Multigain (**Fig. 7**). Based on the evolution of protein and lipid content in the on-grown *Artemia* combined with labor costs, it was concluded that the optimum growth period is 3 days. There was no difference in larval performance. Survival measured as the number of fry at 70 dpff was between 42 and 48% of incubated larvae. Growth, except for the end point, has so far not been measured. However, at the end point there were no differences between the two groups. Both groups showed 100% normal pigmentation and good eye migration (score: more than 2.5/3). In the industry, the routine method is to feed *Artemia* nauplii and it is quite common to produce large fractions of Atlantic halibut larvae with abnormal pigmentation and lack of eye migration, although the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on-grown group had to be fed nauplii to get enough food. As the fish grew, more feed

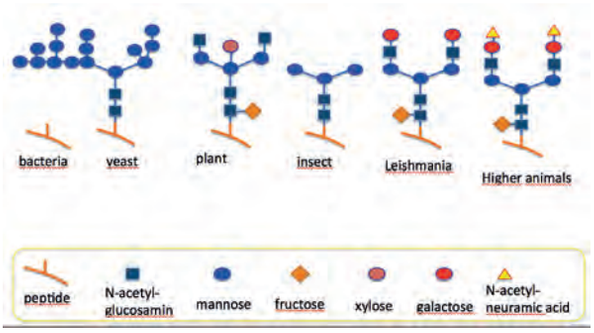


Figure 8. Glycosylation of proteins in different organisms, showing the extent of glycosylation.

was needed and due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as planned. These are all possible reasons that no differences between the groups were detected.

HEALTH

One of the diseases that can affect Atlantic halibut culture is caused by the Viral Neural Necrosis (VNN) virus, which is also known as betanodavirus. The work included in DIVERSIFY was targeted at the development of a vaccine, using recombinant nodavirus protein. Recombinant capsid protein from nodavirus expressed in *E. coli* has been shown to induce protection when formulated in a vaccine and administrated by injection. However, bacterial cells do not glycosylate the expressed protein, as do

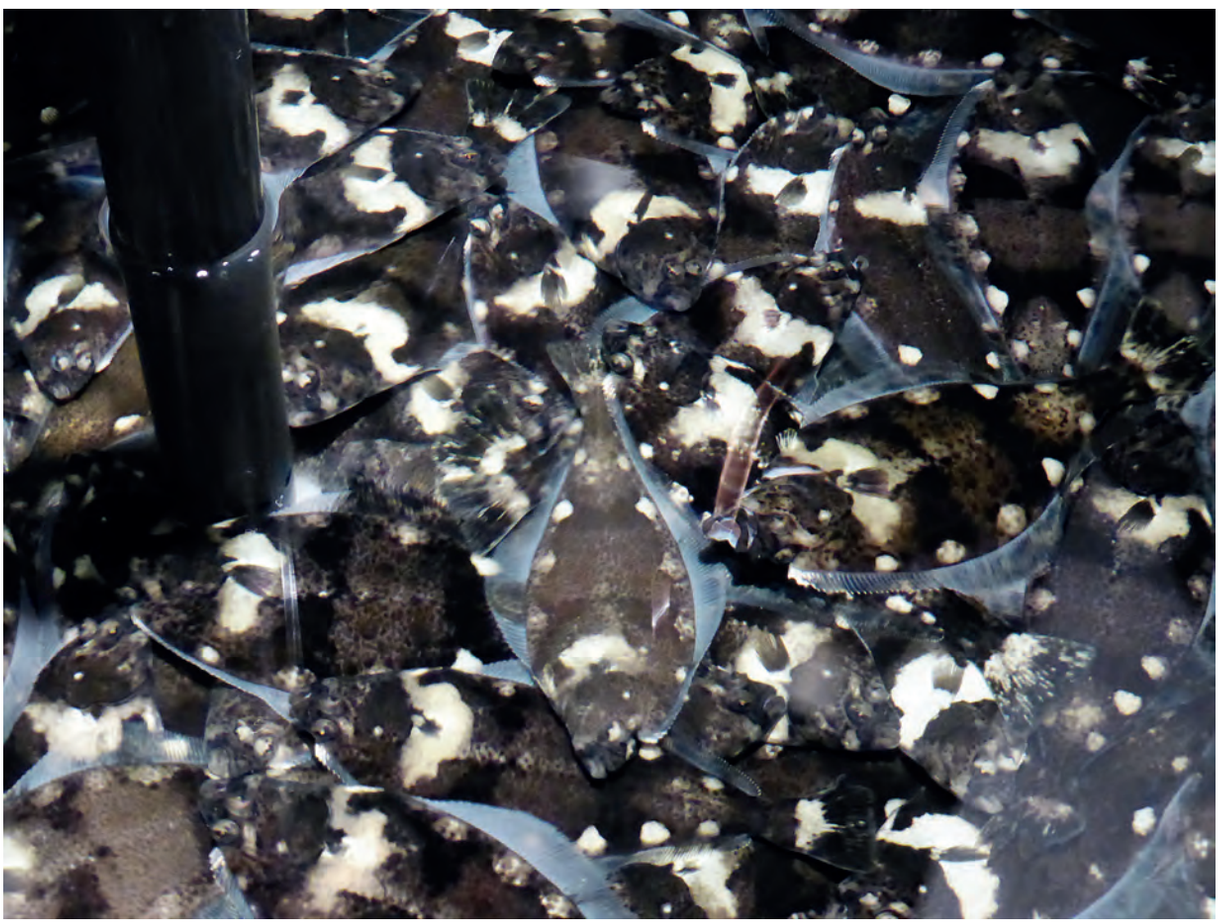


Fig 10. Farmed Atlantic halibut juveniles. *Photo: Sterling White Halibut.*

Figure 9. Atlantic halibut larvae produced at the IMR, Austevoll facilities. Ontogeny of lymphoid organs during larval stages has been characterized in detail to get an understanding of when the larvae are immune-competent and can be stimulated with immune-stimulants or vaccines. *Photo: Sonal Patel, Institute of Marine Research.*

higher eukaryotes. It might be that other expression systems (**Fig. 8**) may provide antigens more similar to the native viral proteins produced after viral infection. By expressing the capsid protein of nodavirus recombinantly in different Systems, it should be possible to find out if post-translational modifications influence antigenicity, thereby affecting its ability to induce protection when used as an antigen in a vaccine.

Assessment of the use of several expression systems for production of nodavirus capsid protein such as two eukaryotic expression systems; microalgae and a protozoan (*Leishmania tarentolae*), in addition to *E. coli* and tobacco plant was carried out. In addition, expression in microalgae is being assessed. Expression of the nodavirus capsid protein could be achieved in all three tested systems. However, it was only in the *E. coli* system that we achieved sufficient and high expression for further use of the protein as antigen for vaccination purposes. This protein has previously been shown to give partial protection in halibut (Øvergård et al 2013), but further optimization for sufficient expression in plant and protozoan systems and a method for purification of the recombinant protein is necessary.

In general, nodavirus infections and disease outbreaks in halibut are seen in larval and early juvenile stages (**Fig 9**). Thus it is important to have knowledge about when the larvae are immune-competent to plan the time-point for vaccination and avoid immune-tolerance (Patel et al 2009, Øvergård et al 2011). Moreover, size of the fish to be stimulated is a hindrance for traditional injection vaccination, especially during early larval stages. An alternative is to bath vaccinate or deliver the vaccine orally. To achieve oral vaccination the antigen has to be presented in a way that the target fish will accept and ingest. If we succeed to get uptake of the antigen by the *Artemia* offered as food item, we anticipate that it can act as a vector for oral uptake to the larvae. Vaccination of halibut during the late larval stages would provide protection during the transition period from live- to commercial- feed, and thus protection from VNN outbreak during some of the phases when halibut are prone to get the disease outbreak can be achieved. Ongoing work is focusing on two subsequent objectives. The first is to test the delivery of vaccine candidates to *Artemia* and then to Atlantic halibut larvae. The second objective includes the monitoring and assessment of the immune response and protection in the Atlantic halibut juveniles.

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 37 partners from 12 European countries –including 9 SMEs, 2 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from the project site at www.diversifyfish.eu, the Atlantic halibut leader Dr Birgitta Norberg (birgitta.norberg@imr.no) and the Project Coordinator Dr. Constantinos C. Mylonas (mylonas@hcmr.gr).

Co-funded by the Seventh Framework Programme of the European Union

REFERENCES

Hendry, C.I., Martin-Robichaud, D.J., and Benfey, T.J. (2003) Hormonal sex reversal of Atlantic halibut (Hippoglossus hippoglossus L.). Aquaculture 219 (1-4): 769-781

Babiak, J., Babiak, I., Harboe, T., Haugen, T., van Nes, S., and Norberg, B (2012) Induced sex reversal using an aromatase inhibitor, Fadrozole, in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 324-32 5: 276-280.

Larsson, D.G.J, Mylonas, C.C., Zohar, Y., Crim, L.W., 1997. Gonadotropin-releasing hormone analogue (GnRH-A) induces multiple ovulations of high-quality eggs in a cold-water, batch-spawning teleost, the yellowtail flounder (*Pleuronectes ferrugineus*).Can. J. Fish. Aquat. Sci. 54.9 : 1957-1964

Mylonas, C.C., Fostier, A., Zanuy, S. 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol.165(3): 516-534.

Norberg B, Valkner V, Huse J, Karlsen I., Lerøy Grung G., 1991. Ovulatory rhythms and egg viability in Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture 97(4): 365-371.*

Olsen, AI; Attramadal, Y; Jensen, A; Olsen, Y. (1999) Influence of size and nutritional value of Artemia franciscana on growth and quality of halibut larvae (Hippoglossus hippoglossus) during the live feed period. Aquaculture 179.1-4: 475-487.

Øvergård, A-C., Patel, S., Nøstbakken, O.J., and Nerland, A.H. (2013). Halibut T-cell and cytokine response upon vaccination and challenge with nodavirus. Vaccine, 31(19):2395-402.

Øvergård, A-C., Nerland, A.H., and Patel, S.(2011). Expression of T-cell markers during Atlantic halibut (*Hippoglossus hippoglossus* L.) ontogenesis, Dev Comp Immunol, 35, 203-213.

Patel, S., Sørhus, E., Uglenes, I.F., Espedal P.G., Bergh, Ø., Rødseth, O-M., Morton HC., and Nerland, A.H. (2009). Ontogeny of lymphoid organs and development of immunocompetance for Ig bearing cells in Atlantic halibut (*Hippoglossus hippoglossus*). Fish and Shellfish immunology, 26 (3), 385-395.

Advances in wreckfish *(Polyprion americanus)* research: the DIVERSIFY project

MYLONAS C.C., PAPANDROULAKIS, N., FAKRIADIS, I., SIGELAKI, I. AND PAPADAKI, M. (HELLENIC CENTER FOR MARINE RESEARCH, HERAKLION, CRETE, GREECE)

PELETEIRO, J.B., ALVAREZ-BLÁZQUEZ B., PÉREZ, E., MARTÍNEZ, J.M. AND LLUCH, N. (INSTITUTO ESPAÑOL DE OCEANOGRAFIA, CENTRO OCEANOGRÁFICO DE VIGO, SPAIN)

VILAR, A. (AQUARIUM FINISTERRAE, A CORUÑA, SPAIN)

LINARES, F., RODRÍGUEZ, J.L. AND PAZOS, G. (XUNTA DE GALICIA, CENTRO DE INVESTIGACIÓNS MARIÑAS, VILANOVA DE AROUSA, SPAIN)

FAUVEL, C. (INSTITUT FRANÇAIS DE RECHERCHE POUR L'EXPLOITATION DE LA MER, FRANCE)

Why wreckfish?

Wreckfish is one of the largest Serranid species, reaching a size of 100 kg (**Fig. 1)**. It is a deep-water fish found almost throughout the world and is characterized by an extended pelagic juvenile phase (Ball et al., 2000). Wreckfish is one of the most interesting new species for aquaculture diversification, due to its fast growth, late reproductive maturation, high market price, limited fisheries landings and easy manipulation in captivity (Suquet et al., 2001). Its large size makes this fish suitable for processing and development of value added products. However, there are major bottlenecks for its incorporation into the aquaculture industry, such as the difficulty in acquiring wild fish for initial broodstock formation, the lack of reproduction control in captivity and the lack of any larval rearing protocols (Fauvel et al., 2008; Papandroulakis et al., 2004; Papandroulakis et al., 2008). Reproduction and larval rearing of a very close relative, the hapuku (*Polyprion oxygeneios*) has been achieved recently in New Zealand (Anderson et al., 2012) providing some information that may be relevant to the wreckfish rearing efforts.

Figure 1. Wreckfish *(Polyprion americanus)* broodstock at the Aquarium Finisterrae, A Coruña Spain.

The EU FP7-funded DIVERSIFY project (www.diversifyfish.eu) begun in December 2013 in order to acquire the necessary knowledge for the diversification of the European Aquaculture production with some new/emerging finfish species. The project has a total budget of 11.8 million € for its 5 year duration (2013-2018), making it one of the largest research projects in the area of aquaculture funded by the

European Commission. In the case of wreckfish, DIVERSIFY examines the potential for wreckfish aquaculture, bringing together almost all partners involved so far in Europe in wreckfish domestication (**Fig. 2**), in order to acquire the necessary knowledge and develop the required procedures for the production of fertilized eggs and high quality juveniles to launch commercial production of this species. This article provides some highlights from the first 3 years of the DIVERSIFY project.

Reproduction

The research activities of DIVERSIFY regarding wreckfish reproduction focus on four objectives:

- Increase the availability of broodstocks,
- Describe the reproductive cycle in captivity,
- Develop spawning induction protocols for tank spawning, as well as artificial fertilization,
- Develop protocols for Computer Assisted Sperm Analysis (CASA) and sperm cryopreservation.

Table 1. Biometric data of wild wreckfish captured by the commercial fishery in the Azores Islands (Atlantic Ocean, Portugal).

	Min-Max	Mean	std
Total length (cm)	54-98	75.36	7.39
Standard length (cm)	48-99	65.99	7.66
Perimeter (cm)	40-81	55.13	6.14
Body weight (kg)	2.6-18.0	7.25	2.22
Eviscereated weight (kg)	2.4 – 16.0	6.73	2.00
Perivisceral fat (g)	0-339.3	70.42	71.93
Stomach weight (g)	54.2-457.2	147.98	72.12
Intestine length (cm)	61-144	96.48	13.96
Intestine weight (g)	34.2-274.0	94.48	61.57
GSI females (%)	0.05-0.65	0.29	0.17
GSI males (%)	0.01-0.54	0.11	0.11
Viscerosomatic index (VSI)	2.40-16.02	7.26	2.11

The collection of wild fish to establish new broodstocks has been carried out along the Galician coast of Spain (**Fig. 3**). It has been hindered by the scarcity of wild wreckfish and unfortunately until now only a limited number of fish (n = 5, 1-4 kg body weight) have been collected. However, this small number of fish adapted easily in captivity and showed resistance to handling, which is very encouraging for the future development of wreckfish aquaculture. Biometric data were obtained from a large number of fish captured by the commercial fishery in the Azores Islands (Atlantic Ocean), and sold fresh at the market in Vigo, Spain (**Table 1**). There was a sexual dimorphism in body size (**Fig. 4**) and fish were mostly immature.

During the years 2014, 2015 and 2016, broodstocks in different locations were followed in order to describe the reproductive cycle in captivity. These fish were maintained in a variety of environmental conditions in regards to tank size and photothermal regime, including indoor and outdoor tanks with natural photothermal conditions, and indoor tanks with simulated natural photothermal conditions or constant temperature. Maintaining these fish for a long period of time (starting well before the beginning of the project), it was quite apparent that this species exhibits a fast rate of growth (**Fig. 5**), and easy adaptation to the captive environment and handling procedures.

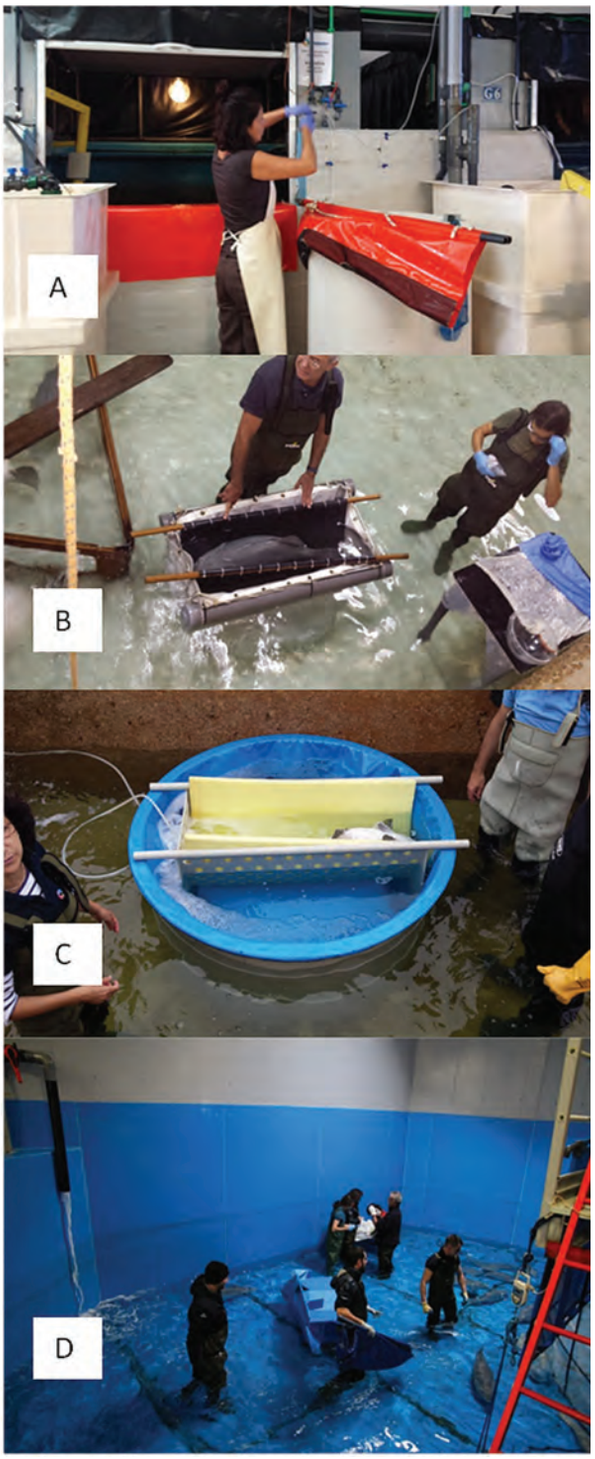


Figure 2. Different facilities working with wreckfish broodstock. (A) Hellenic Center for Marine Research (HCMR) Greece, (B) Institute of Oceanography, (IEO) Spain, (C) Xunta de Galicia (CMRM, IGAFA), Spain, (D) Aquarium Finisterrae, (MC2) Spain.

Monthly or bimonthly samplings were performed and blood, ovarian biopsies and sperm samples were

Figure 3. Wreckfish captured in the West of Corrubedo Cape, La Coruña, Spain.

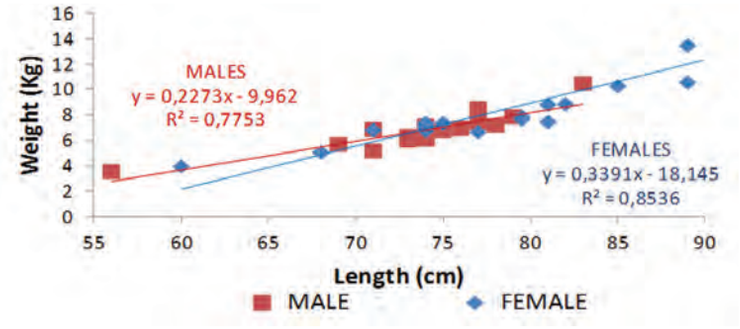


Figure 4. Length-weight relationship of wild wreckfish showing a sexual dimorphism.

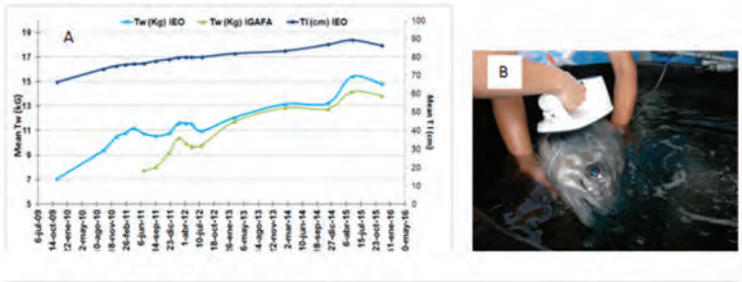


Figure 5. A) Mean total length (cm) and body weight (kg) of two wreckfish broodstocks maintained in captivity since July 2009 at IEO and CMRM (IGAFA), Spain. B) All breeders were individual tagged with PIT tags.

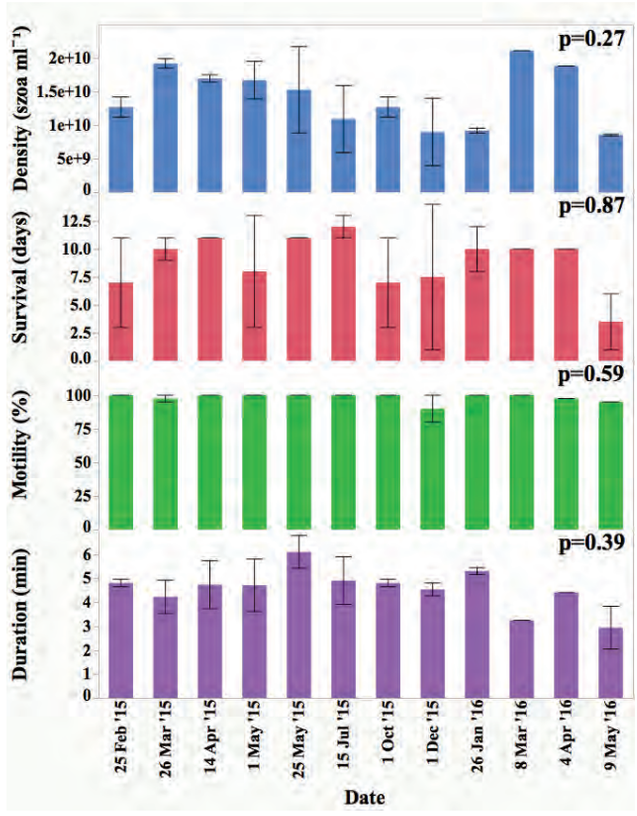


Figure 6. Sperm quality parameters of captive wreckfish that were in spermiating condition throughout the year. The fish were maintained under constant temperature (15-16°C), but simulated natural photoperiod at HCMR (Greece).

obtained. The ovarian biopsies were examined on site, as well as after histological processing, and the blood samples are currently being analyzed for their sex steroid content (testosterone, 11-ketotestosterone, 17β-estradiol, and 17,20β-dihydroxyporgesterone), which are involved in the process of gametogenesis and maturation. Based on the results obtained from the various broodstocks over the 3-year period, it has been demonstrated that males exhibit good sperm quality with large amounts of expressible sperm during an extended reproductive period (April-July), while a proportion of males were shown to be spermiating throughout the year (**Fig. 6**). Gonadal recrudescence in females begins in the fall, but the main part of vitellogenesis takes place in the Winter (Dec-Feb), and oocyte maturation in captivity starts in March and peaks between April and June. Vitellogenesis continues until the oocytes reach a size of ~1200-1400 μm in diameter, at which time oocyte maturation may take place (**Fig. 7**). The vitellogenic process is long, probably related to the low water temperatures that this species can be found and also to the relatively large egg size for a marine fish.

Spontaneous spawning was observed, even though in an unpredictable pattern, mostly at the IEO and Aquarium Finisterrae facilities. At the HCMR facilities, fish that were in the appropriate reproductive maturation were treated with ethylene-vinyl acetate (EVAc) implants loaded with gonadotropin releasing hormone agonist (GnRHa), and were induced to undergo oocyte maturation and ovulation successfully. Both spontaneous spawning in different tank conditions and artificial fertilization were tried as different methods of viable egg production, since batches of eggs with low fecundity and fertilization rates were produced if the fish were allowed to spawn spontaneously in the tank. This may be indicative of a breeding behavior dysfunction –similar to what has been reported in Senegalese sole (*Solea senegalensis*) (Guzmán et al., 2009)- since the males always produced large volumes of high quality sperm. Using artificial insemination at the HCMR, a number of fertilized eggs were delivered to the hatchery for larval rearing (**Fig. 8**). Unfortunately, the exact timing of ovulation after the hormonal treatment and the post-ovulation survival of the eggs are currently not known and might be the reasons for the low fertilization of the artificially inseminated eggs.

On the other hand, spontaneous spawning in the IEO and Aquarium Finisterrae stocks produced a large number of fertilized eggs and achieved satisfactory

fertilization success (**Fig. 9**). During the spawning season of 2015, a total of 10 spawns were obtained from the IEO broodstock between March and June. The majority of spawns were spontaneous, except for one artificial stripping from the IEO and two from the Aquarium Finisterrae broodstock. During 2016, from April to the end of May, 7 spontaneous spawns were obtained from the IEO, and 12 spontaneous spawns and two by stripping from the Aquarium Finisterrae broodstocks.

Overall, spontaneous spawns were achieved in two of the four stocks (IEO, Aquarium Finisterrae) while both spawning after GnRHa therapy and by artificial stripping and fertilization was achieved in three of the four stocks (HCMR, IEO and Aquarium Finisterrae), with a fertilization success ranging between 49 and 100 % (**Fig. 10**). The wreckfish eggs have a large diameter (1.996±0.034 mm), with a large lipid droplet allowing them to float. Hatching takes place after 5 d of incubation at 16±0.8°C (**Fig. 11**)

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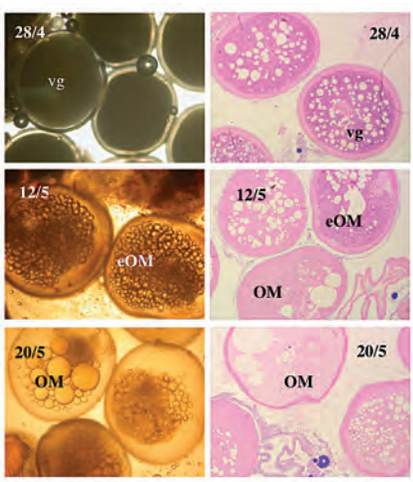


Figure 7. Wet mount and histological sections of biopsies from wreckfish during the 2014 reproductive season (dates on each photo). eOM = early oocyte maturation, OM = oocyte maturation, Vg = vitellogenic oocytes

Figure 8. Artificial insemination of wreckfish at the HCMR facilities.

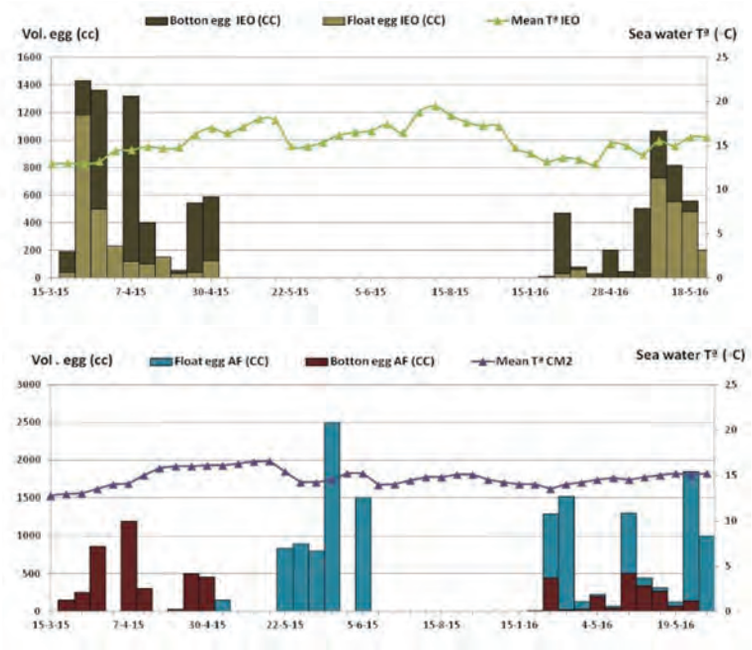


Figure 9. The volume (cubic centimetres cc) of viable floating and non-viable sinking eggs of wreckfish obtained from spawns at the IEO (upper) and Aquarium Finisterrae (lower) facilities between March 2015 and May 2016. A number of spawns were incubated and larvae were obtained.

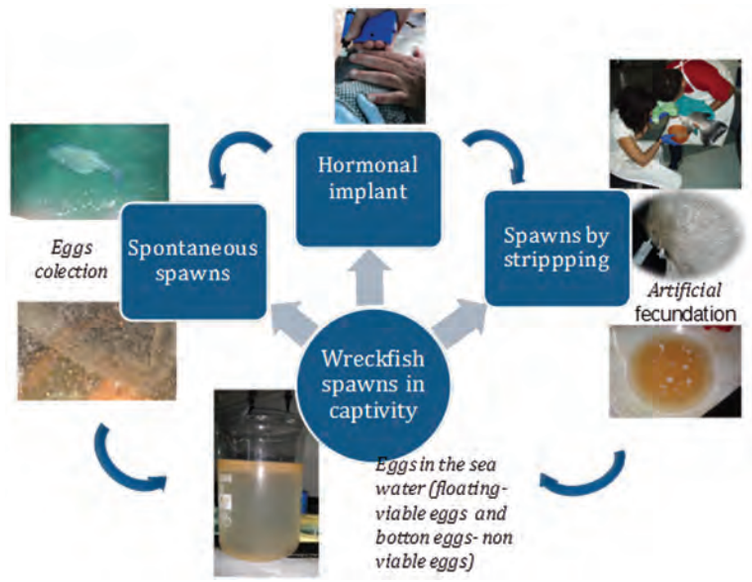


Figure 10. Wreckfish spawned spontaneously in captivity, and after a hormonal therapy with GnRHa implants eggs could be obtained either after tank spawning or using artificial fertilization. In all cases the eggs were collected for evaluation and subsequent incubation and larval rearing.

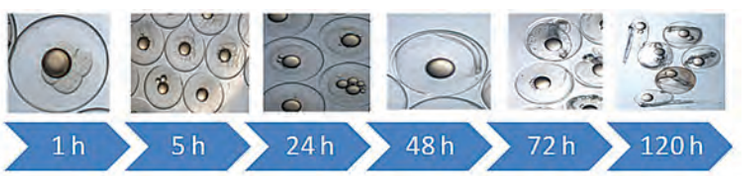


Figure 11. Embryonic development of wreckfish using eggs obtained from captive breeders.

As far as the development of the CASA (**Fig. 12)**, wreckfish sperm exhibited a high percentage of motile cells at activation and one of the highest initial speeds recorded for fish sperm. This high speed was associated with a long swimming duration compared to other marine fish. The long duration exhibited a double trajectory shape. The first trajectory was straight (associated with the search of target eggs) and then the trajectory began bending, which was interpreted as a phase of searching for the micropyle on the egg surface. Cryopreservation of wreckfish sperm was achieved, while chilled storage does not seem to be a good solution for the management of sperm for artificial fertilization. The performance of frozen/thawed wreckfish sperm was half compared to samples of fresh sperm in terms of percentage of motile sperm and duration of swimming, while the velocity in modified Leibovitz medium was similar to that of fresh sperm. Since wreckfish produce large volumes of high quality sperm in terms of concentration, velocity and duration of motility, the loss of sperm quality due to freezing may be compensated by increasing the number of spermatozoa used per egg, as is usually practiced in other species.

Larval husbandry

The main objectives of DIVERSIFY are to understand the larval requirements in order to establish a rearing protocol. In particular, the effect of rearing temperature was studied and the description of the ontogeny of the digestive system was considered as a prerequisite for the development of an appropriate feeding protocol.

Progress during the last year was made towards the optimization of the environmental parameters. Taking advantage of the improved spawns and the availability of eggs, which allowed us to perform several trials, testing different incubation temperatures, it was shown that the optimal incubation temperature is 16±0.8°C. At this temperature range we obtained the best results regarding normal embryonic development and hatching rate of the eggs that reached 65%.

Regarding larval rearing, the results were not satisfactory, as the maximum period that the larvae survived never exceeded 27 days post hatching (dph). Several larval rearing trials were performed during the project's life. In all cases, similar rearing results were obtained by both the HMCR and IEO (**Fig. 13**). Larval total length was 4.70±0.27 mm at 1 dph. Yolk sac was consumed by 11 dph at 14-17°C and by 8 dph at 17-20°C seawater temperature. Mouth opening occurred at 7 and 4 dph at 14-17°C and 17-20°C, respectively. Following mouth opening, larvae were fed with enriched rotifers and *Artemia* nauplii, using different enrichment protocols.

During rearing, some malformed individuals were observed (**Fig. 14**). This problem could be related to inadequate nutrition, environmental conditions, oxidative stress, and husbandry conditions. We expect that in the coming year further studies will produce better results, towards the development of an efficient larval rearing protocol for this species.

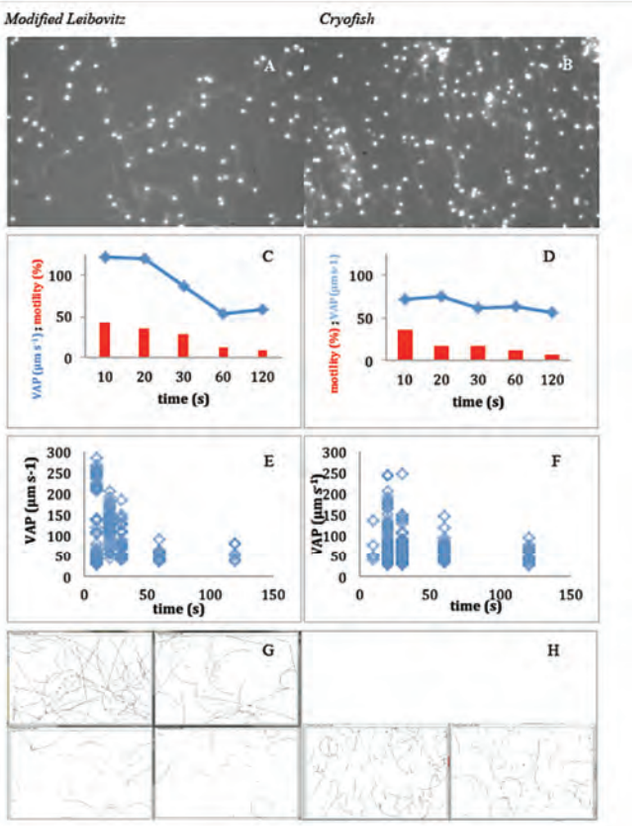


Figure 12. Wreckfish sperm status after cryopreservation in modified Leibovitz and Cryofish media. A and B: Pictures of the spermatozoa diluted in the different media (extracted from video records), Cryofish samples (B) show aggregations of spermatozoa unlike samples cryopreserved in modified Leibovitz (A). C and D: Mean velocity decrease and variations of the percentage of motile spermatozoa with time in the different media. E and F: individual velocities of spermatozoa recorded in the different media showing that modified Leibovitz (E) allows a high recovery of a larger number of spermatozoa compared to Cryofish (F). G and H: illustration of tracks generated by CASA for the spermatozoa stored in the two media: Leibovitz (G) and Cryofish (H).

Nutrition

Wreckfish nutritional requirements and optimum diets are missing. There is only limited information related to feeding habits and rates of wild-caught fish reared in captivity. The research in DIVERSIFY focuses on two main important aspects. Firstly, broodstock nutrition to determine the influence of broodstock feeds on fecundity and spawning quality. Secondly, larval nutrition to test the effectiveness of live prey and the influence of enrichment on wreckfish larval performance.

A comparative study on the composition of wild fish *vs* captive-reared wreckfish broodstocks was conducted. Analysis of tissues of wild and captive-reared wreckfish showed that cultured fish have more lipids in the muscle (27.5% DW) and liver (62%) than wild fish, which have 7% in their muscle and 40% in liver. In contrast, protein content is higher in the muscle of wild wreckfish than in captive-reared fish and some differences were also observed in the fatty acid profile with higher values of polyunsaturated fatty acids (PUFA) and n-3 PUFA in wild than in captive-reared wreckfish. The docosahexaenoic acid (DHA) values represent 11% in the muscle of captive-reared fish and 26% in wild fish **(Table 2)**.

Some commercial broodstock feeds were analyzed, showing that they have a high amount of fat for wreckfish broodstock and a new dry food was formulated on the basis of the data obtained from wild fish (**Table 3**). Our initial results showed that broodstock feed must contain high amounts of protein, low lipid content and a large amount of n-3 highly unsaturated fatty acids (HUFA), and the eicosapentaenoic acid (EPA) arachidonic acid (ARA) ratio must be around 1.5, similar to what has been observed previously in wild wreckfish. A comparison of feeding of broodstock with semi-moist diet and the new formulated diet was conducted, and a clear relationship between fatty acid profile of oocytes from the females and the two diets was found. Furthermore, some differences were observed in the fatty acid profile of oocytes from females of different wreckfish broodstock showing that there is a relationship between fatty acid content and oocyte development.

continued on page 18

Table 2. Biochemical composition of muscle, liver and gonad of wild and captive-reared wreckfish.

	Muscle		Liver	
	Wild fish	Captive-reared	Wild fish	Captive-reared
Proteins(%DW)	84.41±7.34	75.92±8.88	37.94±13.66	31.10±9.42
Lipids(%DW)	6.92±3.39	27.49±10.06	40.19±15.25	61.76±12.18
Fatty acids (% total)				
SAFA´s	28.83±1.28	24.46±1.25	26.11±3.51	22.44±2.27
MUFA´s	32.09±5.43	44.98±1.02	56.23±8.80	60.50±5.45
ARA	3.11±0.79	1.32±0.38	1.55±0.88	0.58±0.12
EPA	4.55±0.70	8.11±1.17	3.09±1.37	2.92±0.19
DHA	26.38±3.33	10.85±2.66	9.31±5.05	7.29±1.71
PUFA´s	39.08±4.41	30.57±0.58	17.66±8.19	17.07±3.32
Total n-3	34.51±3.75	23.78±1.68	14.93±7.01	13.44±2.00
Total n-6	4.08±0.81	6.02±1.85	2.55±1.23	3.48±1.99
n-3/n-6	8.50±1.18	4.51±2.39	5.79±1.42	4.97±3.31
DHA/EPA	5.69±1.23	1.38±0.49	2.99±0.91	2.48±0.47
EPA/ARA	1.54±0.37	6.58±2.15	2.13±0.60	5.16±1.00

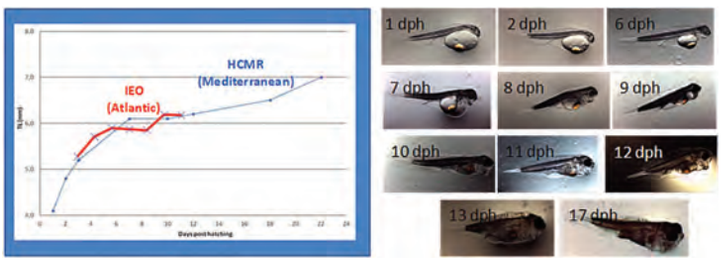


Figure 13. Wreckfish larval growth obtained in the trials that took place at HCMR and IEO (A). Development of larvae for the first 17 days post hatching (B).

Figure 14. Wreckfish larvae with malformations, similar to what has been referred to as "swollen yolk sac syndrome" or the "blue sack disease".

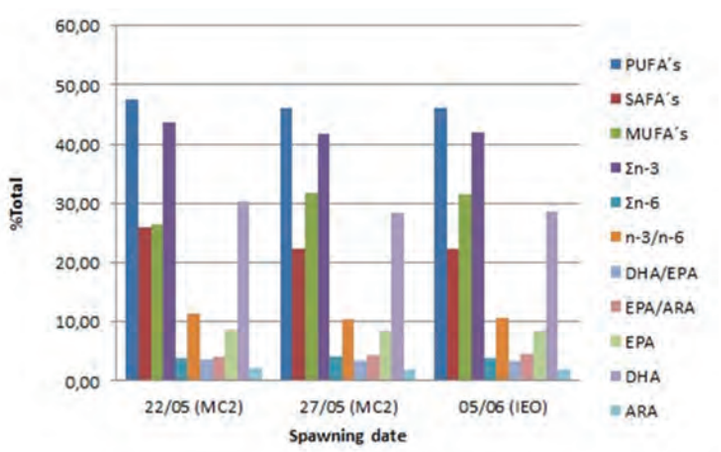


Figure 15. Fatty acid composition of wreckfish larvae at 10 dph, from three different spawnings (two from Aquarium Finisterrae broodstock and one from IEO broodstock).

Table 3. Composition of a new formulated dry feed from wreckfish broodstocks.

Ingredients	Dry food (%)
Fishmeal 70 LT FF Skagen	25.0
CPSP 90	10.0
Squid meal	34.2
Krill meal (Aker Biomarine)	7.5
Wheat Gluten	7.0
Wheat meal	7.25
Tuna oil	1.0
Algatrium 70% DHA	0.2
Incromega DHA 500TG	1.0
VEVODAR	1.3
Vit & Min Premix PV01	2.0
Lutavit E50	0.05
Soy lecithin - Powder	1.5
Macroalgae mix	1.0
Antioxidant powder (Paramega)	0.2
Antioxidant liquid (Naturox)	0.2
SelPlex - Se yeast	0.02
Carophyll Pink 10% - astaxanthin	0.05
Nucleotides (Nucleoforce)	0.03
L-Taurine	0.5
Total	100.0

Related to larval nutrition a new enrichment medium for larval wreckfish was designed on the basis of analyses of wreckfish eggs and gonads, and it will be tested in the next years. In addition, the fatty acid profile of larvae in the first days of life was described. The main fatty acids of wreckfish larvae at 10 dph are shown in **Fig. 15**. It appears that PUFA, saturated fatty acids (SAFA) and mono-unsaturated fatty acids (MUFA) values have a little variation in the first days of life.

The results obtained so far in DIVERSIFY in the studies of wreckfish indicate that acquisition of eggs is difficult but achievable in captivity, either by spontaneous spawns or hormonally induced protocols. However, juvenile production is not yet achieved, and in the following two years of the project it is expected to optimize the larval rearing methods, based on the use of new enrichment media for live food.

References

Anderson, S.A., Salinas, I., Walker, S.P., Gublin, Y., Pether, S., Kohn, Y.Y., Symonds, J.E., 2012. Early development of New Zealand hapuku *Polyprion oxygeneios* eggs and larvae. Journal of Fish Biology 80, 555-571.

Ball, A.O., Sedberry, G.R., Zatcoff, M.S., Chapman, R.W., Carlin, J.L., 2000. Population structure of wreckfish *Polyprion americanus* determined with microsatelite genetic markers. Marine Biology 137, 1077-1090.

Fauvel, C., Suquet, M., Sévère, A., Mylonas, C.C., Papandroulakis, N., 2008. Slow-release GnRHa therapy prevented atresia during vitellogenesis and induced ovulation of captive wreckfish (*Polyprion americanus*). Cybium 32(2) suppl, 191.

Guzmán, J.M., Ramos, J., Mylonas, C.C., Manaños, E., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. Aquaculture 291, 200-209.

Papandroulakis, N., Suquet, M., Spedicato, M.T., Machias, A., Fauvel, C., Divanach, P., 2004. Feeding rates, growth performance and gametogenesis of wreckfish (*Polyprion americanus*) kept in captivity. Aquaculture International 12, 395-407.

Papandroulakis, N., Mylonas, C.C., Syggelaki, E., Katharios, P., Divakaran, S., 2008. First reproduction of captive-reared wreckfish (*Polyprion americanus*) using GnRH implants, European Aquaculture 2008, Krakow, Poland.

Suquet, M., Normant, Y., Sévère, A., Barone, H., Fostier, A., Le Delliou, H., Fauvel, C., 2001. Growth performance of the wreckfish (*Polyprion americanus*) in captivity. European Aquaculture Society, Oostende, Belgium, 586-589 pp.

Co-funded by the Seventh Framework Programme of the European Union

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 2 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from the project site at "www.diversifyfish.eu".

New species for EU aquaculture

Exploring the biological and socio-economic potential of new/ emerging candidate fish species for the expansion of the European aquaculture industry

MYLONAS, C.C., HELLENIC CENTER FOR MARINE RESEARCH, CRETE, GREECE (PROJECT COORDINATOR) MYLONAS@HCMR.GR

ROBLES, R., CENTRO TECNOLÓGICO DE LA ACUICULTURA DE ANDALUCIA - CTAQUA, SPAIN (DISSEMINATION LEADER) R.ROBLES@CTAQUA.ES

ESTÉVEZ, A., IRTA-CENTRE DE SANT CARLES DE LA RÀPITA, SPAIN (MEAGRE LEADER) ALICIA.ESTEVEZ@IRTA.CAT

PAPANDROULAKIS, N., HELLENIC CENTER FOR MARINE RESEARCH, CRETE, GREECE (GREATER AMBERJACK LEADER) NPAP@HCMR.GR

FONTAINE, P., UNIVERSITY OF LORRAINE, FRANCE (PIKEPERCH LEADER) P.FONTAINE@UNIV-LORRAINE.FR

NORBERG, B., INSTITUTE OF MARINE RESEARCH, NORWAY (ATLANTIC HALIBUT LEADER) BIRGITTA.NORBERG@IMR.NO

ALVAREZ-BLÁZQUEZ B., INSTITUTO ESPAÑOL DE OCEANOGRAFIA, CENTRO OCEANOGRÁFICO DE VIGO, SPAIN, (WRECKFISH LEADER) BLANCA.ALVAREZ@VI.IEO.ES

KOVEN, B., NATIONAL CENTER FOR MARICULTURE, IOLR, EILAT, ISRAEL (GREY MULLET LEADER) BMKOVEN@GMAIL.COM

GEMMA TACKEN, STICHTING WAGENINGEN RESEARCH, NETHERLANDS (SOCIOECONOMICS LEADER), GEMMA.TACKEN@WUR.NL

Figure 1: A group photo during the latest Annual Coordination Meeting, which was held at Palau Macaya, Barcelona Spain (17-19 January 2017).

The European Commission project DIVERSIFY (FP7-KBBE-2013, GA 603121) started in December 2013, with the objective of carrying out focused research in a number of new/emerging finfish species, in order to support the diversification of the European aquaculture industry and thus contribute to its sustainable expansion. The project has a total budget of 11.8 million € for its 5-year duration and it is coordinated by Dr. Constantinos C. Mylonas of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC), one of the three institutes of the Hellenic Center for Marine Research (HCMR). The DIVERSIFY consortium includes more than hundred senior scientists (**Fig. 1**) from twenty research and academic institutions, three Large Enterprises, eight Small and Medium Enterprises (SME), five Professional Associations and one consumer NGO (**Table 1**).

The project DIVERSIFY (**www.diversifyfish.eu**) has identified a number of new/emerging finfish species, based both on their biological and economical potential, to cover the entire European geographic area and to stimulate different aquaculture types. Although the emphasis is on Mediterranean cage-culture, fish species suitable for cold-water, pond/extensive and fresh water aquaculture have been included as well *(Aquaculture Europe 39(1) March 2014)*. These new/emerging species are fast growing and/or large finfishes marketed at a large size and can be processed into a range of products to provide the consumer with both a greater diversity of fish species and new value-added products. The fish species studied include **meagre** (*Argyrosomus regius*) and **greater amberjack** (*Seriola dumerili*) for warm-water marine cage culture, **wreckfish** (*Polyprion americanus*) for warm- and cool-water marine cage culture, **Atlantic halibut** (*Hippoglossus hippoglossus*) for marine cold-water culture, **grey mullet** (*Mugil cephalus*) a euryhaline herbivore for pond/extensive culture and **pikeperch** (*Sander lucioperca*) for freshwater intensive culture using recirculating aquaculture systems (RAS).

In the three-and-a-half years that DIVERSIFY has been running, a number of research activities have been carried out in the scientific disciplines of Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health, Socioeconomics and Final product quality. As the project is approaching its conclusion in November 2018, significant progress has been made in all six species. To speed up the dissemination of these results to the interested stakeholders and the aquaculture industry, one-day species-specific workshops are being organized for 2018, to be carried out at different locations around Europe (see later for more information). Moreover, four promotional workshops are being organized in four European countries, to create awareness of the project findings in the area of socioeconomic, marketing and product development. These events are targeted for specialized audiences in the fish market sector, such as fish producers, processors and retailers, consumer organizations, and fisheries and aquaculture authorities.

A full-day special session for research results from DIVERSIFY is also planned during the Aquaculture Europe 2017 conference in Dubrovnik, Croatia (17-20 October 2017). In the present article, we are presenting some highlights of the major achievements of the project so far.

Participating organizations in DIVERSIFY

Greece: Institute of Marine Biology, Biotechnology and Aquaculture (HCMR/IMBBC); ARGOSARONIKOS FISHFARMS SA; AQUACULTURE FORKYS SA; IRIDA SA; Galaxidi Marine Farms S.A.; Hellenic Research House AE; VAS. GEITONAS & Co Ltd; Federation of Greek Maricultures.

Spain: Institut de Recerca i Tecnologia Agroalimentaries (IRTA-San Carles de la Rapita); Parque Científico y Tecnológico de la Universidad de Las Palmas de Gran Canaria; Centro Tecnológico de la Acuicultura de Andalucia (CTAQUA); Universidad de la Laguna; Instituto Español de Oceanografía; Asociación Empresarial de Productores de Cultivos Marinos-APROMAR; Consellería do Medio Rural e do Mar-Xunta de Galicia; Ayuntamento de A Coruña (Museos Científico Coruñeses); CANEXMAR SL; ANFACO-CECOPESCA.

France: French Research Institute for the Exploitation of the Sea (IFREMER); Université de Lorraine

Israel: Israel Oceanographic and Limnological Research-National Center for Mariculture; DOR AQUACULTURE Ltd

Norway: Institute of Marine Research, National Institute of Nutrition and Seafood Research; Skretting Aquaculture Research Center AS; Stirling White Halibut AS

The Netherlands: Stichting Wageningen Research (previously DLO/LEI); Eindhoven University of Technology

United Kingdom: The University of Aberdeen

Italy: Universitá degli Studi di Bari «Aldo Moro»

Belgium: Université de Namur; European Food Information Council; Fish2Be; S.A.

Denmark: Technical University of Denmark, Aarhus University (MAPP Center)

Germany: German Association of Fish Processors (Bundes Verband Fisch, BVFi E.V.)

Hungary: Hungarian Aquaculture Association (Mayar Akvakúltra Szövetség, MASZ)

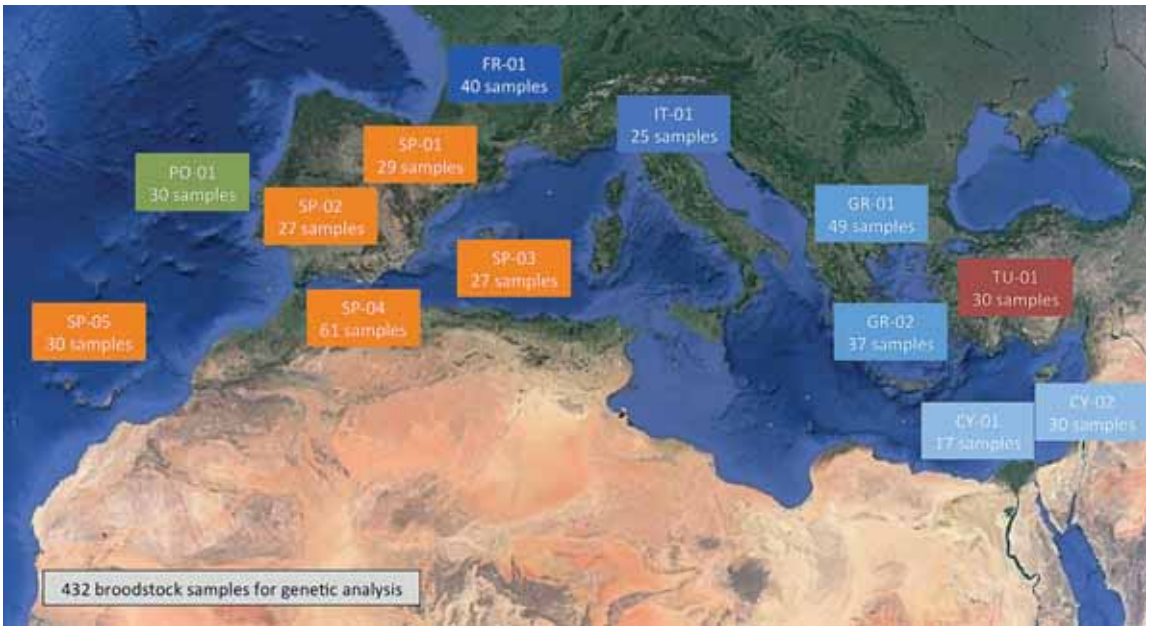


Figure 2: Geographic distribution of meagre samples used in the study of genetic variation in the available research and commercial broodstocks from the Canary Islands to Cyprus.

MEAGRE

Meagre is considered an emerging species that has been cultured increasingly in Europe in the last two decades, though in relatively limited quantities compared to gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Some of the attractive attributes of meagre include **large size, good processing yield, low fat content, excellent taste and firm texture** (Monfort, 2010). A survey of meagre producers carried out during the proposal stage of DIVERSIFY identified the principal bottlenecks to the expansion of the industry *(Aquaculture Europe 40(1) March 2015)*. These include **limited genetic variation of the available broodstocks**, **variable growth rates** and **fish health** related issues, such as the wide occurrence of **systemic granulomatosis** (Elkesh et al., 2012), which may stem from the fact that no specific diets have been developed for this fish. Also, socioeconomic factors have been identified as bottlenecks, including the need for a more expanded market and diversification of available products beyond the whole fresh fish.

To address the issue of limited genetic variation in the broodstocks, DIVERSIFY carried out a genetic characterization of different meagre broodstocks in Europe and evaluation of available variability. Eighteen microsatellite markers (Short Tandem Repeats, STRs) were used to genetically characterize 13 meagre broodstocks held in aquaculture facilities from seven countries, ranging from Gran Canaria in Spain to Cyprus (**Fig. 2**). The analyses indicated that the genetic variation in captive broodstocks is more than adequate to form a base population in breeding programs, even though some broodstocks could benefit from the addition of new breeders. The mean number of alleles and observed heterozygosity were estimated at 3.7 and 0.48, respectively, with the captive populations showing lower mean number of alleles and observed heterozygosity than wild populations (around 3 times and 18% lower, respectively). Population genetics analyses using AMOVA revealed that 18.2% of the variation was found among studied broodstocks, while the remaining 81.8% was located within populations. Moreover, a Factorial Correspondence Analysis showed two clusters; in the first cluster, there seems to be a correlation with the geographical distribution of populations (Atlantic Vs Mediterranean), while in the second there is only the population from Turkey.

The next part of the genetic work for meagre was to characterize for the first time the muscle and liver transcriptome in the species, in order to base future physiology performance. This was done through transcriptome sequencing and RNA-Seq; the assembled transcripts were assigned to a wide range of biological processes including growth, reproduction and behavior. The whole transcriptome has been scanned to identify thousands of markers that may have impact on the functional role of protein-coding genes. The marker search revealed a total of 48,526 high-quality Single Nucleotide Polymorphism (SNP) markers and 20,582 STR markers. The relatively low rate of polymorphism reported might be indicative of inbreeding in the particular broodstock used.

In order to construct a genetic linkage map in meagre and perform preliminary Quantitative Trait loci (QTL) analysis, we used the double-digest restriction-site associate DNA (ddRAD) methodology to genotype two full-sib families and constructed a genetic linkage map that included 731 markers organized in 27 linkage groups (LG), which means 3 LGs-chromosomes more than the haploid number determined in the karyotype of this species (n=24). Comparative genomic analyses through similarity searches revealed conserved synteny with more than one third of the loci having a region homologous to the European seabass genome. Lastly, we completed a genome scan for QTLs that affect body weight (BW) and total length (TL) in fish from five full-sib families using the markers developed for

the linkage map of meagre distributed across 27 LGs. Model mapping from the two larger families identified 5 QTLs on only two LGs (11 and 20) that exhibited significant evidence of linkage at the genome level. Multiple QTLs on LG20 seem to affect both BW and TL, and were located at close positions, suggesting that the same genetic factors may control variability in these traits and are expected to be of great value in future Marker Assisted Selection (MAS) programmes.

Reproduction is no longer considered a bottleneck in meagre aquaculture, since recent studies have produced efficient protocols for the control of reproduction and the induction of spawning in aquaculture (Duncan et al., 2013; Mylonas et al., 2015; Mylonas et al., 2016). Nevertheless, DIVERSIFY developed further techniques to assist in the implementation of breeding selection programs, such as (a) paired-spawning of fish in tanks, and (b) *in vitro* fertilization methods. For the first objective, paired-spawning experiments were completed to determine the potential of paired spawning inductions with male rotation to perform a dialed cross-mating design as the basis of a breeding program. The efficacy of spawning pairs with male rotation was high (76%) and across the three experiments a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success (**Fig. 3**). However, not all paired crosses with male rotation were successful, and a number of females after consecutive successful spawning inductions either failed to spawn or did not present vitellogenic oocytes, preventing their further induction as planned in the dialed cross design. This failure to spawn or maintain maturity status after successive successful spawning inductions appeared to represent a change in spawning kinetics from the prolonged (up to 17 weeks) induced spawning period observed in a previous same-pair experiment and other studies. This change in kinetics may be attributed to the stress of male rotation and consideration should be made that as the number of rotations increases, spawning pairs may fail or induced spawning may not be possible. However, together these experiments have shown that paired spawning of meagre is possible for the production of known families from parents with known phenotypes. Obtaining a large number of families with adequate fecundities that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite for a breeding program.

Since the development of strip spawning with *in vitro* fertilization methods is necessary for the meagre aquaculture industry (as an alternative to paired-spawning), in order to facilitate planned crosses between selected breeders, the following work was also undertaken in DIVERSIFY (**Fig. 4**). Females with advanced stages of maturity were induced to ovulate with a single injection of 15 μg kg^{-1} gonadotropin releasing hormone agonist (GnRHa). The injection was applied at 20:00-22:00 hours and the females held separate from males in darkness until being checked for ovulation. Checks for ovulation were made every 2.5 hours from 35 to 45 hours post GnRHa injection. When ovulated eggs were obtained, *in vitro* fertilization was made and egg quality assessed by determining

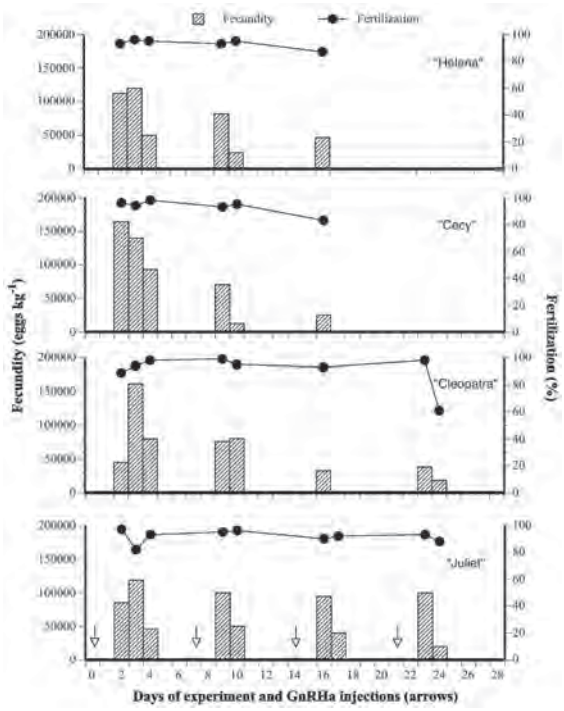


Figure 3. Daily batch relative fecundity and fertilization success of individual meagre females (n=4) induced to spawn with multiple GnRHa injections (n=4, once every week) and paired with four males (named Romeo, Cesar, Peri and Paris). At every GnRHa injection, the males were moved to a different tank, being paired with a different female so at the end all males were paired with all females.

Figure 4. Different phases of artificial fertilization experiments at the facilities of IRTA (Spain): Top left: sperm collection; Top right: egg collection; Bottom left: gamete mixing and activation; Bottom middle: view of 134 individual fertilized batches of eggs from a factorial cross with 3 females and 4 males, and 3 different types of sperm storage (fresh, chilled stored and frozen) in triplicates; Bottom right: devices for the assessment of embryo development after artificial fertilization.

the percentage of developing eggs. An injection of GnRHa was also applied to males, and sperm quality was assessed. Ratios of sperm to eggs were tested, from approximately 3,000 to 500,000 spermatozoa per egg. Ovulated eggs were observed from 35 hours onwards. Optimal egg quality was observed at 38-39 hours after the GnRHa injection. From 35 to 38-39 hours there was a slight increase in egg quality and the ease with which eggs could be stripped, indicating that after 35-38 hours there was a possibility that eggs were not fully ovulated. After 38-39 hours, there was a decline in egg

quality to 43-44 hours. Sperm quality was maintained without decline for up to 7 hours in Leibovitz medium and sperm quality did not appear to affect fertilization success. The *in vitro* fertilization was made by rapidly mixing eggs, sperm and seawater simultaneously, and the first 30 seconds after activation were identified as the optimal period for fertilization. The optimal ratio of sperm to eggs to obtain high percentage of fertilization was above 200,000 spermatozoa per egg. The protocol was used successfully in a large factorial cross of 120 *in vitro* fertilizations using either fresh or cryopreserved sperm.

One of the most important bottlenecks of meagre production is the occurrence of systemic granulomatosis (SG), a pathological condition affecting the majority of farmed populations. Systemic granulomatosis is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. The aetiology of the disease is unknown, however it is suspected that it is related to nutritional factors. One of the objectives of DIVERSIFY is to identify potential nutritional causes of SG via several feeding trials. These included the effect of vitamin D, calcium (Ca) and Phosphorous (P) and Ca/P ratios, and the effect of fishmeal (FM) substitution of the diets with plant proteins (PP) and P supplementation.

The development of SG was not prevented by vitamin D_3 (**Fig. 5**). The organs that seemed to be affected first from SG were the kidney and the liver. The other soft tissues exhibited granulomas after the second month of the feeding trial, mainly visible by microscope. Although the addition of vitamin D_3 did not prevent the development of SG in meagre, the study provided a significant lead concerning the pathophysiology of SG that will further assist the detailed description of this peculiar disease. In terms of the effect of Ca and P, although the fish of all groups exhibited granulomas, high P content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis. Fish fed this diet exhibited a significantly lower percentage of liver and kidney calcification, and there was a significantly higher percentage of fish with no granulomas, compared to those fed the low and medium content of P. As before, the organs mostly affected by granulomatosis were the kidney, the liver and the spleen. This result is in accordance with the hypothesis that granulomatosis could be a metabolic disorder or a nutritional disease. Accordingly, PP in the diets of meagre were found to affect negatively SG, while P supplementation in the PP diets did not affect the overall condition, but had a positive effect in the liver of the fish. Fish fed 60% FM were in a significantly better state regarding the total score of granulomas in all tissues. Furthermore, fish fed this diet exhibited a significantly lower percentage of liver and spleen calcification, and there was a significantly higher percentage of fish with no granulomas in these organs compared to those fed the PP, PP+Medium P and PP+High P diets. Phosphorus supplementation in the PP diets did not affect the overall condition of the fish (as assessed by the granuloma scoring system), but had a positive effect in the liver of the fish. Specifically, fish fed the PP+High P diet exhibited lower percentage of liver calcification and liver with macroscopically visible granulomas compared to those fed the PP and PP+Medium P diets.

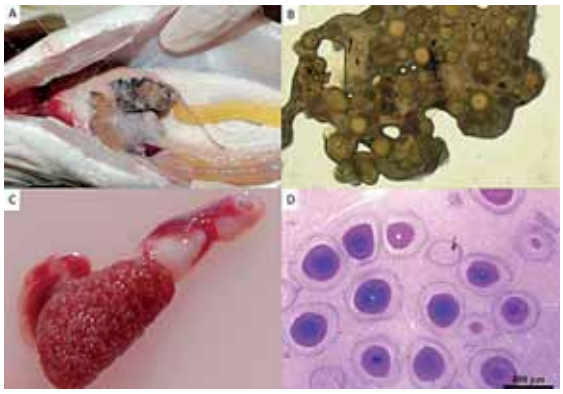


Figure 5. Systemic granulomatosis (SG) in meagre. A. Liver calcification in SG-affected meagre. B. Squash preparation of liver tissue with multiple granulomas. C. Heart of SG-affected meagre with multiple granulomas. D. Histological section of the heart showing the typical appearance of the granulomatous lesions with necrotic center and concentric lamellation.

Despite the fact that the fish of the FM group were in a better state, they also exhibited granulomas in all examined tissues. Some of plant derived ingredients such as wheat and wheat gluten are used as pellet binders in extruded feeds. Thus, FM-based diets also contained small amounts of those, which may be responsible for the appearance of granulomas in meagre. Moreover, the results obtained in our study showed that a reduction of FM from 60% to 14% is possible for juvenile meagre in terms of growth performance, but only in combination with high levels of P supplementation (15 g kg^{-1}).

As it is anticipated that future management of disease issues in meagre will require vaccines as part of the arsenal of approaches used, another objective of DIVERSIFY was to attempt to understand completely the chronology of events that occur –within the context of the immune system- during grow-out. Therefore, work was undertaken towards the characterization of the immune system to identify key immune molecules, as potential markers of immune system development and induction of antiviral and antibacterial responses. For markers of the adaptive immune system, a number of key genes were chosen for cloning, including RAG1/2, Ig and TcR genes, to determine when to vaccinate as the immune system matures. In addition, marker genes of inflammation (IL-1β, TNFα), antibacterial responses (antimicrobial peptides, such as piscidins and defensins) and the antiviral response (interferon, Mx) genes were also chosen. All of the genes initially targeted for this work have been isolated with the exception of transferrin and the expression assays have been established for these genes. Immune markers are now established for the innate, adaptive and inflammatory responses of the immune system as originally proposed. In total, we have 28 assays developed for genes of interest for the study of immune function in this species, and this will be of interest also to other groups and researchers studying this species outside of the DIVERSIFY consortium.

Finally in meagre, trials were conducted in order to improve the fatty acid or micronutrient contents of meagre weaning diets and to achieve early weaning on artificial diets (Campoverde and Estevez, 2017). Despite the fact that meagre larval rearing techniques

have been studied extensively, weaning to dry diets remains an important bottleneck for this species, and it is thought to relate to the variable growth observed from early grow out. Our study has demonstrated the importance of supplementation of meagre weaning diets with 2.4 mg kg^{-1} of vitamin K, since the absence of this vitamin reduced markedly larval survival. However, meagre seemed to be very sensitive to hypervitaminosis D and only mildly sensitive to hypervitaminosis A, since supplementation with these vitamins lead to growth reduction. On the contrary, taurine supplementation did not have any effect in meagre larval performance. Also, based on the results of early weaning trials, meagre larvae can be weaned from live feed to artificial diets as early as 12 days post hatching (dph), but other important aspects for production success including larval performance and survival should be considered (Campoverde et al., 2017). Special care should be taken to avoid cannibalistic behaviour in the rearing tanks, by reducing the light intensity at the water surface and increasing larval feeding rate and daily doses. Early weaning did not affect the incidence of skeletal deformities in meagre, which is of special relevance in terms of assuring fry quality for further on-growing purposes.

GREATER AMBERJACK

This is a cosmopolitan species of great interest to the aquaculture sector due to its **excellent flesh quality** and **worldwide market availability**. Its rapid growth and large size (3 kg in 2 years) makes this species **very suitable for product diversification and development of value added products**. In the Mediterranean (Lovatelli and Holthus, 2008), farming started with capture-based activities using wild juveniles (Crespo et al., 1994), but until recently a very limited commercial activity with hatchery-produced individuals existed in Europe, in spite of the existing interest and efforts by various aquaculture companies in the Mediterranean. The major bottlenecks for the incorporation of greater amberjack in the EU aquaculture industry include lack of **reliable reproduction**, **production of adequate numbers of juveniles** and **fish health** related issues, with monogenean parasites causing mass mortalities in farmed fish (Grau et al., 2003; Montero et al., 2004).

In DIVERSIFY, a major effort in greater amberjack has been channeled toward the study of its reproduction (in the wild and captivity) and the development of reproduction control methods that will allow the production, on demand, of viable eggs of adequate quantity and quality *(Aquaculture Europe 41(1) March 2016)*. This would enable the systematic research on the development of larval rearing methods for the species, and the production of juveniles to supply the aquaculture industry. Our work in greater amberjack reproduction begun with a comparative study looking into the reproductive function of fish in the wild and trying to identify the potential source of the reproductive dysfunctions observed in captivity (Zupa et al., 2017). Wild and captive-reared breeders were sampled in the Mediterranean Sea during three different phases of the reproductive cycle (**Fig. 6**): early gametogenesis (EARLY, late April-early May), advanced gametogenesis (ADVANCED, late May-early June) and spawning (SPAWNING, late June-July). Fish reproductive state was evaluated using the gonado-somatic index (GSI), histological analysis of the gonads and determination of sex steroid levels in the plasma, and correlated with leptin expression in the liver and gonad biochemical composition. The GSI and sex steroid levels were lower in captive-reared than in wild fish. During the ADVANCED period, when the wild greater amberjack breeders were already in spawning condition, ovaries of captive-reared breeders showed extensive atresia of late vitellogenic oocytes and spermatogenic activity ceased in the testes of half of the examined males. During the SPAWNING period, all captive-reared fish had regressed gonads, while wild breeders still displayed reproductive activity. Liver leptin expression and gonad proximate composition of wild and captive greater amberjack were similar. However, the gonads of captive-reared fish showed different total polar lipid contents, as well as specific lipid classes and fatty acid profiles with respect to wild individuals. This study underlines the need for an improvement in rearing technology for this species, which should include minimum handling during the reproductive season and the formulation of a specific diet to overcome the observed gonadal decrements of phospholipids, DHA (22:6n-3) and ARA (20:4n-6), compared to wild breeders.

Figure 6. Sampling of mature broodstock of greater amberjack in the wild (above) and at a commercial facility in Greece (below) at three different times during the reproductive season. The objective of the study was to understand the causes of reproductive dysfunctions that were commonly observed in captive broodstocks of this species.

For the acquisition of viable gametes, a number of different broodstocks were maintained in land-based facilities (Greece and Spain) and sea cages (Greece), and were monitored for reproductive maturation, implementing the principles of broodstock management established earlier. At the expected peak of the reproductive period, the breeders were examined for gonadal development (vitellogenesis and sperm production), and were selected to test different hormonal spawning induction methods. These were based on the use of GnRHa, either in the form of liquid injections or sustained-release delivery systems (implants). Three major experiments have been undertaken. The first (FCPCT,

Table 2. Number of greater amberjack females that spawned, number of spawns and time of natural spawns and latency period for GnRHa injections and implants (FCPCT, Gran Canaria, Spain).

Treatments	Number females that spawned	Number of inductions	Number of spawns	Spawns/ Induction	Latency period (h)
Natural (Control)	2	-	23	-	-
Injected	3	37	29	0.78 ± 0.53[b]	43.06 ± 2.49
Implanted	3	17	38	2.23 ± 1.85[a]	44.19 ± 7.44

* Results are expressed as means ± SD. Different superscripts in the same column indicate significant differences (P<0.01).

Table 3. Number of eggs obtained from greater amberjack broodstocks after treatment with GnRHa injections or implants, in comparison with spontaneously spawning fish (FCPCT, Gran Canaria, Spain).

Treatments	Number of eggs (x 10^6)	Number of eggs per female (x 10^6)	Number of eggs per spawn (x10^6)	Number of eggs per kg of female per spawn (x 10^4)
Natural (Control)	25.60	12.80	1.11±0.32[a]	5.67±1.66 [a]
Injected	12.90	4.30	0.44±0.27[b]	3.72±2.30[b]
Implanted	10.53	3.51	0.28±0.29[b]	2.52 ±2.73[b]

* Results are expressed as means ± SD. Different superscripts in the same column indicate significant differences (P<0.01).

Gran Canaria, Spain) examined the efficacy of different hormonal induction methods on wild-caught breeders from the eastern Atlantic Ocean stock, maintained in tanks. The second (IEO, Tenerife, Spain) examined the efficacy of different doses of GnRHa implants on F1 breeders of the eastern Atlantic Ocean stock. The third set of experiments (HCMR, Greece) examined the timing of application (early, mid, late season), the hormonal induction method (GnRHa injections vs implants) and the dose of GnRHa used, on wild-caught breeders from the Mediterranean Sea stock maintained in sea cages during the year.

For the experiments at FCPCT, breeders with an average weight of 10.7 ± 2.3 kg were distributed in three circular tanks of 40-m^3. In tank 1 (2♀y 5♂), the broodstock was not induced and was allowed to spawn spontaneously. In tank 2 (4♀ y 4♂), three individuals of each sex were selected and were injected with GnRHa at 20 μg kg^{-1} BW. In tank 3 (4♀ y 3♂), three individuals of each sex were also selected and were induced with 500 μg GnRHa implants. Natural spawns started on 1 June 2014 and ended on 18 October, obtaining a total of 23 spawns at temperatures of 21.5-24.5ºC (**Table 2**). The first GnRHa injection was given on 3 June and the last one that resulted in spawning was given on 21 October. The first GnRHa implant was given on 3 June and the last one that resulted in spawning was given on 14 October. The number of spawns per induction was significantly higher in females treated with the GnRHa implants. The number of eggs per spawn in the natural spawnings was greater than those obtained with GnRHa injections and implants (**Table 3**). The quality of the spawns was the best when the fish spawned spontaneously, with a percentage fertilization, hatching and larval survival 1 dph of 84 ± 21, 96 ± 6 and 69 ± 16%, respectively. With GnRHa injections the percentages for the three parameters were, respectively, 58 ± 26, 91 ± 25 and 58 ± 23% and with the GnRHa implants they were 32 ± 34, 77 ± 34 and 49 ± 27%. This study concluded that in the conditions of photoperiod and water temperature of the Canary Islands, it is possible to obtained natural spawnings of a small percentage of wild-caught greater amberjack maintained in captivity during three years in tanks with a volume of 40 m^3. These spawns were better than those obtained by induction with GnRHa injection or implants. However, the majority of breeders would require a hormonal therapy to complete maturation and undergo spawning.

For the experiments at IEO, the broodstock consisted of 14 fish born in captivity (F1 generation) between 2005 and 2009 from a wild-caught broodstock. Fish were maintained during the year in two outdoor covered 50-m^3 tanks, supplied with well water (10 renewals day^{-1}) at natural water temperature and ambient photoperiod. The fish were sampled four times during the spawning season (May, June, July and September) of two consecutive years (2015-2016), and each time (except in September) they were treated with implants at a dose of 25 or 75 μg kg^{-1} GnRHa, respectively, for the two years, in order to examine the effect of treatment dose on spawning performance and egg quality (**Fig. 7**). Using the 75 μg GnRHa dose, a total of 52 spawnings were obtained during a period of 72 days. The number of spawns obtained in the successive post GnRHa-treatment periods decreased, as well as the number of eggs released (**Table 4**). After the 1st treatment, a period of 31 days between the first and the last spawning was observed, while the eggs were collected almost daily (29 spawning events). However, after the 2nd treatment, a total of 15 spawnings were recorded during the first 16 days and no eggs were collected the later days. The eggs released after the 3rd GnRHa treatment were collected after 8 spawning events during the 9 days following treatment. Mean fertilization changed through the spawning period after each treatment, reaching its highest values after the 1st and 2nd GnRHa treatment, and a significant decrease was observed after the 3th treatment. On the other hand, no significant differences were observed in hatching, 1-day embryo survival and 3-day larval survival after successive GnRHa treatment. The study of the lower dose (25 μg kg^{-1} GnRHa in 2016) produced similar results in terms of reproductive performance and egg quality.

The third set of experiments was undertaken at a number of tank and sea cage facilities in Greece,

Figure 7. Induction of spawning of F1 greater amberjack breeders at the IEO facility in Tenerife, Spain. (left) weighing the breeders and (right) evaluating the diameter of the oocytes prior to the administration of the GnRHa implants.

Table 4. Spawning and egg fecundity parameters (mean ± SEM) of greater amberjack broodstock induced to spawn using three consecutive GnRHa implant treatments. No statistically significant differences were observed (P<0.05) between different GnRHa treatments.

Treatment	Spawns (n)	Eggs spawn^{-1}kg^{-1}	Total eggs kg^{-1} (x1000 eggs)	Total eggs (x10^6 eggs)
1	29	2087±218	60.54	7.05
2	15	2679±398	40.18	6.55
3	8	1361±594	10.89	1.35

including the research facilities of HCMR, and the commercial operations of Galaxidi Marine Farms, Argosaronikos Fishfarms S.A. and Aquaculture FORKYS S.A. The first major finding of these studies was that maintaining greater amberjack breeders in land-based facilities supplied with the typical borehole water of commercial hatcheries does not allow full gametogenesis of the fish. This means that males do not spermiate adequately and females do not complete vitellogenesis to the stage of being able to be induced for spawning using GnRHa therapies. On the contrary, fish maintained in sea cages during the year completed gametogenesis adequately, and it was possible to induce them to spawn using exogenous hormones, after moving them temporarily to tanks for spawning (**Fig. 8**).

In one of the trials, wild-captured breeders (mean weight 17.0±2.6 kg) were kept in Argosaronikos Fishfarms S.A. in a 1,000-m^3 cage during the year and were fed with a broodstock diet (Skretting, Vitalis Cal, 22 mm). Females were treated with either a GnRHa injection (20-25 μg kg^{-1}) or GnRHa implant, with an effective dose of 49-69 μg GnRHa kg^{-1}. To enhance spermiation and ensure adequate sperm production, all males were treated at the start of the induction with a GnRHa implant at a dose of 45-70 μg kg^{-1}. After being treated for spawning, fish were transferred to inland facilities, into four 23-m^3 flow-through round tanks (n=3-4 females), at a 1:1 sex ratio. Females in the injected group were given a GnRHa injection every week for 3 weeks, whereas the implanted group was given a second implant after two weeks (a total of 3 GnRHa injections and 2 implants). Tank overflow egg collectors were examined three times a day, and fecundity and fertilization success were estimated immediately after egg collection. Spawning started 1 d after the 1st GnRHa treatment, as some females had oocytes already undergoing maturation (**Fig. 9**).

Figure 8. Acquisition of ovarian biopsies from greater amberjack (top) maintained in sea cages during the year, and evaluation of the stage of development of the obtained oocytes (bottom), in order to select fish for treatment with hormones to induce spawning. After treatment, fish were moved to tanks for spawning and egg collection.

Implanted fish spawned 9-10 times after the 1st implant and only 4 times after the 2nd implant. Injected fish spawned 7, 3-5 and 1-3 times after the 1st, 2nd and 3rd injection, respectively. The highest daily or batch fecundity was produced by the GnRHa implanted fish and was 4,242,000 eggs tank^{-1} 2 days after the

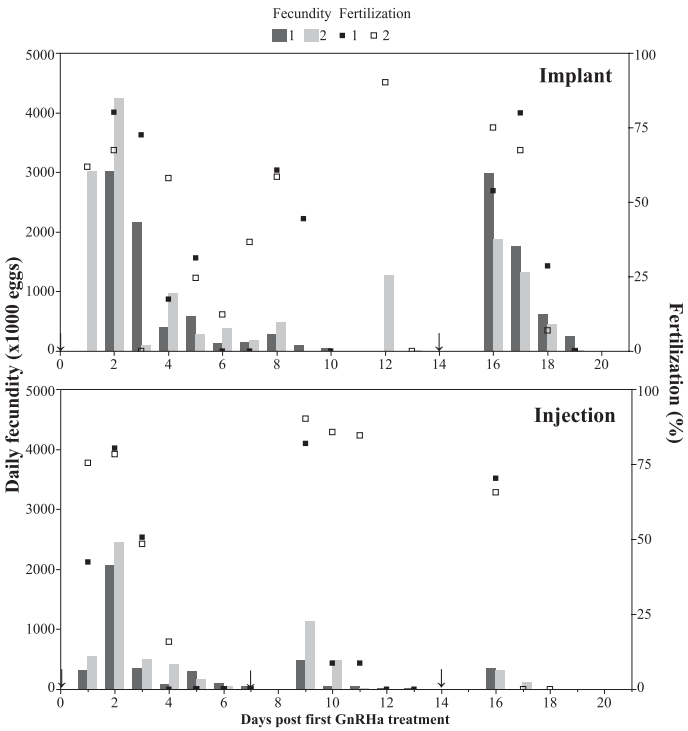


Figure 9. Egg production (per tank, n=2) and fertilization success (%) of greater amberjack maintained in sea cages in Greece during the year, and placed in tanks (numbered 1 and 2 for each treatment) for spawning after treatment with GnRHa injections or implants (arrows on the x-axis).

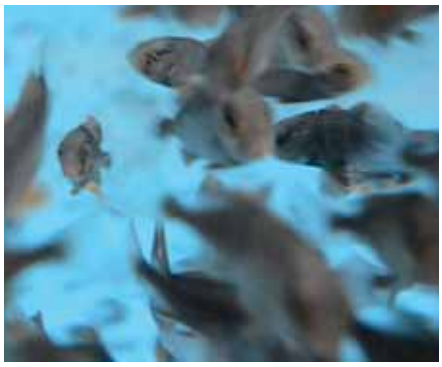


Figure 10. Greater amberjack juveniles produced at HCMR, Greece (above) and transferred to sea cages for grow-out studies (below).

1st treatment, while in the injected fish the highest fecundity was 2,454,000 eggs tank^{-1}. The GnRHa implanted fish produced 2.5X more eggs than the injected fish, with the same number of spawns. On the other hand, no differences were observed among the two treatment methods in terms of fertilization success, embryo survival, hatching or 5-day larval survival. The study demonstrated that GnRHa implants were more effective in this stock to induce spawning, contrary to what was observed for the eastern Atlantic Ocean wild-caught population (see earlier).

In the area of greater amberjack larval rearing, significant breakthroughs have been achieved, allowing the production of large numbers of juveniles for stocking into research and commercial sea cages (**Fig. 10**). The main objective of DIVERSIFY in this area was to improve the survival, growth and performance of greater amberjack larvae by defining the appropriate environmental and feeding conditions adequate for the species. Eggs from the different broodstocks of DIVERSIFY used for the spawning induction experiments, were provided to the larval rearing partners of the consortium to undertake a number of trials.

In one study that has been completed, optimum levels and ratios of essential fatty acids in relation to taurine (Tau) and combined Poly Unsaturated Fatty Acids (PUFA) and carotenoids in enrichment products were examined (**Fig. 11**). A list of the optimum levels and ratios of essential fatty acids and carotenoids that should be included in enrichment products for rotifers to be fed to greater amberjack larvae have been established. The results included the effects of essential fatty acids and carotenoids on (a) larval performance,

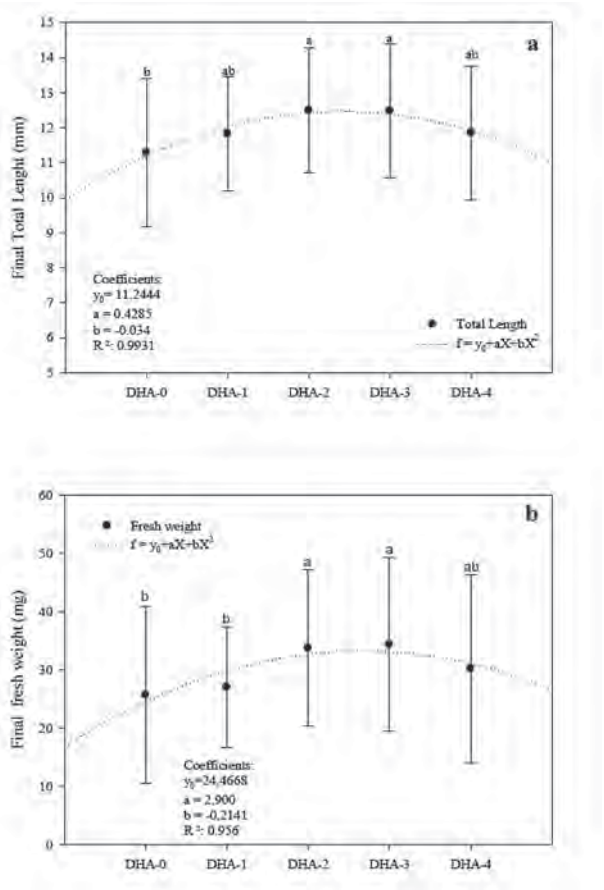


Figure 11. Relationship between (a) total length (mm) and (b) fresh weight (mg) and different levels of dietary *Artemia* DHA (22:6n-3) content in greater amberjack larvae 35 dph (mean ± S.D., n=3). Data are fitted to a quadratic regression analysis (f=y0+ax+bx2).

Figure 12. The mesocosm facilities of HCMR (Greece) where the studies on the effect of semi-intensive rearing of greater amberjack larvae were undertaken.

(b) welfare and (c) fatty acid analysis, lipid classes, and carotenoid profiles of enrichment products, live preys and larvae.

Another study compared the semi-intensive and intensive larval rearing systems **(Fig. 12)**. The gene expression of growth hormone (GH) releasing hormone (GHRH), GH, insulin-like growth factor(IGF) -I and –II, and IGF binding protein (BP)s were not affected by the rearing method. However, there was a gradual increase in their mRNA levels as development proceeded, with statistically significant differences observed at 20 dph with peak levels at 25 and 30 dph of IGF-I and GHRH, respectively. In addition, IGF-II was higher at 5 dph compared to 2 and 10 dph, while GH exhibited higher mRNA levels at 5 and 15 dph. The study determining the effect of stocking density on larval performance showed the optimum egg density for the larval rearing of greater amberjack is between 25 and 50 eggs l^{-1}. There was a marked appearance of a number of different anomalies in the larval stage that could lead to a lower survival. The study of the ontogeny of the greater amberjack larval digestive system showed that the average enzyme activity measured for a particular age range was independent of the larval rearing conditions. In general terms, the pancreatic enzymes amylase and alkaline protease were more active in the youngest larvae compared to the 30 dph larvae, whereas pepsin followed the opposite trend, displaying almost no activity at 12 dph. Intensive rearing seemed to favour amylase, alkaline protease and pepsin activities in the older larvae. Amylase was highly active in the eggs, decreasing at 0-5 dph, while increasing from 5 to 10 dph. From 10 to 30 dph, carbohydrates displayed a less relevant role in larval metabolism. Lipase and alkaline protease activities showed an increasing trend from 0-5 to 5-10 dph. However, although lipase decreased similarly to amylase activity after 10 dph, alkaline protease activity was still high at 10-15 dph, and increased further in the oldest larvae (20-30 dph). Amylase activity was also higher at 12 dph for the intensive system larvae, whereas the opposite trend was observed for alkaline protease and lipase activities. According to the results, greater amberjack seem to use dietary proteins effectively from 20-30 dph.

In another study, the effect of tank hydrodynamics on larval performance was studied by estimating the hydrodynamic field in tanks of 2,000 and 40,000 l. The applied water exchange rates (as % of total water volume) were 10% and 4% per hour, while the airflow was set at 350 and 1400 ml min^{-1} for the 2,000 and 40,000 l tank respectively. The conditions regarding water exchange and airflow were similar to the ones applied during larval rearing. The results showed differences between the conditions, as the higher currents occurred in the 2,000 l tanks followed by the 40,000 l tanks. Significant differences, in total length and body weight, were observed between treatments, with the individuals from the 2,000 l tanks being larger. Results showed higher survival at the end of the experiment in 2,000 l tanks, independent of egg stocking density. This was particularly apparent in 2,000 l tanks stocked with 10 eggs/l^{-1}.

The photoperiod study showed a good performance with the survival of the larvae varying between 6% and 13.6%. The mean survival for the 18L:06D photo phase was slightly higher (10.6±4.2%), than the 24L:00D (8.2±3.1%). In terms of total length, larvae grew with an exponential rate of 0.310 d^{-1} independent of photo phase. The trials revealed that the photoperiod affected the mRNA expression levels of IGF-I with higher levels for the 18L:06D group at 17 and 25 dph compared to the 24L:00D group. Additionally, there was a significant gradual increase in mRNA levels as development proceeded, which was observed only in the 18L:06D group with peak values at 25 dph. The mRNA levels of IGF-BP1 appeared to be generally stable during development, and increased expression levels were observed in the 18L:06D group compared to the 24L:00D group. The IGF-BP2 expression showed a gradual increase throughout development with statistically higher levels at 25 and 30 dph. Additionally, at 30 dph an effect of the photoperiod regime was observed with higher expression levels in the 18L:06D group compared to the 24L:00D group.

The study on the effect of tank color showed no significant differences in the growth of the larvae in terms of total length and body weight. Fish growth was exponential in terms of TL (black: 0.0481 d^{-1}, white: 0.0393 d^{-1}, green: 0.0355 d^{-1}) and wet weight (black: 0.1260 d^{-1}, white: 0.1970 d^{-1}, green: 0.171 d^{-1}). However, significant differences were observed in the survival of the larvae during the 2015 experimental period that were more profound during 2016. The white background resulted in a significantly higher survival rate (22.2±0.7%) compared to green (16.5±0.9%) and to black (8.2±3.1%) backgrounds. The analysis of the 2016 results showed that the fish reared in the white background showed increased levels of the genes implicated in the growth axis system compared to the fish reared in the black and green backgrounds. In particular, IGF-I showed generally higher levels of expression as development proceeds and also it appeared to be affected by the background color, as higher levels were observed in fish reared in the white background at 17 dph and 30 dph compared to fish reared in the black and green backgrounds. The IGF-II did not show a particular pattern during development. However its expression appeared up regulated in a statistically significant

Figure 13. Examination of greater amberjack gills for the presence of monogenean parasites. This breeder was found with a large number of *Zeuxapta seriolae*.

way at 17 dph in fish reared in the white background compared to fish reared in the black and green backgrounds. This preliminary study provides for the first time information on the regulation of the various components of the IGF signaling pathway in greater amberjack and may serve for the better understanding of the complex relationship between background color and fish performance during early ontogeny. The results from the trials with the modified "light environment" improved survival an order of magnitude from any previous trial, reinforcing the validity of the tested hypothesis and indicate a clear technological step forward in the larval rearing of the greater amberjack.

In the area of fish health, progress has been made in all tasks included in the proposal. This included further mesocosm trials for the development of a rapid detection method for epitheliocystis, and screening of gill samples from different Greek fish farms (**Fig. 13**). Studies were undertaken on (a) the morphology and the incidence of monogenean parasites in greater amberjack skin, (b) the determination of environmental conditions that can modulate greater amberjack resistance to parasitic infection, (c) formulation of a diet supplemented with mucus stimulation products, and (d) standardization of monogenean cultures. Primers for the detection of 11 immune genes have been optimized for qPCR, ready for studies of mucosal defenses, with initial pathogen-associated molecular pattern (PAMP) stimulation *in vivo* revealing good induction at mucosal sites such as gills. Further grow out trials have been undertaken, to assess the relationship between monogenean parasite egg number and fish mortality, and the impact of several potential anti-monogenean treatments, with mannose looking promising. Diagnosis of bacterial and viral infections was performed with juveniles, and *Bacillus oceanisediminis* and *Aeromonas* spp. have been detected. Challenge trials were also undertaken to assess relative disease susceptibility to two bacterial species, namely *Listonella anguillarum* and *Photobacterium damselae* subsp. *piscicida*. The fish were found to be refractory to the former.

One of the objectives of the research in this area was to identify the effect of dietary regime on mucus immune barrier and modulate the resistance to parasite infection by adding immunostimulants to the diet. Immune potential of mucus defenses has been studied from the systemic point of view (including lysozyme and bactericidal activities) and from the histological point of view. The utilization of mucus stimulatory substances, such as mannan-oligosaccharides (MOS) or concentrated mannan—oligosaccharides (c-MOS), has been also evaluated. A histological study of the effects of monogenean parasitization on greater amberjack juveniles was conducted, and the potential of immunostimulants to reduce parasitic infection has been also assessed. The study, completed recently, concluded that dietary regime could alter mucus immunological properties. The addition of mucus stimulating products, and especially those based on concentrated mannan-oligosaccharides, enhance mucus immune potential and resistance to the ectoparasite *Neobenedenia girelliae*.

A trial was also conducted in order to determine how aquaculture-associated stressful conditions are affecting selected parameters. The processes selected were manipulation (high and low) and stocking density (high and low), both related with aquaculture practices and are necessary to manage stocks of greater amberjack. At the end of the experimental period, a stress challenge test was conducted. The treatment with the highest manipulation and density had reduced growth performance when compared to the treatment with low manipulation and low density, denoting that greater amberjack is highly sensitive to aquaculture procedures. High stocking density induced a decrease in mucus lysozyme activity. A similar effect was obtained for bactericidal activity of the mucus, but no effects were found in peroxidase activity. These data are part of the results that have shown the immune potential of skin mucus of amberjack. Relative to other species, the mucosal surfaces of greater amberjack include a full repertoire of antimicrobial defenses that can vary with certain environmental conditions.

Pikeperch

This freshwater fish is considered to have the **highest potential in Europe for inland aquaculture diversification**. Pikeperch flesh has a neutral taste, thus lending itself to different forms of preparation, and the filets are without bones --unlike carp, which competes on the same market segment. Year-round production of pikeperch requires constant high temperatures (24-26°C) to ensure relatively high growth rates (*i.e.*, production of 1.2 kg fish in 15 -18 months from non-selected strains), which is only feasible in RAS. These RAS also allow high densities of 80-100 kg m^{-3}. Identified by a survey addressed to fish farmers in preparation for DIVERSIFY *(Aquaculture Europe 40(2) Sept 2015)*, the major bottlenecks for further expansion of pikeperch culture today include (a) **high sensitivity to stressors, handling and husbandry practices** that result in high and sudden mortalities, (b) **low larval survival** (typically 5-10%) and **high incidence of deformities**, and (c) **lack of knowledge of the genetic variability of the used broodstocks**. Identification of genetic relationships among different broodstocks, inbreeding phenomena and loss of heterozygosity is important in aquaculture, since it may result in subsequent reproductive and productive failure (reduced progeny survival, growth, food conversion efficiency and increased frequency of deformities). It is also important to know how the domesticated stocks differ from their wild counterparts, which could potentially be a future source of fish to include in breeding programs. Overcoming the above bottlenecks is very important to reduce production costs and, therefore, expand the aquaculture production of pikeperch in the EU (**Fig. 14**).

Figure 14. Rearing system for pikeperch larvae © Photos: Tatiana Colchen & Elodie Faux.

The first task of DIVERSIFY for pikeperch was to assess the genetic variability of captive broodstocks in commercial farms in Europe operating in RAS, and then compare this variability with that of wild populations. The results have indicated that some broodstocks have adequate genetic variation, but as some of them originate from few fish, attention should be paid in the future to establish breeding programmes. In general, there was agreement with the stock origin and our studies provided evidence that pikeperch populations in Europe are part of at least two genetically differentiated groups. The first group is found in northern Europe from the Netherlands/ Denmark to the West, Poland (at least) to the East, and Finland to the North. The second group comprises all remaining populations in Central Europe to as south as Tunisia (and probably Spain, Italy and northern Greece). Based on this grouping, it can be stated that most analyzed populations seemed to contain fish of a single origin; nevertheless, in few domesticated populations this ratio varied from 5-19%, possibly due to the mixing of fish from multiple sources. The objectives to evaluate the genetic variability of captive pikeperch broodstocks and make a comparison with wild individuals to define future breeding programs have been completed. A total of 21 populations/ broodstocks were sampled and analyzed, which included 13 captive broodstocks and eight wild origin populations. The different stocks were grouped into three populations that were of Hungarian origin, Scandinavian origin and other origins (German, Polish and Tunisian). The different captive broodstock populations presented different levels of genetic variability that ranged from wide variability greater than observed in wild populations to broodstocks that had reduced genetic variability that may have been the result of loss of variability through inbreeding. For these broodstocks with reduced genetic variability, measures should be taken to introduce greater variation into the base population for future breeding programs.

In the area of pikeperch nutrition, trials have shown that pikeperch larvae require both high dietary inclusion levels of phospholipids and Long Chain (LC) PUFAs to perform optimally. A multifactorial screening trial of the importance of eight dietary factors (high or low levels) has been initiated and is still ongoing. Also, adding saline water to rearing does not improve growth, but can change the ability of pikeperch larvae to elongate and desaturate different fatty acids and phospholipids. An experiment investigating the consequence of various phospholipid levels and LC PUFAs on welfare indicators and stress physiology, behaviour and respiratory metabolism is currently ongoing. In the area of grow out, our studies identified the optimal conditions improving growth and welfare of pikeperch in aquaculture and characterized the effects of major husbandry and environmental factors on growth and physiological status of this species (Baekelandt et al., 2017). In a screening experiment, eight factors considered as relevant for the welfare of pikeperch were compared in two modalities using a fractional multifactorial design (2^{8-4}). Each experimental unit represented a combination of eight factors in two modalities, which included grading, stocking density (15 *vs* 30 kg/m^3), feed type (floating *vs* sinking), light intensity (10 *vs* 100 lux), light spectrum (red *vs* white), photoperiod (long *vs* short), dissolved oxygen (60 *vs* 90 %) and temperature (21 *vs* 26°C). Fish sampling occurred on days 36 and 63. Stress markers – glucose, cortisol and brain serotonergic activity – and changes in humoral immune activities and immune gene expression in kidney were assessed. Light intensity and the type of feed clearly appeared as directive factors for pikeperch culture. A strong effect of the feed type was observed on the final individual weight, the specific growth rate and the weight heterogeneity. High light intensity affected survival. The main influence on physiological and immune status was imposed by light characteristics, including intensity, spectrum and photoperiod, as well as temperature.

In the area of larval rearing (**Fig. 15**), one of the objectives was to determine the effects of four environmental factors and their interactions on pikeperch production using a multifactorial approach. One of the most important results of the present study was that different behavioral traits were observed in very young pikeperch juveniles using several behavioural tests (cross maze, social and stressor tests). This implies that some personality traits appeared very early in the life of pikeperch, and could be inheritable characters. We know, for instance, that a gene mutation linked to growth factors may modify fish personality in zebrafish *Danio rerio* (Norton et al. 2011). It is also known that domestication may also act as a selection process for personality traits (Moretz et al. 2007). Most studies carried out on it recognized that personality is defined by behavioural traits consistent through time and/or contexts. In fish, most studies on personality were performed either on juveniles (aged between 6

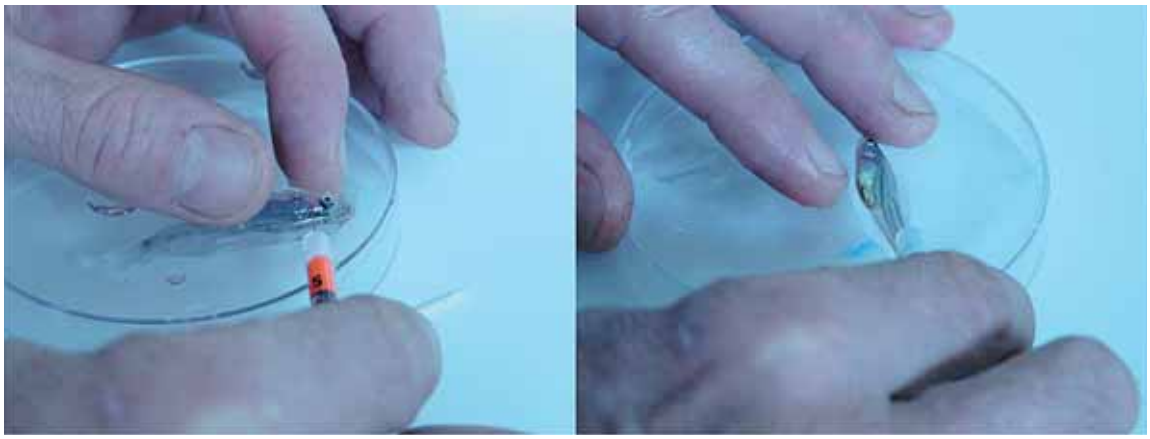


Figure 15. Fish tagged with Visible Implant Elastomers (VIE tag, Northwest Marine Technologies, USA). Two different colours (blue and pink) were used and tags were injected in the operculum and/or in flanks of each fish. © Photos: Tatiana Colchen.

months and 1 year) or adults, while very few focused on the early life stages. The main goal of this study in pikeperch was to characterize behavioural syndromes and to highlight the existence of a personality in young juvenile pikeperch. To study the consistency of behavioural responses of juvenile (50 and 64 days post-hatch) pikeperch (n = 41, TL =5.8 ± 1.0 cm and BW =1.6 ± 0.7 g), we performed three behavioural tests per fish: exploration (cross-maze), dyadic and restraint test. In the cross-maze test, exploratory fish were more active and bolder. In the dyadic test, fish with the highest number of contacts, showed also more approaches, orientations and avoidance behaviours. In the restraint test, bolder fish were more active and tried to escape more often. Consequently, the investigation of the different behavioural responses of each fish highlighted behavioural syndromes in this species. Furthermore, for the first time, we showed with a cross-context analysis that young juvenile pikeperch, responded in the same way to exploration and dyadic test, but their responses were opposite in the restraint test. Our results opened new opportunities for testing individual personality in very young fish that may help solving some aquaculture problems, such as the intra-cohort cannibalism.

Atlantic halibut

The **Atlantic halibut is the world's largest flatfish** and can attain a weight of over 300 kg. It is **highly prized at markets worldwide**, but availability of wild Atlantic halibut has been decreasing. The fish has been classified as endangered on the IUCN red list and a complete ban was imposed on Icelandic fisheries, while stocks along the Norwegian coast are declining and under strict regulation. This has led to a higher market demand for Atlantic halibut than can be met by fisheries alone. Cultured Atlantic halibut has an excellent reputation, but has been rarely available outside specialty restaurants due to low annual production. However, it is now the marine aquaculture industry that has the fastest growth in terms of production, with an increase of 20% from 2015 to 2016. The Atlantic halibut is a semi-fat fish rich in omega-3 fatty acids, with a characteristic flaky white meat with few bones. These characteristics led to the inclusion of Atlantic halibut in DIVERSIFY, as a great candidate for fish species and product diversification in European cold-water aquaculture *(Aquaculture Europe 41(2) Sept 2016)*. Despite a significant research effort between 1985 and 2000, the complicated life cycle of Atlantic halibut made aquaculture progress slow, and very little research funding has been allocated thereafter. The remaining bottlenecks for increased and stable production are related to a **steady supply of fry and a need to decrease the production time**. DIVERSIFY addresses these important bottlenecks with a coordinated research effort in **reproduction, and larval nutrition and husbandry**.

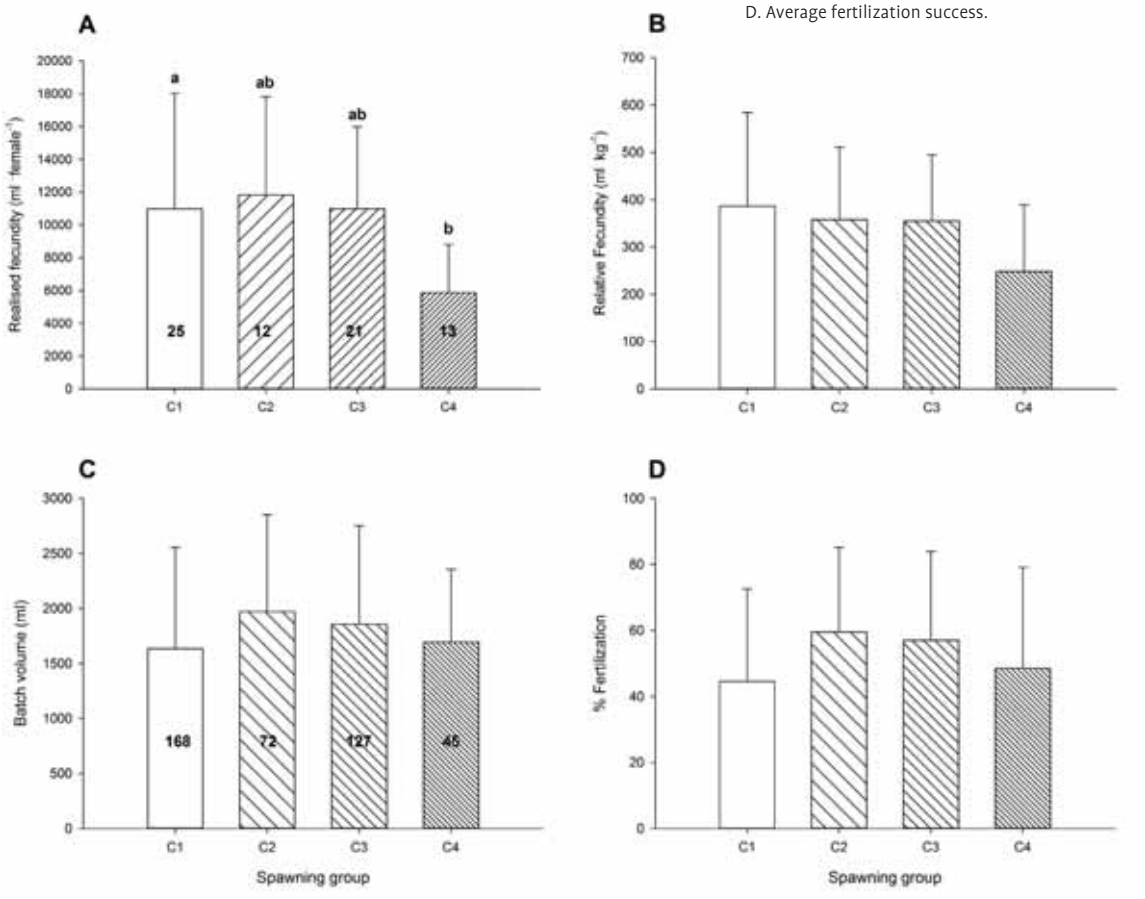
Advances have been made so far in all tasks in the area of Atlantic halibut reproduction (**Fig. 16**). Regarding the documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut, there were actually few differences between fecundity, fertilization, hatching, egg size and hormone content between eggs from wild-caught and farmed females. However, although there were no significant differences, wild-caught females appeared to be more predictable spawners and gave fewer, but larger batches of eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified correctly and stripping was carried out close to ovulation, thus reducing or eliminating over-ripening (**Fig. 17**). However, for commercial as well as breeding purposes, it is not practical to rely solely on wild-caught females. As at both the Institute of Marine Research and Stirling White Halibut AS (Norway) relatively few farmed females produced eggs with fertilization rates >80-85% consistently, it may be necessary to include also wild-caught broodstock in future breeding groups, in order to ensure a broad enough genetic material. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the considerable workload connected with artificial spawning and egg collection in Atlantic halibut. The other approach explored in the task included the use of GnRHa implant therapy as a means to improve reproductive performance, and ensure (and regulate) ovulation using GnRHa implants. The GnRHa implants used did ensure and synchronize ovulations

of the treated females and were found not to affect egg quality or quantity.

In the area of nutrition and larval rearing, a protocol for weaning of Atlantic halibut at 28 days post first-feeding (dpff) has been developed and almost 100% of the larvae fed Otohime diet (Japan) were filling up their guts with feed after a 5 day adaptation period. Gut fullness was lower in the morning than in the evening, possibly because the larvae were measured before and after hand feeding and clay addition in the morning and in the evening, respectively. According to the evening measurements, larvae fed *Artemia* were almost full after 1 day and stayed full for the rest of the experiment. Larvae fed Otohime showed increasing fullness over the whole period and on day 5 almost 100% of the larvae were full in the evening. The fraction of larvae with food in their gut increased more slowly on Gemma and Aglonorse feeds. On the evening of day 5, a total of 12 and 15 larvae, respectively, out of 28 had filled guts, while no larvae had filled guts on these diets.

Left: Figure 16. Atlantic halibut breeders being examined for reproductive maturation at the IMR facilities (Norway).

Below: Figure 17. Egg production results from four farmed Atlantic halibut broodstock groups with different spawning periods at Stirling White Halibut AS, Norway. A. Total fecundity (number in bars = n of females), B. Relative fecundity, C. Average batch size (ml of eggs; number in bars=n of batches) and D. Average fertilization success.



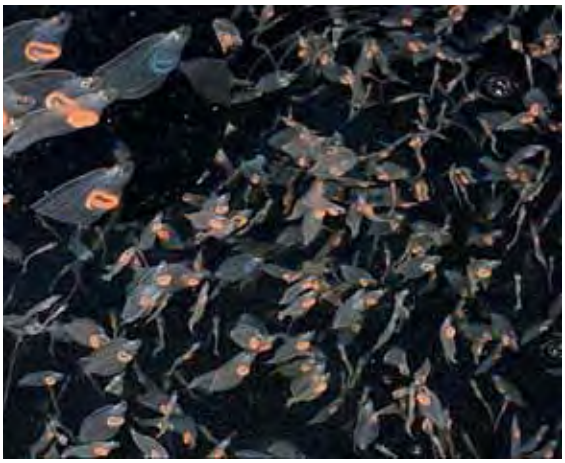


Figure 18. Atlantic halibut larvae at the stage of first feeding, at the IMR facilities (Norway).

A production strategy for on-grown *Artemia* has been established, which improves the nutritional value of *Artemia* with respect to protein, lipid and micronutrient contents. Growth and juvenile quality was excellent in larvae fed *Artemia* nauplii in this experiment, but not improved by feeding on-grown *Artemia* (**Fig. 18**). In the industry, the routine larval rearing method is to feed *Artemia* nauplii, with varying incidence of larvae with abnormal pigmentation and lack of eye migration, although the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. The nutrient concentrations of Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff were similar, except that the on-grown group had a slightly lower level of EPA than larvae fed nauplii, a difference that is probably biologically insignificant. This is another possible explanation of the lack of differences in growth and larval performance between the two treatments. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on grown group had to be fed nauplii to get enough food. As the fish grow, more feed is needed and, due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as was planned. These are all possible reasons that no differences were detected between the groups.

Figure 19. Wreckfish being sampled for ovarian biopsies and blood for the description of the annual reproductive cycle in Aquarium A Coruna (above) and HCMR (below).

Wreckfish

Wreckfish is one of the largest Serranid species, reaching a size of 100 Kg, and it is found in deep-waters almost throughout the world. Wreckfish is one of the most interesting new species for aquaculture, due to its **fast growth**, **late reproductive maturation**, **high market price and limited fisheries** landings. Its large size lends itself to **processing and development of value added products**, and its **cosmopolitan distribution may enable** EU exports. **Lack of reproduction control and established larval rearing protocols are considered major bottlenecks** preventing wreckfish aquaculture, and the clear biological and economical potential of this species justifies allocation of part of the effort of DIVERSIFY in bringing together almost all partners involved in Europe in wreckfish domestication.

Recently, an article has described the work and achievement of the DIVERSIFY project regarding wreckfish (Aquaculture Europe, 42(1) March 2017). Therefore, just a brief mention is made in this article of the latest information. Although significant progress has been made in the area of reproduction during the first 3 years of DIVERSIFY, development of effective larval rearing methods is still not at hand. The reproductive cycle of the species in captivity and the associated profiles of the sex steroids have been characterized recently, using a number of broodstocks from Spain and Greece (**Fig. 19**). Vitellogenesis begins in October and is completed between April-June, depending on geographic location and rearing temperature. Spontaneous spawning of viable, fertilized eggs has been accomplished in a number of broodstocks, but hatching success has been very limited (**Fig. 20**). Natural spawning occurs in a cycle of ~5 days. Males produced very good quality sperm during the reproductive season, and in some stocks they are spermiating for the whole year (under constant 15°C rearing conditions).

Egg quality and larval rearing continued to be problematic during the 2017 reproductive season, and we are still away from the development of a reliable larval rearing protocol for transfer to the industry. Efforts will continue in 2018, the last year of the project, and we hope for a better success.

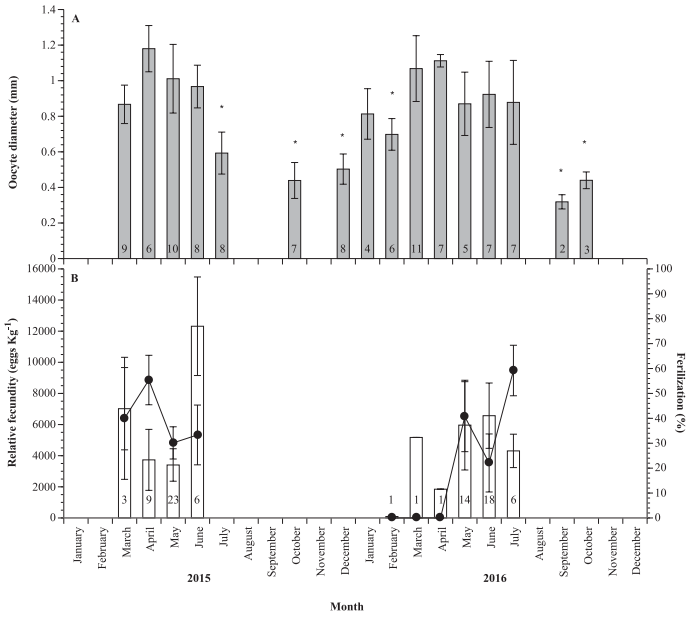


Figure 20. Mean (±SEM) oocyte diameter (A) of wreckfish broodstocks at four different sites in Greece and Spain during the annual reproductive cycles from March 2015 until October 2016. The numbers inside the bars indicate the number of females biopsied each month. Asterisks (*) denote significantly lower values than maximums observed each year (April 2015 and 2016). Mean (±SEM) monthly relative fecundity (bars) and fertilization percentage (lines, B) of natural and induced spawns of 4 wreckfish broodstocks in the reproductive periods of 2015 and 2016. The numbers inside the bars indicate the number of spawns obtained each month.

Grey mullet

Farming of grey mullet has been practiced for centuries, but production of this potentially invaluable source of animal protein in Europe has been small and non-intensive (Crosetti and Blaber, 2016). It is a **euryhaline species, found throughout the world** and is a **rapid-growing, herbivorous species** that can be reared over the wide geographical and temperature range of the Mediterranean basin (**Fig. 21**). As it is detritivorous in the wild, it has been stocked in fishponds to improve sediment quality and avoid oxygen depletion. Therefore, it can be an excellent candidate for the enhancement of aquaculture in **earthen ponds, coastal lagoons, "valli" and deserted Salinas** that exist throughout the EU Mediterranean countries. The development of FM-free feed will reduce the cost of aquaculture fish production, and will be **more sustainable and environmentally friendly**. In this way, grey mullet would be more acceptable to an increasingly aware consumer public that demands sustainability and lower environmental impact. Moreover, grey mullet aquaculture has the advantage of providing not only affordable whole fish and fillets, but also **fish roe or "bottarga" in Italian, a high value product (>100 € kg^{-1})**, whose market is expanding around the Mediterranean. Therefore, grey mullet has a great biological and economical potential for the diversification of fish species and product, and the development of value added products.

The future growth of the grey mullet aquaculture is limited by a number of bottlenecks, which are addressed in DIVERSIFY *(Aquaculture Europe 39(1) March 2014)*. Firstly, **controlling the reproductive cycle and improving egg quality** via broodstock management and nutrition is necessary not only for the production of robust larvae, but also for producing high value bottarga. Secondly, **development of a larval rearing protocol** is necessary to reduce early

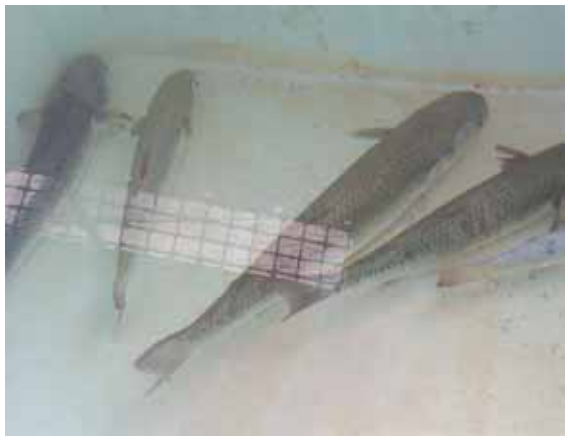


Figure 21. Grey mullet broodstock at the facilities of NCM-IOLR, Israel.

mortalities, size dispersion as well as increasing metamorphic synchrony, which will lead to a supply of high quality juveniles. Finally, development of a sustainable, economical, **FM-free grow out feed** is needed, which would perform well under different environmental conditions of temperature, pond type, and water quality, thus broadening the geographical range of grey mullet aquaculture in Europe.

Lacking the natural spawning environment, captive grey mullet fail to reproduce spontaneously, largely due to a failure to undergo complete gametogenesis (Aizen et al., 2005). Therefore, DIVERSIFY first evaluated the effectiveness of hormone-based treatments on synchronizing gonadal development (**Fig. 22**). A combined treatment consisting of follicle stimulating hormone (FSH) and dopamine antagonist (metoclopramide) on spermatogenesis in males and follicle growth and maturation in females was tested. The methylotrophic yeast (*Pichia pastoris*) expression system was used to produce large quantities

of bioactive recombinant single-chain FSH, which was used in a series of *in vivo* assays. Unlike the controls, the hormonally treated groups (injected with rFSH and metoclopramide during the onset of the reproductive season) demonstrated synchronized gonadal development within and between sexes, with higher rates, over time, of spermiating males and post-vitellogenic females. Once gonadal development was accomplished, we proceeded with the development of hormone-based treatments for inducing spawning. Spawning induction trials that timed the administration of GnRHa and metoclopramide with advanced stages of gamete maturation were relatively successful, producing tens of millions of fertilized eggs. Nevertheless, our results highlight two major problems: (i) the female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) the episodic fertilization success ranging between 0 to 98%, underlining the need to fine-tune further and optimize the hormone-based breeding protocol for captive grey mullet.

In the area of larval rearing, the objectives in DIVERSIFY are to (a) investigate environmental and nutritional factors that affect larval rearing, (b) determine the effect of co-feeding copepods and rotifers on digestive tract maturation and enzyme production and (c) determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding (**Fig. 23**). Some of our results so far indicate that the beneficial effect of "green water" in the rearing tanks for larval grey mullet was derived predominantly from the resultant turbidity on prey ingestion rate (within the turbidity levels measured in this study) and less so to the algal type or biochemical content (*i.e.* fatty acid profile). Nevertheless, ingested algae by the larvae may have stimulated and improved gut maturation in early developing larvae, resulting in markedly improved survival during the juvenile stage. The algal treatments given to 2-23 dph larvae did not have a significant effect on older larvae and juveniles in terms of pancreatic and digestive tract enzyme activities. On the other hand, diet composition may have influenced the lipase and total alkaline protease specific activities. Nevertheless, overall the ontogeny and activity of the pancreatic and digestive tract enzymes measured appeared to be genetically programmed. The enzymatic activity of Alkaline Phosphatase and leu-ala peptidase individually and

Figure 22. Acquisition of an ovarian biopsy from a grey mullet breeder, in order to evaluate reproductive stage of development (IOLR-NCMR, Israel).

Figure 23. The larval rearing facilities at IOLR-NCM, Israel, where the majority of the work with grey mullet is undertaken.

in ratio indicated gut maturation around 61 dph and an increasing amylase capacity to at least 79 dph. This suggests (a) the capacity to feed on micro- and macroalgae, as well as benthic organisms when the fish move to the lower saline and shallower waters of estuaries at this age and (b) when to include significant levels of low cost starch in prepared feeds in order to efficiently grow grey mullet following gut maturation. Also, studies on the taurine requirement at different stages of development during the larval rearing of grey mullet showed a significant effect of dietary taurine on larval growth and survival. This effect is strongest during rotifer feeding compared to *Artemia* feeding, which also influences significantly growth in later stages of larval development. Nevertheless, the results indicated that larvae fed both high taurine enriched rotifers and *Artemia*, survived and grew significantly better.

Socioeconomics (including new product development)

Besides the technical improvement of the selected species, the socio-economic research in DIVERSIFY includes applied market development approach solutions on perception of aquaculture products, market demand, buyer preferences, new product development, value adding and market development (Banović et al., 2016; Grigorakis, 2017; Lazo et al., 2016; Reinders et al., 2016). These outcomes will help the EU aquaculture sector and the supply industry in targeted marketing and improvement of its international competitive position.

The combination of biological and socioeconomic research activities planned in DIVERSIFY *(Aquaculture Europe 39(1) March 2014)* are expected to **support the diversification of the aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets**. Specifically, the socioeconomics work has three main objectives: (a) find out the consumer market opportunities for the six new species (*i.e.* greater amberjack, pike perch, meagre, wreckfish, Atlantic halibut and grey mullet), (b) examine the business-to-business market opportunities for the species above and (c) develop business models for the new species on the basis of an online market test. These insights are being generated for the five largest European fish markets:

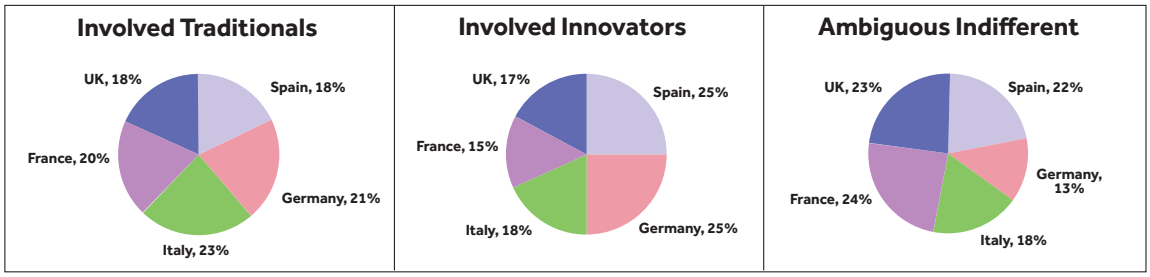


Figure 24. Consumer segments and country membership according to a DIVERSIFY study.

France, Germany, Italy, Spain and the United Kingdom. Not all activities are finished yet, but some general insights are visible already.

A quantitative online study with 2,500 consumers in the above five focal countries demonstrated that some consumer segments are open to try new species (**Fig. 24**). Especially in Germany and Spain, consumers from the segment «involved innovators» are very open to new fish species. On the other hand, French and Italian consumers are interested in new fish species although more traditional in their fish choices (*i.e.* the «involved traditional» segment) and British consumers are less involved and more ambiguous in the product choices they make (*i.e.* the «ambiguous indifferent» segment).

The market analysis demonstrated that buyers (*i.e.* retailers) in the five countries find it very difficult to position the six new species in relation to the current species in the market. Both as wild catch and aquaculture products, they are all fairly unknown. However the buyers are open to welcome new species under the following conditions: (a) the product must be cultured in a sustainable way, (b) the product should be available as a fresh product (southern-Europe) and as a frozen product (especially Germany), (c) the product must be easy to prepare and/or ready to eat, and (d) the product must be priced competitively. All these issues have been covered in DIVERSIFY. The feasibility study based on real cost prices of production is still going on. Sustainable production is covered in the reported biological research work packages (presented above), and in the socio economic work package convenience is included in the consumer oriented product development.

A qualitative study with 10 focus groups consisting of six participants each, undertaken across the five study markets (*i.e.* two focus groups per country) has identified the most promising product ideas for new fish products per investigated country (**Table 5**). In terms of general recommendations for new product development of selected fish species, the most important drivers and barriers for the choice of the new product ideas that are most relevant for consumers have been identified (**Fig. 25**).

On the basis of this study, a long list of product ideas has been developed for the different countries. However, not all products were practically possible with the different fish species. Therefore, only a selection of products has been sensory-tested in the five countries among regular consumers of fish. This sensory test showed that all the products were well accepted, except for fish pate. Products with a lower degree of processing were those who generated higher expected scores and higher acceptability in the blind test. It seems reasonable to infer that products having a higher degree of processing would be more appropriate for consumers who do not like fish because of its taste, presence of bones, odour, etc. In these cases, the existence of different processed alternatives could be a good solution for those individuals looking for a more convenient and less "fishy" product.

Table 5. Preferred fish products per country, based on the six new/emerging fish species included in the DIVERSIFY project.

Country	Description of the created and best voted product ideas
France	Fresh fish Carpaccio that can be used as starter for a hot meal or as sandwich filling. This Carpaccio is seasoned with ginger and chili and presented as scales of the fish. The product is produced environmentally sustainable. The packaging is a plate that looks like a round box with the compartments and transparent wheel on the top that you can turn to reach different sections.
Germany	Fresh fish fillet covered with herbs and spices in the transparent packaging. Different fillet size in the packaging conveying the product message through images and voice: 'For him – Fish for the triathletes'; 'For her – vacation in Provence'.
Italy	Fresh fish steak for grilling in the pan. Transparent packaging with a label that guarantees the origin of the product and communicates its quality, signs and references to tradition and respect for the environment.
Spain	Fish sausages and fish burgers. The main advantage of this product is that the product has no bones. The seasoning is very mild and therefore this product is therefore suitable for children. The product is produced environmentally sustainable.
UK	Fresh fish fillet with different 'healthy' seasoning and marinades separately packed that consumer can choose and vary depending on the occasion. This product is sold with recommendation for the appropriate vegetables and wine to accompany the dish. Product message: 'Not two same dishes in a row; 'You have it ready for you, healthy but still have the hectic lifestyle.'

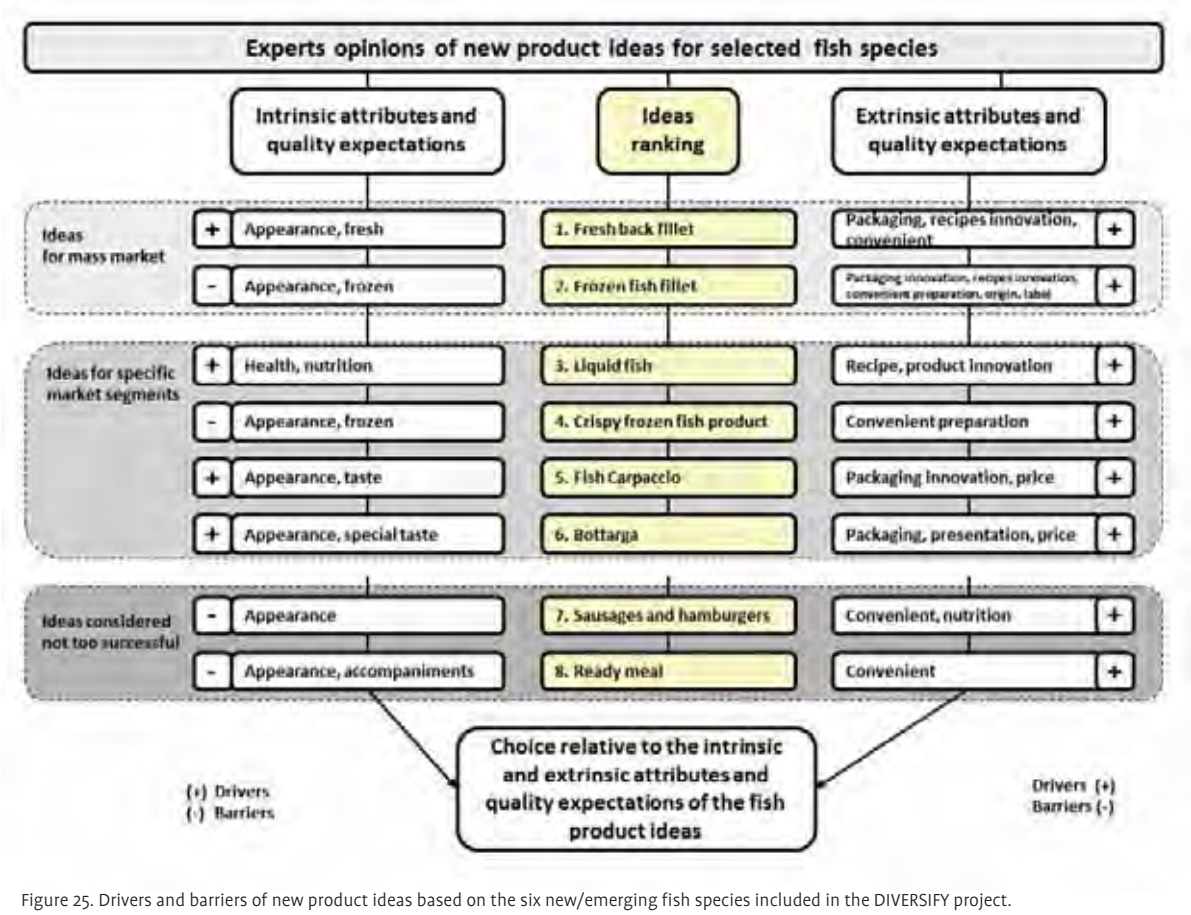


Figure 25. Drivers and barriers of new product ideas based on the six new/emerging fish species included in the DIVERSIFY project.

Upcoming "DIVERSIFY One-day Species-specific Workshops" for stakeholders

To speed up the dissemination of the project's results to the interested stakeholders and the aquaculture industry, one-day species-specific workshops are being organized for the Spring of 2018, to be carried out in different locations around Europe (Greece, Italy, France, Spain, Belgium, Norway). The location will be selected based on the aquaculture potential of each species and the Workshops will be will be hosted by the species leaders within the DIVERSIFY consortium, as follows:

1. Meagre, Alicia Estevez, IRTA, Spain
2. Greater amberjack, Nikos Papandroulakis, HCMR, Greece
3. Pikeperch, Pascal Fontaine, Uni. Lorraine, France
4. Atlantic halibut, Birgitta Norberg, IMR, Norway
5. Wreckfish, Blanca Alvarez, IEO, Spain
6. Grey mullet, William (Bill) Koven, IOLR-NCM, Israel

The workshops will be announced in the website of the project **(www.diversifyfish.eu)** at the end of 2017, and will be also advertised in relevant websites. In each workshop, researchers from within the DIVERSIFY consortium will present a summary of the work carried out and the production methods developed, in the different areas (Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health, Final product quality and Socioeconomics). Relevant researchers from outside the consortium will also be invited to present their work. This will ensure that the participants are provided with the State-of-the-art of the scientific knowledge for each of the species, coming not only from DIVERSIFY, but also from other European or National initiatives. The workshops will be **free of charge** and will be open to any interested researcher, farmer or regulator, on a **first come-first served basis**.

An online experimental choice study with product mock-ups developed from the created product ideas from the qualitative study (**Fig. 26**) was conducted in the five study countries to identify the optimal intrinsic-extrinsic product quality profiles for earlier identified consumer segments (*i.e.* the involved innovators and the involved traditionals). This study showed that country-of-origin and price come first when choosing new fish products, followed by quality certification (*i.e.* Aquaculture Stewardship Council – ASC logo), while nutrition and health claims appear to have varying and minimal impact, which is highly dependent on the type of product and level of processing, and country. Thus, a certain degree of customisation is needed for certain products, depending on the level of processing and countries.

Figure 26. Different products developed by DIVERSIFY for testing with consumers.

REFERENCES

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

Baekelandt, S., Redivo, B., Mandiki, S.N.M., Bournonville, T., Schmitz, M., Krauss, D., Ledoré, Y., Fontaine, P., Kestemont, P., 2017. Multifactorial analyses revealed mild- or major- aquaculture stressors affecting growth, physiology and immune status of pikeperch *Sander lucioperca* General and Comparative Endocrinology, in press.

Banović, M., Krystallis, A., Guerrero, L., Reinders, M.J., 2016. Consumers as co-creators of new product ideas: An application of projective and creative research techniques. Food Research International 87, 211-223.

Campoverde, C., Estevez, A., 2017. The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae. Aquaculture 478, 16-24.

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A., 2017. Early weaning in meagre Argyrosomus regius: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research 1, 1-11.

Crespo, S., Grau, A., Padrós, F., 1994. The intensive culture of 0-group amberjack in the western Mediterranean is compromised by disease problems. Aquaculture International 2: 262-265.

Crosetti, D., Blaber, S., 2016. Biology, Ecology and Culture of Grey Mullet (Mugilidae). CRC Press, Boca Raton, 529 pp.

Duncan, N.J., Estévez, A., Fernández-Palacios, H., Gairin, I., Hernández-Cruz, C.M., Roo, F.J., Schuchardt, D., Vallés, R., 2013. Aquaculture production of meagre (*Argyrosomus regius*): hatchery techniques, ongrowing and market, in: Allan, G., Burnell, G. (Eds.), Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited, Cambridge, UK, pp. 519-541.

Elkesh, A., Kantham, K.P.L., Shinn, A.P., Crumlish, M., Richards, R.H., 2012. Systemic nocardiosis in a Mediterranean population of cultured meagre, *Argyrosomus regius* Asso (Perciformes: Sciaenidae). Journal of Fish Diseases 36: 141-149.

Grau, A., Crespo, S., Pastor, E., Gonzalez, P., Carbonell, E., 2003. High infection by *Zeuxapta seriolae* (Monogenea: Heteraxinidae) associated with mass mortalities of amberjack *Seriola dumerili* Risso reared in sea cages in the Balearic Islands (western Mediterranean). Bulletin of the European Association of Fish Pathologists 23: 139–142.

Grigorakis, K., 2017. Fillet proximate composition, lipid quality, yields, and organoleptic quality of Mediterranean-farmed marine fish: A review with emphasis on new species. Crit Rev Food Sci Nutr 57, 2956-2969.

Lazo, O., Claret, A., Guerrero, L., 2016. A comparison of two methods for generating descripting attributes with trained assesors: check-all-that-apply (CATA) vs. free choice. Journal of Sensory Studies 31, 163-176.

Lovatelli, A., Holthus, P.F., 2008. Capture-based aquaculture; Global overview. Food and Agriculture Organization of the United Nations, Rome, 298 pp.

Monfort, M.C., 2010. Present market situation and prospects of meagre (*Argyrosomus regius*), as an emerging species in Mediterranean aquaculture, Studies and Reviews. General Fisheries Commission for the Mediterranean No. 89. Food and Agriculture Organization of the United Nations, Roma, pp. 28.

Montero, F.E., Crespo, S., Padrós, F., De la Gándara, F., García, A., Raga, J.A., 2004. Effects of the gill parasite *Zeuxapta seriolae* (Monogenea: Heteraxinidae) on the amberjack *Seriola dumerili* Risso (Teleostei: Carangidae). Aquaculture 232: 153–163.

Moretz, J.A., Martins, E.P., Robison, B.D., 2007. The effects of early and adult social environment on zebrafish (*Danio rerio*) behavior. Environ. Biol. Fish 80, 91–101.

Mylonas, C.C., Fatira, E., Karkut, P., Sigelaki, I., Papadaki, M., Duncan, N., 2015. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity III. Comparison between GnRHa implants and injections on spawning kinetics and egg/larval performance parameters. Aquaculture 448, 44-53.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture 464, 323-330.

Norton, W. H., Stumpenhorst, K., Faus-Kessler, T., Folcher,t A., Rohner, N., Harris, M.P., Bally-Cuif, L., 2011. Modulation of Fgfr1a signaling in zebrafish reveals a genetic basis for the aggression–boldness syndrome. J. Neurosci. 31, 13796-13807.

Reinders, M.J., Banović, M., Guerrero, L., Krystallis, A., 2016. Consumer perceptions of farmed fish: A cross-national segmentation in five European countries. British Food Journal 118, 2581-2597.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., Corriero, A., 2017. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLoS ONE 12, e0169645.

Co-funded by the Seventh Framework Programme of the European Union

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). For further information contact C.C. Mylonas at mylonas@hcmr.gr and R. Robles at r.robles@ctaqua.es

Figure 1.- Commercial size individuals of *Múgil cephalus*.

Advances in larval and juvenile grey mullet (*Mugil cephalus*) culture: The DIVERSIFY project

W. KOVEN[1], O. NIXON[1], I. MEIR-ASHKANAZI[1], R. ROTEM[1], A. TANDLER[1], D. ISRAELI[1], H. BRACHA[1], A. CORREIRO[2], E. GISBERT[3], H. ROSENFELD[1], M. BANOVIC[4], L. GUERRERO[5], G. TACKEN[6], R. ROBLES[7]

[1]Israel Oceanographic And Limnological Research, P.o.b.1212, Eilat 88112, Israel.

[2]University Of Bari, Aldo Moro, Dept. Of Emergency And Organ Transplantation, Section Of Veterinary Medicine And Animal Production, Bari, Italy.

[3]Irta, Centre De Sant Carles De La Ràpita (Irta-Scr), Unitat De Cultius Experimentals, Crta. Del Poble Nou Km 5.5, 43540 Sant Carles De La Rápita, Spain.

[4]Aarhus Universitet, Nordre Ringgade 1, Aarhus C 8000, Denmark

[5]Irta Food Technology, Finca Camps I Amet, S/N. 17121 Monells, Girona, Spain.

[6] Stichting Wageningen Research. Afdeling Consument & Gedrag, Postbus 356700 A, Wageningen, The Netherlands.

[7]Ctaqua, Muelle Comercial S/N, 11500 El Puerto De Santa Maria, Cádiz, Spain.

The grey mullet *(Mugil cephalus)* (Fig. 1) is one of 6 species selected for the European program DIVERSIFY (FP7, GA 603121), a five-year project to advance our knowledge and its practical application in the culture of new and emerging finfish species, with the potential of satisfying an expanding sustainably the European market for a variety of fresh sea food. There is increasing interest in the culture of the omnivorous grey mullet as a high quality source of protein and as a species that requires little or no dietary fishmeal (FM). Moreover, the salted and dried roe (bottarga) from gravid females is considered a highly prized delicacy in the southern Mediterranean and an added value product from the culture of this species.

The future growth of the grey mullet aquaculture is limited by a number of bottlenecks, which have been addressed in DIVERSIFY. The control of the reproductive cycle and improving egg quality via broodstock management and nutrition was necessary not only for the production of robust larvae, but also for obtaining high value bottarga. A very important issue has been the development of a larval rearing protocol necessary to reduce early mortalities and size dispersion, as well as increasing metamorphic synchrony, which can lead to a supply of high quality juveniles. Finally, development of a sustainable, economical, FM-free grow out feed providing good performance under different environmental conditions of temperature, pond type, and water quality, thus broadening the geographical range of grey mullet aquaculture in Europe, is under investigation.

Reproduction control

Captive grey mullet fail to reproduce spontaneously, largely due to a failure to undergo complete gametogenesis (Aizen et al., 2005). DIVERSIFY first evaluated the effectiveness of hormone-based treatments on synchronizing gonadal development (**Fig. 2**). A combined treatment consisting of follicle stimulating hormone (FSH) and dopamine antagonist (metoclopramide) on spermatogenesis in males and follicle growth and maturation in females was tested. The methylotrophic yeast (*Pichia pastoris*) expression system was used to produce large quantities of bioactive recombinant single-chain FSH (rFSH), which was used in a series of in vivo assays. Unlike the controls, the hormonally treated groups (injected with rFSH and metoclopramide during the onset of the reproductive season) demonstrated synchronized gonadal development within and between sexes, with higher rates, over time, of spermiating males and post-vitellogenic females. Once gonadal development was accomplished, we proceeded with the development of hormone-based treat-

Figure 2. Grey mullet breeder *(left)* ready for an ovarian biopsy *(right)* to evaluate reproductive stage of development (IOLR, Israel).

ments for inducing spawning. Spawning induction trials that timed the administration of gonadotropin releasing hormone agonist (GnRHa) and metoclopramide with advanced stages of gamete maturation were quite successful, producing tens of millions of fertilized eggs. Nevertheless, our results highlighted two major problems: (i) the female's failure to ovulate in 42% of the spawning induction trials and (ii) the variable fertilization success ranging between 0 to 98%, underlining the need to fine-tune further and optimize the hormone-based breeding protocol for captive grey mullet.

Larval Husbandry

In the commercial rearing of marine fish larvae, tanks are frequently "greened" with microalgae such as *Nannochloropsis oculata* or *Isochrysis galbana*. It is widely believed and demonstrated that the provision of these algae to the tanks improves significantly larval performance and has become an inseparable part of commercial rearing protocols in fish farms around the Mediterranean basin and in Europe. On the other hand, it remains speculative how algal supplementation contributes to larval growth and survival or if different algal species are equally effective. The biochemical composition of algal species (e.g. fatty acids) varies considerably and it is entirely possible that species-specific compounds secreted from the algal cell (e.g. polysaccharides) and/or are released during digestion might stimulate the immune system or enhance the digestive process. In addition, water turbidity from specific algal concentrations may provide optimal backlighting for larvae to facilitate live prey identification (e.g. rotifers), and, thereby, enhance hunting success.

In DIVERSIFY trials, the effect of two tank turbidity levels has been tested (0.76 and 1.19 NTU (Nephelometric Turbidity Units)) from two algal species (*N. oculata* and *I. galbana*) compared to the no-algae control (0.26 NTU) in 2-25 days post hatching (dph) grey mullet larvae. This study demonstrated that the higher turbidity (1.19 NTU) increased rotifer consumption independently of algal type **(Fig. 3a),** while the pattern of survival in 51 dph juveniles (3 weeks after the addition of the algal treatments to the tanks) was strikingly simi-

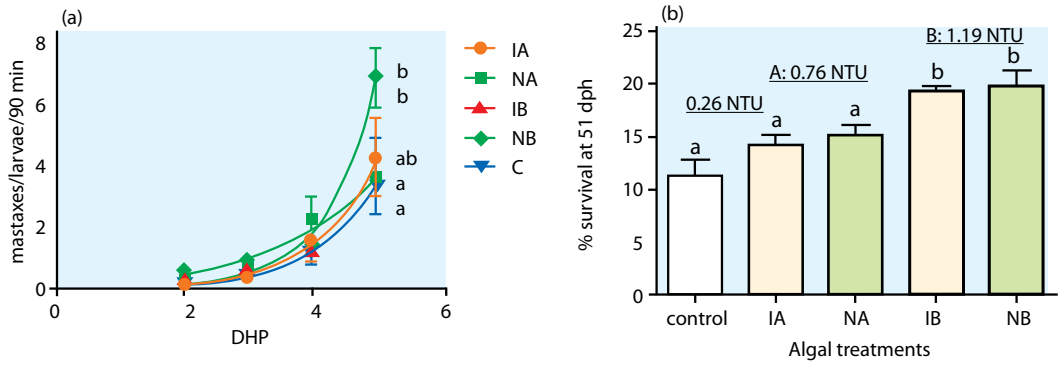


Figure 3. The effect of turbidity treatments; no algae (control), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) on (a) average rotifer (number of mastaxes) consumption larva-1 found 90 min after feeding from 2-5 dph and (b) percent (%) larval survival at 51 dph. Mastax number at 5 dph and percent (following arcsine transformation) survival values of the algal treatments on 51 dph having different letters were significantly (P<0.05) different.

lar to rotifer ingestion at 5 dph **(Fig. 3b)**. This suggested the importance of rotifer feeding during early larval development on later juvenile survival in grey mullet. However, it was still unclear if turbidity or background lighting was the main factor influencing rotifer consumption and larval growth or common biochemical factors between these algal species were influencing larval performance.

When potter's clay (red or white) was added to the larval rearing tanks at the same turbidity (1.2 NTU) as *N. oculata*, rotifer consumption **(Fig. 4a)**, as well as growth in 30 dph larvae and survival in 50 dph juveniles **(Fig. 4b, c)** was markedly (P<0.05) lower and was similar to the lower turbidity level of *N. oculata* (0.76 NTU). This suggested a further advantage that live algae provide, in addition to its ability to produce turbidity in the larval rearing of grey mullet. Moreover, in another study the benefit of algal addition at higher turbidity was not altered if the algae were freeze dried. This means that the costly culturing of live algae could be replaced by more economical off-the-shelf algal products in the rearing of grey mullet, which would translate into a significant saving in energy and labour.

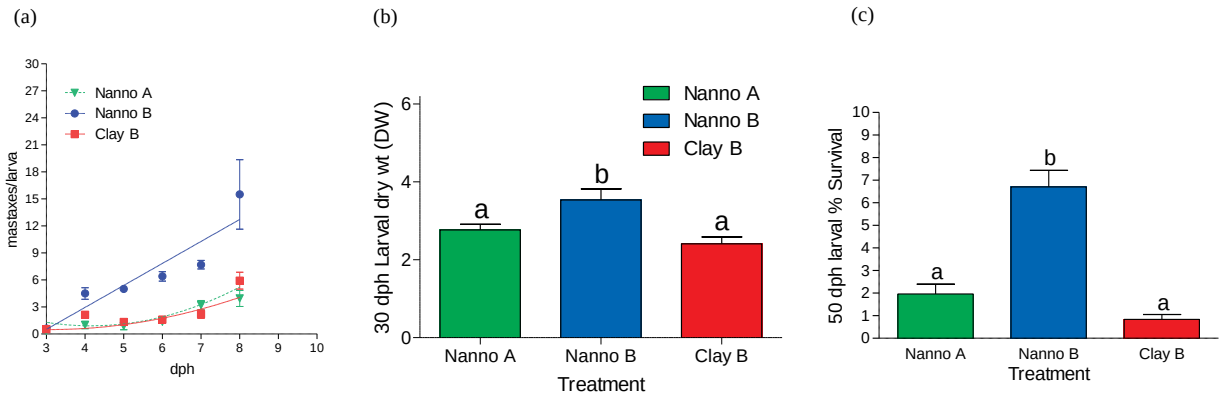


Figure 4. (a) Average number of mastaxes consumed per larvae 90 minutes after feeding. The Nanno B curve was significantly (P=0.0004) different than the Nanno A and Clay B curves while the latter two curves were not significantly (P>0.05) different from each other. The effect of the Nanno A, Nanno B and Clay B turbidity treatments on (b) 30 dph larval dry weight and (c) 50 dph juveniles are shown. DW values having different letters were significantly (P<0.05) different.

Juvenile Weaning

The grey mullet larvae, as in all early developing marine teleost fish, are strict carnivores feeding on zooplankton such as rotifers and Artemia in commercial hatcheries. However, after the mullet larvae have metamorphosed into juveniles, they begin to change their mode of feeding from a carnivorous to an herbivorous/omnivorous diet as the fish begin to search out less saline estuaries with higher primary productivity of micro and macroalgae. We demonstrated that the digestive tract reaches full maturation around 61 dph and considerable pancreatic amylase production exists at 79 dph, while maintaining alkaline protease activity, as the grey mullet adapt to a high carbohydrate, low protein diet. This contrasts to other marine species such as the gilthead sea bream (Sparus aurata) and the European sea bass (Dicentrarchus labrax), which remain carnivorous throughout their life and consume a high protein, low carbohydrate diet. From 24-38 dph, grey mullet juveniles at the IOLR (Israel) facilities can be incrementally weaned off live Artemia and onto a dry, more energy dense starter diet. As the weaning period appears to overlap the transition period where the mullet juveniles change their mode of feeding, the question remains if weaning diets should be carnivorous, herbivorous or omnivorous in nature in order to maximize growth and survival. In the DIVERSIFY studies, we demonstrated that mullet juveniles grew significantly (P<0.05) less when fed only a macroalgae (Ulva lactuca) based herbivorous weaning diet compared to a commercial carnivorous feed (Caviar, Bernaqua, Belgium), while fish fed the 1:1 omnivorous mix of these diets exhibited markedly (P<0.05) superior growth than all the treatments **(Fig. 5a).** Fish fed the herbivorous diet demonstrated significantly (P<0.05) higher numbers of smaller fish (<100 mg), than the carnivorous and omnivorous diet fish. Conversely, 200-300 mg carnivorous and omnivo-

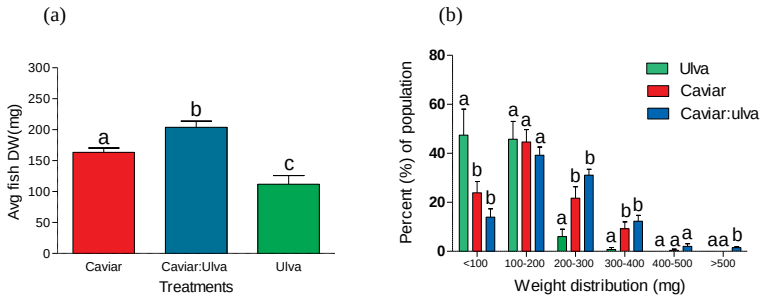


Figure 5 The effect of the commercial starter diet Caviar (Bernaqua, Belgium), macroalgae Ulva lactaca (Ulva) and the 1:1 mix Caviar: Ulva on (a) dry weight (DW) at the end of the experiment and (b) weight distribution. Weight (mg) values having different letters were significantly (P<0.05) different.

rous treatment fish represented a significantly (P<0.05) higher percentage of the population than in the herbivorous diet fed fish (**Fig. 5b**). Grey mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch-rich micro and macroalgae, as well as benthic protein rich organisms characterizing the lower salinity estuarine waters they move into at this developmental stage. Furthermore, the high amylase and maltase activity in fish fed the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing, resulting in improved growth. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds.

These results were generally reinforced in another study, which weaned fish onto compound diets that differed in their levels (50 and 75%) of FM substitution with a plant-based meal blend (corn gluten, wheat gluten, soy bean meal and soy protein concentrate) supplemented with L-lysine and DL-methionine free amino acids. This study showed that diets with 75% fish meal substitution can be successfully used for weaning and on-growing wild fry without any detrimental effect on fry performance and condition (see Gisbert et al. 2016, Aquaculture 462, 92-100).

Larval and juvenile DHA requirement

It is well documented that the long chain n-3 polyunsaturated fatty acid (LC-PUFA) docosahexaenoic acid (DHA; 22:6n-3) promotes growth more effectively than the other LC-PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA;20:4n-6) in marine fish larvae. The benefit of DHA to weight gain lies in its contribution to membrane fluidity and function primarily in the neural membranes of the eyes and brain, as well as its involvement in immune function and gene expression. Optimum DHA levels in larval feeds to promote growth and survival range from approximately 0.5 to 2.5% DW diet. We found no dietary DHA effect on larval grey mullet wet weight gain and rotifer consumption rate above the 5.5% DHA level (analyzed at the IOLR) in the commercial enrichment preparation "Red Pepper" (Bernaqua, Belgium) suggesting that, in terms of DHA content, this product is suitable for the larval rearing of grey mullet. Interestingly, the 5.5% DHA

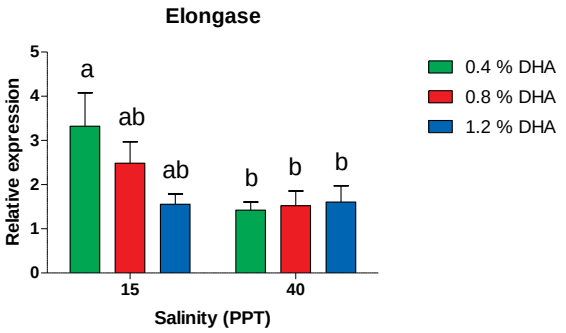


Figure 6. The effect of salinity and DHA treatment on elongase gene expression. Bar values having a different letter(s) were significantly (P<0.05) different.

DW diet level resulted in significantly (P<0.05) improved larval survival over the other higher DHA treatments when fish were 40 dph or 15 days after the rotifer treatments had been discontinued. This emphasizes the importance of feeding an effective level of DHA at the rotifer stage on survival in later development stages. Similarly, there was no dietary DHA benefit when increasing DHA from 0.4% to 0.8 and 1.2% DW diet during juvenile mullet growth, where all treatments demonstrated excellent survival (92.4, 88.8 and 97.6%, respectively).

Although there was no apparent DHA benefit at levels greater than 0.4% DW diet to juvenile mullet reared in 40‰ seawater, the question remained if a DHA requirement would be influenced by lower salinity seawater when, in nature, mullet juveniles are moving into lower saline estuaries. Our studies showed that a seawater salinity of 15 ‰ appears to trigger desaturase and elongase capability. In fact, elongase gene expression markedly (P<0.05) increased, when fish were fed low DHA dietary levels (0.4% DW diet) (**Fig. 6).** Overall, these results suggested that it may be more efficient and economical to grow grey mullet from juveniles to market weight in lower salinity seawater while feeding them relatively low levels of dietary DHA.

Larval and juvenile taurine requirement

The β-amino sulfonic acid taurine, which is not incorporated into proteins, plays an array of critical roles in its free form. These include involvement in bile salt synthesis, anti-oxidative defense, cellular osmoregulation, as well as contributing to visual, neural and muscular function. In general, taurine cannot be synthesized in carnivorous teleosts and must be provided in the diet. However, it is unclear if omnivorous/herbivorous species have taurine synthesis capability, as their natural plant-based diet would likely be taurine deficient. Our studies found not only a significant (P<0.05) taurine requirement during rotifer feeding **(Fig. 7a),** but the benefit of ingesting this nutrient during early feeding was still apparent in much later stages of juvenile growth **(Fig. 7b)**.

On the other hand, Artemia nauplii have considerable natural levels of taurine and we found no benefit feeding taurine enriched Artemia on larval growth and survival. Moreover, we showed that juvenile grey mullet have a 0.5% DW dietary taurine requirement **(Fig. 8)**, which is within the range of the requirement for this nutrient in a variety of marine species such as the Florida pompano (*Trachinotus carolinus*), California yellowtail (*Seriola lalandi*), cobia (*Rachycentron canadum*), common dentex (*Dentex dentex*), Japanese flounder (*Paralichthys olivaceus*) and red sea bream (*Pagrus major*). Despite this taurine requirement, there was a taurine dose-dependent response on liver CSD (cysteine sulfonate decarboxylase) expression, which increased 9.2 times from 0 to 1% taurine DW diet, but then decreased substantially in the highest taurine diet (2% DW diet) **(Fig. 9a)**. The synthesis of taurine in the liver, when levels of this nutrient are increasing in the diet, seems counter intuitive. However, taurine can function as an osmolyte to maintain cell volume. Conceivably, increased taurine in the blood circulation of the liver, due to higher dietary taurine, may stimulate increased synthesis within liver cells to reduce osmotic pressure across the membrane, in order to prevent cell shrinkage and changes in intracellular hydro-mineral balance. This suggests that the overall taurine requirement might be higher than 0.5%, as part of the taurine requirement appears to be satisfied through endogenous synthesis of this nutrient. On the other hand, fish fed the 2% taurine diet may be ingesting excessive levels of taurine resulting in decreased production of endogenous taurine.

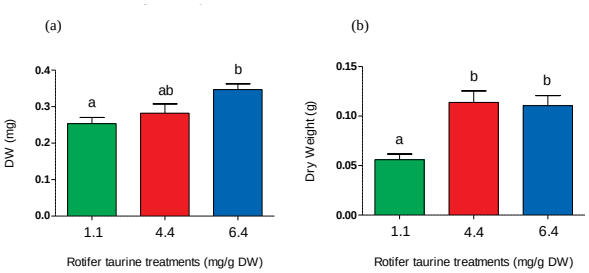


Figure 7. The effect of rotifer taurine treatments on dry weight (DW) in (a) 19 dph larvae and (b) 44 dph juveniles. Values having a different letter(s) were significantly (P<0.05).

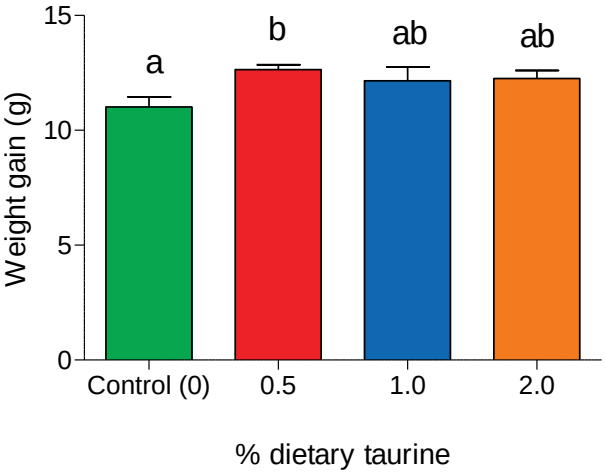


Figure 8. The effect of percent (%) dietary taurine on weight gain in juvenile grey mullet. Bar values having different letter(s) were significantly (P<0.05) different.

One of the major roles for taurine is to conjugate with bile acids such as cholic acid or chenodeoxycholic acid in the liver, which is then stored in the gall bladder. Conjugated bile acids, when released into the lumen of the intestine after feeding, emulsify fats to make them more accessible for digestion and absorption. The enzyme 7α-hydroxylase (CYP7A1) is the rate limiting enzyme in bile salt synthesis and has been associated with dietary taurine and its growth promoting properties. The synthesis of this enzyme did not change markedly with dietary taurine level, which suggests that endogenous taurine synthesis

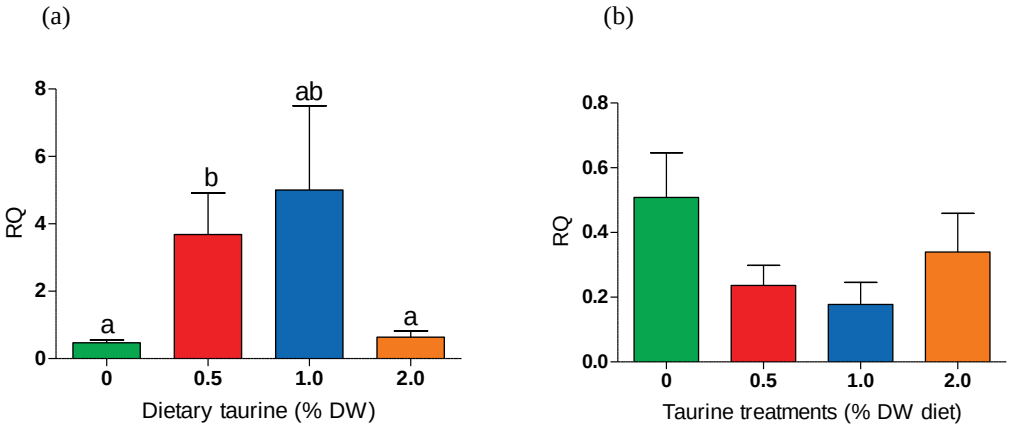


Figure 9. The effect of percent (%) dietary taurine on (a) CSD and (b) Cyp7a gene expression (RQ). Bar values having different letter(s) were significantly (P<0.05) different.

is sufficient to produce adequate levels of bile acids **(Fig. 9b)** and that the dietary lipid requirement for this species may not be high. Furthermore, these results suggest that the growth promoting effect of taurine is not primarily due to improved lipid digestion and absorption, but possibly its contribution to other physiological pathways such as muscle function and growth.

Juvenile production and grow-out of grey mullet

During October of 2017, grey mullet eggs at the IOLR were stocked (30-54 eggs/l) in three 6 m^3 V-tanks and were reared using the improved grey mullet larval rearing protocol, based on the results mentioned above, to 70 dph before being transferred to the nursery. The survival rate to this age ranged between 12.5 to 16.6% and produced in excess of 44,000 high quality juveniles. Nevertheless, during the grow out of juveniles to market weight in monoculture, there was a wide size distribution of the population that appears to be amplified with increasing stocking density, although survival remains high. This presents a significant obstacle to the successful culture of this species and is currently being studied.

A farm trial performed in earthen ponds in the South of Spain (CTAQUA) has also shown an effect of density on the size distribution of grey mullet juveniles during grow out (**Fig. 10).** These results are being evaluated at the moment of this article publication.

New fish product development

Besides the technical improvement of the selected species, DIVERSIFY project has a substantial socio-economic work package. This work package includes not only scientific sound but practical market development solutions on perception of aquaculture products, market demand insights, buyer preferences understandings , new product development ideas and value adding designs (Banović et al., 2016; Grigorakis, 2017; Lazo et al., 2016; Reinders et al., 2016). These outcomes can help the EU aquaculture sector and the supply industry in targeted marketing and improvement of its international competitive position.

The socioeconomic work of DIVERSIFY has three main objectives: (a) find out the consumer market opportunities for the six new species (i.e. grey mullet), (b) examine the business-to-business market opportunities for the species and (c) develop business models for the new species on the basis of an online market test. These insights are being generated for the five largest European fish markets: France, Germany, Italy, Spain and the United Kingdom. Although these insights are generated for the six species producers, suppliers and traders of other species might find consumer insights in this project that are interesting for their products too.

A quantitative online study with 2,500 consumers in the above mentioned five countries demonstrated that some consumer segments are open to try new species and/or products ("involved innovators" and "involved traditional"). Especially in Germany and Spain, consumers from the segment «involved innovators» are very open to new fish species and/or products. On the other hand, in France and Italy "involved traditional" consumers are the ones most interested in new fish species despite their traditional fish choices. British consumers were much less involved towards offerings from new fish species and more ambiguous in their product choices making the "ambiguous indifferent" segment.

Figure 10. Top: weight sampling of on-grown grey mullet. Bottom: Harvest of grey mullet at the pond farm in the South of Spain.

A qualitative study with focus groups undertaken across the five study markets has generated the most promising product ideas for new fish products per investigated country. On the basis of this study, a long list of product ideas has been developed for the different countries. However, not all products were practically possible with the different fish species. Therefore, only a selection of products has been sensory-tested in the five countries among regular fish consumers. The developed products for grey mullet were: 1) fresh filet with healthy seasoning, 2) thin smoked filet and 3) fish filets in olive oil (**Fig. 11).** All the grey mullet products were prepared by the DIVERSIFY partner Ctaqua.

The sensory test showed that the two tested products from grey mulet were well accepted. **(Fig. 12)**. Products with

Figure 11. The three grey mullet products developed. From less processed product (left): fresh filet with healthy seasoning; medium process (center): thin smoked filet and higher processing (right): grey mullet filet bottled in olive oil.

a lower degree of processing were those who generated higher expected scores and higher acceptability in the blind test. It seems reasonable to infer that products having a higher degree of processing would be more appropriate for consumers who do not like fish because of its taste, presence of bones, odor, etc. In these cases, the existence of different processed alternatives could be a good solution for those individuals looking for a more convenient and less "fishy" product.

An online experimental choice study with product mock-ups (**Fig. 13)** was conducted to determine which product attributes should be communicated when selling the fish product (optimal extrinsic product quality profiles). This test was done for the created product ideas from the qualitative study and it was conducted in the five study countries. The study showed that country-of-origin and price come first when choosing new fish products, followed by quality certification, eco-label (i.e. Aquaculture Stewardship Council – ASC logo), while nutrition and health claims appear to have varying and minimal impact, which is highly dependent on the type of product and level of processing, and country. Thus, a certain degree of customization is needed for certain products, depending on the level of processing and countries.

Concerning the market analysis, it has been demonstrated that buyers (i.e. retailers) in the five countries of the study, find it very difficult to position the six new species (e.g. grey mullet) in relation to the current species in the market. Species such as grey mullet are unknown as aquaculture products as well as wild catch. However the buyers are open to welcome new species under the following conditions: (a) the product must be cultured in a sustainable way, (b) the product should be available as a fresh product (southern-Europe) and as a frozen product (especially Germany), (c) the product must be easy to prepare and/or ready to eat, and (d) the product must be priced competitively. All these issues have been covered in DIVERSIFY.

Figure 13. Example of products mock-up developed by DIVERSIFY for the online experimental choice study.

The feasibility study based on real production cost prices and business plan development is planned for the last year of the project.

KNOW-HOW TRANSFER SEMINAR

Among the dissemination activities of DIVERSIFY, full-day seminars on "Know-how Transfer" of the aquaculture of each of the DIVERSIFY species are being organized. At the moment of this publication, 5 seminars have been completed already for grey mullet (May 2018 in Bari, Italy), pikeperch (June 2018, Nancy, France), wreckfish (July 2018, Vigo, Spain), Atlantic halibut (September 2018, Hjelmeland Spa, Ryfylke, Norway) and greater amberjack (September 2018, Electra Metropolis Hotel, Athens, Greece). In October the following seminar is planned: meagre: 9th October (Palau Macaya, Barcelona, Spain). All the information on the seminars, as well as the presentations given, can be found in the DIVERSIFY web site https://www.diversifyfish.eu/species-workshops.html

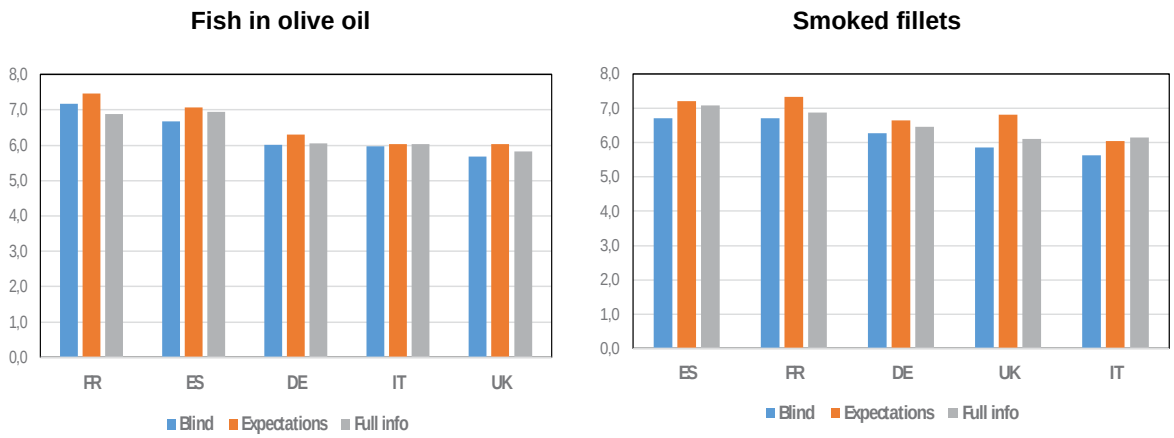


Figure 12. Results of the consumers' acceptance tests for grey mullet new developed products performed in 5 European countries. Consumers were not informed about the product (blue bar). They were asked about their expectation once informed about the products (orange bar) and finally they had the full information before tasting the product (grey bar).

The seminars include several presentations on selected aspects (*e.g.* reproduction and spawning induction, final product diversification and quality, socioeconomic issues and marketing, etc.), given by DIVERSIFY partners. In addition, presentations are also given by scientists and authorities in the species (European and world-wide), whose work is relevant although it has not been part of the project. Farmers and producers, but also European aquaculture support companies (feed, pharmaceutical, equipment, engineering, etc.), researchers and educators, government organizations and other institutions are invited to attend these meetings.

The grey mullet know-how seminar was organized by Dr. Aldo Corriero from the University of Bari, Italy and Dr. Bill Koven, grey mullet Species leader from IOLR, Israel. The workshop was addressed to the aquaculture industry, with the objective of transferring the knowledge acquired by the project on the various aspects of grey mullet rearing to enable any commercial aquaculture operation to include this species in their production.

In total 14 presentations on the species were given during the seminar. Apart from the presentations given by the DIVERSIFY partners, five invited speakers (Dr. Donatella Crossetti (Institute for Environmental Protection and Research, Rome, Italy), Dr. Ken Leber (MOTE Marine Laboratory, Florida, USA), Dr. Sherif Sadek (Aquaculture Consultant Office, Cairo, Egypt), Dr. Dario Vallanic (International Marine Center, Cagliari, Italy) and Dr. Antonella Rosa (University of Cagliari, Italy), all of them authorities in the species, provided relevant insights on key aspects of the species such as the need for restocking programs (Dr. Leber), the cultural heritage of grey mullet culture (Dr. Crossetti) and the nutraceutical properties of mullet bottarga (Dr. Rosa).

The seminar ended with a round-table discussion providing a summary on the main issues for the species:

- Grey mullet has a high potential as sustainable aquaculture species.
- There is need for a stock assessment in the Mediterranean area.
- It is essential to have fingerlings available at affordable prices, given the low cost of wild-caught juveniles, which is still allowed in many countries (*e.g.* Spain, Egypt and Israel).
- There is a need for further and more in depth research on the different grow out culture systems.
- It is crucial to define and establish the species business model.
- Designing of a market strategy for the species is of outmost importance.

The DIVERSIFY project has addressed some of the above points such as the need for further and more in depth research on the different grow out culture systems, the definition and establishment of the business model and the design of a market strategy for the species.

DIVERSIFY
New species for EU aquaculture

Co-funded by the Seventh Framework Programme of the European Union

Breakthrough in the reproduction and larval rearing of wreckfish in the DIVERSIFY project

Fig 1. Wreckfish juveniles at the IGAFA facilities.

Fig 2. The wreckfish (*Polyprion americanus*).

MYLONAS, C.C. (Hellenic Center for Marine Research-HCMR, Heraklion, Crete, Greece)

RODRÍGUEZ, J.L (Instituto Galego de Formación en Acuicultura-IGAFA, Illa de Arousa, Spain); Linares, F., and Pazos, G. (Xunta de Galicia, Centro de Investigacións Mariñas, Vilanova de Arousa, Spain)

PÉREZ-RIAL, E. LLUCH, N., PEREZ, M. AND ÁLVAREZ-BLÁZQUEZ, B. (Instituto Español de Oceanografía-IEO, Centro Oceanográfico de Vigo, Spain)

VILAR, A. (Aquarium Finisterrae, A Coruña, Spain)

R. ROBLES, CTAQUA, El Puerto de Santa Maria, Cadiz, Spain

The first wreckfish (*Polyprion americanus*) juveniles have been produced in Galicia, Spain, after years of research in the reproduction and larval rearing of the species in the framework of the DIVERSIFY project (www.diversifyfish.eu). Early this year, researchers in Vigo, Illa de Arousa, Vilanova de Arousa and A Coruña have finally seen their efforts coming to fruition with the consistent acquisition of large quantities of fertilized eggs after spontaneous tank spawning of their captive reared broodstocks. Given the continuous supply of eggs, the planned research efforts for the development of larval rearing methods have been implemented and the result is a small number of hatchery-produced juvenile wreckfish happily swimming in the tanks of IGAFA and IEO (**Fig. 1)**. The results of the wreckfish research of DIVERSIFY have been presented in a special workshop on the 19 of July, 2018 at the facilities of IEO, Vigo, Spain and a Technical Manual is available for downloading at the web site of the project (https://www.diversifyfish.eu/wreckfish-workshop.html).

The cosmopolitan wreckfish is one of the largest Serranid species, reaching a size of 100 kg (**Fig. 2)** and it is one of the most interesting new species for aquaculture diversification, due to its fast growth, late reproductive maturation, high market price, limited fisheries landings and easy manipulation in captivity (Suquet et al., 2002; Papandroulakis et al., 2004; Rodríguez et al., 2017). However, until this year the lack of consistent reproduction control in captivity (Fauvel et al., 2008; Papandroulakis et al., 2008) and the limited trials for the development of larval rearing protocols (Álvarez-Blázquez et al., 2017) prevented the production of any significant results towards the production of juveniles for grow out.

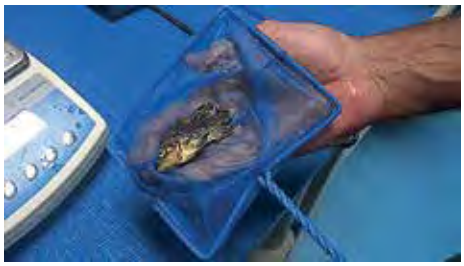
The EU FP7-funded DIVERSIFY project begun in December 2013 with the objective of acquiring the necessary knowledge for the diversification of the European Aquaculture production with some new/emerging finfish species (meagre, greater amberjack, pikeperch, grey mullet, Atlantic halibut and wreckfish). This project is the largest and most multidisciplinary effort made so far for the acquisition of knowledge for the aquaculture of wreckfish, in many ways a unique species for aquaculture. The project had a total budget of 11.8 million € for its 5 year duration, making it one of the largest research projects in the area of aquaculture funded by the European Commission. In the case of wreckfish, DIVERSIFY brought together almost all partners involved up to date in Europe in wreckfish domestication, in order to acquire the necessary knowledge and develop the required procedures for the production of fertilized eggs and juveniles to launch commercial production. After 5 years of work, the project has succeeded in achieving its target and the future looks promising!

Three different broodstocks have been maintained for the last 5 years in the facilities of the Spanish Institute of Oceanography in Vigo (IEO), the Instituto Gallego de Formacion en Acuicultura (IGAFA) of the Xunta de Galicia and the Aquarium Finesterrae in A Coruña (**Fig. 3**). The research carried out during the first years of the DIVERSIFY project on (a) the description of the reproductive cycle in captivity, (b) the development of methods for the induction of spawning using hormonal methods and (c) the production of an appropriate broodstock diet for this species have finally paid off! The IEO broodstock begun spawning first in January 2018 and produced a total of 43 spontaneous spawns in the course of 5 months, with one female spawning 10 times in the course of the season. Then the stock of IGAFA begun spawning and produced 30 spawns in 5 months. Finally, the broodstock at the Aquarium Finisterrae was induced to spawn using hormonal implants produced by the Hellenic Center for Marine Research, Greece and has produced more than 15 natural and spontaneous spawns until the end of July 2018. Spawning periodicity was every 3-5 days in all stocks and the time of spawning was mainly between 05:00 and 08:00 h, with some exceptions that took place at midday. Fertilization success was between 50 and 100% with better quality eggs towards the mid or end of the spawning season for each female. In the case of the males, a single male was noted to spawn for 30 times, in a period of 5 months.

Fig 3. Wreckfish breeders at the Aquarium Finisterrae, A Coruña

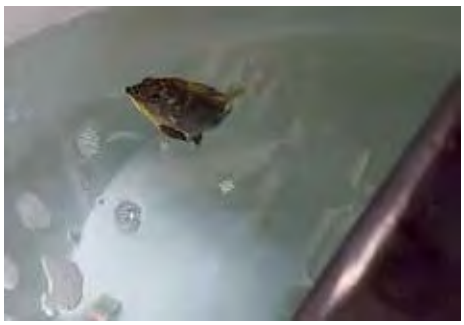
Fig 4. Seven-day-old larvae of wreckfish.

In the area of larval rearing, the objectives of DIVERSIFY were to establish a rearing protocol. In particular, the effect of rearing temperature was studied and the description of the ontogeny of the digestive system was considered as a prerequisite for the development of an appropriate feeding protocol. Having done these, once egg availability ceased to be a bottleneck this year, a number of larval rearing trials were initiated in 2018 with very good larval hatching (42-82%). Larval length was 4.7 mm at one day post hatching (dph) and 7.2 mm at 22 dph, and yolk sac consumption was completed at 11 dph at 14-17°C sea water temperature and 8 dph at 17-20°C. The moment of mouth opening was at 7 dph at 14-17°C and 4 dph at 17-20°C. Larvae were fed with rotifers and *Artemia* nauplii (**Fig. 4**).



A small number of juveniles were weaned to artificial feed and they are swimming in the tanks of IGAFA, at >100 dph and a mean weight of 4.5g. Also in the Aquarium Finisterrae and IEO, larval culture trials are currently being carried out with good expectations. It is the first time in the project that we succeeded in producing juveniles weaned to inert food, which is a milestone in the efforts to produce wreckfish under aquaculture conditions. This trial provides important data on the growth parameters of the species and increased our knowledge about the feeding protocol and the specific behavior and metamorphosis of wreckfish larvae.

So, an important step in the production of juvenile wreckfish has been achieved as promised in the DIVERSIFY project. Based on this important development, we expect that the efforts towards the aquaculture of this great species will intensify, both at the national and European level, and we hope to be able to offer **farmed wreckfish** to the European consumer in the not-so-far future!



References

Fauvel, C., Suquet, M., Sévère, A., Mylonas, C.C., Papandroulakis, N., 2008. Slow-release GnRHa therapy prevented atresia during vitellogenesis and induced ovulation of captive wreckfish (*Polyprion americanus*). Cybium 32(2) suppl, 191.

Papandroulakis, N., Suquet, M., Spedicato, M.T., Machias, A., Fauvel, C., Divanach, P., 2004. Feeding rates, growth performance and gametogenesis of wreckfish (*Polyprion americanus*) kept in captivity. Aquaculture International 12, 395-407.

Papandroulakis, N., Mylonas, C.C., Syggelaki, E., Katharios, P., Divakaran, S., 2008. First reproduction of captive-reared wreckfish (*Polyprion americanus*) using GnRH implants, European Aquaculture 2008, Krakow, Poland.

Suquet, M., La Pomèlie, Ch., 2002. Le cernier (*Polyprion americanus*): biologie, pêche, marché et potentiel aquacole. Plouzané: IFREMER, cop. 2002. 279 h. (Ressources de la mer). ISBN 2-84433-075-4.

Rodriguez, J.L., Alvarez-Blázquez, B., Pérez Rial, E., Martínez, J.M., Pazos, G., Fátima Linares. 2017. Evaluation of wreckfish P*olyprion americanus*) growth in Galicia (Spain) Aquaculture Europe 2017 (EAS), 17-20 October, Dubrovnik, Croatia.

Álvarez-Blázquez, B., Rodríguez, J.L., Vilar, A., Mylonas, C., Papandroulakis, C., Pérez Rial, E., N. Lluch, G. Pazos and F. Linares. 2017. Progress in the wreckfish intensive culture. new candidate species for aquaculture. Aquaculture Europe 2017 (EAS), 17-20 October, Dubrovnik, Croatia.

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 2 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from the project site at "www.diversifyfish.eu".

DIVERSIFY
New species for EU aquaculture

Co-funded by the Seventh Framework Programme of the European Union

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Fundación Canaria Parque Científico Tecnológico de la Universidad de Las Palmas de Gran Canaria (FCPCT), Spain

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Hellenic Center for Marine Research (HCMR), Greece

Hellenic Research House (HRH), Greece

Hungarian Aquaculture and Fisheries Inter-branch Organization (MA-HAL), Hungary

Institut de Recerca i Tecnologia Agralimentàries (IRTA), Spain

Instituto Español de Oceanografía (IEO), Spain

Institut Francais de Recherche pour l'Exploitation de la Mer (IFREMER), France

Institute of Marine Research (IMR), Norway

Irida SA (IRIDA), Greece

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Stichting Wageningen Research (SWR/DLO), the Netherlands

Technische Universiteit Eindhoven (TU/e), the Netherlands

The University of Aberdeen (UNIABDN), United Kingdom

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Universitá degli Studi di Bari Aldo Moro (UNIBA), Italy

Université de Lorraine (UL), France

Université de Namur ASBL (FUNDP), Belgium

Vas. Geitonas & Co LTD EE (GEI), Greece

Project Coordinator

Dr Constantinos C Mylonas, Research Director
Institute of Marine Biology, Biotechnology and Aquaculture
Hellenic Center for Marine Research P.O. Box 2214, Iraklion, Crete 71003, Greece
Tel: +30 2810 337878
Email: mylonas@hcmr.gr

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