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Is it possible to successfully rear meagre (*Argyrosomus regius* Asso 1801) larvae without using rotifers?

Said Sedki[1,2] | Jawad Kassila[1] | Hassan Nhhala[1] | Kamal Chebbaki[1] | Houda Akharbach[1] | Mohamed Id Halla[3] | Hassan Er-Raioui[2]

[1]Centre Spécialisé en Aquaculture, INRH, M'diq, Morocco

[2]Faculté des Sciences et Techniques Tanger, Morocco

[3]Unité de Recherche et Développement en Aquaculture, INRH, Casablanca, Morocco

Correspondence
Said Sedki, Centre Spécialisé en Aquaculture, INRH, M'diq, Morocco.
Email: saidsedki@gmail.com

Summary

In hatcheries, meagre *Argyrosomus regius* larvae still depend on an adequate supply of rotifers and *Artemia*, as no artificial diet can totally fulfil their nutritional requirements. However, production of live feed is highly expensive and demands intensive labour and specific facilities. This study investigated the effect of a dietary regime without the use of rotifers, to simplify the meagre larval rearing protocol. Two feeding treatments (T_1 & T_2) are compared to investigate their effects on survival and growth of meagre larvae. In T_1, larvae were fed rotifers from 2 to 5 days post hatch (dph), and *Artemia* from 4 to 15 dph. In T_2, larvae were kept under dark conditions and fed *Artemia* from 6 to 15 dph. Standard larval length (SL) was significantly higher in T_1 ($p < .01$) until 8 dph in comparison with larvae reared initially without rotifers. No significant difference in SL was found among treatments ($p = .187$) at 15 dph. Significant difference was found among treatments in survival rate at 15 dph ($p < .003$). The survival rate observed at 15 dph in T_2 (30 ± 4.2%) represents an important finding, although the highest survival rate was observed in T_1 (45.0 ± 3.4%). This study showed that it is possible to conduct larval rearing of meagre without using rotifers. Nevertheless, further research efforts are still needed to improve these results in comparison with the common larval rearing protocol.

KEYWORDS
feeding protocol, larval diet, length growth, live prey, survival, treatment

1 | INTRODUCTION

Meagre (*Argyrosomus regius* Asso, 1801) has been proposed as one of the most promising candidates for marine finfish diversification on commercial aquaculture in Mediterranean and Eastern Atlantic coasts (Mateos, 2007). It is highly fecund (Sadovy & Cheung, 2003) and fast growing (Gil, Grau, Basilone, Ferreri & Palmer, 2013) and presents good food conversion ratios (Jiménez, Pastor, Grau, Alconchel & Cárdenas, 2005). There has also been increasing interest in studying meagre for restocking the depleted natural population (Gil, Palmer, Grau & Pérez-Mayol, 2014).

Recent studies about *A. regius* have been focused on pond culture (El-Shebly, El-Kady, Hussin & Hossain, 2007), food characteristics (Piccolo et al., 2008) and dietary lipid/protein ratio (Chatzifotis, Panagiotudou & Divanach, 2012) in juvenile and/or adult fish. Recently, the ontogeny of the digestive system in meagre from hatching to metamorphosis using a mesocosm rearing system has been described by Papadakis, Kentouri, Divanach and Mylonas (2013). Suzer et al. (2013) determined the ontogenetic development and pattern of main digestive enzyme (pancreatic and intestinal) activities of *A. regius* larvae fed on live prey and compound microdiet.

Meagre is already being produced at a small scale in several Mediterranean countries. The low survival, during and after larval weaning, has been the main bottleneck for meagre commercial production (Vallés & Estévez, 2013). The success of meagre culture is contingent upon development of hatchery techniques that improve

growth and survival, particularly during the critical switch from endogenous to exogenous feeding. This step is crucial in larval rearing, as after the exhaustion of yolk reserves, the delay or deprivation of food has been associated with massive mortality in marine fish culture (Gisbert, Conklin & Piedrahita, 2004; Yufera & Darias, 2007). To obtain a predictable year-round production of high-quality fry, hatchery techniques must be improved to optimize culture performance and larval quality. Recent studies have been focused on identifying the best conditions for larval rearing of meagre such as live prey feeding sequences (Roo, Hernández-Cruz, Borrero, Schuchardt & Fernández-Palacios, 2010) and light conditions (Vallés & Estévez, 2013), to standardize the culture protocols. Other authors are applying feeding protocols adapting gilthead sea-bream culture techniques (Estévez, Treviño & Gisbert, 2007; Pastor & Cárdenas, 2007; Roo, Hernández -Cruz, Borrero, Fernández-Palacios & Schuchardt, 2007). Generally, rotifers (*Brachionus sp*.) are used at 5–10 rotifers/ml, either enriched with commercial products or with microalgae and fed twice a day as first feeding from 2 to 15–20 dph. Thereafter, brine shrimp (*Artemia*) are introduced from 8 to 30 dph to rear meagre larvae (Fernández- Palacios, Schuchardt, Roo, Hernández-Cruz & Duncan, 2009; Pousão-Ferreira et al., 2013; Roo et al., 2007, 2010).

The intensive production of microalgae and rotifers is expensive and often unreliable (Nash & Novotny, 1995). The designs used for experimental and pilot-scale units are usually inappropriate for a larger system because of logistical problems and prohibitive cost of materials, which affect scale-up performance (Boeing, 2000). Routine maintenance and cleaning of culture units become a major problem with increased scale. During the past three decades, enormous efforts have been made to develop microdiets to replace live feed, both rotifers and *Artemia*, as complete or partial diets for marine fish (Kolkovski, Curnow & King, 2004; Koven, Kolkovski, Hadas, Gamsiz & Tandler, 2001). Many studies investigated the suitability of different diets for larviculture of meagre (Gültepe et al., 2016; Saavedra et al., 2016). Although there were substantial achievements in reducing the reliance on live feeds, microdiets cannot completely replace them. Roo et al. (2007, 2010) have shown that early introduction of *Artemia* was reflected in a lower survival rate and higher larval length of meagre larvae. Other feeding experiments showed very good results of growth and survival using only rotifers (days 2 to 20) and artificial diets (day 18 onwards) (Durán et al., 2009). However, no feeding test suggested *Artemia* as first feeding for meagre larvae.

In Morocco, the production of meagre fry started in 2009 in the single public hatchery of the Aquaculture Research Centre in M'diq (National Institute of Fisheries Research [INRH]), using similar rearing techniques to sea-bream (*Sparus aurata*) and sea-bass (*Dicentrarchus labrax*). Rotifers and *Artemia* are usually used successively during the first weeks, followed by co-feeding live prey (*Artemia*) and microdiets until the larvae are completely weaned. This dependence on live prey increases the production costs during early larval rearing. In this study, we tested a larval rearing protocol using directly *Artemia* as a first feeding. The main objective was to reduce the reliance on live prey and to determine whether omission of rotifers in larval diets is possible while maintaining good growth and survival.

2 | MATERIALS AND METHODS

2.1 | Broodstock maintenance

The rearing of breeders was undertaken in the hatchery facilities of INRH's Aquaculture Research Centre (M'diq, Morocco). They were kept in 10 m^3 fibreglass reinforced plastic (FRP) tanks and were maintained under natural conditions in terms of water temperature and photoperiod. FRP tanks were supplied with flow-through seawater at salinity of 36.0 ± 1.0 g/L. Broodstock were fed until apparent satiation with fresh or frozen fish, squid and shrimp 5 days/week.

2.2 | Broodstock selection

To date, meagre broodstock held in hatchery facilities of INRH's Aquaculture Research Centre has not exhibit natural spawning. Eggs were obtained using hormone treatment, which is a current practice in reproduction of captive meagre (Duncan et al., 2012; Mylonas et al., 2013a) and other fish. In the present experiment, the spawning was induced using one hormonal injection of LH-RHa (L4513, Sigma Aldrich, USA). Females and males were injected with 15 and 10 μg/kg of LH-RHa, respectively. Females and males (F_3 generation cultured specimens) were first examined for their maturity status on the 31st of July 2015. Then, spermiating males (releasing substantial amounts of sperm after a gentle abdominal pressure) and females showing oocytes (obtained through ovary biopsies technique), with a diameter ≥ 500 μm and very little atresia (Mylonas, Mitrizakis, Papadaki & Sigelaki, 2013b; Pastor et al., 2013) were selected for hormonally induced spawning. The selected breeders were measured for weight and length, then transferred individually to an anaesthetic bath (0.07 g/L of MS-222) for complete sedation 5–10 min. Females (n = 9) had a mean standard length and body weight of 84.2 ± 3.1 cm and 6,635.5 ± 857.2 g, respectively. Males (n = 15) had a mean standard length and body weight of 78.9 ± 3.7 cm and 5,244.0 ± 605.1 g, respectively. After treatment, fishes were kept in 10 m^3 tank and were allowed to spawn. The temperature range during the spawning period was 21 ± 0.5°C. Salinity was 36.0 ± 1.0 g/L, while light conditions were 700 lux in intensity and 12- hr L:12- hr D in photoperiod.

2.3 | Embryonic and larval rearing

An egg collector was placed on the outflow of the spawning tank, to collect the spawned eggs. The hormonal induction resulted in two successive spawns that occurred 36 and 60 hr after injection. Eggs were collected 12 hr after each spawn to ensure that the entire batch was collected. The fecundity was estimated by counting the total number of eggs in three subsamples of 10 ml. Fertilization rate was evaluated at the same time by examining each of the eggs within replicates. Only eggs with regular cleavage (usually at the

blastula stage) were considered fertilized (Dahlke, Politis, Butts, Trippel & Peck, 2016). High batch fecundity was obtained in the first spawn with 32,853 eggs/kg with and 54% fertilization rate. The fertilized eggs were buoyant and had a mean diameter of 986 ± 44 μm. Their mean lipid globule's diameter was 258 ± 14 μm. Fertilization rate increased to 72% in the second spawn with a relative fecundity of 18,821 eggs/kg. The fertilized eggs had a mean diameter of 952 ± 22 μm.

Fertilized eggs were incubated at ambient temperature (21 ± 0.5°C) in 200–L conical tanks supplied with flow-through seawater. The tanks were aerated (supplied with air) to ensure homogenous distribution. Once hatching was completed (48 hr post-fertilization), larvae were counted volumetrically to determine the hatching rate, expressed as the total number of larvae divided by the total number of fertilized eggs. The hatching day was taken as 0 dph of the experiment. The mean hatching rate was estimated to be 46 ± 12%.

2.4 | Experiment

Two treatments with two replicates each were used in this study. Larvae were counted volumetrically and transferred to the four experimental tanks (200 L). The initial density was kept at 50 larvae/L. These tanks were kept at ambient temperature (21 ± 0.5°C) with a flow-through system and gradually increasing seawater renewal rates. Indeed, daily water exchange varied between 20% (1–4 dph) and 50% at the end of experiment (15 dph). The tanks were aerated to ensure homogenous distribution of prey.

In treatment 1 (T_1), the followed larval rearing protocol was quite similar to the one used for sea-bream. Therefore, in T_1, meagre larvae were first fed enriched *Brachionus sp.* (Sparkle, INVE, Belgium) from 2 to 5 dph, twice a day to keep a density of 5 rotifers/ml using the green water technique. In fact, live cultured phytoplankton (*Nannochloropsis sp.*) was added to maintain a concentration of 120.000 cells/ml. Then, nauplii of *Artemia sp* were introduced from 4 to 15 dph (*Artemia* was given as first feed when larval mean length was over 3 mm). This treatment was conducted under 12–hr L:12–hr D artificial photoperiod and a light intensity of 300 lux just above the water surface.

In treatment 2 (T_2), the followed larval rearing protocol was quite similar to the one used for sea-bass. Therefore, in T_2, meagre larvae were reared in clear water from 1 to 5 dph, under a dark environment (by covering tanks with a black polyethylene sheet) and without using rotifers as first feed. The hypothetical purpose of keeping larvae in dark conditions was to reduce their motility, decrease their energy waste and to allow a more efficient of their endogenous nutritive reserve (yolk sac) for growth. The dark environment method is commonly used for sea-bass larval rearing in many commercial hatcheries, frequently referred to as the "French technique" (Büke, 2002; Moretti, Fernandez-Criado, Cittolin & Guidastri, 1999). It successfully allows skipping the rotifers phase. Larvae were fed *Artemia sp* after the opening of their mouth when their digestive system became functional and their yolk sac was highly depleted. They were fed from 6–15 dph under the same lighting conditions (12 hr L:12 hr D, 300 lux) as in T_1.

2.5 | Data collection

Larvae were sampled (N = 15) daily from to 1 to 10 dph and at 15 dph. They were first anaesthetized with MS–222 and then measured for standard length (SL) using a Nikon profile projector (V–12B). Survival rates were assessed on a daily basis from 2 to 5 dph. Sampling was performed using a rigid PVC pipe fitted with a one-way valve to collect three integrated water column samples in each tank. The surviving larvae were counted daily, and the survival rate (%) of each day was calculated by dividing the number of surviving larvae by the initial larval number on 1 dph. Final survival was determined at 15 dph. Larvae were inspected under a microscope and considered dead if there was no visible heartbeat via ×20 magnification.

2.6 | Statistical analyses

Data were analysed using XLSTAT statistical analysis software (v.5.1). Differences in standard length and survival among the treatments (T_1 & T_2) were performed using one-way ANOVA. A value of $p < .05$ was considered to indicate statistical significance. Data were transformed to meet the assumptions of normality and homoscedasticity. Total length was *log10* transformed, while survival was *arcsin* transformed, prior to performing the parametric test.

3 | RESULTS

3.1 | Experiment

The length of the newly hatched larvae was 2.46 ± 0.1 mm (Figure 1). Larvae from T_1 showed a rapid increase in length within the first 48 hr (+38%), whereas larvae from T_2 increased slightly in SL (+17%) under darkness (0–hr L:24–hr D). At 2 dph, SL was 3.40 ± 0.12 and 2.88 ± 0.17 mm in T_1 and T_2, respectively. No significant growth in length was recorded from 2 to 5 dph within treatments (p = .39 and p = .12 in T_1 and T_2, respectively) despite the addition of rotifers in T_1. At 2 dph, the mouth of the larvae started to open in T_1, although it still lacked movement. It became functional on 3 dph and rotifers were observed in the gut of numerous individuals from T_1.

The swim bladders of larvae began to be inflated on 7 dph, and by 10 dph, 100% of larvae had a functional swim bladder. When larvae in both treatments began to feed *Artemia sp*, SL nearly doubled during the 10–day period (from 6 to 15 dph). The statistical analysis showed that SL was significantly different among treatments only until 8 dph. As larvae in T_2 have grown considerably from 9 to 15 dph, no significant difference was found at the end of the experiment (p = .187). Figure 1.

Larval survival is shown in Figure 2. At 2 dph, no significant difference in survival was recorded among treatments (p = .200). From 3 to 15 dph, T_2 presented significantly lower survival than

T_1 ($p < .02$). Despite that the curves showed same patterns, the survival fell sharply from 1 to 5 dph in T_2, in comparison with T_1. From 5 to 15 dph, 78% of larvae in both treatments survived when they were fed *Artemia sp*. By the end of the trial, T_1 showed significantly higher survival than T_2 ($p < .003$); 45.0 ± 3.4% was recorded in T_1, whereas 30 ± 4.2% was recorded in T_2. Figure 2.

4 | DISCUSSION

The mature females released a total of 51,674 eggs/kg in the two spawning events. This fecundity is low compared to those obtained in the Spanish hatcheries (Duncan et al., 2012; Pastor et al., 2013). This was probably due to the late spawning induction achieved in August 2015. Pastor et al. (2013) have also observed low fecundity in females induced late in relation to the natural spawning season of meagre. The dose of injection and oocyte size are the two most important aspects of a successful hormone therapy (Ibarra-Castro & Duncan, 2007; Mañanós, Duncan & Mylonas, 2008). The females used in this study contained adequate oocytes with a diameter greater than 500 μm, in accordance with Mylonas et al. (2013b). The hormone doses applied in our spawning induction (10–15 μg/kg) were very close to those used by Fernández-Palacios, Hernández-Cruz, Schuchardt, Izquierdo and Roo (2009), Fernández-Palacios et al., 2014). They obtained best spawning quality with lower doses of 10–20 μg/kg. Pastor et al. (2013) have observed that hormonal injections performed with low doses (only 5–10 μg/kg) gave eggs with highest quality, while females induced late in relation to the natural spawning season of meagre, with high doses (25–50 μg/kg), had low fecundity.

The relative fecundity values could also vary depending on the number of repetitive injections. Pastor et al. (2013) obtained, in LIMIA (Balearic Islands) and IFAPA (Andalusia), values ranging between 20,691.9 eggs/kg of a female with only a single injection and 232,849.23 eggs/kg obtained from a single female injected three times in 15- day period. Moreover, Duncan et al. (2012) reported values ranging between 198,200 eggs/kg obtained on five consecutive spawns of two hormonal-induced females and 276,200 eggs/kg obtained on 14 spawns of two females in response to treatment with GnRHa implants.

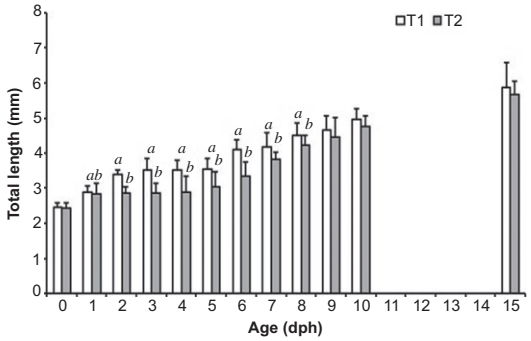

FIGURE 1 Standard length (SL) of larvae reared using two feeding treatments (in T_1: rotifers+*Artemia* and T_2: only *Artemia*) in the period 1 to 15 dph. Results are given as mean ± *SD* ($n = 15$). Data were analysed using a one-way ANOVA after log_{10} transformation. Letters indicate significant differences ($p < .05$) among the treatments

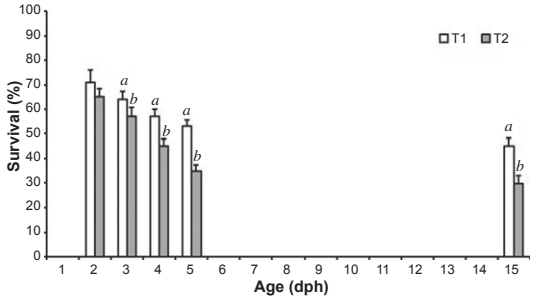

FIGURE 2 Survival of larvae reared using two feeding treatments (in T_1: rotifers+*Artemia* and T_2: only *Artemia*) in the period 2 to 15 dph. Results are given as mean ± *SD* ($n = 15$). Data were analysed using a one-way ANOVA after *arcsin* transformation. Letters indicate differences ($p < .05$) among the treatments

4.1 | Experiment

Regarding feeding protocols, survival and growth showed an opposite trend, with negative correlations. The difference in length among treatments at 2 dph was most probably due to the lighting conditions, whereas survival was not affected. As no rotifers were added in T_2, larvae were reared under complete darkness until 5 dph to limit larval mobility and food-searching activity. Rearing in darkness seems to have reduced the growth of meagre larvae in T_2. Larvae of some other species develop better in darkness like the Atlantic halibut (Bolla & Holmefjord, 1988) and gilthead sea-bream (Sahin, Kürat & Cüneyt, 2001). In T_1, larvae reared at 300 lux using 12–hr L:12–hr D photoperiod grew normally. Similar growth was obtained by Pastor et al. (2013) using a medium light intensity (500 lux). Vallés and Estévez (2013) obtained highest growth of meagre larvae with 16- hr and 24- hr light using a mid-light intensity (500 lux), although causing lowest survival rates. Moreover, Downing and Litvak (1999) demonstrated the impact of light intensity on growth of haddock (*Melanogrammus aeglefinus*) larvae. High light intensities may also modify prey and larval behaviour (Downing & Litvak, 2001). Furthermore, European eel (*Anguilla anguilla*) larvae reared in low light intensity had higher survival than those reared in high light intensity (Politis, Butts & Tomkiewicz, 2014). Thus, the effect of light intensity should be studied in detail to be incorporated into models of larval growth and to assist in developing husbandry protocols to maximize larval survival.

Larval length was potentially also affected by the time of first exogenous feeding. From 3 to 8 dph, the differences in SL among treatments were probably a consequence of a delay in starting feeding in T_2. Different species of fish differ in their ability to withstand delay in first feeding of food deprivation (Dou, Masuda, Tanaka & Tsukamoto, 2004; Yufera & Darias, 2007). The low survival recorded

in T_2 from 3 to 5 dph could be related to the absence of rotifers in the larval diet. Furthermore, a large part of the starved larvae suffered irreversible damage; the survival was lower than in the standard protocol using rotifers. Gisbert et al. (2004) have found that food deprivation of halibut larvae resulted in a progressive deterioration of the larval digestive system and atrophy of skeletal muscle. The high mortalities reported after 5 days of starvation suggest that the meagre larvae were likely close to the point of irreversible starvation. Klimogianni, Pagoulatou, Trageli and Hotos (2013) also demonstrated that meagre larvae are close to the point of no return (PNR) at 5 dph. The lipid droplet still carried generally until 5 dph by meagre larvae could be insufficient to satisfy the nutrient requirements of the starved larvae. The lower survival observed in T_2 could also be due to rearing in clear water as recent studies have demonstrated enhanced larval survival due to microalgae and their potential antibacterial effects, both under light and dark conditions (Kokou, Makridis, Kentouri & Divanach, 2012; Salvensen, Skjermo & Vadstein, 1999). In fact, Salvensen et al. (1999) reported that the use of microbially matured water, to which microalgae was added, could lower opportunistic bacteria proliferation and allow viable and fast-growing turbot larvae. Also, Kokou et al. (2012) reported that microalgae cultures inhibited the growth of bacteria and their antibacterial activity was not influenced by the presence or absence of light.

The early introduction of *Artemia* at 4 dph in T_1 was reflected in higher larval length. Big larvae seemed to quickly adapt to *Artemia* feeding, whereas small and weaker larvae died in the absence of rotifers. Roo et al. (2010) reported early *Artemia* introduction at 8 dph had a positive correlation with meagre larval growth but negative correlation with their survival. They also reported high mortalities of meagre larvae due to their swim bladder hyperinflation in early *Artemia* introduction at 8 dph. A positive relationship has been demonstrated between age-at-transfer from rotifers to *Artemia* (from 5 to 25 dph) and rearing performance of intensively cultured Atlantic cod (*Gadus morhua*) (Shields, Irwin, Smith & McEvoy, 2003). Furthermore, cod larvae transferred to *Artemia* at 5 dph suffered a high incidence of swim bladder over-inflation and high mortality during metamorphosis. Based on their findings, they recommended that intensively reared Atlantic cod larvae should continue to receive rotifers until completion of metamorphosis. In the present experiment, final length in both treatments was almost similar after the *Artemia* feeding phase. The lack of rotifers in T_2 was offset by providing *Artemia* at 6 dph. The compensatory growth reduced the difference in size with T_1 larvae. This growth is defined as a phase of accelerated growth when favourable conditions are restored after a period of growth depression (Ali, Nicieza & Wootton, 2003).

Regarding survival, about 22% of larvae died from 5 to 15 dph in both treatments. The early use of *Artemia* (at 4 dph in T_1 and at 6 dph in T_2) was reflected in a higher survival rate, contrary to the results found by Roo et al. (2007, 2010) who have shown that earlier introduction of *Artemia* (at 8 dph) was reflected in a lower survival rate. In this study, using rotifers and *Artemia* together (T_1) enabled a higher survival rate (45.0 $\pm$ 3.4%) at 15 dph. The early use of *Artemia* in T_1 gave best results, which reflect the high acceptability of *Artemia* by meagre larvae since 4 dph. This treatment was more suitable in terms of survival, but using only *Artemia* might be more cost-effective for larval rearing of meagre. In fact, even without using rotifers, T_2 showed a relatively interesting and promising survival rate that could be improved by more efforts in further studies and research.

5 | CONCLUSION

This study underlines the possibility to rear meagre larvae without the use of rotifers until 5 dph and with the use of *Artemia* as first exogenous feed since 6 dph. The rotifer absence in larval diets could simplify the rearing protocol of meagre and reduce the fry production cost. However, survival at 15 dph was lower (30 $\pm$ 4.2% at 15 dph) compared to the standard protocol using rotifers and *Artemia* together (45.0 $\pm$ 3.4%). The larvae have suffered heavy losses probably to the starvation period of 5 days. Thus, it is needed to understand the consumption pattern of endogenous energy reserves of meagre larvae to optimize the rearing protocol, while not using rotifers. As the fast growth of meagre larvae allows the earlier introduction of *Artemia*, the other method using a short rotifer period (2 – 5 dph) and early *Artemia* introduction (4–15 dph) gave important results in terms of growth and survival. Thus, it seems promising to conduct subsequent studies for a successful implementation at industrial scale and to therefore help solving the lack of meagre fry.

In conclusion, this experiment showed it is possible to conduct larval rearing of meagre without using rotifers although more studies are encouraged to improve the larval rearing protocol and to determine the suitable time of larval first feeding.

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