Deliverable Report

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Task Title:	Effectiveness of stocking density and anti-oncomiracidia attaching substances in the control of monogenean parasites.			
Other beneficiaries:	P15. ULL	P5. UNIABDN		
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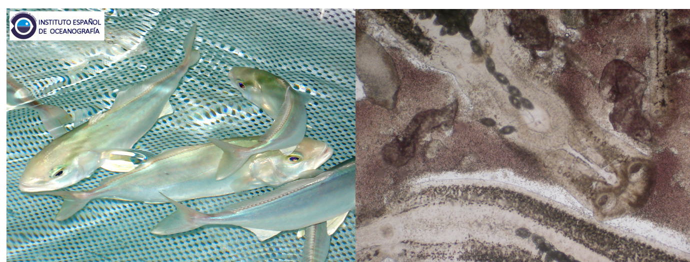
Objective: Rearing protocol against monogenean parasites: The implementation of measures, including biosecurity practices, inhibition of oncomiracidia attaching capacity, as well as reinforcement of the fish immune system against stressful rearing conditions was developed to control the incidence of infected fish. A treatment protocol against monogenean parasites is proposed based in novel results attained through the control of some environmental rearing factors, as well as by the assay of anti-oncomiracidia attaching substances and other measures targeting the reinforcement of the fish immune system. The results are presented to also include information of (a) intensity and sites of preference for infection, (b) survival, (c) fish growth, (d) haematological, immunological and biochemical indicators of health and welfare. In addition, results of the evaluation of some indicators of the functional integrity of osmoregulatory epithelia (gills and gut) are presented.

Description: Different measures and methods against the monogenean parasites (*Neobenedenia seriolae* and *Zeuxapta seriolae*) of juveniles of greater amberjack were tested, including biosecurity practices, inhibition of oncomiracidia attaching capacity, as well as reinforcement of the fish immune system. The previous information obtained during the development of grow-out trials allowed the reconsideration of the original plan regarding the stocking density assays, while the natural presence of the parasites also impacted the development of initially planned experiments.



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1. Introduction

One of the most important bottlenecks in the commercial production of greater amberjack (*Seriola dumerili*) is the high incidence of pathologies in the culture conditions (De la Gándara, 2006). In fact, this is a common scenario for the different reared *Seriola* species (*S. quinqueradiata, S. lalandi, S. rivoliana*, and *S. dumerili*), which show pathological problems related to both bacteria and parasites. In the Mediterranean, the first pathological problems cited relate to parasites such as the protozoon *Cryptocaryon irritans,* and digenean *Paradeontacylix sp.*, and more recently with the monogenean *Benedenia seriolae* and *Zeuxapta seriolae* (De la Gándara, 2006; Montero, 2001; Montero *et al*., 2004). These parasites are the first cause of massive mortalities in wild and reared *S. dumerili* (Alcaide *et al*., 2000; Lia *et al.,* 2007; Montero, 2001) having the most severe negative effects in warm periods and in younger individuals (Repullés-Albelda *et al*., 2013). The impact of these parasites on *S. dumerili* has also been seen in the Canary Islands, although the presence and effects of *Z. seriolae* is less frequent, with the highest mortalities related to skin flukes caused by the monogeneans *Neobenedenia girellae and Neobenedenia melleni*, species with a similar morphological aspect but genetically different (Cejas *et al*., 2015).

The monogeneans of the genus *Neobenedenia* are obligate parasites with direct life cycles and short generation times (Hirazawa *et al*., 2010). Unlike other species of monogenean that show a greater host specificity, *N. melleni* has been cited as a virulent parasite that causes serious problems in the culture of many and different species of teleost fish from around the world (Bondad-Reantaso *et al*., 1995; Deveney *et al*., 2001; Ogawa *et al*., 2006; Rückert *et al*., 2008; Whittington and Horton, 1996; Whittington, 2012). Juveniles and adult parasites of *Neobenedenia* sp. attach to the skin, fins and eyes of the fish using the sucker-like haptor. They advance along the epithelial surface of fish, scraping the epidermis of the host and causing epithelial hemorrhages, inflammation and hyper production of mucus (Ogawa *et al*., 1995, 2006), increasing the risk of secondary bacterial infections (Buchman and Lindenstrom, 2002). Adult parasites release eggs into the water column, which have a tetrahedral capsule with a long filament that allows them to adhere to any surface including the surface of the fish. The hatching of eggs leads to ciliate larvae (oncomiracidia) that can re-infect quickly (Brazenor and Hutson, 2015; Whittington, 2012). The duration of the egg development depends on the temperature, with hatching occurring at 4, 5-6, 7 and 8 days at temperatures between 27-30, 25, 20 and 18ºC, respectively (Hutson *et al*., 2012). The duration of the life cycle of this species lasts between 12 and 16 days in tilapia (*Oreochromis mossambicus*) (Kishimori *et al*., 2015), being similar to the cycle duration (15-17 days at 25°C) reported by Bondad Reantaso *et al*. (1995).

Severely parasitized fish can stop feeding, show a dark body, swim erratically and rub against the wall of tanks or nets causing skin ulceration that favors the action of bacteria, fungi and viruses (Brazenor and Hutson, 2015; Thoney and Hargis, 1991). The direct life cycle, short generation times and filamentous eggs, which become entangled in any surface, cause difficulties in the management of the infections and facilitate the presence of a large number of parasites in a culture system in a short period of time (Ogawa *et al*., 1995, 2006).

The most used treatment against the monogenean is bathing of fish in solutions of hydrogen peroxide, formalin or freshwater (Ogawa *et al*., 2006), with the use of pharmacological substances such as praziquantel (Hirazawa *et al*., 2013; Sharp *et al*., 2004) nowadays forbidden for commercial purposes. More specifically, to treat *Neobenedenia* sp., the most common methods used are formalin or freshwater baths (Fajer-Ávila *et al*., 2007; Thoney and Hargis, 1991). However, these treatment methods affect the adult parasite only and have no effects on the eggs and embryo stages, so their efficacy is restricted (Militz *et al*., 2013; Sharp *et al*., 2004; Whittington, 2012) and the fish are susceptible to reinfection (Diggles *et al*., 1993; Yoshinaga *et al*., 2000). In addition, the application of these bath treatments increases stress (Brazenor and Hutson, 2015; Shirakashi *et al*., 2013) and requires a lot of time and work, increasing the cost of maintenance in the farming facilities (Ernst and Whittington, 1996).

Treatment with praziquantel has shown promising results but its efficacy has been variable between species (Brazenor and Hutson, 2015; Hirazawa *et al*., 2013) and its inclusion in food causes palatability problems (Hardy-Smith *et al*., 2012). In addition, the administration of drugs, in general can cause residues in tissues and can affect the environment (Lee and Gao, 2012), so their use in organisms intended for human consumption must be legislated (Sharp *et al*., 2004). For instance praziquantel has not been approved by the **U.S.** Food and Drug Administration for use on fish but can be used legally in some situations (Bader, 2017; Iles *et al*., 2012).

In the last few years, research has been mainly focused in two ways: study of the behavior of the parasites under different environmental factors, and the search of alternative methods for the prevention and/or treatment of pathologies, that are more effective/ less stressful for fish, and more safety and environmentally friendly. Knowledge of the effects of environmental variables such as light, temperature and salinity, on the parasites biological cycle could provide relevant information for a better management of the parasite infections (Hirazawa *et al*., 2009). Changes in temperature and salinity influence the success of the parasite's reproductive strategy, affecting maturation, reproduction, hatching and re-infection (Hoai and Hutson, 2014). In recent years, the search for natural products has also been promoted as an alternative for the treatment of different pathologies, evaluating the potential of plants as an alternative therapy against parasites that affect aquaculture (Bulfon *et al*., 2015; Lee y Gao, 2012; Trasviña-Moreno *et al*., 2017). In fact, allicin, a phytochemical agent of garlic, *Allium sativum* L., has been demonstrated to exhibit antibacterial activity against several pathogenic bacteria in fish (Lee and Gao, 2012) and has been tested against all life stages of the parasite *Neobenedenia* sp. (Militz *et al*., 2013). Another way to fight against parasites is to study the mechanisms responsible for the specificity of the monogeneans that parasitize teleosts (Buchmann and Lindenstrøm, 2002). Monogeneans are generally highly specific for the host, suggesting the existence of chemical, mechanical and behavioral mechanisms responsible for this specificity (Buchmann and Lindenstrøm, 2002; Ohashi *et al*., 2007).

There is evidence to suggest that host factors, including mucus, are important players in the system. In this sense, prebiotics, including mannan oligosaccharide (MOS) by-products, are commonly used in the animal production industry due to their effects on the immune system leading to pathogen protection (Guerreiro *et al*., 2017; Torrecillas *et al*., 2014). Several actions performed within DIVERSIFY´s WP25, led by partners P5. UNIABDN and P2. FCPCT aimed at studying the “Impact of dietary regime on parasite resistance and mucosal defenses of greater amberjack juveniles” (D25.3) and the “Impact of oral administration of greater amberjack with mucus stimulation products on immune resistance to parasitic infections and development of molecular markers for its evaluation” (D25.5). Their recent results concluded that the utilization of dietary concentrated mannan—oligosaccharides (c-MOS) at 2 g kg^{-1} increased protection against *N. girellae* after 90 days of feeding, by reducing the parasite level and parasite total length. This protection was associated with up-regulation of several proinflammatory cytokines, AMPs, MUC-2 and IgT genes in skin and enhanced

serum bactericidal activity. The union of the oncomiracidia of *N. girellae* to its host is initiated by several carbohydrates (Yoshinaga *et al*., 2000). The most known molecules responsible for attachment are the lectins, a diverse group of proteins that have selective affinity for a carbohydrate or a group of carbohydrates (Vázquez-Mendoza *et al*., 2013), that are found in fish serum and mucus (Nakamura *et al*., 2000; Newton, 2000; Zhang *et al*., 2000), with different carbohydrates present in monogenean parasites. Mannan and mannose binding lectins (MBL) are included in this group. On the other hand, the microbial communities of the intestine are also being studied, because they participate in a wide range of key biological processes, including the absorption of nutrients (Semova *et al.,* 2012), development (Sommer and Bäckhed, 2013), metabolism (Nicholson *et al.,* 2012), immune modulation (Geva-Zatorsky *et al.,* 2017), and defense against pathogens and diseases (Schuijt *et al.,* 2016).

In order to reinforce this microbial community to combat diseases, there is an alternative technique called faecal microbiota transplantation (FMT), which involves the introduction of a faecal suspension derived from a healthy donor into the gastrointestinal tract of a diseased individual. This technique is being investigated to combat the global epidemic disease caused by the bacterium *Clostridium difficile* in humans. It is also being tested in pigs (Pang *et al.,* 2007), and in fish, mainly in zebrafish (*Denio rerio*) (Pham *et al.,* 2008) and more recently in the killifish *Nothobranchius furzeri* (Smith *et al.,* 2017).

All these approaches have been considered during the last 4 years by partner P8.IEO in collaboration with P15.ULL to provide a useful rearing protocol against monogenean parasites. The present deliverable includes studies designed to evaluate the effects of two environmental parameters such as temperature and photoperiod, on behavior and success of the reproductive strategy of *N. melleni* in greater amberjack to develop alternative control methods against its infection in farming facilities. In addition, some methods based on the mechanisms responsible for the specificity of the binding between the parasite and the host fish, and others, focused on testing methods for reinforcement of the immune system of *S. dumerili*, through fecal microbiota transplantation (FMT), were also tested. The influence of some of these treatments were evaluated on growth, survival, plasma metabolites, humoral immune parameters, hepatic oxidative status and on gut and gill osmoregulatory epithelium integrity, to give an overall picture of the main physiological processes affected by the parasite infection.

Some preliminary experiments, summarized in previous reports, were performed according to the initial working plan established in the DOW and useful information was attained concerning the efficacy of baths with anti-oncomiracidia substances and the stocking density of greater amberjack juveniles. In this document, we present all the findings from these preliminary trials performed during the course of the project and that allowed us to obtain relevant information that was useful for further planning of trials as summarized below:

(i) Design and testing of an egg collector device as a method to detect and quantify the level of infestation by skin and gill monogenean parasites (*Z. seriolae, B. seriolae, N. melleni*) in the fish rearing tank without the need to manipulate the fish.

(ii) Genetic identification of *Z. seriolae* and *N. melleni*, and preliminary information of interest about the biology and behavior of these parasites related to temperature to allow a better environmental management of the infestation, that shows the duration of egg development until hatching at 20°C is 4-6 days for *Z. seriolae* and 7-9 days for *N. melleni*.

(iii) Weekly monitoring of the infestation level by monogeneans in grow out trials of 180 juveniles of *S. dumerili* (262.1 ± 55.5 g) brought from a Mediterranean farm (FUTUNA BLUE ESPAÑA S.L.), distributed in 12 indoor tanks (mean initial density of 3.8 kg m^{-3} per tank) over 120 days and fed at 1, 2, 3 and 7 times day^{-1} (**Sub-task 21.2.3 Definition of feeding pattern)** which showed that the fish in 2015 were infested by *Z. seriolae*, whereas eggs from other monogeneans, such as *N. melleni*, were not recorded. The first fish mortality was recorded after four weeks, and the total mortality registered during this grow out period was 27%, with the highest number of dead fish and the peak in egg number coinciding (**Fig. 1a**).

(iv) In a grow out trial performed in 2015-2016, with 5 g juveniles of *S. dumerili*, born at IEO facilities in Tenerife, initially stocked at three different densities (**Sub-task 21.3.2 Definition of optimal stocking**

density) in triplicate (0.17±0.02, 0.28±0.01 and 0.46±0.07 kg m^{-3}), the weakly monitoring during 120 days showed the presence of *Z. seriolae* and *N. melleni* eggs, but their presence did not coincide in time (**Fig. 1 b**). No differences in the number of collected eggs of *N. melleni* and *Z. seriolae* were encountered between densities, during this grow out period.

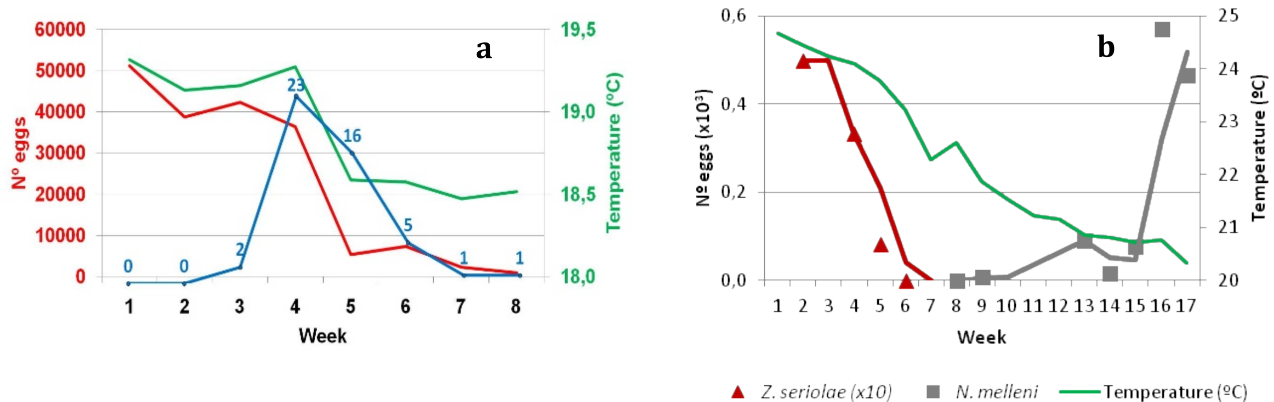

Figure 1. Weekly means of *Z. seriolae* collected eggs (red), number of dead fish (blue), and temperature (green) during grow out trials of 180 g juveniles of greater amberjack fed 1, 2, 3 and 7 meals day^{-1} (**a**), and of trials with 5 g juveniles of greater amberjack stocked at three different densities (**b**).

In a third grow out trial (**Sub-task 21.3.2 Definition of optimal stocking density)** carried out in 2016-2017 with 480 *S. dumerili* juveniles (175.7 ± 56.4 g), born at IEO facilities in Tenerife, initially stocked at four different densities, 1.3 (T1), 1.7 (T2), 2.4 (T3) and 3.2 (T4) kg m^{-3}), the weakly monitoring up to 120 days only showed the presence of *N. melleni* eggs, and no mortality was registered during the experimental period.

The weekly average number of *N. melleni* eggs tended to decrease (P=0.08) with the increase of the rearing density (**Fig. 2a**). However, the average number of eggs related to the number of fish stocked in the tanks significantly decreased (P<0.05) with the increase in culture density (**Fig. 2b**).

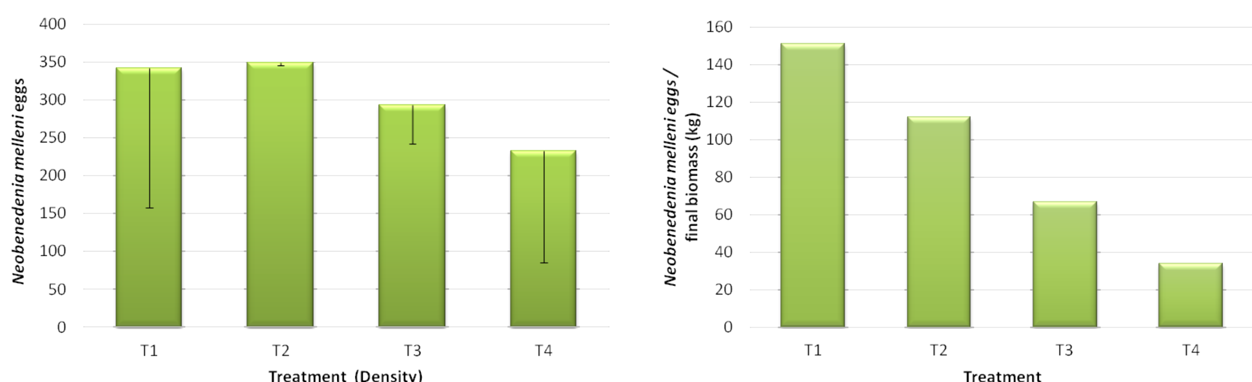

Figure 2. Average number of eggs of *N. melleni* collected at each rearing density **(a)** and with respect to the final biomass of fish **(b)** during a 120-day trial.

(v) Regarding the effects of anti-attachment substances (sucrose, glucose and mannose), a preliminary assay was performed to test the effects on the attaching capacity of *Z. seriolae* to the gills. A total of 4-6 samples of adult parasites from the external arch and 2-4 from second and third gill arches were obtained.

The parasites were filtered using a nylon mesh and several gill-attached parasites were observed for 5-6 h after they were incubated with different media (glucose-seawater, sucrose-seawater and mannose-seawater).

The parasites were very active under glucose and sucrose seawater medium during this period. However, the parasites incubated in mannose-seawater medium (0.25 M) showed inactivity and were released from gill tissue after 2-5 min of exposure. Moreover, the gill isolated cells showed a high viability rate (over 90%) according to the Trypan blue exclusion test, 18 h after isolation **(Fig. 3),** that was independent of the degree of infestation of the gill arches.

Figure 3. Greater amberjack gill arch (left) and gill fluke *Zeuxapta seriolae* (right).

Based on these previous results, a new trial was performed with different experimental treatments consisting of anti-attachment/prebiotic substances (mannose and black cumin oil) in order to determine their effects on the greater amberjack degree of parasitosis by *N. melleni*.

A total of 51 greater amberjack individuals (40.0 ± 20.7 g) were divided in three groups (17 fish per group), stocked at 1 m^3 tanks, and maintained for 106 days with constant seawater renewal and aeration (20.5 ± 1.3ºC and 94.0 ± 0.4% oxygen saturation). Fish groups were subjected to the following experimental treatment protocols:

- **Control**: bath in seawater for 3 min and moved to a new tank (S1).
- **Black cumin oil**: bath in seawater and black cumin oil (4 ml l^{-1}) diluted in 60 ml of ethanol. After 3 min the fish were moved to a new tank (S2).
- **Mannose**: bath in a mannose dilution (30 mM) in seawater (5.4 g of mannose l^{-1}) and after 3 min moved to a new tank (S3).

After the treatments, egg collector devices were placed for 24 h in each of the new tanks and checked at 1, 2, 3, 6 and 8 days after treatment.

The control fish (S1) increased the number of monogenean eggs during the following 8 days post bathing in seawater (532% more eggs than 10 days before), whereas both the black cumin (S2) and mannose (S3) groups showed a reduction in the number of eggs collected at days 2 and 8 days post treatment, respectively. At the end of the experiment, the number of eggs, with respect to the initial values, increased by a 48% in the black cumin treated fish, and decreased about 45% in fish treated with mannose (**Fig. 4**). The mannose concentration and the frequency of application could also improve the anti-attachment effects of this molecule.

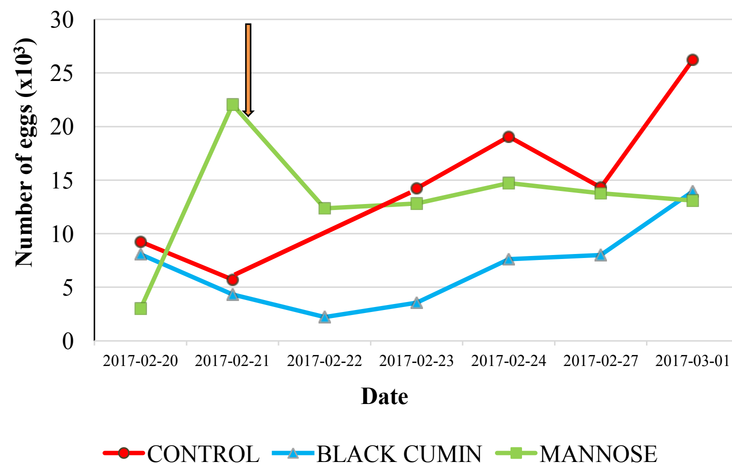

Figure 4. Number of *Neobenedenia melleni* eggs collected from tanks of juvenile greater amberjack untreated (control) and treated with black cumin oil and mannose. The arrow indicates the day of application of the treatment.

According to this information it can be concluded that:

- The fish are not parasitized by skin and gill monogeneans at the same time, at least not in the same acute way. The presence of one species was always been greater than that of the other. This fact has not allowed working with both parasites at once.

- Each of the parasites showed its own rhythm of development apparently related to environmental variables, an aspect that could be useful for environmental management of parasite infections.

- The rearing density does not seem to greatly influence the level of parasite infestation of fish.

- Treatment with mannose, as an anti-attachment substance, seemed to reduce the level of parasitosis by *Z. seriolae* and *N. melleni.* Therefore, further insights should be address on these findings.

Based on these results, new trials were developed in two ways:

1.-To study the influence of the environmental parameters, temperature and photoperiod, on the *N. melleni* biology for possible application in the management of parasite infections in greater amberjack.

2.-To study the anti-attachment effect of treatments performed by immersion in mannose solution and other novel methods of possible interest for the control of *N. melleni* parasites.

As a result, two academic documents written in Spanish were produced. The first one was the final Degree Dissertation of the ULL student Yefermin Jesús Darias Dágfeel, “External parasites in the rearing of the greater amberjack (*Seriola dumerili*) in Tenerife”, supervised by Gonzalo Lozano Soldevilla (ULL) and Salvador J. Jerez Herrera (IEO). The second one, a Master Thesis Dissertation of the ULL student Andrea Villena Rodríguez, “Incidence and control of monogenean parasites in *Seriola dumerili*”, supervised by Salvador Jerez Herrera (IEO) and José A. Pérez Pérez (ULL), both defended on July 2018 (see pdf at DIVERSIFY´s website).

2.1. Effects of environmental parameters on the biological cycle of *Neobenedenia melleni* and degree of greater amberjack infestation.

A set of trials were carried out in order to test the effect of photoperiod on greater amberjack parasitized by *N. melleni* and the effects of photoperiod and temperature on the biological cycle of this monogenean parasite.

2.1.1. Material and methods

Experimental conditions

A total of 81 juveniles of greater amberjack (296.4 ± 19.9 g and 24.4 ±0.6 cm) born in the IEO-COC facilities were distributed in 9 groups of 9 fish each. Fish were maintained in fiberglass tanks (1 m^3) with constant renewal (15 l min^{-1}) and aeration, under natural conditions of salinity (37.5 psu), temperature (19.1 ± 0.9ºC) and oxygen saturation (86.9 ± 3.8 %), and three photoperiod regimens (in triplicate) for 68 days as follows:

Treatment **Control:** natural photoperiod and maximal light intensity of 200 lux.

Treatment **24L:0D**: continuous artificial light for 24 h at 1000 lux.

Treatment **0-3L:24-21D**: dark (< 2 lux) for 24 h from day 0 to 34 and light (1000 lux) for 3 h and dark for 21 h from day 34 to 68.

Fish were fed daily (8:00 h) with a commercial pellet for turbot (5 mm diameter; Skretting Ltd, Norway; composition in % dry weight was: 52 % crude protein, 20 % crude fat, 8.7 % ash, 1.7 % crude cellulose and 1.4 % total phosphorus). Uneaten food was recovered from the bottom of the tank 30 min after its administration to quantify the daily feed intake (FI). Dead fish during the trial were recorded daily.

The level of parasitosis by monogeneans was monitored by dish traps placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014).

Egg-laying rhythm

The rhythm of eggs released by adults of *N. melleni* parasitizing fish maintained at different photoperiod conditions (Control, 24L:0D and 0L:24D) was monitored over 24 h using mesh dishes submerged for 3 h in each tank (8 dish per tank) and the number of eggs entangled counted.

Temperature and photoperiod effects on egg parasite hatching

A total of 27 mesh dishes were submerged at the same time in a single tank for 20 min, in order to obtain *N. melleni* eggs to incubate at three different temperature (15, 20 and 24ºC) combined to three different photoperiod conditions (12L:12D, 24L:0D and 0L:24D) in triplicate. The total number of eggs and eggs hatched were recorded daily during 8 days.

Fish sampling

All fish in each tank were sampled for weight and length at the beginning (0 days) and at 34 and 68 days. Two fish per tank were selected for blood sampling from the caudal vessel using heparinized syringes. Plasma samples were separated after centrifugation at 1400 rpm for 20 min and stored at -80°C until analysis.

During the study, specific growth rate (SGR, % day^{-1}), condition factor (CF, g cm^{-3}), survival (S, %) and feed intake (FI, % body weight) were calculated as below:

SGR=100 × (ln final Body weight (g) - ln initial Body weight (g)) × $days^{-1}$

CF=100 × Body weight (g) × TL (cm^{-3})

S=100 × final fish number × initial fish $number^{-1}$

FI=100 × feed consumption (g) × average $biomass^{-1}$ (g) × $days^{-1}$

2.1.2. Results

The Specific Growth Rate (SGR) and Condition Factor (CF) of fish maintained in continuous darkness (0L:24D) were significantly lower (P<0.05) during the period 0-34, while no differences were observed in the period 34-68, and in the overall period (0-68) between treatments (**Fig. 5**).

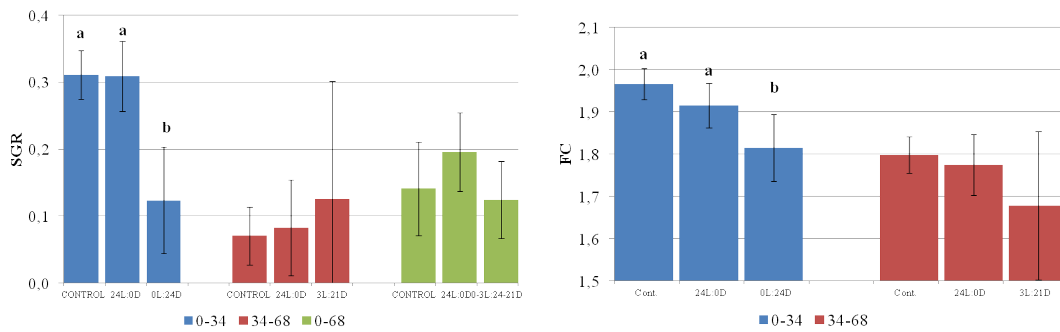

Figure 5. Specific growth rate (SGR, % day^{-1}) and Condition Factor (CF, g cm^{-3}) at different periods and over the entire duration (68 days) of fish maintained under different photoperiod treatments. Different letters indicate significant differences (P<0.05).

The feed intake (% of body weight day^{-1}) was significantly lower (P<0.05) in the fish maintained in continuous darkness during the period 0-34 days and 3L:21D in the period 34-68, and in the overall period (0-68) **(Fig. 6)**.

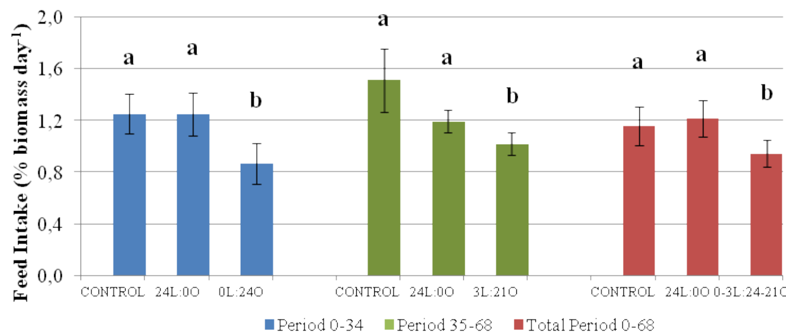

Figure 6. Feed intake (% body weight day^{-1}) at different periods and over the entire duration (68 days) of fish maintained under different photoperiod treatments. Different letters indicate significant differences (P<0.05).

No fish mortality was recorded in the period 0-34 days in any of the different photoperiod treatments, but the fish maintained in 3L:21D from 34 to 68 days, previously maintained from 0 to 34 days under continuous darkness (0L:24D), exhibited the lowest survival rate (P<0.05) **(Fig. 7)**.

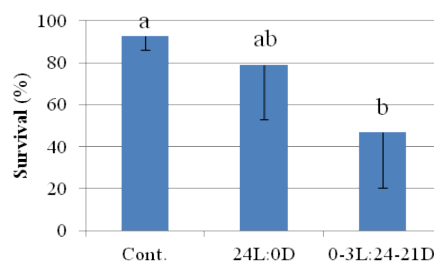

Figure 7. Survival (%) of fish maintained at different photoperiod treatments over the entire duration (68 days) of the experiment. Different letters indicate significant differences among groups (P<0.05).

Regarding parasite behavior, the number of eggs of *N. melleni* during the first 3 weeks was significantly ($p<0.05$) lower in fish maintained in 24L:0D (41±45 eggs d^{-1}) than 0L:24D (179±73 eggs d^{-1}). The Control group showed an intermediate number of eggs (107±37 eggs d^{-1}). Even so, the number of eggs recorded was low in all groups, and the subsequent reinfection treatment applied increased significantly the number of eggs collected from that moment.

Two weeks after reinfection fish maintained in continuous darkness (0L:24D) showed the highest number of eggs per day, and fish maintained in continuous light (24L:0D) the lowest, to the end of the trial **(Fig. 8 a)**. The mean number of *N. melleni* eggs per day after reinfection to the end of the trial (68 days) was significantly higher in fish maintained in photoperiod treatments of 0-3L:24-21D than in fish under continuous light (24L:0D) (**Fig. 8 b**).

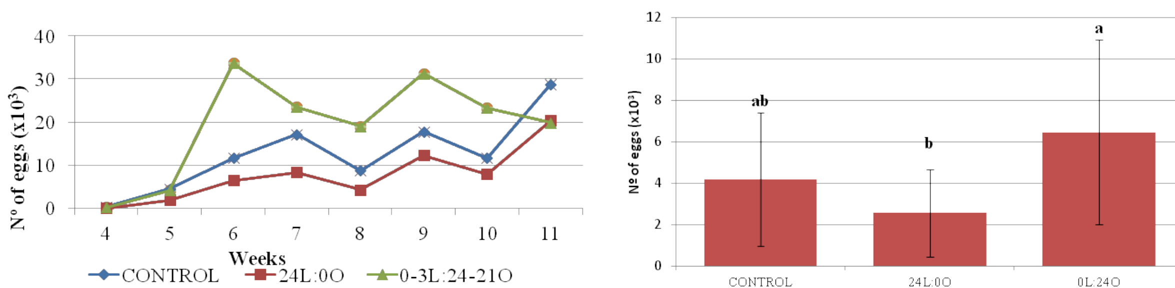

Figure 8. Number of eggs of *N. melleni* collected from greater amberjack maintained in different photoperiod treatments following reinfection (week 3) to the end of the trial (68 days) (a), and mean number of eggs over the whole period (b). Different letters indicate significant differences ($P<0.05$).

Hematology and blood biochemistry

Hematological and biochemical parameters measured at 34 and 68 days of the trial for the three different photoperiod treatments assayed are shown in **Table 1.**

At 34 days, all hematological parameters studied remained constant in all groups of fish and only the hematocrit of fish maintained in the 0L:24D photoperiod was significantly lower ($P<0.05$) compared to the other treatments. At the end of the growth period (68 days), significant differences were absent between groups maintained with different photoperiods.

Plasma biochemical parameters were homogeneous between fish groups at 34 and 68 days, except for plasma triglycerides that were significantly higher in the Control group compared to the 24L:0D fish group during the period 0-34 days.

Table 1. Effect of feeding frequencies on erythrocytes (10^6 μl^{-1}), leucocytes (10^6 μl^{-1}), hematocrit (%), Mean Corpuscular Volume (MCV, μm^{-3}), triglycerides (mg dl^{-1}), cholesterol (mg dl^{-1}) and glucose (mg dl^{-1}). Data collected at 34 and 68 days. Different letters indicate significant differences among groups ($P<0.05$).

Treatment	CONTROL	24L:OD	0L:24D	CONTROL	24L:OD	3L:21D
Period		0-34			34-68	
Erythrocytes	5.9 ± 0.5	5.1 ± 0.3	4.8 ± 1.4	1.9 ± 0.4	1.6 ± 0.6	1.9 ± 0.8
Leucocytes	1.4 ± 0.1	0.9 ± 0.3	1.0 ± 0.4	0.3 ± 0.1	0.5 ± 0.3	0.5 ± 0.1
Hematocrit	59.5 ± 7.8 a	60.0 ± 2.2 a	43.7 ± 6.9 b	44.3 ± 12.4	42.9 ± 8.1	45.5 ± 6.4
MCV	264.7 ± 4.2	286.4 ± 15.7	199.9 ± 57.6	243.6 ± 72.8	270.3 ± 118.1	297.7 ± 137.3
Glucose	77.7 ± 16.6	88.7 ± 4.0	86.6 ± 10.3	113.2 ± 30.1	150.0 ± 74.5	155.9 ± 97.9
Cholesterol	159.5 ± 24.8	149.5 ± 7.4	143.3 ± 17.7	86.4 ± 46.6	78.5 ± 34.6	55.0 ± 36.9
Triglycerides	289.6 ± 71.8 a	111.9 ± 21.1 b	148.4 ± 29.4 ab	266.9 ± 7.5	211.8 ± 72.1	178.3 ± 75.7

The egg daily releasing rhythm of adults of *N. melleni* maintained at the different photoperiod treatments were adjusted to the following cosine model:

Control: Nº eggs=474.5+298.8 × cos (2π × (Time (h) - 18)/24); r=0.85, p<0.01, n=16

24L:0D: Nº eggs=391.9+429.2 × cos (2π × (Time (h) - 18)/24); r=0.89, p<0.01, n=16

0L:24D: Nº eggs=561.1+457.2 × cos (2π × (Time (h) - 18)/24); r=0.83, p<0.01, n=16

The release of eggs by adults of *N. melleni* occurred between 7:00 and 19:00 h, reaching the highest number of released eggs between 16:00 and 17:00 independently of the photoperiod treatment **(Fig. 9)**.

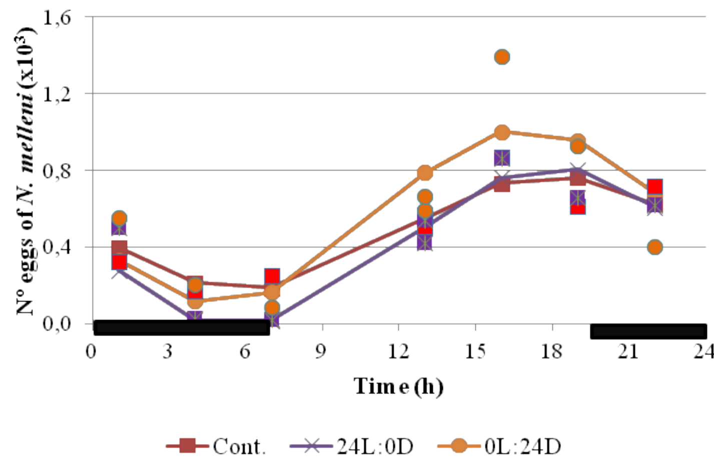

Figure 9. Daily egg releasing rhythm of *N. melleni* parasitizing fish maintained under three different photoperiod treatments. Black bars indicate the natural darkness hours.

The hatching rate of *N. melleni* eggs was significantly lower in continuous light conditions (24L:0D) regardless of temperature. However, a significantly lower hatching rate occurred at 15ºC **(Fig. 10)**.

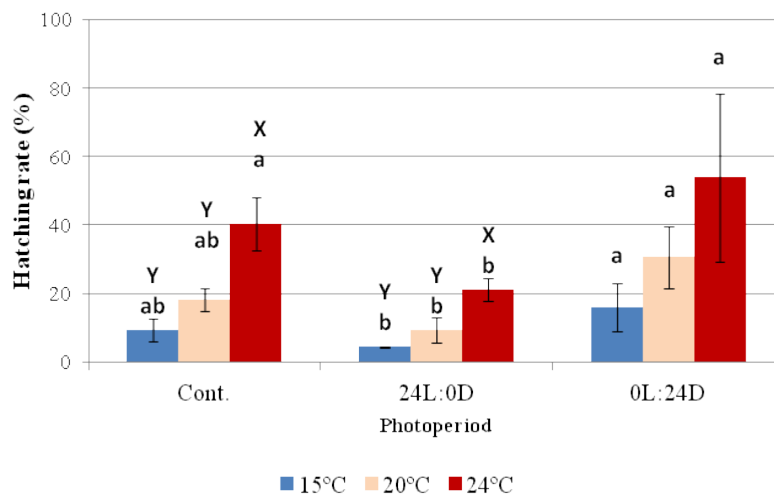

Figure 10. Total Hatching rate (%) of eggs of *N. melleni* incubated at different photoperiods (Control (Cont.), continuous light (24L:0D) and continuous darkness (0L:24D)) and temperature (15, 20 and 24ºC). Different lowercase letters indicate significant differences among photoperiods for a particular culture temperature; different uppercase letters denote significant differences among temperatures for a particular photoperiod.

The highest temperature decreased the time to hatching **(Fig. 11**). The eggs began to hatch on day 2 when incubated at 24 °C while at 15 °C hatching started after 5 day of incubation.

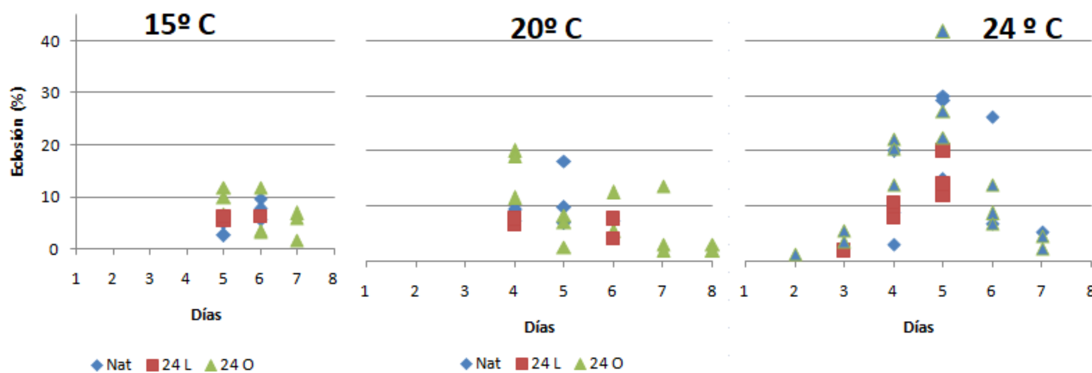

Figure 11. Daily hatching rate (%) of eggs of *N. melleni* incubated under different temperature and photoperiod conditions for 8 days after being released.

2.2 Effects of anti-attachment substances and novel methods to reinforce the immune system of greater amberjack.

A set of experiments were carried out in order to test the effects of anti-attachment substances such as mannose and a novel method of Fecal Microbiota Transplantation (FMT) to reinforce the immune system of greater amberjack, in order to improve their defences against infection by *N. melleni*.

2.2.1. Material and methods

Experimental conditions

A total of 96 juveniles of greater amberjack (210.0 ± 68.6 g) born in the IEO-COC facilities and parasitized by *N. melleni*, were randomly distributed in 12 homogeneous groups (8 fish per tank). The groups were maintained in fiberglass tanks (1 m^3) for 4 months with a constant water exchange (0.25 ± 0.04 l s^{-1}) and aeration, under natural conditions of photoperiod, water salinity (37.5 psu), temperature (19.0 ± 0.3ºC) and oxygen saturation (91.2 ± 1.1%). Fish were fed a commercial pellet for turbot (3-5 mm diameter; Skretting Ltd, Norway; composition in % dry weight was: 52% crude protein, 20% crude fat, 8.7% ash, 1.7% crude cellulose and 1.4% total phosphorus). The fish groups were daily fed at a feeding frequency of 2 times daily (8:00 and 18:00 h) during the first month and once daily afterwards (8:00 h). Food left uneaten was recovered from the bottom of the tank 30 min after its administration to quantify the daily feed intake. Dead fish during the trial were recorded daily.

The level of parasitism by monogeneans was monitored by dish traps placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014) for 4 months (January-May). The absence of *Zeuxapta seriolae* throughout the study allowed the collection of *N. melleni* eggs. Mesh traps were placed in the tanks 1-2 times per week and retrieved 2-3 days later to count the eggs entangled in the dish traps.

After 4 weeks of monitoring, juveniles of greater amberjack were sampled and blood of 12 fish collected (1 fish per tank) in order to obtain the hematological and biochemical parameters. Haematocrit was determined using micro haematocrit capillaries filled with blood and centrifuged at 12000 rpm for 5 min. The results were expressed as percentage (%) of total blood volume. Red blood cells (RBC) and white blood cells were counted in 1/100 dilutions of blood using a Neubauer haemocytometer and Natt and Herricks (1952) solution. Mean Corpuscular Volume (MCV) was calculated by the equation: MCV (μ m^3) = 10 x Hematocrit (%) / RBC ($x10^6$ μl^{-1}).

The remaining blood was centrifuged, and the blood plasma was removed and frozen at -80 °C for later analysis.

Plasma levels of glucose, triglycerides, and total cholesterol, were measured in duplicate using commercial kits ByoSistems, and plasma lactate, chloride, sodium and potassium using standard spectrophotometric commercial kits SPINREACT. The assays were performed with a PowerWaver microplate spectrophotometer (Bio-Tek Instruments, Vermont, USA), except for sodium and potassium where a UV-visible cuvette spectrophotometer (Shimadzu, Kyoto, Japan) was used.

Mannose treatment

The immersion or "baths" of the fish in mannose solutions was carried out in three increasing doses (30, 50 and 70 mM), and in sea water (control), resulting in 4 treatments in triplicate (M0, M30, M50 and M70), for 5 min. The fish in the M0 treatment were subjected to a bath in sea water without mannose. After each bath, the water was sifted for counting of the detached adults of *N. melleni* by loupe and the fish returned to the tank.

Fecal microbiota transplantation, FMT

The Fecal Microbiota Transplantation (FMT) treatment was performed as a method for the reinforcement of the immune system of fish. Four treatments were applied in triplicate: Control (buffer PBS); transfection with warthog faeces (*Phacochoerus africanus*) provided by IRTA (Instituto de Investigación y Tecnología Agroalimentarias de Cataluña) (FMTP), and transfection with faeces of gilthead seabream (*Sparus aurata*) maintained at the IEO-COC facilities, with a previous dose of antibiotic (single dose of sulphanilamide, 125 mg kg^{-1}) (FMTGS) and without a previous dose of antibiotic (FMTG). The objective of antibiotic application was to eliminate native bacterial flora in an attempt to make the exogenous flora (donor) installation easier.

Inoculation of the feces transplant was carried out orally by syringe for 3 consecutive days. The dose was 0.3 ml of stool suspension per fish per day.

Fish sampling

At the beginning (day 0), and at 7, 15, 21 and 34 days post inoculation of feces, all fish in each tank were anesthetized and measured for weight and length, and samples of feces of all fish of each tank obtained by cannulation. At each sampling time, 2 fish per tank were then selected randomly for blood collection from the caudal vessels using heparinized syringes. Plasma samples were separated after centrifugation at 1400 rpm^{-1} for 20 min and stored at -80°C until analysis.

At the end of the trial (day 34), a total of two fish per tank (6 fish per treatment, 24 total fish) were sampled to determine biometric parameters (viscerosomatic and hepatosomatic index) and to obtain samples of gill and gut and to the isolation of gill, and hepatic (hepatocytes) and intestinal (enterocytes) epithelial cells for the evaluation of their viability and functional integrity (viability test with Trypan blue exclusion and ATPase activity). Samples of gut tissue and digestive tract content were also frozen in liquid nitrogen and stored at -80°C until later analysis of digestive enzymes.

The gut, including pyloric caeca, was rapidly removed from the carcass, cleaned of adhering adipose tissue, and contents removed with cold (4 °C) physiological saline solution III (Dópido *et al*., 2004). The intestine was then cut and incubated in HBSS with collagenase (1 mg ml^{-1}). Hepatocytes were prepared basically as described by Rodríguez *et al*. (2002). The gill arches were finely chopped in a beaker with HBSS solution containing collagenase (1 mg ml^{-1}). Both gut and liver preparations were incubated with collagenase for 15 min at 25 °C with shaking. The resultant cell suspensions were filtered through a 60–100 µm nylon mesh and centrifuged. The isolated cells were then frozen at -80ºC until analysis of ATPase and digestive enzyme activities.

Cell viability was also assessed by the Trypan-blue dye exclusion test. In this test, 50 µl cell suspensions were diluted with 200 µl 0.4% Trypan blue solution, stained for 5 min at room temperature, and viable cells (dye excluded) counted using a Neubauer haemocytometer. Data were expressed as mean percentage of total viable cells.

Na^+-K^+-ATPase activities were measured in duplicate as the difference in inorganic phosphate production from ATP in the presence or absence of 1 mM ouabain under steady-state conditions, as described previously (Almansa *et al*., 2001; Díaz *et al*., 1998). Brifely, 25 µl of protein suspension, containing 50-100 µg protein, was added to 1 ml of incubation media in which the composition was adapted for each tissue in order to attain optimal Na^+-K^+-ATPase activities according to previous data reported by our group (Almansa *et al*., 2001). The reaction was started by the addition of 50 µl (5 mM final concentration) of vanadate-free ATP and incubated for 10 min at different temperatures ranging from 4 to 50 ºC. Corrections for unspecific ATP hydrolysis were made by measuring the amount of Pi liberated in the absence of protein samples at each temperature tested. Specific Na^+-K^+-ATPase activities were expressed as µmol Pi/mg prot.h.

For digestive enzyme quantification, samples were homogenized by means of an Ultra-turrax T8 (IKA-Werke, GmbH & Co.KG, Staufen, Germany), in 5 volumes (w:v) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4 ºC, and the supernatant aliquoted in 1.5 ml Eppendorfs and stored at -80 ºC until use. Enzymatic determinations for total amylase, lipase and alkaline proteases activities were based on methods previously performed and described by Gisbert *et al*. (2009).

Protein content of samples was determined by means of the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. All measured enzymatic activities were normalized with the protein content. The absorbance was read using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Number of adult parasites

In order to evaluate the intensity and sites of infection preference and to quantify the number of adult parasites of *N. melleni*, the sacrificed fish were submerged in fresh water to cause the detachment of adult parasites attached to the skin. The detached parasites of each fish were collected with a plastic Pasteur pipette, and deposited in individual Petri dishes. Subsequently, the number of adult parasites present in each fish was recorded. The surface area of each greater amberjack fish was estimated (Ohno *et al.*, 2008) and the number of parasites per fish surface determined as:

Number of parasites cm^{-2} of fish surface area = Parasites number × $(2 \times 0.158 \times TL^{2,089})^{-1}$

During the study, specific growth rate (SGR, % day^{-1}), condition factor (CF, g cm^{-3}), survival (S, %) and feed intake (FI, % body weight) were calculated as below:

SGR=100 × (ln final Body weight (g) - ln initial Body weight (g)) × $days^{-1}$

CF=100 × Body weight (g) × TL (cm^{-3})

S=100 × final fish number × initial fish $number^{-1}$

FI=100 × feed consumption (g) × average $biomass^{-1}$ (g) × $days^{-1}$

Additionally, to check if the transfection caused an immediate or longer-term effect on the production of *N. melleni* eggs, the level of parasitism was monitored in the tanks by dish traps. For this, data were collected during the 3 days of stool inoculation, and subsequently 1 or 2 times per week during the 34 days of the experiment. Due to the small number of eggs of *N. melleni* attached to the dishes, where values ranged between 23 and 27 eggs collected every 24 h, it was decided to carry out a reinfection or guided infection of the 12 experimental tanks in order to ensure a higher level of parasitosis.

Previous methodological procedures tested

Reinfection method

The mesh dishes were also used as the reinfection method to increase the level of parasitosis of fish. To this purpose, 12 dishes were placed in one culture tank of *S. dumerili* intensively parasitized by *N. melleni* that was existing in the facilities. After being submerged for 4 days to allow egg collection from the adult

parasites, each of these 12 dishes was placed in the 12 experimental tanks for 14 days, with the aim of achieving reinfection.

Feces sampling and inoculation, antibiotic dose and anesthesia frequency

To optimize the application and effectiveness of the different treatments, a series of preliminary tests were carried out in juvenile gilthead seabream (*Sparus aurata*) of similar size to the greater amberjack maintained at IEO facilities, related to resistance to handling, the method of application and the optimal dose of antibiotic. The inoculation of faces into gilthead seabream over 3 consecutive days verified the viability of the method to be used in greater amberjack.

In addition, because the sulfanilamide solution is colorless, the antibiotic concentrations selected (75, 100 and 125 mg sulphanilamide kg^{-1}, according to Rigos and Troisi (2005)), had stain added using food colouring and were tested to determinate the maximum volume that could be supplied without being expelled, with the dose dependent on the color intensity of the feces.

The food colouring solutions were administered orally with the help of a syringe, and the effectiveness was evaluated by taking samples of feces the following day. The intensity of the colour indicated the effect of each treatment. The effectiveness of inoculation of oral antibiotic by syringe was proven, and the maximum volume that could be supplied without being expelled was determined to be 0.5 ml, thus establishing that the optimal dose for the transfection experiment to ensure an effect was the highest concentration (125 mg sulfanilamide kg^{-1}). In addition, the resistance of the fish to 4 consecutive days of anesthesia and handling was confirmed, and the amount of feces that could be obtained, using a cannula, from fish of that size was also determined.

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 19.0 for Windows (SPSS, www.spss.com). All values, presented as percentages, were arcsine transformed. Values were checked for normality and homogeneity of variance, using the Shapiro-Wilk and the Levene tests, respectively.

To compare means, data of the number of parasite eggs, biometric, haematological and plasmatic parameters were statistically tested using a two-way ANOVA with repeated measurements followed by one-way ANOVA and the Tukey test.

A one-way ANOVA followed by a multiple range test (Newman–Keuls) was used for feed intake, number of adult parasites per fish surface and parasite size (length and width) to examine significant differences ($P<0.05$) among various treatments. When variances were not homogeneous, a non-parametric Kruskal–Wallis test was carried out.

The correlation between the level of triglycerides and the number of eggs emitted daily was carried out by a linear regression. Differences were considered significant at $P<0.05$.

A cosine model was used to describe the relationship between treatment (photoperiod and time) and response variables (number of eggs) and a linear model was used to describe the relationship between number of eggs and cholesterol. Data were expressed as mean ± SD.

2.2.2. Results

Bath treatments with mannose

Bath treatments with different concentrations of mannose (30 mM, 50 mM and 70 mM) did not cause a significant detachment of adults of *N. melleni* from greater amberjack.

The increase in mannose concentration did not affect (p>0.05) the number of eggs emitted per day by the parasite and collected in any of the subsequent samplings. The number of eggs collected the day after baths (day 1) with mannose, treatments M30 and M70 showed similar values to those registered at the beginning (day 0), while the number of eggs in control (C) and M50 treatment tended to increase (**Fig. 12**).

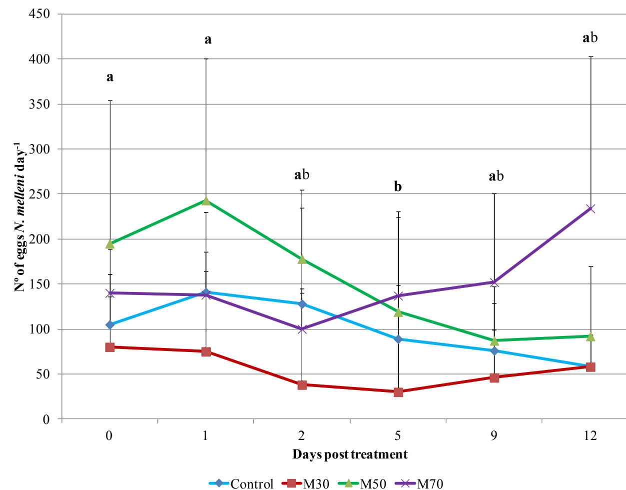

Figure 12. Number of *N. melleni* eggs collected per day over the experimental period (0, 1, 2, 5, 9 and 12 post-treatment days). C, Treatment control; M30, bath with 30 mM of mannose; M50, bath with 50 mM of mannose; M70, bath with 70 mM of mannose. The values are averages (+SD) (n=3). Different letters indicate significant differences (p<0.05) in each treatment at different sampling days.

In all tested treatments, the number of eggs recorded at day 5 were significantly lower (p<0.05) than those registered at day 0 and 1, showing the M30 treatment had the largest decrease followed by the M50 treatment. From day 6, the number of eggs collected was stable for all tested mannose concentrations.

Reinfection of fish

The number of eggs per day collected increased significantly three weeks after reinfection in all groups of fish, showing this was a successful method to infect fish (**Fig. 13**).

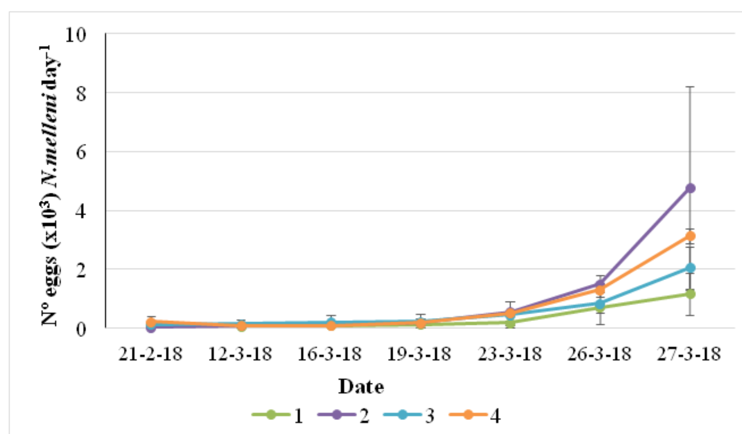

Figure 13. Number of *N. melleni* eggs per day collected after the reinfection experiment in all groups of fish. C, control treatment; M30, bath with 30 mM of mannose; M50, bath with 50 mM of mannose; M70, bath with 70 mM of mannose.

Fecal Transfection treatments

Figure 14 shows the number of *N. melleni* eggs per day released during the experimental period after the application of transfection treatments. The statistical comparison was made among the different sampling days (0, 7, 15, 21 and 34).

There was no differential effect among treatments during the experimental period ($p>0.05$). At 7 day post-transfection, the number of eggs collected was similar to that recorded at the beginning in all tested treatments. However, at days 15 and 21, the eggs per day collected decreased significantly ($p<0.05$), reaching values at the end of the trial (day 34) that were similar to those recorded at the beginning (**Fig. 14**).

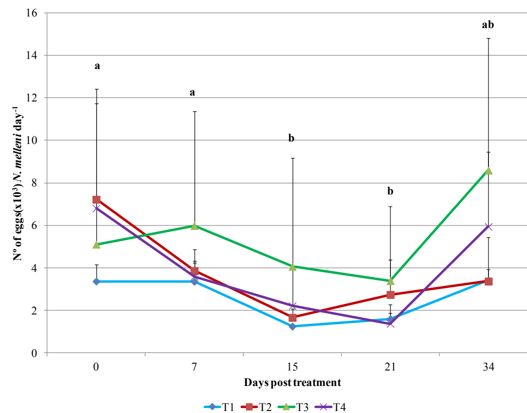

Figure 14. Number of *N. melleni* eggs per day released during the experimental period (0, 1, 2, 5, 7, 12, 15, 19, 21, 26, 33 and 34 post-transfection days). Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4). Different letters indicate significant differences ($p<0.05$) at days 0, 7, 15, 21 and 34 post-transfection.

The growth (SGR) and condition factor (CF) of greater amberjack after the transfection did not change significantly during the experimental period for any of the tested treatments (**Fig. 15**).

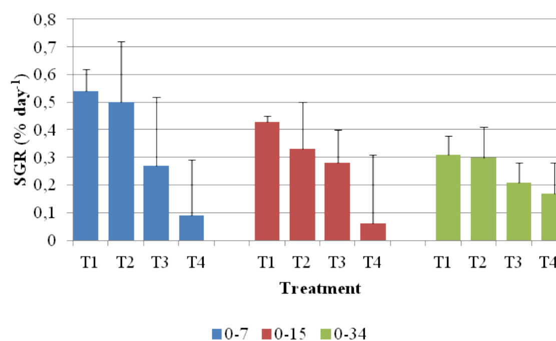

Figure 15. Specific Growth Rate (SGR) of greater amberjack in each treatment group during the experimental periods, 0-7, 0-15 and 0-34 days post-transfection. Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4). Values are means + SD (n=3).

Feed intake ratio also did not show differences ($p>0.05$) during the overall experimental period (0-34 day) between different treatments (**Fig. 16**).

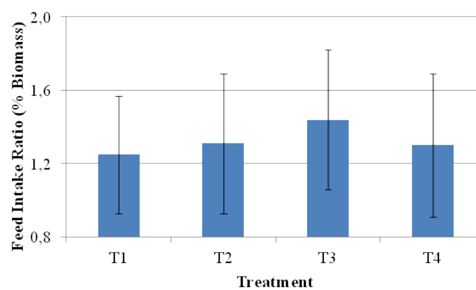

Figure 16. Feed Intake (% Biomass) of greater amberjack in each treatment group during the experimental periods, 0-7, 0-15 and 0-34 days post-transfection. Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4). Values are means ± SD (n=3).

The number of erythrocytes increased, but not significantly, during the experimental period in the Control (T1) and T2 treatments with respect to the initial values (0 day). In contrast, treatments with transfection of seabream feces without (T3) and with a previous dose of antibiotic (T4) showed final values similar to those recorded at day 0 (**Table 2**).

Table 2. Hematological parameters (erythrocytes and leucocytes, 10^6 cells μl^{-1}), hematocrit (%) and mean corpuscular volume (MCV, μm^3) of greater amberjack at 0, 7, 15, 21, and 34 days post-transfection. Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4). Different letters indicate significant differences (p<0.05) in each treatment at 0, 7, 15, 21 and 34 days post-transfection.

	Day	**0**	**7**	**15**	**21**	**34**
Parameter	**Treatment**					
Erythrocytes	**T1**	1.8 ± 0.7	2.4 ± 0.2	2.7 ± 0.6	2.5 ± 0.3a	2.6 ± 0.4a
	T2	1.7 ± 0.1	2.2 ± 1.0	1.8 ± 0.4	2.1 ± 0.1ab	2.8 ± 0.1a
	T3	1.7 ± 0.1	2.2 ± 0.2	1.8 ± 0.3	1.4 ± 0.5b	1.6 ± 0.2b
	T4	1.8 ± 1.4	2.0 ± 0.5	1.7 ± 0.2	1.5 ± 0.1b	1.3 ± 0.3b
Hematocrit	**T1**	43.6 ± 6.6	39.4 ± 2.5	39.3 ± 3.3	47.1 ± 4.3	36.4 ± 3.1
	T2	39.4 ± 4.2	42.9 ± 14.0	35.4 ± 3.2	40.5 ± 4.9	41.4 ± 4.5
	T3	38.2 ± 3.4	33.6 ± 6.0	41.1 ± 5.0	35.3 ± 5.8	36.0 ± 2.9
	T4	42.6 ± 7.4	40.4 ± 1.7	40.9 ± 2.6	38.7 ± 1.7	39.5 ± 1.7
MCV	**T1**	260.1 ± 67.6	162.3 ± 17.6	163.6 ± 35.4	132.6 ± 12.1b	156.3 ± 20.7b
	T2	244.4 ± 56.0	143.0 ± 37.7	210.7 ± 60.8	195.1 ± 15.5a	146.7 ± 13.3b
	T3	227.4 ± 3.7	213.3 ± 41.3	229.2 ± 37.9	254.4 ± 46.7a	251.3 ± 36.0a
	T4	241.5 ± 61.8	189.8 ± 39.2	246.7 ± 32.7	240.2 ± 20.4a	310.0 ± 76.7a
Leucocytes	**T1**	1.1 ± 0.4	0.8 ± 0.1	0.3 ± 0.1	0.9 ± 0.4	0.8 ± 0.2
	T2	1.4 ± 0.4	0.9 ± 0.1	0.3 ± 0.1	0.8 ± 0.3	0.7 ± 0.3
	T3	1.2 ± 0.4	0.9 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
	T4	1.4 ± 0.4	0.9 ± 0.0	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.3

Values are shown as means (±SD) (n=3). Different letters indicate significant differences (p<0.05) at the different sampling days between treatments.

At day 21, the number of erythrocytes was significantly higher (p<0.05) in the T1 treatment than in treatments T3 and T4, while at day 34, values from treatments T1 and T2 were significantly higher than treatments T3 and T4.

Tested treatments did not significantly affect the hematocrit during the 0-34 period, or the mean corpuscular volume (MCV) during the 0-15 period. However, the MCV decreased in control (T1) and the transfection with warthog feces treatment group (T2) at day 21 compared with values at day 0, with the MCV significantly higher in Treatments T3 and T4 than in T1 and T2 at day 34 (**Fig. 17**).

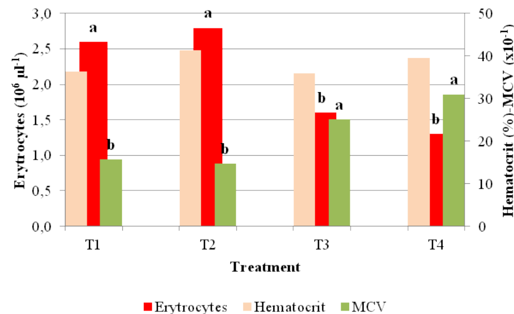

Figure 17. Erythrocytes (10^6 µl^{-1}), hematocrit (%) and mean corpuscular volume (MCV, µm^3) of greater amberjack in each treatment group at 34 days post-transfection. Treatment control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4). Values are means (n=3). Different letters indicate significant differences between treatments for a particular hematological parameter ($p<0.05$).

The number of white blood cells did not change during the experimental period in any treatment assayed and there were no significantly differences ($p>0.05$) among treatments at any sampling day.

There were significant differences in cholesterol and triglyceride plasma levels during the experimental period (**Table 3**). Cholesterol was higher at day 15 post-transfection than day 0, and triglyceride levels were significantly higher at days 15 and 34, than day 7, regardless of the treatment. However, there were no differences between treatments in these two metabolites at any sampling days post-transfection. Glucose and lactate levels did not show significant differences between treatment or sampling days during the trial.

Table 3. Levels of cholesterol (mg dl^{-1}), triglycerides (mg dl^{-1}), glucose (mg dl^{-1}) and lactate (mg dl^{-1}) in greater amberjack plasma at 0, 7, 15, 21, and 34 days post-transfection. Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4).

Parameter	Treatment	Day 0	Day 7	Day 15	Day 21	Day 34
Cholesterol	T1	199.0 ± 22.1b	259.1 ± 26.0ab	360.4 ± 26.6a	263.1 ± 48.0ab	289.5 ± 78.3ab
	T2	213.2 ± 17.6b	213.1 ± 101.8ab	308.3 ± 3.1a	296.6 ± 35.9ab	279.2 ± 57.9ab
	T3	241.1 ± 8.0b	337.0 ± 58.4ab	265.2 ± 57.6a	255.9 ± 47.8ab	338.3 ± 51.1ab
	T4	225.8 ± 42.7b	183.3 ± 59.0ab	323.3 ± 49.9 a	277.1 ± 17.4ab	334.1 ± 70.1ab
Triglycerides	T1	554.6 ± 426.8ab	202.5 ± 37.0b	354.1 ± 106.2a	422.1 ± 160.3ab	828.2 ± 425.2a
	T2	777.9 ± 380.8ab	143.1 ± 123.2b	351.3 ± 78.8a	332.0 ± 58.0ab	568.2 ± 198.4a
	T3	917.4 ± 521.2ab	233.2 ± 51.1b	276.7 ± 115.0a	314.2 ± 269.1ab	1096.4 ± 360.8a
	T4	1261.5 ± 412.8ab	83.5 ± 57.2b	785.4 ± 157.3a	719.9 ± 404.3ab	970.1 ± 615.3a
Glucose	T1	63.6 ± 0.0	82.7 ± 15.7	70.8 ± 10.1	67.6 ± 27.4	53.8 ± 27.2
	T2	49.0 ± 5.8	62.1 ± 18.6	64.6 ± 16.0	57.9 ± 21.4	65.1 ± 33.7
	T3	55.1 ± 9.4	114.1 ± 30.0	56.4 ± 28.8	50.7 ± 4.4	91.8 ± 33.0
	T4	39.7 ± 0.3	72.1 ± 0.7	78.5 ± 22.2	59.2 ± 10.2	84.5 ± 12.9
Lactate	T1	150.7 ± 0.0*	36.1 ± 21.5c	114.4 ± 31.2a	90.6 ± 17.9b	78.7 ± 17.5b
	T2	80.6 ± 11.1*	35.2 ± 5.6c	110.3 ± 11.7a	89.3 ± 13.9b	80.8 ± 28.5b
	T3	77.8 ± 27.6*	64.7 ± 10.0c	195.4 ± 17.5a	117.6 ± 26.0b	107.9 ± 26.0b
	T4	85.0 ± 14.2*	45.0 ± 7.3c	145.0 ± 24.2a	96.2 ± 30.9b	50.7 ± 11.7b

Values are means (±SD) (n=3). Different letters indicate significant differences ($p<0.05$) at the different sampling days between treatments. * indicates that statistical differences could not be verified at different days between treatments.

The overall relationship between triglyceride level and the number of eggs per day collected at the different sampling days during the trial in all treatments assayed was linear with the following equation:

Triglyceride level (mg dL^{-1}) = 218.5 + 0.09 Nº eggs *N. melleni* x $24h^{-1}$ (r= 0.53; p<0.05).

Sodium, potassium and chloride fluctuated during the experimental period in all treatments assayed, but not significantly (**Table 4**). Only the level of sodium was significantly (p<0.05) higher in treatment T3 than in the Control group at the end of the trial (day 34).

Table 4. Levels of sodium, potassium and chloride **(**mmol l^{-1}) of greater amberjack at 0, 7, 15, 21, and 34 days post-transfection. Treatment control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4).

Parameter	Treatment	Day 0	Day 7	Day 15	Day 21	Day 34
Sodium	T1	148.7 ± 24.7	101.8 ± 65.0	152.5 ± 3.5	145.8 ± 6.0	120.0 ± 34.7
	T2	72.6 ± 49.4	173.8 ± 29.0	150.1 ± 18.8	129.1 ± 27.0	152.1 ± 9.2
	T3	160.7 ± 4.3	177.0 ± 3.4	145.5 ± 33.3	148.4 ± 8.8	176.6 ± 9.4
	T4	160.5 ± 3.0	144.4 ± 28.0	186.0 ± 16.5	135.5 ± 34.8	156.3 ± 7.6
Potassium	T1	5.2 ± 0.1a	0.9 ± 0.5b	6.5 ± 0.8a	4.4 ± 1.1a	4.1 ± 1.9ab
	T2	4.3 ± 1.1a	1.8 ± 0.8b	6.2 ± 1.0a	4.2 ± 0.4a	5.5 ± 0.9ab
	T3	4.6 ± 1.0a	0.9 ± 0.2b	6.4 ± 0.9a	4.1 ± 0.7a	2.2 ± 1.4ab
	T4	4.8 ± 0.1a	1.2 ± 0.4b	6.8 ± 3.1a	4.0 ± 0.8a	4.2 ± 0.8ab
Chloride	T1	177.9 ± 0.0*	189.8 ± 27.5ab	192.7 ± 26.6a	117.8 ± 31.4b	187.9 ± 95.7a
	T2	260.6 ± 4.9*	166.2 ± 26.2ab	172.3 ± 21.2a	94.9 ± 15.4b	159.8 ± 65.1a
	T3	263.3 ± 24.2*	191.2 ± 55.5ab	184.7 ± 28.0a	101.6 ± 37.3b	209.9 ± 35.4a
	T4	292.0 ± 49.1*	167.5 ± 20.1ab	206.7 ± 4.7a	128.1 ± 5.7b	171.1 ± 33.9a

Values are means (±SD) (n=3). Different letters indicate significant differences (p<0.05) at the different sampling days between treatments. * indicates that statistical differences could not be verified at different days between treatments.

The fish given a transfection with seabream feces after a previous dose of antibiotic treatment (T4) showed a higher number of parasites detached from fish, and had a significantly lower number of parasites per fish surface than treatment without antibiotic supplementation (T3) (**Table 5**).

The preferred sites of parasite attachment on greater amberjack were the eyes and the area located above the lateral line. Detached adult parasites measured 2.52 to 2.61 mm in length and 1.20 to 1.23 mm wide. There were no significantly differences between treatments.

Table 5. Number of adult parasites detached from fish, by fish surface (cm^{-2}) and length (mm)/ width (mm) of adult parasites at the end of the experimental period. Treatments: control (T1); transfection with warthog feces (T2); transfection with seabream feces (T3); transfection with seabream feces with a previous dose of antibiotic (T4).

Treatment	T1	T2	T3	T4
Nº parasites per fish	42.0 ± 0.0	38.7 ± 22.0	55.7 ± 8.3	23.3 ± 1.5
Total surface of fish (cm^2)	221.1 ± 0.0	225.5 ± 37.4	249.4 ± 33.7	282.5 ± 36.0
Nº parasites fish $surface^{-1}$ (cm^2)	0.20 ± 0.01ab	0.17 ± 0.08ab	0.22 ± 0.02a	0.08 ± 0.01b
Parasite length (mm)	*	2.54 ± 0.54	2.61 ± 0.40	2.52 ± 0.10
Parasite width (mm)	*	1.22 ± 0.32	1.20 ± 0.23	1.23 ± 0.05

Values are shown as means (±SD) (n=3). Different letters indicate significant differences (p<0.05) between treatments.* indicates it was not possible to make a statistical comparison.

Viability of isolated enterocytes and gill cells was not significantly different between the two treatments, with more than 91 % viability achieved even under severe *N. melleni* infections. As shown in **Table 6,** ATPase activity in gill cells was not affected by any transfection treatment whereas this activity was significantly reduced in enterocytes when this transfection was based upon *S. aurata* microbiota. **Table 7** displays data corresponding to the main digestive enzyme activities measured in both the gut tissue and the corresponding inner contents of *N. melleni* infected fish. Transplantation with *P. africanus and S. aurata* induced a significant change - higher activity for the tissue alkaline protease.

Table 6. ATPase activity (µmol mg protein h^{-1}) in branchial cells and enterocytes of greater amberjack (*Seriola dumerili*) juveniles infested with *Neobenedenia melleni* and treated with fecal microbiota transplantation

	Gill cells	Enterocytes
T1 *Control*	2.29 ± 0.10	2.08 ± 0.37 b
T2 *P. africanus*	1.65 ± 1.31	0.72 ± 0.39 a
T3 *S. aurata*	1.44 ± 0.20	0.73 ± 0.13 a
T4 *S.aurata+antibiotic**	1.58 ± 0.07	0.67 ± 0.48 a

Data are means ± SD (n=3). Different letters within a column denote significant differences among treatments (P<0.05). * Single dose of sulphanilamide, 125 mg kg $fish^{-1}$

Table 7. Specific digestive enzymatic activity (U/mg protein) of greater amberjack (*Seriola dumerili*) juveniles infested with *Neobenedenia melleni* and treated with fecal microbiota transplantation

	Amylase		Alkaline proteases		Lipase		Pepsin	
	Tissue	Inner	Tissue	Inner	Tissue	Inner	Tissue	Inner
T1 *Control*	1.80±0.1	2.71±0.1	0.33±0.0 ab	2.88±0.4	34.17±16.03	65.71±22.38	5.62±1.2	39.82±13.47
T2 *P. africanus*	2.15±1.3	2.64±0.4	0.91±0.3 b	2.75±0.4	43.90± 8.88	73.50±15.15	5.12±0.4	41.81± 7.77
T3 *S. aurata*	2.44±0.3	3.37±0.7	0.68±0.3 b	2.96±1.0	38.42±11.61	75.67± 4.05	5.15±1.0	33.73± 5.16
T4 *S. aurata+antib.**	1.39±0.3	1.97±0.7	0.26±0.0 a	2.15±0.8	25.60± 8.87	43.78±19.46	4.36±0.6	25.55± 8.22

Values are expressed as means ± SD (n=3). Different letters within a column denote significant differences among treatments (P<0.05). * Single dose of sulphanilamide, 125 mg kg $fish^{-1}$.

3. Discussion and conclusions

Continuous darkness had negative effects on growth, behavior and condition of greater amberjack. The slight change to only three hours of light caused some recovery of the growth and condition index. However, the growth and condition of fish maintained under continuous light conditions (24 h of light) were similar to that recorded for fish under a natural photoperiod. The growth performance and condition of the greater amberjack showed a clear relationship with feed intake. Continuous darkness caused a decrease in feed intake and periods of light as short as 3 h slightly improved it. The positive effect of daylight hours is limited because continuous light conditions did not cause higher intake or better growth.

In general, fish with diurnal dietary habits grow better under photo phase conditions (Spieler and Noeske, 1984) while those with nocturnal habits do better when fed in scot phase conditions (Sundararaj *et al*., 1982). In addition, the level of locomotor activity is often increased gradually before dawn in fish with diurnal eating habits (Sánchez-Vazquez and Tabata, 1998).

Greater amberjack has been described as a highly dependent on vision for food capture, showing a clear and rigid feed rhythm with maximal activity between 1-3 h after sunrise (Chen *et al.*, 2007). In addition, the absence of locomotor activity before sunrise has been described which further reinforces the idea that feeding is very much dependent on vision (Bolliet *et al*., 2001), unlike that described for closely related species such as the Japanese yellowtail*,* which changes its feeding behavior based on photoperiod conditions (Kohbara *et al*., 2003).

No mortality was registered in any of the treatments applied, despite recording the lowest levels of intake, growth and condition in the greater amberjack subjected to continuous darkness in the first period. The higher mortality occurred in the fish that in the second period changed from total darkness to 3 h of light conditions, probably due to a deleterious effect on the health that occurred during the previous period. This highlights the importance of maintaining optimal rearing conditions during the whole growing period.

The number of eggs of *N. melleni* depended on the photoperiod applied during the trial. During the first three weeks, the number of parasite eggs was low but the behavior was practically the same as that recorded later, with the lowest number of parasite eggs collected in fish subjected to continuous light conditions and the highest in darkness conditions. The success of reinfection allowed high levels of parasitosis and the number of eggs collected confirmed this pattern and relationship with light conditions. In addition, the reduction in the number of eggs under continuous light conditions was corroborated by the fact that the application of light for only 3 h a day caused a reduction of 30.3% in 7 days.

In the stage prior to reinfection, the number of eggs collected in continuous light conditions decreased progressively, reaching zero values in only three weeks. Considering that the life cycle duration of the parasite has been estimated at 21 days (Hoai and Hutson, 2014), our results could indicate that a three-week continuous light pulse could be effective against parasitism, even when the total parasite population is low.

The dark conditions negatively affected the hematocrit more than the number of red blood cells, giving rise to similar values of mean corpuscular volume (MCV) in all treatments tested. This could indicate that the lower hematocrit was related to a smaller size of the red blood cells in the fish subjected to continuous darkness. In general, the hematocrit and number of red cells decreased in the second period with respect to first period, regardless of photoperiod. The behavior of both parameters together with the values of MCV in the second period could indicate that the decrease in the number of red blood cells was compensated by an increase in the size of these cells to reduce the effect of a possible anemia.

It is known that parasite infections can affect the hematological parameters of the host fish, resulting in anemic states as a consequence of hemorrhages in epithelia caused by ectoparasites, as seen for *S. dumerili* parasitized by *Z. seriolae* (Montero *et al*., 2004) and by *N. girellae* (Hirazawa *et al*., 2016).

The number of white blood cells was similar in each period regardless of treatment applied, but decreased in an important way between the first and second period of this study. This marked decrease can be related to an immunodeficiency state caused by the high level of parasitosis and duration. Previous studies have related the variations in the number of white blood cells with agents that cause chronic or repetitive stress such as parasitism, which depending on the duration can cause immunosuppression (Barton, 1997; Barandica and Tort, 2008). These changes have also been linked to infestation with *Z. seriolae* in *S. dumerili* (Jerez *et al*., 2017).

Physiological activities in many organisms show rhythms related or synchronized with diverse factors such as the photoperiod. The non-completion of any of the two phases (24 h of uninterrupted light) or changes in these rhythms can affect the welfare of fish and therefore the blood constituents, both inorganic and organic and hormonal (Pavlidis *et al*., 1997).

In this study, the glucose level increased in the second period with respect to the first period in all treatments. These higher levels could be related to a greater stress of the fish, regardless of the treatment. Elevated glucose levels in teleosts have been linked to a higher level of cortisol, so glucose is used as a secondary indicator of stress (Benfey and Biron, 2000). In addition, only the level of triglycerides showed significant differences between treatments, during the first period, where the lowest plasma concentration was seen in fish subjected to continuous light conditions and which also coincided with a lower number of *N. melleni*

eggs collected. In fish, the increase in the concentration of triglycerides in plasma has been related to the activation of lipid metabolism in response to stressors, such as parasites, which cause great energy demands (Montero *et al*., 1999).

Here, the daily release of eggs of *N. melleni* parasitising the greater amberjack took place in natural daylight hours, between 8:00 and 19:00, regardless of the photoperiod applied. In Australia, Hoai and Hutson (2014) observed a nocturnal egg emission rate of *Neobenedenia sp*. This different pattern of egg emission could be due to differences related to the parasite species, the area of study and/or factors other than the hours of light.

At present, the parameters to which the egg emission rhythm of the parasites is associated (Mooney *et al*., 2008) are uncertain. The different parameters that affect egg emission include temperature, age of the parasite, health of the host, immune status and breeding conditions such as light regime, frequency, feeding time and fish density (Jackson and Tinsley, 1988; Kohbara *et al*., 2003).Temperature greatly affects the life cycle of monogenean parasites (Ernst and Whittington 1996; Ernst *et al.,* 2005; Tubbs *et al*., 2005), and these effects have to be known and understood to develop effective strategies to interrupt their cycle. The reinfection of fish can be prevented if the environmental variables such as temperature and salinity are strategically coordinated.

The positive effects obtained of continuous darkness (0L:24D) on development and hatching rate of eggs of *N. melleni* corroborate the great success of parasitising greater amberjack kept in continuous darkness, while the increase in the number of hours of light reduces the egg viability and hatching, and as a consequence the possibilities of reinfection.

In addition, the higher temperatures increase the hatching rate of eggs of *N. melleni* and favors that it takes place in a shorter period of time, making the parasite's life cycle complete more quickly. A higher level of parasitosis and rapid reinfestation due to the increase in temperature has also been suggested in previous studies (Brazenor *et al*., 2015; Hirazawa *et al*., 2010).

Our present results show that the synchronization of low temperature and the increase of the hours of light significantly reduce the embryonic development of the parasite *N. melleni*, while an increase of temperature and the lack of light increases the success of the embryonic development and hatching making the parasite's life cycle complete more quickly. Taking into account these results, it is foreseeable that the life cycle of the parasite will be completed earlier in summer, increasing the success of parasite infection, than in winter, as has been mentioned in previous studies (Brazenor *et al*., 2015).

This study provides relevant knowledge about the biology of *N. melleni*, in relation to the effect that the combined variation of light and temperature has on the embryonic development and hatching of eggs, showing the best conditions that help to reduce the levels of parasitism in *S. dumerili*. Thus, photoperiod may be used to optimize the environmental strategies to control and reduce parasitosis in greater amberjack culture.

Regarding to the effects of anti-attachment methods, the present study shows the results obtained using different experimental methods to treat *S. dumerili* against the monogenean parasite *N. melleni* in order to control the parasite infestation in the commercial culture of this species. The mannose baths based on the specific attachments between parasite and host, had shown hopeful results in previous trials where an *in vitro* test on *S. dumerili* gills parasitized by *Z. seriolae* and incubated at a concentration of 0.25 M detached parasites in 2-5 min, without affecting tissue viability. Furthermore, Yoshinaga *et al*. (2000) showed that the binding of the oncomiracidia of *N. girellae* to the host is started by several carbohydrates. The work hypothesis for these mannose baths-trials was that this carbohydrate might occupy the binding sites for mannose of the parasite to the mannose-binding lectin (MBL) of the host, causing the detachment of the *N. melleni*.

Under the assayed conditions used, mannose did not cause the parasites to detache but could prevent the attachment of further individuals as the application of the treatment maintained the number of eggs constant until 15 days later, when the new oncomiracids were mature.

In our study, the number of eggs collected two days after applying the mannose treatments was reduced compared to those collected before applying the baths, with the greatest reduction seen with the lowest concentration of mannose (T2, 30mM). The fact that mannose baths did not show a clear effect on the number of eggs collected could be due to a low level of parasitosis. The number of eggs collected in this study (< 200 eggs per day) was much lower than that recorded in the previous assay (22050 ± 2460 eggs per day) (Salvador Jerez, personnel communication).

On the other hand, there was no enhanced effect on the number of eggs collected with further increase in mannose concentration. The lack of clearer effects of mannose baths could imply the existence of more carbohydrates other than mannose involved in the junction of *N. melleni* at the MBL of the host. In the rainbow trout, lectins have been detected to react with mannose, galactose and lactose, and these lectins were similar to those detected in the monogenean skin fluke *Gyrodactylus derjavini* that parasitized these fish (Buchmann, 2001; Buchmann and Lindenstrøm, 2002). In fact, lectins are known to be a diverse group of proteins that have selective affinity for a carbohydrate or carbohydrate group (Vázquez-Mendoza *et al.*, 2013).

Another possibility could be that the adherance of adult *N. melleni* to *S. dumerili* is not mediated by a single MBL, but involves a combination of several molecules. Ohashi *et al.* (2007) tried to clarify the mechanisms involved in the specificity of the *N. girellae* host through studying *in vitro*the binding capacities of the oncomiracidia to skin extracts of *Paralichthys olivaceus* (olive flounder) with and without mixing with 12 different lectins. The study determined that the lectins Con A (concanavalin A), WGA (wheat germ agglutinin) and PSA (*Pisum sativum* agglutinin) suppressed the binding capacity of the oncomiracidia of *N. girellae* to mucus extracts of skin, with Con A being the most effective. They showed that the substance responsible for the binding of the parasite to the mucus extracts of the host fish was a glycoprotein, of which there are many present in the mucus of teleost fishes. In addition, they indicated that the glycoproteins had side chains of sugar, as was seen by their affinity to Con A, WGA and PSA. The lectin Con A binds specifically to sugar chains high in α-mannose and α-glucose, and PSA also has specificity for α-mannose, thus both are MBL. By ion exchange chromatography the authors suggested that there could be two or more types of glycoproteins (lectin type) responsible for the binding of the oncomiracidia to the host fish.

As the present results were not conclusive, in future studies the parasite load of the fish should be increased to check whether this gives a clearer effect of the mannose, and also, if the effectiveness is greater at mannose doses lower than 30 mM which showed the greatest effect.

On the other hand, it would be necessary to perform more *in vitro* studies to determine the lectin/s present in the skin of *S. dumerili* involved in the binding to *N. melleni*, and if they present specificity for a single mannose-type carbohydrate or there are several involved. This would allow the developement of more effective immersion treatments or "baths" using the specific carbohydrates involved in binding to the lectin/s of the host fish.

The use of egg collectors has proved an efficient tool to allow both a closer control to the evaluation of infection success as well as for fish reinfection with *N. melleni* that may be applied in further challenge trials.

Fecal microbiota transplantation (FMT) is used to reinforce the immune system in terrestrial species, such as pig. Diao *et al.* (2016) tested this method in mice using different species of pigs as donors and concluded that the intestinal characteristics were transmissible throughout FMT. This technique has also been trialled in fish, mainly in zebrafish (*Danio rerio*) (Pham *et al.,* 2008) and more recently in *Nothobranchius furzeri* (Smith *et al.,* 2017). Based on this knowledge, not only the pig was taken as a donor but also gilthead seabream resistant to the parasite, in order to check the effect on *S. dumerili*. To this purpose, different indicators were determined, including the number of eggs emitted daily, the growth and condition of the cultivated specimens, their hematological and biochemical parameters indicators of animal welfare, as well as some biomarkers of functional integrity of the osmoregulatory epithelium, including enterocytes and gill cells.

No immediate effect was recorded after the application of the different FMT treatments on the number of eggs of the *N. melleni* parasite collected during the experimental period. A significant decrease was found on

day 15 and 21 after applying all of the treatments. This behavior could be due to the life cycle of the parasite, with a duration of 12-17 days as previously has been mentioned by Bondad Reantaso *et al*. (1995) and Kishimori *et al*. (2015). In addition, the variation in the number of eggs recorded on days 5, 12, 19 and 26 post transfection in all treatments coincides with the duration of embryonic development of 7 and 8 days at 20 and 18 ºC respectively (Hutson *et al*., 2012), temperatures similar to those recorded in this study (19.1 ± 0.3ºC).

The parasitosis can cause physiological disorders and nutritional stress, negatively affecting the growth, survival and even reproduction of the host (Barber *et al*., 2000; Gauldie and Jones, 2000). In our study, the SGR were similar in all treatments and lower than in non parasitised fish of similar weight range (0.51±0.03 % day^{1}) cited by Tomás *et al*. (2005). The feed intake was also unaffected and similar in all fish group treatments.

The negative effects of parasitism may be apparent in hematological and plasma parameters of the host. The study of these parameters can provide information regarding the health status of the fish (Bahmani and Kazemi, 2001; De Pedro *et al.*, 2005). Anemia is frequently one of the negative effects of parasitosis. Hematological parameters such as hematocrit, number of red cells and their relation, the mean corpuscular volume (MCV), provide reliable information on metabolic disorders, deficiencies and chronic stress state, allowing the diagnosis of possible anemia due to parasitosis (Bahmani and Kazemi, 2001). Previous studies in *S. dumerili* have shown that high levels of *N. girellae* cause a significant decrease in hematocrit with respect to non-parasitized fish due to skin hemorrhages (Hirazawa *et al.*, 2016). In our study, the hematocrit and number of red cells remained stable in all treatments. However, the mean corpuscular volume (MCV) decreased over time in the control and transfection group given feces of warthog (T2), while in the transfection treatments with gilthead seabream feces with and without a previous dose of antibiotic (T3 and T4) the opposite trend was observed. The increase of this parameter in these two treatments, T3 and T4, could be related to a better response of fish to anemia by manufacturing more red cells.

The number of white blood cells can also be affected by the parasites. However, there are not enough studies that show a complete picture of this indicator in fish blood (Tort *et al*., 2005). In the present work, a slight decrease in the number of white blood cells was observed in all treatments throughout the experimental period. In general, these values are much lower than those obtained by Armuelles (2016) in *Seriola lalandi* with leukopenia (3.72-4.09 106 cells μl^{-1}), but perhaps indicate that our experimental fish had a depressed immune system due to at high parasite load. Immunosuppression is a common effect resulting from a chronic or repetitive stress situation, such as a parasitosis, over time (Barandica and Tort, 2008; Barton, 1997).

In fish rearing, many factors including parasite infections can activate endogenous stress systems and induce changes in the physiology and metabolic responses of fish (Barton, 2002; Vargas-Chacoff *et al*., 2017). To identify these possible stress states, primary response indicators, such as catecholamine and cortisol levels, which induce rapid changes at the cardiovascular and metabolic levels are analyzed (Tort *et al.*, 2005). However, metabolic indices of the secondary response, such as glucose, lactate and plasma ions, also provide information on the level of stress in reared fish (Rotllant and Tort, 1997; Rotllant, *et al.*, 2000).

Fish mobilize substrates for the energy demand that takes place in cellular processes (Wells *et al.*, 2006). In particular, teleost fishes exposed to parasitic stress can mobilize energetic metabolites to modulate immune responses against pathogens (Barton, 2002), with glucose and lactate being the most important. Stress can contribute to the increase of glucose levels obtained from glycogen stores. In addition, plasma lactate levels generally increase in stressed fish, mainly if the stressor requires more energy or causes a decrease in oxygen availability, since the anaerobic lactate production pathway is activated (Grutter *et al*., 2000; Thomas *et al*., 1999). For greater energy demands, fish can activate lipid and protein metabolism, mobilizing triglycerides, proteins and amino acids among other substrates (Montero *et al*., 1999).

Infection of Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) by the copepod *Caligus rogercresseyi* caused an increase in plasma glucose levels in the first day post-infection, but as the parasitic load increased, glucose levels decreased and plasma proteins, amino acids, triglycerides and lactate levels increased as a consequence of the chronic energy demand (González *et al*., 2015; Vargas-Chacoff *et al.*, 2017). Here, glucose levels were similar in all treatments tested, and did not vary throughout the

experimental period, while plasma lactate increased from day 7 post-transfection in all groups. These higher levels of lactate in greater amberjack could be due to the strong parasitism by *N. melleni* that could be causing anemia, reducing the number of red blood cells and with it the capacity to supply oxygen to the body, which would lead to the activation of anaerobic metabolism and the consequent production of lactate (Wells and Pankhurst, 1999).

Lipid metabolism could also be activated as a consequence of this chronic energy demand, since the plasma levels of triglycerides and cholesterol recorded on day 34 post-transfection were higher than those recorded on day 7, being significantly increased in the case of triglycerides. The above is supported by the fact that the plasma triglyceride level correlated with the number of *N. melleni* eggs collected every 24 h.

The parasitosis caused an imbalance of ions in the host that varied throughout the study. However, the plasma levels of sodium and potassium at the end of the trial were similar to the initial ones, while the chloride levels were similar to those of day 7.

Skin flukes such as *N. girellae* feed mainly on the epithelial cells (Hirayama *et al.*, 2009) causing an osmotic imbalance in the host (Sato *et al.*, 2008). In a recent study on the incidence of this parasite on *S. dumerili*, significantly higher plasma levels of sodium were obtained in groups of severely infected fish with respect to the control groups, while potassium levels were similar (Hirazawa *et al.*, 2016). The parasite load in this study could be the reason why no differences in plasma ions were detected. However this apparent imbalance of plasma ions seems to be caused by parasite-induced skin injuries since both the gut and gill osmoregulatory epithelium seemed to be in good condition in the infested fish. This is evident by both the high viability of isolated cells and the consistent activities of Na^{+}-K^{+} ATPase and gut digestive enzymes.

A higher number of *N. melleni* adults detached per fish surface was obtained in fish of T3 (transfection with seabream feces), coinciding with a higher number of eggs per day collected (8592 ± 6227 eggs). A previous study in greater amberjack showed similar values for number of parasites of *N. girellae* per cm^2 of fish. However, the size of the parasites was larger than the size of *N. melleni* obtained in this study. In addition, the size of parasites was significantly lower in fish highly parasitized compared to those from the least parasitized fish, perhaps relating to a lower availability of skin to feed on (Hirayama *et al.*, 2009).

The site preference of *N. melleni* on greater amberjack is in accordance with previous studies where the eyes and the area above the lateral line are preferable sites for *N. girellae* on greater amberjack (Hirayama *et al.*, 2009).

In humans affected by the bacterium *Clostridium difficile* (CDI), the fecal microbiota of the receptor before and after FMT was also evaluated. Following sequencing of the RNA gene, it was shown the patient's fecal matter was surprisingly similar to that of the donor after the transplant, suggesting that the donor's stool had helped to restore a healthy microbiome to the colon (Owens *et al.*, 2013). The objective of the transfection was to alter the microbial flora of fish in order to improve the immune response that would allow the fish to defend itself from the parasites. The overall results do not seem to show significant effects on *S. dumerili* parasitism by *N. melleni*. However, the treatments did not adversely affect the growth and survival of the farmed fish. It is possible that the duration of the trial and the level of parasitism did not give an opportunity to reach conclusive results, because taking into account the results obtained in the trial to study photoperiod on greater amberjack parasitism by *N. melleni*, mortality started after 34 days subjected to the worst conditions (0L:24D), when more than 20,000 eggs per day were collected. On the other hand, we cannot conclude that transfection caused differences in the microbial flora of the fish although transfection with *S. aurata* feces seemed to increase the alkaline protease activities.

A study applying FMT in the fish *Nothobranchius furzeri* showed successful results when comparing intestinal microbiota communities through isolation and sequencing techniques of genetic material (Smith e*t al.*, 2017). For this reason, both intact digestive tracts and corresponding feces were also sampled from all treatments and are currently being analysed.

Another possible improvement of the transfection experiment would be to perform treatments with different amounts of feces, that is, to increase the ratio of feces / glycerol buffer in the suspension used to inoculate, since the amount of feces could have been insufficient to allow the installation of the microbial communities

of the donor. Indeed, in a study of FMT carried out in people with CDI (Gough *et al*., 2011; Owens *et al*., 2013) it was observed that patients who received less than 50 g of feces had four times the relapse rate of those who were inoculated with a higher amount of stool.

Therefore, due to the novel nature of these alternative techniques of control and reinforcement of the immune system, future studies are necessary to continue optimizing the effective defense methods against parasites such as *N. melleni* that are causing serious problems in aquaculture.

4. Conclusions

The continuous light conditions (24L:0D) did not improve the growth performance of greater amberjack, but negatively affected the number of eggs produced by *N. melleni*, which was correlated to a lower fish parasite load. However, the difference in the photoperiod is not the only factor that seems to influence the rhythm of egg release of the *N. melleni* parasite. The embryo development and hatching of *N. melleni* was increased in dark conditions and high temperature, increasing the reinfection success of the parasite.

A concentration of 30 mM mannose was the most effective dose tested in the "baths" applied to greater amberjack parasitized with *N. melleni*, in trying to reduce the number of eggs emitted by the parasite.

The use of mesh dish devices for the evaluation of the level of infestation and reinfection of greater amberjack with *N. melleni* gave highly satisfactory results. This device allowed the application of control measures or to increase the number of eggs emitted per day by more than 20 times over a period of two weeks, in the challenge trials.

Fecal microbiota transplantation (FMT) as a method of improving the immune system of greater amberjack had no immediate effects on the number of eggs emitted by *N. melleni* in any of the treatments tested. However, the increase of the mean corpuscular volume (VCM) in the treatment group undergoing transfection with gilthead seabream feces with previous dose of antibiotic, and the gut alkaline protease activity could indicate a decrease in the anemic state of the parasitized fish and modulation of gut microbiota.

The high levels of lactate, triglycerides and plasma cholesterol obtained regardless of the treatment used, suggest a possible activation of lipid metabolism in infected specimens as a consequence of the stress associated with parasitosis by *N. melleni*.

The significant reduction of triglycerides and plasma cholesterol on the seventh day post transfection with feces from gilthead seabream previously treated with antibiotic could indicate a degree of improvement in the regulation of lipid metabolism due to infection by *N. melleni*.

5. General considerations and recommendations

The infestation with skin and gill monogeneans such as *N. melleni* and *Z. seriolae*, respectively, is an important problem that affects negatively the development of industrial rearing of greater amberjack. These monogeneans have a direct life cycle and produce free eggs that often become entangled in nearby surfaces - such as culture tanks or sea cage nets- leading to high re-infection rates amongst fish, in a relatively short period of time. Fluke infections require regular monitoring to estimate the parasite load in the culture facilities and timing of the application of treatments in accordance with the optimal period regarding the parasite development stage. In addition, the fluke life cycle proceeds rapidly when the environmental conditions such as sea water temperature and photoperiod are optimal, so it is critical to obtain the parasite data over time, to interpret and act accordingly.

Taking the above into account, the results from this project can aid management practices for monitoring parasite prevalence in the following ways:

- To detect the number of eggs of *N. melleni* and *Z. seriolae* and to estimate the level of parasitism of reared fish, a simple submerged mesh disc system to which the eggs adhere, can be used. The weekly monitoring of the number of eggs of the parasites with this system is sufficient to estimate the level of parasitosis.
- Taking into account the time necessary between the release of the egg and the sexual maturation of the parasite, it would be possible that monitoring every two or three weeks will provide reliable information. This could be dependent upon the synchronization of the parasite population and the environmental conditions.
- In *N. melleni*, the embryo development of the egg and the percentage of hatching are favored by high temperatures and photoperiod of a few hours of light. The periods of the year in which this occurs would be the most critical times for a more exhaustive control.
- The time of immersion of the egg collector device must be at least 24 h, since the rate of egg emission can be modified with environmental conditions. If the surface of the culture facilities is covered by natural or artificial artefacts that favor the adhesion of the eggs, the estimation of the population of parasites with the discs is completely different to a situation where the surface is clear.

Simple and routine operations such as the management of the fish in culture facilities can favor the parasite infection of healthy specimens through the sampling nets because the mucus and adult parasites stay attached to net and infect healthy fish captured with the same equipment if it is not properly cleaned previously.

A series of general rules should be adopted to minimize the risk of parasite transmission and spread in the facility:

- do not exchange or mix water, equipment and instruments among sectors in the facility
- establish a routine for daily and weekly cleaning procedures to ensure that all equipment and materials are kept under the best hygienic conditions
- select equipment and instruments and establish working procedures for their use

The use of baths of mannose at concentrations between 30 and 70 mM for the inhibition of the binding capacity of monogenean parasites could be used without negative effects on fish. The baths with a mannose concentration of 30 mM caused a decrease in the number of eggs of *N. melleni* emitted. The effectiveness of these baths in *Z. seriolae* could be increased, so that the use of these baths has greater impact as an anti-attachment substance. However, further research is needed.

Fecal microbiota transplantation (FMT) can be a suitable method to modify the intestinal microbiota and strengthen the immune system of greater amberjack. Microbiota from the gut and feces taken during the trials are currently under analysis.

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Deviations: In the description of this task, stocking density would be a parameter to test. However, the monitoring of parasites made in previous grow out trials showed no effects on parasite infection, at least under our culture conditions and with *Nobenedenia melleni*. On the other hand, the presence of *Zeuxapta seriolae* was more limited in the time than *N. melleni*.

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