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Transmission and maintenance of *Sparicotyle chrysophrii* infection in gilthead sea bream (*Sparus aurata*) using a recirculating aquatic system

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1. Introduction

1.1 Aquaculture in the European and global framework

The world faces the formidable challenge of feeding the rapidly growing human population. Vegetable and animal productivities have to improve enormously to keep pace with this growth, and aquaculture is one of the most promising ones, since it remains one of the fastest-growing food-producing sectors and now provides more than half of all fish for human food. In fact, in 2015 aquaculture has surpassed wild fisheries, reaching 53.1% of the fish production worldwide (FAO, 2015). This contribution is projected to increase to 62% by 2030 (FAO, 2014) in front of a non-sustainable overexploitation of fisheries that has led to a depletion of natural stocks and biodiversity in the oceans during the modern ages (Swaan,1992; Hilborn et al., 2003).

The main farmed fish species produced in the EU-28 are by value: Atlantic salmon, rainbow trout, gilthead sea bream, European sea bass, and common carp (JRC, 2013). Europe is also the main world producer of turbot (besides China), with a yearly production around 11,000 t. Europe is the largest market for fish and the highest importer of fish products in the world (JRC, 2013), and the aquaculture industry serves to fill this gap between production and consumption. This sector is also important because it provides quality food and food security, and jobs and economic growth in rural and coastal areas, these areas often being threatened by forces causing migration to urban areas. The EU aquaculture sector gave direct employment to more than 80,000 people in Europe in 2011(JRC, 2013).

1.2 Spanish and European gilthead sea bream production

Spain is one of the leading Mediterranean countries in the European marine fish farming, producing mainly gilthead sea bream (*Sparus aurata*), sole (*Solea senegalensis*), European sea bass (*Dicentrarchus labrax*), meagre (*Agyrosomus regius*), turbot (*Scophthalmus maximus*) and tuna (*Thunnus thynnus*), which are among the most demanded fish at the European and Spanish markets. The culture of gilthead sea bream (GSB) in Europe is well developed and extended to many European countries. The total production of European GSB during 2016 was estimated in 195,853 tons that generated 1,116 million of euros in economic terms. The principal Mediterranean GSB producers are: Turkey with 67,612 tons, Greece with 59,000 tons, Egypt with 17,000 tons and Spain with 13,740 tons (APROMAR, 2017). During 2017, 17,800 tons of GSB were produced in Spain, with a small increase in comparison to the previous year. The Spanish region leading GSB production is the Valencian Community with 5,619 tons followed by Murcia with 3,368 tons, Canary Islands with 2,492 tons, Andalucía with 1,605 tons and Catalonia with 730 tons (APROMAR., 2017) (Fig.1)

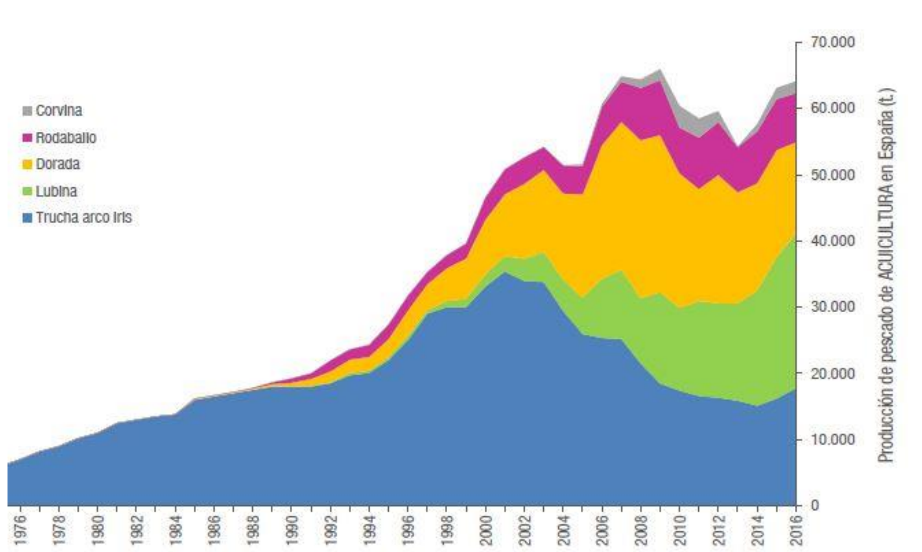

Figure 1. Graphic showing the evolution of Spanish finfish aquaculture production from 1976 to 2016 (APROMAR, 2017).

1.3 Pathological constrains of aquaculture: parasitic diseases in focus

The two main limiting factors of modern aquaculture are the high costs of raw materials to produce aquafeeds and diseases.

During the last decades, the massive increase of aquaculture production has been due in part to a dramatic increase of farming densities and massive intensive production methods. These procedures have also increased diseases, that when handled improperly they can evolve into unmanageable proportions and contribute to economic losses (Lafferty et al., 2015). The impact of pathogens on aquaculture is substantial; the financial losses are estimated to be roughly 20% of the total production value. Diseases due to bacteria and virus have been studied during decades and some of them are relatively under control by vaccination and antibiotics. However, parasitic diseases have received less attention and less funding to be studied so intensively and are far from being controlled in fish farms

It is estimated that the world annual grow-out loss due to parasites in finfish farming ranges from 1% to 10% of harvest size, with an annual cost of $1.05 to $9.58 billion (Shinn et al., 2015). Although data on the economic impact of parasites in aquaculture is scarce, the impact of some parasite species is well documented. The highest economic costs for parasite control in European aquaculture accrue to sea lice infecting Atlantic salmon(Costello et al., European nations and associates spend 170 M€ annually to control sea lice, with annual global losses estimated to exceed 300 M€(Costello.,2009).Proliferative kidney disease caused by *Tetracapsuloides bryosalmonae* continues to be a major production constraint for the rainbow trout industry in Western Europe. The disease has been estimated to cost the UK trout industry approximately 3 M€ per year (Okamura et al.,2011) with mortalities ranging between 20-100% depending on water temperature. Amoebic gill disease caused by *Paramoeba perurans* resulted in more than 36 M€ in lost revenue in Scottish salmon farms during 2012 and forced earlier harvesting times to avoid further losses (The Herald Scotland).

The myxozoan *E. leei*, has arrested the industrial production of some promising new aquaculture species, such as sharpsnout sea bream (*Diplodus puntazzo*) and *E. scophthalmi* and *P. dicentrarchi* have forced the closing of several inland turbot farms (Sitjá-Bobadilla A and Palenzuela O., 2012)(Piazzon et al., 2014) *Saprolegnia* infections in salmon are responsible for at least 10% losses in salmon farming annually(Van der Berg et al., 2013) ,but also produce losses in other hosts. *Ichthyophthirius multifiliis* produces severe morbidity and mortality in European rainbow trout and carp farms, with estimated annual losses around 102 M€. European sea bass parasites like *Lernathropus kroyeri* and *Caligus* species cause delays of approximately two months to reach commercial size.

In the Mediterranean, parasitic diseases produce economical loses that in most cases are not quantified. These loses are due not only to direct mortality, but also to morbidity, poor growth performance, low reproduction efficacy, increased susceptibility to other diseases, high cost of treatments and decreased value or marketability and lifespan of fish products (Sitjà-Bobadilla, 2010). The current study is focused on one of the main parasitic diseases that affect GSB production in sea cages, sparicotylosis, which is widely established in the Mediterranean basin. This type of production can favour the presence and spreading of certain parasites because of different factors:

- Water quality cannot be controlled, thus changes in water conditions (temperature, salinity, turbidity, etc.) and access of enzootic parasites are hard to control in the cage environment.
- The introduction of foreign animals from different production sites can lead also to stablish new exotic parasitic infections, because the outsiders can carry different forms and parasitic stages.
- Some parasite stages benefit of the existence of biofouling on the nets, by developing part of their cycles on them and therefore having easy and convenient access the fish within the cages.
- Fish within sea cages are exposed to natural reservoirs of parasites, such as some wild fish that are usually swimming around the cages or even can enter the cages, attracted by the food.
- Parasitic cycles can be maintained or favoured by the emission of fish faeces, skin and intestinal mucus or carcasses that can facilitate the transmission both within the farmed fish and to wild fish.
- Pharmacological treatments are very expensive and difficult in terms of logistics because of the large size of farms and the increasing diameter of sea cages. There is also a concern on the possible pollution impact of the chemicals that are used to control parasitic diseases.

Currently, fish diseases are regulated by the European directive 2006/88/CE and in Spain by the transposed Royal Decree of 1614/2008 published at BOE 242/2008, but nowadays there are no fish parasites included in any of the public mandatory declarations for fish health. In terms of parasites, the only law to be applied is the European directive 93/140/EC, in which it is stated the main actions to be done when fish specimens are carrying high numbers of parasites or when their meat is deteriorated, that involves the removal from market.

The future actions that will improve parasitological management are:

- Increase of the knowledge of the parasites cycles and hosts interaction, genetic characteristics and phylogeny of parasites.
- Development of vaccines and treatments against parasites.
- Establishment of *in vitro* assays and experimental infections in closed system to provide a continuous source of parasitic stages for research.
- Improvement of the preventive strategies and periodic controls, screening of infections.
- Establishment of periodical samplings (including hematologic analysis), early diagnosis and predictive modelling.
- Determine fish genes involved in resistance to the pathogens, which can be used to select fish strains.

1.4 The gilthead sea bream case: impact of diseases, parasites and ectoparasites

Bacterial, viral, parasitic and other non-infectious diseases affect GSB production under different types of production. Colorni & Padrós (2010) recently reviewed the main pathological problems for this species (Table 1).

Viral diseases
• Lymphocystis disease (LCD) • Red sea bream iridovirus disease (RSID) • Viral encephalopathy and retinopathy
Bacterial diseases
• Vibriosis • Photobacteriosis *Photobacterium damselae* ssp.*piscicida* *Photobacterium damselae* ssp. *damselae* • Infections by *Pseudomonas anguilliseptica* • Infections by *Tenacibaculum* spp. • Streptococcosis • Staphylococcosis • Mycobacteriosis • Infections by Epitheliocystis agent
Fungal diseases
• Ichthyophonosis • Ochroconis
Non infectious diseases and multifactorial diseases
• Systemic granuloma • Winter syndrome • Petechial rash • Neoplasia • Larval enteropathy • Larval “White Stripe Disease”

Table 1. The most important non-parasitic diseases affecting GSB culture. Data from Colorni & Padrós, 2010.

Among the parasites that affect GSB culture there are both ecto and endoparasites (Table 2). Endoparasites are those that live in the tissues and internal cavities. They can be found in all the fish organs and tissues. Table 1 summarizes the most important ones.

	Parasite group	Species	Affected tissue	Reference
ENDOPARASITES	Platyhelmintha	Digenea: *Cardicola aurata*	Blood vessels, Gi, Kd	Holzer et al. (2008) Padrós et al. (2001b)
	Myxozoa	*Ceratomyxa sparusaurati*	GB, Int	Sitjà-Bobadilla et al. (1995)
		Enteromyxum leei	Int	Diamant et al. (1994)
		Henneguya sp.	Gi, Heart	Caffara et al. (2003)
		Kudoa iwatai	Mu (Syst)	Diamant et al. (2005)
		Leptotheca sparidarum	Kd	Sitjà-Bobadilla and Álvarez-Pellitero (2001)
		Polysporoplasma sparis	Kd	Sitjà-Bobadilla and Álvarez-Pellitero (1995)
	Microsporea	*Pleistophora* sp.	Mu	Abela et al. (1996), Athanassopoulou (1998)
		Glugea sp.	Sk, Mu (Int)	Mathieu-Daude et al. (1992)
		Enterospora sp.	Int	Palenzuela et al. (2011)
	Apicomplexa	*Cryptosporidium molnari*	Sto, Int	Álvarez-Pellitero and Sitjà-Bobadilla (2002)
		Eimeria sparis	Int	Sitjà-Bobadilla et al. (1996)
		Goussia sparis	Int	Sitjà-Bobadilla et al. (1996)

Table 2. Summary of the main endoparasites of gilthead sea bream. Abbreviations: gills (Gi), skin (Sk), mouth cavity (MC), kidney (Kd), muscle (Mu), intestine (Int), stomach (Sto), systemic (syst). Taken from Estensoro, 2013.

Ectoparasites are those that live in/on the skin, gills or the oral cavity. They are mainly by protozoans, crustaceans and monogeneans. Some of them are true pathogens and other have an opportunistic lifestyle. Most of them have direct cycles or include stages developing in organisms found in the biological fouling of the cages, thus the transmission and prevalence of these parasites in the cages can be very high and can have a fast spreading (Sitjà-Bobadilla, 2010). Some ectoparasites are easy to diagnose by magnification lenses or stereomicroscope, or by direct observation of fresh gills, skin or mucus smears at the microscope. Although some of them are extensively studied, the knowledge of life cycle and the details of the intermediate hosts and the dynamics of the infections are quite limited for others. The main ectoparasites of GSB are summarized in Table 3. The protozoans are represented by ciliates and flagellates and the metazoans by worms and crustaceans. The current work is focused on the monogenean worm *Sparicotyle chrysophrii.*

	PARASITE GROUP	SPECIES	AFFECTED TISSUE	REFERENCE
ECTOPARASITES	Ciliophora	*Cryptocaryon irritans*	Gi, Sk, Eye	Colorni (1985)
		Trichodina spp. (peritrich ciliate)	Gi, Sk	Álvarez-Pellitero et al. (1995)
		Scuticociliates	Wounds, Gi, MC (Syst)	Fioravanti et al. (2006)
		Porpostoma notatum (scuticociliate)	Sk	Paperna (1984)
		Brooklynella hostilis	Gi, Sk	Diamant (1998a)
	Flagellata	Mastigophora: *Amylodinium ocellatum*	Gi, Sk	Paperna (1980)
		Kinetoplastida: *Ichthyobodo sp.*	Sk, Gi	Álvarez-Pellitero et al. (1995)
		Cryptobia spp.	Gi	Blanc et al. (1989) Diamant (1990)
	Platyhelmintha	Monogenea: *Sparicotyle chrysophrii*	Gi	Faisal and Imam (1990)
		Furnestinia echeneis	Gi	Reversat et al. (1992)
		Gyrodactylus sp.	Body surface	Paladini et al. (2009) Paladini et al. (2011)
		Neobenedenia melleni	Body surface	Colorni (1994)
	Crustacea	Copepoda: *Caligus minimus*	MC	Paperna (1984)
		Ergasilus spp.	Gi	Fioravanti et al. (2006) Dezfuli et al. (2011)
		Isopoda: *Ceratothoa oestroides*	MC, Gi	Mladineo (2003a)
		Ceratothoa parallela	MC, Gi	Papapanagiotou and Trilles (2001)

Table 3. Summary of the main ectoparasites of gilthead sea bream. Abbreviations: gills (Gi), skin (Sk), mouth cavity (MC), systemic (syst). Taken from Estensoro, 2013.

1.5 An helminthic thread for gilthead sea bream: *Sparicotyle chrysophrii*

The Phylum Platyhelminthes (flatworms) is a major subdivision of the Animal Kingdom. Many flatworms are free-living, but they also comprise three groups with parasitic life styles. It includes the Cestoda (tapeworms), the Digenea (flukes) and the Monogenea. The last group is mostly restricted to the skin and gills of marine and freshwater fish and has relatively simple life cycles, lacking intermediate hosts. Depending on the configuration of anchoring apparatus or opistohaptor, the mogenean taxonomy further splits in Monopisthocotylea (MONO) and Polyopistocothylea (POLY), which is highly adapted to the morphology and physiology of their hosts. In the case of blood-feeding polyopisthocotylean monogeneans, they are too large to fit between secondary lamellae. They have acquired remarkable clamp-like organs which are capable of gripping one or two secondary gill lamellae. The two opposing jaws of each clamp are supported by a framework of hard sclerites.

Monogeneans produce important diseases in the cage-cultured fish species in Europe and worldwide, that can also produce serious economic loses and difficulties in aquaculture. The most important ones in Europe are depicted below:

- *Gyrodactylus salaris* (MONO): it is a freshwater parasite that reaches the highest levels of infections in fluvial ecosystems, but it can also be found in sea water or brackish water. Gyrodactiliosis is one of the most dangerous parasitic infections in freshwater salmonids and it is well spread around the north of Europe where the large salmon producers are located. The mortalities are mostly suffered by fingerings and juveniles. There are some different strains of *Gyrodactylus salaris* with different pathogenicity and also resistant populations of fishes that can be found in the parasite environment (Sterud et al., 2002). This parasite was included in the first version of the European directive 2006/88/CE regulating diseases in aquaculture.
- *Diplectanum aequans* (MONO): it infects European sea bass gills and is widely spread among Mediterranean and Atlantic areas coinciding with the distribution of its host. The highest impact is on sea bass juveniles and broodstock (Gonzalez-Lanza et al., 1991). In Italy, during 2006 the losses attributed to this parasite reached 110-150 tons valued in 1.85 million dollars (Shinn, et al 2015).
- *Furnestinia echeneis* (MONO): it can be found in the gills of GSB around the Mediterranean basin. This parasite is not considered very pathogenic but it can increase its impact when present with *Sparicotyle* in coinfections, a very common occurrence.
- *Zeuxapta seriolae* (POLY): it infects the greater amberjack (*Seriola dumerili*) and it is associated with very high mortalities in aquaculture settings. The pathogenicity of this infection is very severe as it spreads and grows very fast, being one of the biggest problems in the domestication of these species in the Mediterranean basin (Montero, et al 2004). In Australia, during 2003 the loss of production was 39 tons, and an economic cost of 0.53 million dollars (Shinn et al., 2015).
- *Hexostoma thynni* (POLY): it affects the gills of the most valuable fish worldwide, bluefin tuna (*Thunnus thynnus*). The infections rarely end in fish death but they can affect the condition factor and fattening of the fish with serious impact on the market value (Nowak B., et al 2006).

- *Sciaenacotyle panceri* (POLY): it affects the meagre (*Argyrosomus regius*) in a similar way to *Sparicotyle* in GSB, provoking anaemia and mortality episodes at the sea cages (Merella et al., 2009).

In the Mediterranean basin, the most important and dangerous ectoparasite for GSB production is the helminth *Sparicotyle chrysophri*, that blooms during the Spring-Summer period, being responsible for serious economical loses in sea cages. This monogenean parasite can result in mortality of up to 15% of the cage and even total losses if the cage is not treated (Alvarez-
This polyopistocothylid was first described more than a century ago (Van Beneden & Hesse, 1863). The adult stage length ranges between 0.5 and 0.7 mm, it has a transparent body, two pairs of eyes and the vitellaria are visible since the juvenile stage. It has an anchor of 60/72 pairs of pins (Van Beneden and Hesse, 1863) that attaches to the gill lamellae of the host. Every adult produces about 20 eggs that are released as a bundle (Repullés-Albelda et al., 2012). The hatching time after deposition varies from 5 to 10 days. After hatching, free living oncomiracidia can survive up to 52 hours before finding a fish to attach to. When the host is found, the post-larvae stages start feeding, and, depending on temperature, in about 5 days they will develop into juvenile with the first visible vitellaria. Adult size is reached after at least 26 days (Repullés-Albelda et al., 2011).

S. chrysophrii anchors itself by clamps and suckers to the gills and lamellae producing significant direct damage, typically erosive lesions, fusion of lamellae or hypertrophies/hypotrophies of the gills with necrosis and haemorrhages. The infection also produces tissue loss and malfunction of the gas exchange surfaces. The adults are the most harmful; as they are hematophagous and they can thrive in severe anaemia (Sitjà-Bobadilla & Alvarez Pellitero, 2009). Due to this, heavily infected fish usually show lethargy and slow swimming, pale-coloured or white gills, weight loss and low visceral fat among other clinical signs. *Sparicotyle* infection also facilitates co-infections, normally by bacteria or virus that takes advantage of the weakened status to produce a new infection that can aggravate the health status of the fishes (Mladineo, I. 2018). This helminthic parasite is not zoonotic, but it impacts the consumer's perception of quality in commercial fishes with pale gills or reduced product lifespan.

2. Objectives

The purpose of the current work is to establish an experimental model of *Sparicotyle* infection in GSB held in captivity, using the effluent of water from tanks holding naturally infected individuals, within a closed water-recirculating system (RAS). Additional objectives of the study are also to get insights on the infection dynamics and how the infection impact on growth performance and haematology. The final aim is to continuously maintain *in vivo* the infection for research purposes, such as the possible development of vaccine and oral or bath treatments. Hence, closing the life cycle of this parasite in captivity can provide an invaluable resource for many biological fields, such as phylogenetics, genomics, transcriptomics, chemotherapeutics, etc. These research lines are currently constrained by the dependence on natural (heterogeneous and unpredictable) infections by this parasite.

The maintenance of the infection inside a RAS allows having the ability to screen, monitor and control different water parameters such as temperature, flow, oxygen and other important aspects related with the infection. The RAS also allows keeping the infection isolated from the rest of the tanks of the facility avoiding a possible unwanted infection.

3. Materials and Methods

3.1- Design, setup and maintenance of RAS for effluent infection

The experimental design of the RAS was developed at the IATS-CSIC facilities in the pathology building, using tanks located within the quarantine area. The inflow water in the Pathology Unit comes from the general seawater pumped on shore pump with a flow of 20 L/m. The outflow water was disinfected by high chloramine concentrations before going to the main waste water effluent.

The main RAS setup is composed by:

- A monophasic Astral pool sucking pump with a flow of 8 m^3/h with a power of 0.5 HP and a renovation flow of 50 L/h.
- Two modular “CANTABRIC” filters of Astral pool with a flow of 6 m^3/h, one of them was filled with bio-balls and the other with sand. The later was by-passed in order to avoid loss of parasite free-living stages on the main system that could be trapped in the sand bed. The filters were cleaned and rinse functions with water from outside the system.
- Two fibre-glass tanks of 500 L, used as donor tanks.
- One main reservoir tank of 250 L.
- Three recipient tanks of 90L, used as recipient tanks
- Six aeration flutes.
- One UV sterilizer lamp.

Additional material used in the experimental trials was:

- Fishing nets identified for each different infection condition of the fish
- Oximeter (OxyGuard): calibrated every 15 days.
- Red Sea Marine Care water test for water parameters(NH3/Nh4,No3,pH,KH)
- Maintenance and cleaning material (broom, scrubbers, etc.)
- Disinfection liquid (Bactocide).

The RAS setup was designed in order to easily control the infection and standardize the infection maintenance in captivity. It also attempted to adapt to the generation, development and survival of the parasite larvae as previously described (Repullés-Albelda et al., 2011), removing the granular filter to enhance the chance of post larvae infection. For this same reason, the UV sterilizer was off during the trials. Water salinity was 37.5 ‰ and temperature follow natural fluctuations along the trial, never higher than 28ºC. The oxygen saturation content was higher than 85% during the whole trial. The renovation and replacement of cleaning water were the main sources of new water for the RAS.

Fig. 2 describes the flow of water in the RAS setup, in which the water from donor tanks (D) and Fig. 3 shows a RAS similar to the one used for the experimental trial.

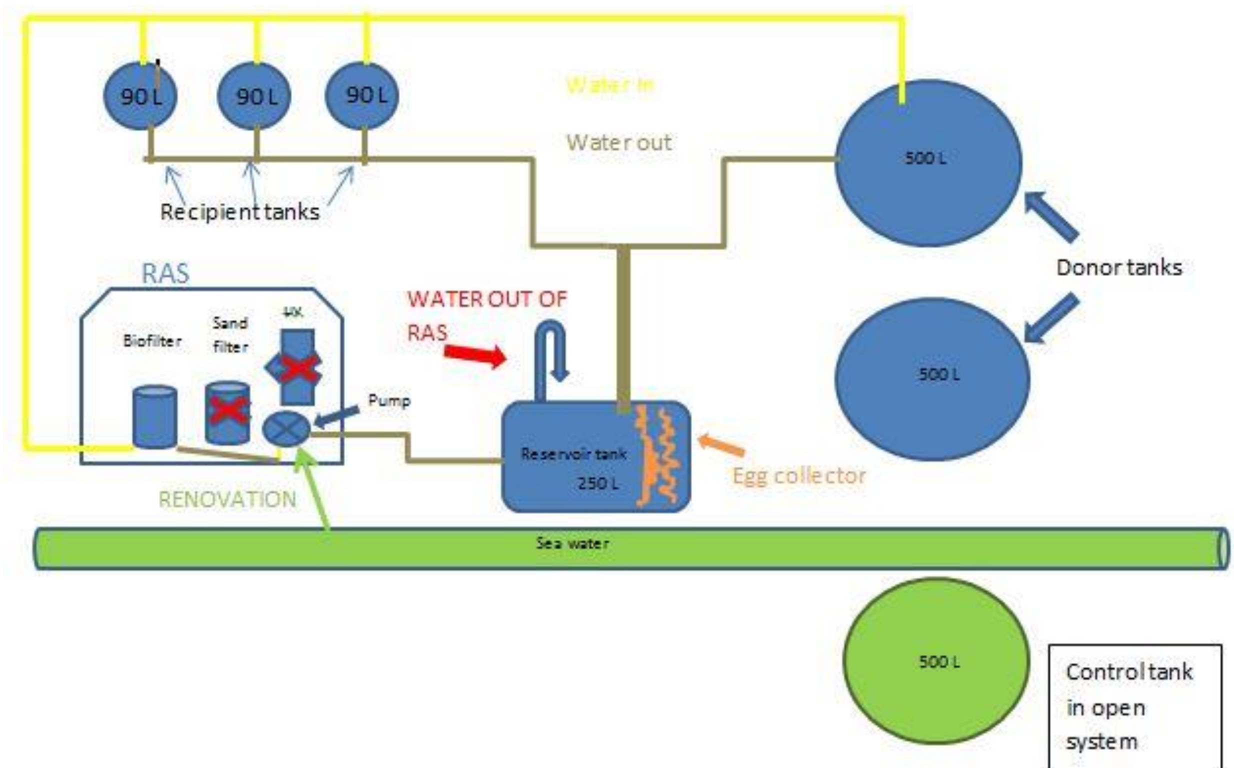

Figure 2. Schematical drawing of the RAS set up for the effluent infection trial.

Figure 3. RAS model used for the *Sparicotyle* experimental trial at IATS.

The daily maintenance routine consisted of the measurement of water NH3/NH4, oxygen content and temperature. A daily check of the system was carried out and cleaning of the main reservoir tanks was made when needed. The flow and the level of water of the pump where checked every Friday and the cleaning of the filters was done every ten days if there were not issues. After a first week of testing of the RAS with a low number of naïve healthy fish, naturally infected fish were entered in the donor tanks and the cleaning frequency increased due to the higher amount of organic waste. The fish tanks were cleaned once a week. Carcasses of death fish were removed rapidly to minimize pollution of the water in the RAS.

An experimental egg collector was designed to mimic the dynamics seen in the sea cages (Mladineo, 2005) and let the released eggs from the fish hosts to attach to a surface from where oncomiracidia could hatch and find a suitable fish to parasitize. The devices consisted of rectangular pieces of porexpan (5 x 20 cm) used for buoyancy, to which four microfiber broom filaments (25 cm length) were attached and acted as an egg trap as figure 4 shows. A total of eight devices where placed floating in the main reservoir tank to have an adequate attachment surface between the tanks included in the RAS system.

Figure 4. Broom filaments taken from the experimental collector devices, examined at the stereomicroscope for searching eggs of *S. chrysophrii*.

As previously described (Repullés-Albelda et al., 2012), one of the most critical stages of *Sparicotyle* life cycle is the hatching, thus these devices were used as a enhancer of the attachment and hatching rate inside the RAS, providing a continuous source of freshly released eggs and hatched larvae.

3.2. Fish and experimental set up

Three groups of GSB were established:

- Donors (D): 300 GSB naturally infected by *S. chrysophrii* were obtained from a commercial fish farm close to IATS facilities. They were transported alive in two 250 L transport tanks with pure O_2 added. They were allocated in two 500 L tanks; one tank (n

= 200) was connected to the RAS and the other (n = 100) was kept in open flow as a backup for the RAS D tank. The initial mean weight of the donor fish was 100 g. Upon arrival, mortality started due to the high infection level of the fish and initial problems. These dead or moribund fish were screened to check the initial level of infection of the D stock.

- Recipients (R): Naïve healthy GSB with a mean initial weight of 60 g and kindly donated by the Nutrigenomics and Endocrinology of Growth group of IATS were allocated in two replicated 90L fibre tanks connected to the RAS (n = 28 fish/tank).
- Controls (C): An equivalent group of naïve healthy GSB of the same origin and stock as R fish (n = 46) was allocated in another tank out of the RAS to serve as controls of the infection and to compare their growth performance and haematology with R fish.

On day 0 of the experimental trial, R fish started to receive water from D fish in the RAS, whereas C fish received normal open flow water. All the groups were fed with the same diet (Biomar, Efico, 4.5 mm granule size). The biomass of each tank was calculated and the fish were fed at a ratio of 2 % biomass. Fish were manually fed once a day at 10.30 am on weekdays. Feeding was not carried out on weekends.

3.3. Fish sampling procedures

Before the infection trial started and four weeks after the effluent infection, fish were sampled for different purposes. D fish were sampled along the acclimation period to check their infective status as they were dying of the infection. The gills of C fish were initially sampled to check that they were free of the parasite and together with R fish sampled 4 weeks after exposure to the RAS. At this moment, biometrical and blood data were also obtained from 8 fish from each group (4 from each replicate). Table 4 summarizes the timing of the different events along the experimental trial for the different groups.

Date	Days p.e.	Action	Samples of
28/6/2018	-1	Transport and acclimation (D)	-
29/6/2018	0	Entrance of water from RAS in R tanks	-
2/7/2018	5	Sample of dead D fish, parasite diagnosis	Gills from 3 D
3/7/2018	6	Sample of dead D fish, parasite diagnosis and biometry	Gills from 4 D
5/7/2018	7	Sample of dead D fish, parasite diagnosis and biometry	Gills from 3 D
13/7/2018	15	Sample of dead D fish, parasite diagnosis and biometry	Gills from 5 D
19/7/2018	21	Sample of dead R fish, parasite diagnosis and biometry	Gills from 2 R
26/7/2018	28	4 weeks p.e., sacrificed R, C, D: biometry, haematology and parasite diagnosis	Gills and blood from 8 R, 8 C, 1 D

Table 4. Schedule of the 4 weeks RAS *Sparicotyle* trial, indicating the actions and type of samples taken at each time point.

3.3.1. Blood sampling and haematological determinations

At 4 weeks p.e, blood was drawn from the caudal vessels of 8 R, 8C and 1D (for comparison purposes of very high naturally infected fish) anesthetized fish with 1 ml heparinised syringes and collected in heparinised 1.5 ml eppendorft tubes (Fig. 4).

Figure 4. Blood collection from causal vessels of GSB with a heparinised syringe.

Blood was kept on ice and used immediately for the measurement of haematological parameters as previously described (Sitjà-Bobadilla & Alvarez-Pellitero, 2009) The haematocrit level (Hc) was measured after centrifugation of the blood in heparinised capillary tubes at 1500 *g* for 10 min. Haemoglobin concentration (Hb) was determined with a Hemocue B-Haemoglobin Analyser® (AB, Leo Diagnostic, Sweden), which uses a modified azide methemoglobin reaction for haemoglobin quantification. Blood was drawn into disposable microcuvettes which contain reagents in dried form that produce the red blood cell lysis and the conversion of haemoglobin to methemoglobin by sodium nitrate, which is then combined with azide. The absorbance of the azide methemoglobinis is then photometrically measured at 565 nm and 880 nm. Red blood cell (RBC) counts (2 per fish) were made in a Neubauer chamber using and isotonic solution (1% NaCl). Mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin content (MCH) and mean cellular volume (MCV) were also calculated.

3.3.2. Gill sampling, parasite diagnosis and isolation

Parasite diagnosis was done on the dissected gill arches of dead or sacrificed alive fish. For alive fish, they were anaesthetised MS-222 (1g/10L of water), weighed, measured, blood taken and then scarified by a sharp and quick knife cut of the head. Then all the gills arches were removed and separated using Right and Left side codes, plus another annotation of their location in the gill cavity: I (for the most external), II, III, and IV(for the deepest). Each gill arch was placed in small petri dishes with filtered (10 µm) marine water for their posterior analysis as can be seen at figure 5.Each gill arch was examined individually with a 10 X Wild-Heerbrug Stereomicroscope. The parasite stages were classified as: A (Adults), AE (Adult with eggs), J (Juveniles), PL (Post Larvae) and E (eggs). The attachment site of each parasite stage inside the

gill was recorded to achieve information for further research. The prevalence of infection (number of infected fish/number of sampled fish*100), and the mean intensity of infection (mean number of parasites/infected fish), and the abundance (mean number of parasites/sampled fish) were calculated.

Figure 5. Gill arches of a D GSB in a Petri dish under the stereomicroscope. Note their pale colour.

The isolation of live parasites from the gill lamellae was carefully done using handmade tools and laboratory material consisting of insect needles and fine pipette tips attached to with a handler. While sampling, the isolated parasites were placed also in filtered (10 µm) marine water. Dead parasites were not further used and serve only for parasite diagnosis. The living parasites and eggs were isolated, sorted out by parasite stage, counted and placed in 1.5 ml Eppendorf tubes with different media depending on downstream uses. Some parasites were kept in ethanol for genomic studies of *S. chrysophrii* and others in RNAlater solution for transcriptomic studies of the different stages. Each tube was tagged with the code of the parasite stage, date and fish origin.

In order to obtain freshly hatched oncomiracidia, eggs were obtained from adults and from the floating devices of the reservoir tank, under a stereomicroscope to assure the collection of embrionated ones. Several groups of eggs were placed in a 24 well-plates containing UV and 10 µm mesh-filtered sea water, at 23ºC during 24-48 hours in the dark, as previously recommended (Repullés-Albelda et al., 2012). The plates were examined under an inverted microscope and the free swimming oncomiracidia were photographed, measured and also collected for further transcriptomic studies.

3.4. Statistics

A student *t*-test or a Mann-Whitney rank sum test were used to compare the biometrical and haematological values of C and R fish at 4 weeks p.e. A Pearson Product Moment Correlation

was run to test the possible correlation between the haematological, biometrical values and the intensity of infection (number of parasites per fish).

All statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL), and the minimum significance level was set at $P<0.05$.

4. Results

4.1. Performance of RAS

During the experimental trial, the RAS performance was successful, without significant incidences and with routine cleaning and maintenance. The flow and the water movement were enough for the volume and biomass that the RAS was supporting, and the effluent generated infection in R fish. The functioning of the pump and the biological filter was optimum as reflected in the values of water parameters. The key for this success was a proper cleaning and maintenance of the RAS during the experience. The water parameters after 4 weeks, shown below, are compatible with good water quality for keeping the animals:

Average temperature	26.2ºC
Average (NH3/NH4) concentration	0.23 mg/L
Average O_2 concentration	5.8mg/L

4.2. Egg collecting devices

The design and functioning of egg collectors was optimum during the study, as large amounts of alive or embryonated eggs were found attached on the microfibers (Fig. 6).

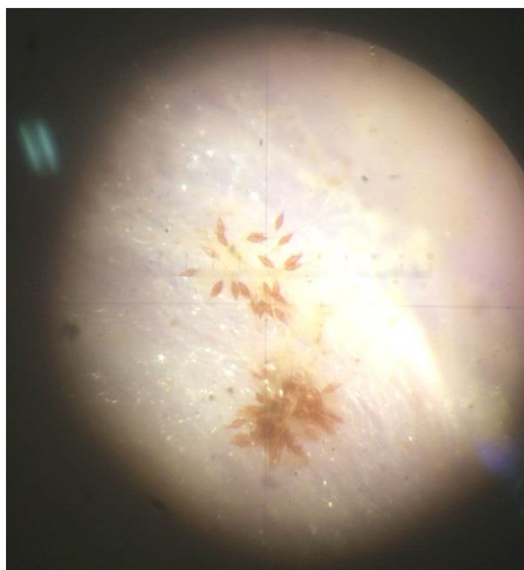

Figure 6. Several bundles of eggs of *S. chrysophrii* isolated from the experimental collector devices.

The fibres acted as a trap for the eggs, avoiding their loss with the waste water of the RAS. This is the first time this type of surface is used for monogenean egg attachment, and we can consider successful. During the whole experience, no other parasitic stages were not found attached to the collector fibres, but some copepods and ciliated organisms were found swimming through the fibres.

4.3. Infection status of donor fish

The initial level of infection of D fish was assessed from the examination of 10 naturally infected dead fish (sampled during the first days after the transport). The prevalence of infection was 100 % and the mean intensity of infection was 59 parasites/fish, being the highest number of parasites found in a single fish 88. The average stages per fish were: 39 adults and 20 juveniles per fish. These levels of infection were considered extremely high and compromising the health of the donor fishes. They presented pale coloured or almost white gills indicating the high pathogenicity of the infection, and were consistent with the associated the high mortality (see below).

4.4. Mortality

The cumulative mortality of D fish in the RAS at the end of the 4 weeks of experimental trial was 81.5% with 163 dead fishes from a total of 200 fishes. The peak of mortality was reached at day 15 days p.e with 29 casualties. The mortality of D fish on open flow (OF) was lower, probably due to the lower initial density and for the higher renovation of the water. By contrast, the mortality of R fish was 3.31% and no fish died in C group (Fig. 7).

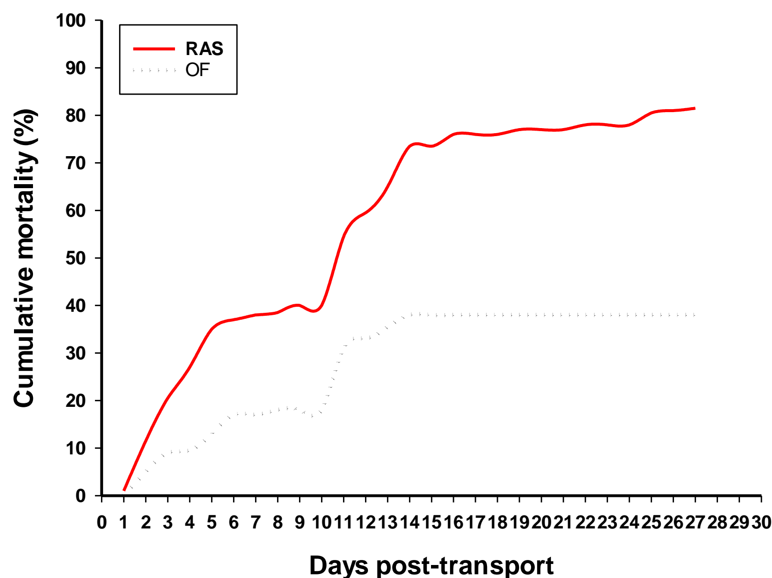

Figure 7. Mortality of Donor fish at the RAS compared to the one in open flow (OF).

4.5. Parasite distribution pattern

The gill examination of parasitized D fish showed a clear distribution pattern among de gill lamellae, with adults positioned at the proximal parts of the lamellae, whereas larvae and

juveniles where mostly occupying the space around the gill cartilage in the inner part of the lamellae (Fig. 8).

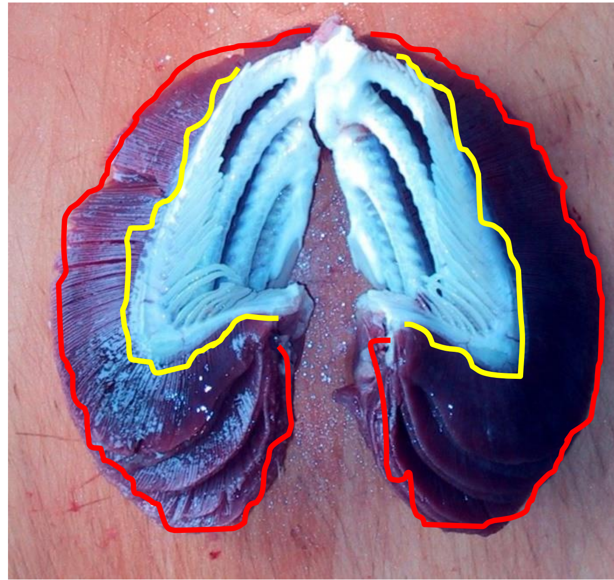

Figure 8. Macroscopic image of GSB gill arches showing the attachment sites along the gill lamellae: in yellow the location of the juveniles and in red colour that of adults.

4.6. Observation of parasite stages

Adult, juveniles and post larvae of *S. chrysophrii* were isolated from the gills of D fish, whereas eggs were mainly obtained from the microfibers of the collecting devices located in the reservoir tank (Fig. 9).The most abundant *Sparicotyle* stages were the eggs, to the extent that their number could not be registered. Besides the eggs, the most abundant stages were adult worms (65.41%), followed by juveniles (32.91%) and post-larvae (1.68%). Oncomiradia were observed occasionally, but their small size and fast movement prevented their counts.

The trial of egg hatching was very successful and large quantities of oncomiracidia were found alive at the 24 well-plate after the incubation period. The oncomiracidia had very fast movements in the well water column and their swimming was erratic with spasmodic, uncontrolled and cyclic movements.

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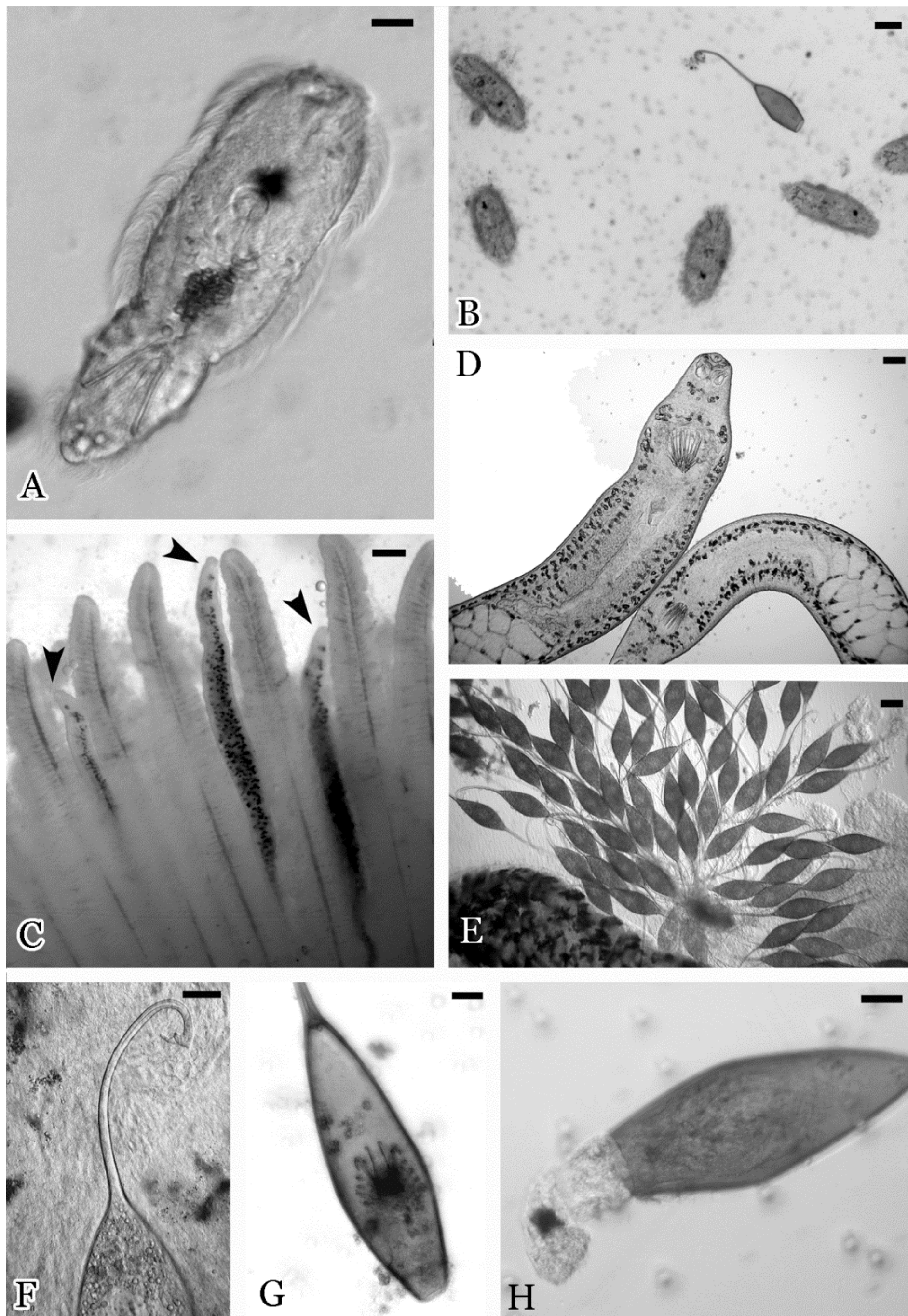

Figure 9. *Sparicotyle chrysoprhii* stages as the ones observed in the trial. A = post-larvae, B = just hatched oncomiracidia, C = juvenile in the gills, D = Gravid adults, E = bundle of eggs, F = anchor of an egg, G = embryonated egg, H = hatching eggs (from Sitjà-Bobadilla et al., 2006.)

4.7. Results of effluent exposure by RAS

4.7.1 Parasite infection

All the R fish were infected after 4 weeks of exposure to the RAS, thus prevalence of infection was 100.0%. All the infection values can be seen in Table 5 As all the fish were infected, abundance and mean intensity of infection have the same value. As can be observed, only one adult stage harbouring and two eggs were found in one fish, and the most abundant stage was the juvenile. There was a high individual variation in intensity of infection, ranging from 2 to 17 parasites per fish.

Parameter	value
n	8
Prevalence of infection (%)	100
Mean intensity of infection	8.38
Median intensity	5.5
Abundance	8.38
Max	17
Min	2
Adults	1
Juveniles	62
Post-larvae	2
Eggs	2
Total number of parasites counted	67
Parasite stages/Arch 1	14
Parasite stages/Arch 2	23
Parasite stages/Arch 3	21
Parasite stages/Arch 4	9

Table 5. Summary of the main parameters of the infection.

Parasite distribution by gill arches is also shown in table 5. The inner arch (4) harboured the lowest amount of parasites and, interestingly, the outer arch (1) was not the most infected, but the second and third arches, which showed similar high values.

4.7.2. Effect of infection on haematological values

Table 6 shows the individual values of the haematological parameters of C and R fish 4 weeks p.e. and the values of one D fish as a reference of high infection. Fig. 10 shows a macroscopic view of the collected blood corresponding to the different normal and anaemic stages of the fish. The statistical analysis revealed that haemoglobin concentration, haematocrit, and erythrocyte counts were significantly lower in R fish than in C fish (Fig. 11). Thus, the fish at this time point was clearly anaemic, even though their intensity of infection was moderate and most of the stages were juveniles.

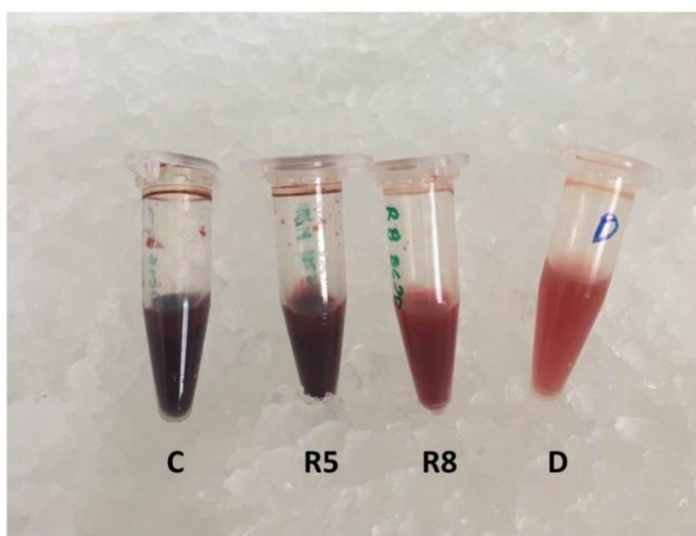

Figure 10. Macroscopic view of blood samples taken 4 weeks p.e. Note the different colour of the blood of a C fish (non-anaemic) from that of two R fish with different levels of anaemia, and that of a D fish (the most watery blood).

Label	Tank #	Fish #	Weight (g)	Lenght (cm)	CF	Hb (g/dl)	Hc (%)	RBC/ µl x10-6	MCHC (pg/100 µm^3)	MHC (pg/cel)	MCV (µm^3)
C1	B4	1	57.5	12.75	2.774	9.2	44	3.165	20.91	29.07	139.02
C2	B4	2	71.5	14	2.606	8.6	44	2.870	19.55	29.97	153.31
C3	B4	3	53	13	2.412	9.6	47	3.535	20.43	27.16	132.96
C4	B4	4	81.5	14.75	2.540	9.2	40	3.575	23.00	25.73	111.89
C5	B4	5	61.5	14	2.241	9.3	40	2.895	23.25	32.12	138.17
C6	B4	6	66.5	14	2.423	9.6	43	3.280	22.33	29.27	131.10
C7	B4	7	51	13	2.321	8.9	41	3.325	21.71	26.77	123.31
C8	B4	8	69.5	14	2.533	9.6	48	2.940	20.00	32.65	163.27
MEAN C			**52.0**	**13.7**	**2.5**	**9.3**	**43.4**	**3.2**	**21.4**	**29.1**	**136.6**
R1	B17	1	61	13.5	2.479	6.7	35	2.270	19.14	29.52	154.19
R2	B17	2	61	13.5	2.479	6.7	35	2.810	19.14	23.84	124.56
R3	B18	3	55.5	14	2.023	5.7	34	1.640	16.76	34.76	207.32
R4	B18	4	68	14.5	2.231	5.4	27	2.370	20.00	22.78	113.92
R5	B18	5	46.5	13	2.117	7.8	31	1.390	25.16	56.12	223.02
R6	B18	6	59	13.5	2.398	6.1	23	2.470	26.52	24.70	93.12
R7	B17	7	58.5	14	2.132	4.8	27	1.740	17.78	27.59	155.17
R8	B17	8	73	14.75	2.275	2.5	18	0.580	13.89	43.10	310.34
MEAN R			**64.4**	**13.7**	**2.3**	**5.7**	**28.8**	**1.9**	**19.8**	**32.8**	**172.7**
D	B2	1	**101**	**16.25**	**2.354**	**0.7**	**3**	**0.410**	**23.33**	**17.07**	**73.17**

Table 6. Biometrical and haematology values 4 weeks p.e. Mean corpuscular haemoglobin concentration (MCHC); Mean corpuscular haemoglobin content (MCH); Mean cellular volume (MCV); CF = Condition Factor; Hb = haemoglobin; Hc = haematocrit.

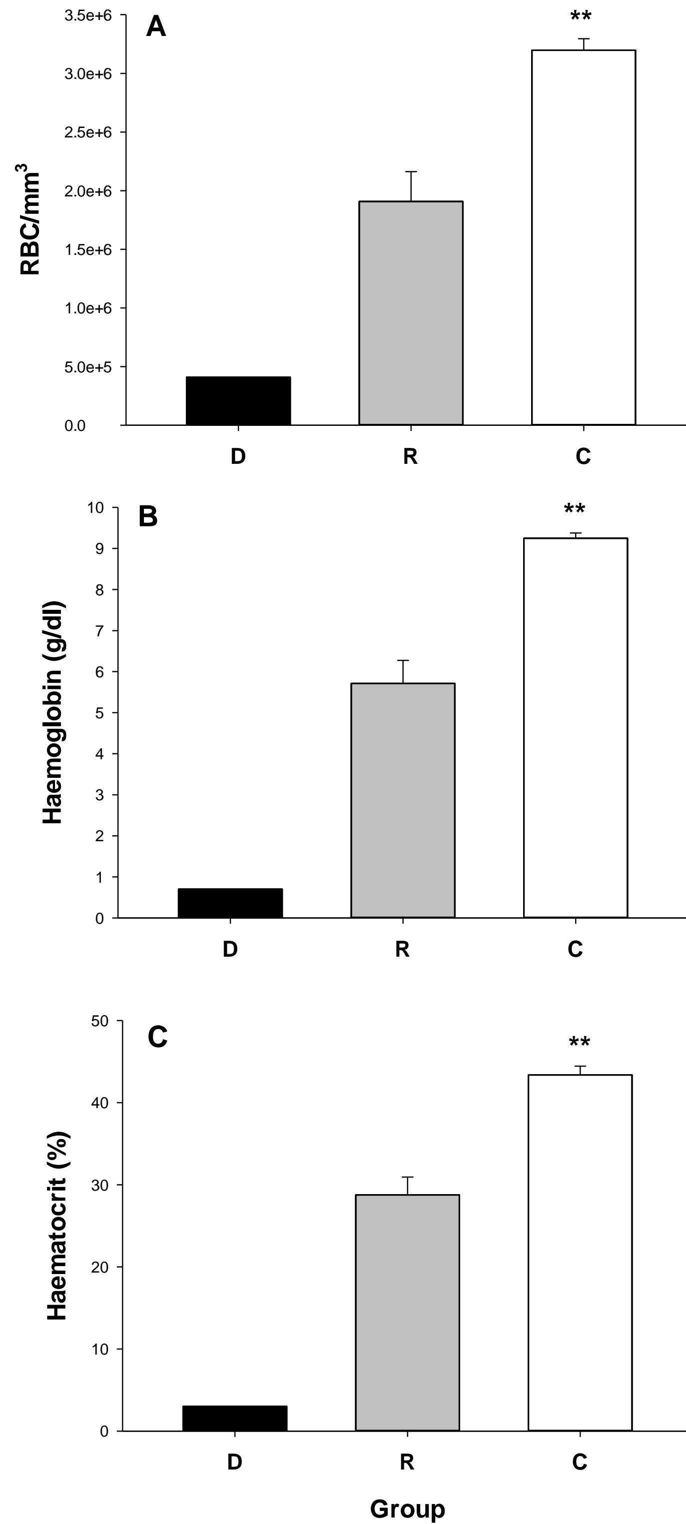

Figure 11. Red blood cells counts (RBC) (A), haemoglobin (B) and haematocrit (C) of D, R and C fish at 28 days post exposure to *Sparicotyle* by effluent. Values are mean + SEM for C and R and only one value is represented for D. ** = statistical significant difference between R and C group at $P < 0.001$.

4.7.3. Effect of infection on biometry

Table 6 also shows the individual biometrical data of C and R fish. After four weeks of exposure, R fish did not show significant changes in weight and length compared to C fish fed the same food ratio. However, the condition factor (CF) was significantly decreased (P = 0.025) (Figure 12). The biometrical values of the single donor fish are just shown as a reference, but are not comparable, since they have a different nutritional background and origin.

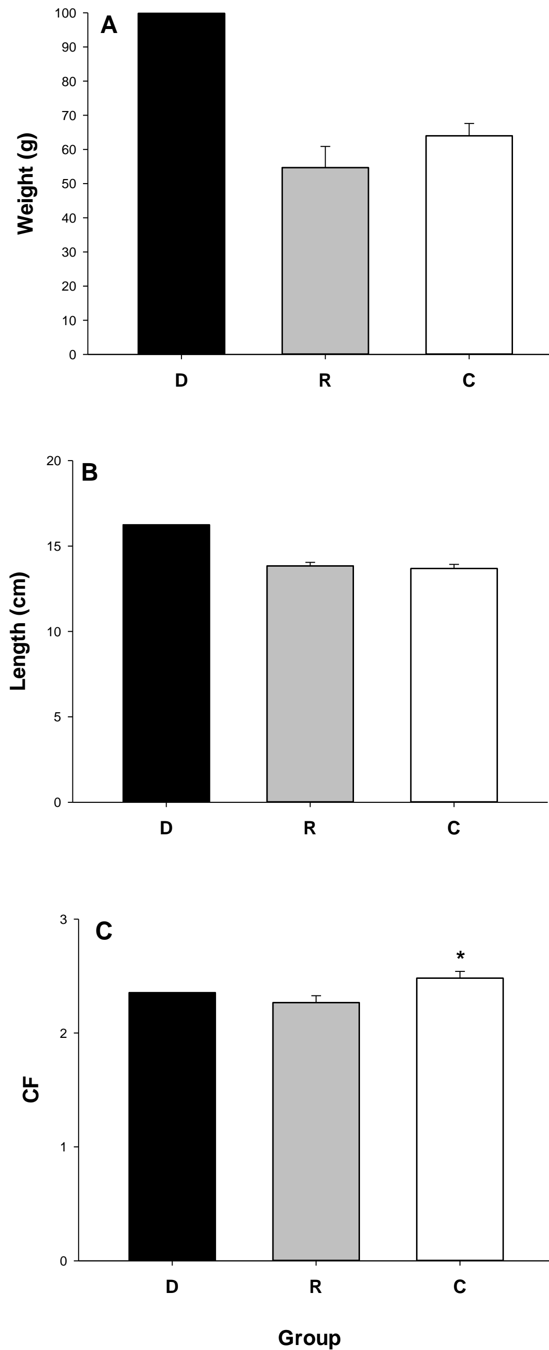

Figure 12. Weight (A), length (B) and Condition Factor (C) of D, R and C fish at 28 days post exposure to *Sparicotyle* by effluent. Values are mean + SEM for C and R and only one value is represented for D. * = statistical significant difference between R and C group at P < 0.05.

4.7.3. Correlations between infection, haematology and biometry

As expected, there was a highly significant (P<0.001) positive correlation between the haemoglobin, the haematocrit and the number of erythrocytes. There was also a significant positive (P<0.05) correlation between the condition factor and the three haematological indicators. Thus, the higher the CF, the higher those factors. Concerning the intensity of infection, a significant negative (P<0.05) correlation was found with the Hb and the Hc. Thus, the higher the number of parasites per fish, the lower the Hb and the Hc.

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Variable	CF	Hb	Hc	RBC
Intensity of infection	-0.239	-0.511	-0.546	-0.437
	0.372	0.0432	0.0288	0.0906
	16	16	16	16
CF		0.505	0.54	0.621
		0.0458	0.0308	0.0103
		16	16	16
Hb			0.917	0.848
			0.000000598	0.000033
			16	16
Hc				0.8
				0.000197
				16

Table 7. Pearson product moment correlation between haematological, biometrical and intensity of infection by *Sparicotyle*. For each pair of variables the correlation coefficient, the P value and the number of samples are shown. Values in red indicate significant negative correlation and those in green significant positive correlation.

5. Discussion

The infection of *Sparicotyle chrysophrii* was successfully transmitted to healthy fishes by the exposure to effluent from naturally infected fish using a specific RAS setup. The eggs attached to the special designed collectors were capable to mature and hatch correctly inside the RAS and the released oncimiracida survive and infect R fish. The parasite reproduction followed Hutson et al (2018) guidelines for the establishment of the hatching method. The timing of the trial was designed after consulting the existing data on S. *chrysophrii* oncomiracidial development (Repullés-Albelda et al., 2012) and the previous experimental infections under other conditions (Sitjà-Bobadilla & Alvarez-Pellitero, 2009) and. The initial level of infection of D fish was very high and the experimental set up of the RAS acted as a perfect spreader for the parasite infective stages. However, although the prevalence of infection at 4 weeks p.e in R fishes was 100%, the number of parasites after the exposure was clearly lower (average of 8.8 parasites/fish) than in naturally infected donor (average of 59 parasites/fish). However, this mean intensity of infection is close to that established for some fish farmers to start bath treatments in the sea cages. A similar situation was described when exposing *Sebastes schegeli* to eggs of *Microcotyle sebastis* (Kim & Choi., 1998). Furthermore, 100% of prevalence is

clearly higher than that of natural infections of GSB in Adriatic sea cages (Mladineo & Maršić-Lučić, 2007).

In addition, although 4 weeks exposure were enough for achieving a 100 % prevalence, it was not sufficient to sustain itself infecting more fish, because the parasites isolated were mostly juveniles. It is likely that a higher intensity could be achieve after a longer period, as seen in previous cohabitation trials in *Sparus aurata* (Sitjà-Bobadilla & Alvarez Pellitero, 2009), where the highest numbers of parasites were achieved at 8 and 20 weeks at the same range of temperatures of the current RAS effluent experiment. The number of juvenile parasites is also a good indicator for the infection active status because higher numbers of juveniles than adults are common in young populations as seen also in cohabitation trials (Sitjà-Bobadilla & Alvarez Petillero, 2009). Thus, the intensity of infection and the type of parasites stages found could be just due to the timing of the sampling. Considering the published data on the timing of development of the entire *S. chrysophrii* life cycle (Sitjà-Bobadilla & Alvarez Pellitero, 2009, Rigos et al., 2015, Henry et al., 2015), 4 weeks could not be enough for the infection to be completely established in a new setting, and a longer period would be needed to multiply the populations exponentially. The lower biomass density inside the RAS (due to the high mortality found in D fish), and the unavoidable losses of eggs and oncomiracidia could also account for the moderate intensity of the infection achieved in the trial. In any case, it would be challenging to obtain a farm level of infection in a RAS setup because of the poor survival of the donors with dramatically high levels of infection under these conditions. During the trial, the cumulative mortality among D fish was 81.5%, clearly indicating that the health of these fish was compromised by the infection.

The haematological data showed solid effects of the infection on Hb, Hc and RBC as they were significantly lower in R fish than in C fish. It is widely understood that this is due to hematophagous behaviour of these parasites. None of one of the indicators of the amount of haemoglobin in red blood cells MCHC and MCH was significantly modified in R fish. However, in previous transmission experiments (Sitjà-Bobadilla & Alvarez-Pellitero, 2009), it was found that erythrocytes were hypochromic. Hypochromic anaemias usually overlap with microcytic anaemias, but this was not the case then, neither in the current experiment, since MCV was not decreased in infected fish, just the opposite, it was increased. The higher (but not significantly) MCV values of R fish could be due to the response of the host creating more new cells to compensate the anaemia.

The clear anaemia produced by the infection with *Sparicotyle* was found even with a moderate intensity infection, such as the one generated in our RAS trial. The amount of Hb, Hc and RBC critically decreased with the infection, macroscopically correlated with gill paleness and watery blood, producing dysfunctions of oxygen transport, osmoregulation, plasma metabolite changes, reduced swimming performance and finally the death of the fish (Sitja-Bobadilla, 2010). As observed in the haematological values of D fish, a long infection time with much higher parasites/fish decreased even more Hb, Hc and RBC values, affecting the health of the fish in a higher grade.

The high negative correlation found between the parasite numbers per fish and Hb and Hc, was previously observed for Hb levels in Japanese flounders (*Paralichthys olivaceus*) infected by the diclidophorid *Neoheterobothrium hirame* (Mushiake et al., 2001) and *S. lalandi* infected by *Z.seriolae* (Mansell et al., 2005) and for *S. aurata* infected with even lower parasite numbers of

S. chrysophrii than in the current trial (Sitjà-Bobadilla & Alvarez-Pellitero, 2009). In the latter, Hb concentration was significantly lower in R than in C fish at 5 and 10 weeks p.e., in small gilthead seabream harbouring an average of 8 and 4.7 monogenean specimens, respectively, and also in larger R juveniles at 8 weeks p.e., with infection levels as low as 2.2 specimens/fish. Furthermore, more recently a negative correlation was found between Hb and parasite load in GSB after 10 weeks of infection by cohabitation with a mean number of parasites per fish of 6.84 (but only counting the 2 most external gill arches) (Henry et al., 2015). These authors also found a positive correlation between Hb and weight, and other immune factors such as lysozyme, bacterial killing activity, anti-protease activity, nitric oxide and total immunoglobulins. They considered that haemoglobin was the best indicator of fish health. Johnstone et al. (2017) also considered that haematological values could be health and condition indicators for the detection of early stages of infection.

Considering all the results and previous published information, haematological analysis, especially Hb, could be a useful monitoring tool for commercial fish farms, to check the health status of fish suffering sparicotylosis, as the Hb values can be affected even at low parasite loads, when no external clinical signs or changes in behaviour are seen. Thus, it could be an anticipatory prognostic marker. Blood sampling is easier and faster than screening all the gill arches under a stereomicroscope and could be even done non-lethally, as just a small amount of blood is necessary to analyse Hb with the miniaturized device used in the current trial. Furthermore, fish can recover normal blood values in just 8 days after experimental bleeding (Montero et al., 1995). Further studies at large scale are needed to obtain normal reference values of GSB of different sizes, ages, reared under different conditions and seasons, to establish a threshold level that could be used as an alert of infection.

6. Conclusions

In summary, the 4 weeks of RAS exposure resulted in 100 % prevalence of infection, moderate parasite loads and pathological effects in terms of growth and anaemia in R fish. The anaemia was produced by the hematophagous nature of the parasite and the haemorrhages produced to the tissue and the vascular disruption, even at this moderate intensity of infection (8.8 parasites/fish). At this time of exposure, all the parasite stages were observed (though juveniles were the most abundant), which indicates a faster development than the ones achieved for *Sparicotyle* and other species of Microcotylidae studies (see Repullés-Albelda et al., 2010). There was a strong negative correlation between some blood parameters (Hb, Hc) and the parasite load even with this intensity levels and juvenile infection. The effect on growth was detected in the CF, but not in length of weight, probably due to the relatively short time of exposure and the "young" status of the infection.

To conclude, the present study clearly demonstrates that the establishment of *Sparicotyle chrysophrii* infection is feasible by the effluent of water from naturally infected fishes using a RAS setup, mimicking some conditions found in sea cages, as the presence of substrates (floating devices) for the attachment of eggs. The RAS is valid to create, maintain and reproduce the infection, as it can sustain the parasite cycle, providing high numbers of parasites for further studies on the parasite and the hosts. The possibilities of control and monitoring of the system can also provide data to improve the knowledge of the parasite cycle and will help to discover critical development events in order to control the infection. It will facilitate having a

source of homogenously infected fish for drug testing or vaccine challenges. The isolation of the RAS from the remaining tanks in a facility is also an advantage to avoid cross-infections.

This experimental trial is a continuation of the work conducted by the Fish Pathology group of IATS (Sitjà Bobadilla & Alvarez-Pellitero in 2009), providing more information to the previous one obtained by cohabitation or egg exposure by bath. Most probably longer terms are necessary to fully achieve multiplication of the parasites in the system. Future samplings and improvements of the system will be done. The parasite material collected from D and R fish will be used for genomic and transcriptomics studies that would help to decipher drug targets and design drugs, so needed in the GSB culture.

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