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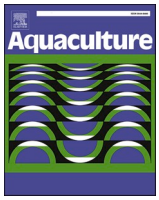

# *Sparicotyle chrysophrii* experimental infection of gilthead seabream (*Sparus aurata*): Establishment of an *in vivo* model reproducing the pathological outcomes of sparicotylosis

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ABSTRACT

*Sparicotyle chrysophrii* (Microcotylidae) is considered the most threatening pathogen affecting the gilthead seabream (GSB; *Sparus aurata*) off-shore farming due to its economic impact. This study explores the best experimental conditions to set up an *in vivo* infection model capable of mimicking the sparicotylosis signs observed in farmed diseased fish. The experimental setup for parasite transmission consisted of a recipient (R) fish tank with naïve GSB receiving water from two *S. chrysophrii*-infected donor tanks in a recirculating aquaculture system (RAS). Egg collectors, consisting of a polyester mesh in a supporting plastic frame, were placed in the R tank in order to monitor the progression of the parasitosis. An additional tank with control unexposed naïve fish was maintained in parallel, with open water flow and disconnected from the RAS. After a preliminary trial, infective pressure in the R tank was increased by placing an additional egg collector already loaded with entangled parasite eggs, and by maintaining the fish number throughout the experiment.

Adult *S. chrysophrii* parasite load correlated with most of the evaluated biotic and abiotic factors. Haemoglobin and haematocrit significantly dropped around 40 days after exposing GSB to *S. chrysophrii*. Furthermore, the abundance of eosinophilic granular cells and goblet cells in gill filaments, and splenic melanomacrophagic centres increased. In contrast, hepatic fat was depleted in *S. chrysophrii*-infected GSB. This study provides an advancement not only for studying *S. chrysophrii*'s biology and its interaction with its host, but also for further studying the disease under experimental conditions in search of treatment alternatives and prophylactic measures.

## 1. Introduction

Mediterranean aquaculture has notably diversified in the past decade. However, the gilthead seabream (*Sparus aurata*; GSB) remains the most farmed species in the area and ranks second among marine farmed fish in Europe (FAO, 2022; FEAP, 2020). Parasitic infections are a growing concern in mariculture (Shinn et al., 2015), more specifically, monogeneans are among the most threatening ectoparasites in aquaculture due to their impact on economically relevant finfish species, including GSB (Grau et al., 2003; Jahangiri et al., 2022; Merella et al., 2009; Ogawa et al., 1995; Sitjà-Bobadilla et al., 2010; Ternengo et al., 2010; Tu et al., 2015).

Sparicotylosis is caused by *Sparicotyle chrysophrii* (Microcotylidae), a polyopisthocotylean monogenean infecting the gills of GSB. Sparicotylosis represents the number one health concern in GSB livestock units under off-shore farming conditions across the Mediterranean Sea (Muniesa et al., 2020; Vendramin et al., 2016). This disease has been associated with lethargy due to hypoxia, severe anaemia and emaciation, and histopathological findings such as lamellar synechiae, clubbing and shortening, epithelial hyperplasia resulting in secondary lamellae fusion, and proliferation of chloride cells have been described (Riera-Ferrer et al., 2022; Sitjà-Bobadilla et al., 2006; Sitjà-Bobadilla and

*Abbreviations:* C, Control; D, Donor; dpe, Days post-exposure; EGCs, Eosinophilic granular cells; GSB, Gilthead seabream; Hb, Haemoglobin; Hct, Haematocrit; MMs, Melanomacrophages; MMCs, Melanomacrophage centres; R, Recipient; T1, Trial 1; T2, Trial2.

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Álvarez-Pellitero, 2009).

This parasite has a direct life cycle, which can be divided into a resistance, an infective and a pathogenic stage (Fig. 1). Bundles of entangled eggs shed by gravid adults into the water column constitute the resistance stage. These eggs get hooked on biofouling and fibrous surfaces such as the net pen lines (Sitjà-Bobadilla et al., 2006) or on fish gill filaments. Eggs with fully developed embryos hatch when the egg operculum opens, releasing the oncomiracidia into the water column (Repullés-Albelda et al., 2012). The infective stage, the oncomiracidia, are free-swimming larvae propelled by cilia and able to swim in both vertical and horizontal planes. This stage has a short lifespan, and larvae die if they do not encounter their type host within hours (Repullés-Albelda et al., 2012). When oncomiracidia come across their host, they attach to the gill filaments and develop into post-larvae, which will locate between gill filaments in a proximal plane close to the branchial arch. Then, they will undergo several growth phases in which the development of the parasite's haptor and other vital structures, such as the pharynx and gut, takes place (Repullés-Albelda et al., 2011). Post-larvae develop into juveniles, which entail the pathogenic stages. This stage has a minimally pigmented gut, lacks post-larval hooklets and uses its clamps to attach to the gill filaments, inflicting mechanical trauma to the branchial tissue. Juveniles develop into adults with a more heavily pigmented gut, and their pathogenic effect becomes more evident. Finally, gravid adults shed the eggs into the water column, closing the cycle. Many authors have highlighted that the monogenean's development is temperature-dependent, where high temperature plays a key role in speeding egg development, embryonation rates, shedding and hatching, and therefore, timings in the parasite's development may differ throughout the year (Ernst et al., 2005; Hoai, 2020; Lackenby et al., 2007; Repullés-Albelda et al., 2013; Repullés-Albelda et al., 2012; Sitjà-Bobadilla and Álvarez-Pellitero, 2009; Valles-Vega et al., 2019; Villar-Torres et al., 2018; Zhang et al., 2022).

Many aspects still remain obscure despite the scientific and technical efforts to better understand *S. chrysophrii*'s biology, the effects on its host, and to find possible treatment alternatives and husbandry strategies to mitigate and prevent this disease(Aguado-Giménez et al., 2022; Fioravanti et al., 2020; Merella et al., 2021; Mladineo et al., 2021; Sitjà-Bobadilla et al., 2006).

Thus far, several attempts to keep *S. chrysophrii* in an *in vivo* model have been performed (Ormad-García, 2018; Rigos et al., 2015; Sitjà-Bobadilla and Álvarez-Pellitero, 2009). However, these models were not standardised and plausible later improvements in the methodology have not been addressed in scientific literature. *In vivo* experimental models are not only convenient for having a constant source of parasites to study their biology, the host-pathogen interactions and the impact on the host, but also for testing alternative treatments under *in vitro* conditions that can then be scaled up to *in vivo* models. The success of *in vivo* models is hampered by obtaining an initial source of naturally infected fish, usually harbouring concomitant infections, and only available seasonally.

The current study aims to determine the best experimental conditions to maintain an *in vivo* infection of *S. chrysophrii* in GSB, capable of mimicking the natural sparicotylosis signs observed in farmed GSB in terms of biometry, haematology and histopathology.

## 2. Materials and methods

### 2.1. Donor and recipient fish

In December 2019, GSB displaying growth arrestment and pale gill syndrome ($n = 64$) were harvested from commercial sea cages in the Western Mediterranean Sea and brought alive to the Fish Pathology facilities at the Institute of Aquaculture Torre de la Sal (IATS, CSIC).

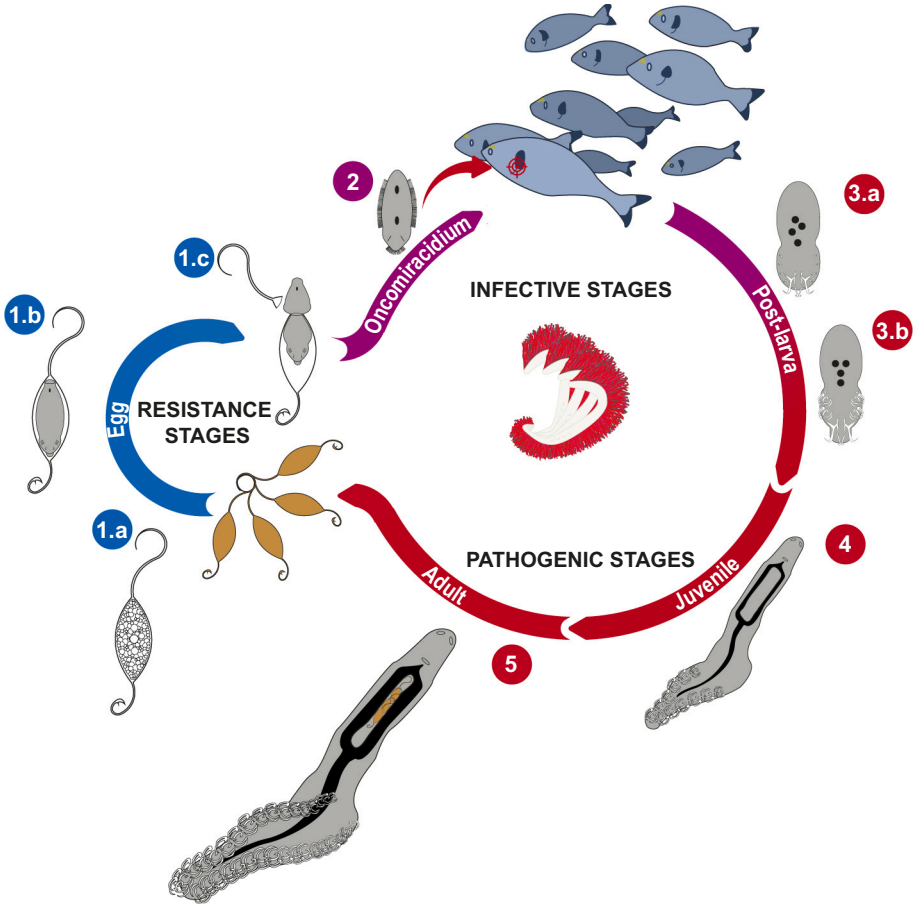


**Fig. 1.** Schematic representation of *Sparicotyle chrysophrii* life cycle. Resistance stage: 1.a: Embryonated egg; 1.b: Egg with a fully developed embryo and displaying a conspicuous operculum; 1.c: Oncomiracidium hatching from the egg. Infective stages: 2: Free-swimming oncomiracidium searching for the gills of nearby gilthead seabream; 3.a: Post-larval phase with haptoral posterior hooklets and hamuli and postero-lateral and lateral hooklets; 3.b: Post-larval phase with hamuli, two pairs of clamps and anteriorly displaced hooklets; 4: Juvenile stage ($\leq$ 6 pairs of clamps and no hooks); 5: Adult stage.

Upon arrival, a subsample of GSB ($n = 10$; 90 ± 14.4 g; mean weight ± SD) was euthanised by anaesthetic overexposure (MS-222, 0.1 g·L$^{-1}$; Sigma-Aldrich), gill arches were dissected and checked under a stereomicroscope, and fresh smears were mounted and observed under a light microscope. Prevalence of infection in gills was 100% for *S. chrysophrii*, 100% for *Furnestinia echeneis*, 90% for epitheliocystis and 80% for lymphocystis. In the following months, these fish were allocated into two 500 L tanks ($n = 32$ each) with open water flow and parasite egg collectors (described in section 2.3; Supplementary Fig. 1), consisting of a polyester mesh in a supporting plastic frame, placed in the tank. Donor (D) GSB were gradually euthanised, gill arches dissected, inspected under a stereomicroscope and *S. chrysophrii* adult specimens retrieved alive. The daily harvested worms were rinsed with filtered seawater and poured into two 200 L tanks with $N = 20$ healthy GSB each (82 ± 11.9 g; mean weight ± SD), held for up to 3 h without water renewal. Additionally, *S. chrysophrii* eggs were retrieved from collectors, rinsed and poured into the same two tanks together with the *S. chrysophrii* harvest. GSB held in these tanks were subsequently used as D fish bearing the "purified" *S. chrysophrii* infection.

Naïve GSB juveniles used as unexposed control (C) and recipient (R) (details in Table 1), were purchased from a Mediterranean hatchery (Avramar, Burriana, Spain) and acclimatised to the indoor experimental facilities of IATS, CSIC under natural photoperiod and temperature conditions of the latitude (40°5′N; 0°10′E). Upon arrival, a subset of these animals was tested by microscopy, histology and molecular diagnosis for the absence of gill and intestinal parasites. Water parameters were monitored; oxygen saturation was kept above 85%, and unionized ammonia below 0.02 mg·L$^{-1}$ in all tanks.

All experiments were carried out according to current Spanish (Royal Decree RD53/2013) and EU (2010/63/EU) legislation on the handling of experimental fish. All procedures were approved by the Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal (IATS, CSIC, Castellón, Spain), CSIC and "Generalitat Valenciana" (permit number 2018/VSC/PEA/0240).

### 2.2. Experimental set-up: Parasite transmission

Two experimental infection trials were conducted in a recirculating aquaculture system (RAS). The first trial (T1) was a preliminary approach, and in light of its infection outcome, modifications were carried out to standardise the experimental setup in the second trial (T2). The core common experimental setup consisted of an R fish tank (200 L) with naïve GSB receiving water from two D tanks (200 L) in a closed recirculation water flow. An additional tank with C unexposed naïve fish, was maintained in parallel, with open water flow (*i.e.*, disconnected from the RAS but under the same temperature and oxygen conditions). A clean and empty egg collector was placed in the R tank in T1 and T2 to register the egg production and drift in the RAS on a weekly basis, as described in detail in the following section 2.3.

T1 was performed in winter 2020/21 (November–January) using R GSB receiving effluent water from D tanks. Fish were periodically sampled (Table 1) and after each sampling, euthanised R fish were not replaced; therefore, the biomass in the R tank decreased along the trial. T2 took place in spring 2021 (April–May) using smaller R GSB, individually tagged with passive integrated transponders (ID-100A/1.4 Mini transponder, Trovan, Spain) and receiving effluent water from D tanks. A collector, harbouring 388 *S. chrysophrii* eggs obtained from a D tank, was placed into the R tank to increase the infective pressure. In addition, after each sampling, removed R were replaced by adding the same number of D to the R tank. Consequently, in T2, as the GSB number in the R tank remained constant, biomass increased due to growth, and the infective pressure by tank cohabitation was maintained throughout the experiment. Details on both trials are included in Table 1.

**Table 1**
Details on *S. chrysophrii* experimental infections in gilthead seabream Trial 1 (T1) and Trial 2 (T2).

| | T1 | | | T2 | | |
|---|---|---|---|---|---|---|
| Period | Nov 2020-Jan 2021 | | | April 2021–May 2021 | | |
| Water temperature range (°C; initial → final) | 17.60 → 11.50 | | | 16.00 → 23.40 | | |
| Water temperature (°C; mean ± SD) | 15.61 ± 2.66 | | | 20.42 ± 2.18 | | |
| Daily water temperature variation (°C; mean ± SD) | 1.40 ± 0.90 | | | 1.05 ± 0.32 | | |
| Photoperiod (h; light:dark; initial → final) | 10:14 | | | 13:11 → 14:10 | | |
| Initial weight (g; mean ± SD) | 118.50 ± 11.90 | | | 20.22 ± 3.04 | | |
| Initial length (cm; mean ± SD) | 16.30 ± 0.45 | | | 9.35 ± 0.44 | | |
| Final weight (g; mean ± SD) | 157.30 ± 10.25 | | | 43.20 ± 5.28 | | |
| Final length (cm; mean ± SD) | 18.20 ± 0.46 | | | 12.10 ± 0.56 | | |
| Samplings (dpe) | 0, 11, 20, 32, 41, 61, 81 | | | 0, 14, 28, 42, 50, 58 | | |
| Fish groups | C | R | D | C | R | D |
| Fish number | 23 | 28 | 40 | 20 | 28 | 52 |

C = control; R = recipient; D = donor; SD = standard deviation; dpe = days post-exposure.

In both trials, *S. chrysophrii* egg shedding from experimentally infected GSB and its successful drift through the RAS was monitored using a custom-made device modified from the egg quantifying method previously described by Buchmann (1988) and Merella et al. (2021) and named "egg collector". Clean 12 cm in diameter plastic frames holding a polyester mesh (pore size = 4 mm$^2$), a hook, and a sinker (Supplementary Fig. 1) were submerged in R tanks. These collectors were temporarily removed and checked under a stereomicroscope every 7 days in both trials. Ten random fields at 2.5 X magnification, corresponding to a total polyester-mesh surface of 18 cm$^2$, were checked, and the number of entangled eggs per cm$^2$ was determined.

### 2.4. Samplings and parasite diagnosis

Five C were sampled at 0 days post-exposure (dpe), whereas 3 C and 5 R were sampled during the following sampling points (Table 1), except at 81 dpe in T1, when only 3 R were sampled. In each sampling, GSB were euthanised by tricaine methanesulfonate (MS-222) overexposure (0.1 g·L$^{-1}$). Individual biometric data was registered, and GSB were bled from the caudal vein using heparinised syringes. Haemoglobin (Hb) values were immediately measured (HemoCue® Hb 201+ AB, Ängelholm, Sweden), and haematocrit (Hct) values were determined by standard microhematocrit capillary centrifugation in a haematocrit centrifuge (myLab HC-01, AHN®, Germany) at 10,000 *x g* for 10 min.

The four right-sided gill arches of each R fish were dissected to carry out *in situ* juvenile, and adult *S. chrysophrii* counts under a stereomicroscope to determine the infection intensities. These counts were extrapolated for the 8-gill arches of each GSB according to Riera-Ferrer et al. (2021a). The left gill arches, head kidney, spleen and liver samples were fixed in Bouin's solution and processed for routine paraffin histology.

### 2.5. Histopathology

Tissue sections (4 μm-thick) were stained with Giemsa, haematoxylin and eosin (H/E), or periodic acid-Schiff stain (PAS) and observed under a Leitz Dialux 22 light microscope connected to a digital Olympus DP70 camera. Relevant histopathological findings determined in the preliminary T1 were analysed in depth in T2 and registered according to the following semiquantitative scoring criteria. The abundance of eosinophilic granular cells (EGCs) and goblet cells in gills was scored ranging from 0 (absence) to 3 (very abundant, meaning 25–30 cells/microscope field at 500 X magnification). In liver sections, lipid and glycogen storage was scored from 0 (absence) to 3 (pervasive) by Giemsa or PAS

staining, respectively. The abundance of melanomacrophage centers (MMCs) was estimated in the spleen according to a semiquantitative scale ranging from 0 (absence) to 3 (very abundant).

### 2.6. Statistical analysis

A Spearman's rank correlation coefficient test was run with the *corrplot R* package (Wei and Simko, 2021) to identify the experimental setup parameters significantly affecting parasite transmission in T2. The correlation analysis included infection intensity (total, juvenile and adult parasite load·fish$^{-1}$) and egg counts in collectors *versus* biotic and abiotic factors: weight, length, condition factor (*CF*) and biomass, haematological parameters (Hb, Hct), dpe and daily mean water temperature.

Mean data of haematological parameters, mean infection intensity, and histological scorings were compared among samplings within each experimental group (C or R) for each trial by One-way ANOVA, followed by Student-Newman-Keuls *post-hoc* test. Normality of the data was checked by Shapiro-Wilks test and homogeneity of variances by Brown-Forsythe test. When normality was not met, Kruskal-Wallis on ranks followed by Dunn's method was used instead. Differences between C and R groups were determined by Student's *t*-test or Mann-Whitney test when normality was not met. Differences were considered significant at a $P < 0.05$. These statistical analyses were carried out using SigmaPlot v14.5 software (Systat Software Inc., San Jose, CA, USA).

## 3. Results

### 3.1. Infection outcome

*Sparicotyle chrysophrii* infection was first confirmed after 11 and 14 dpe in T1 and T2, respectively. Initially, juvenile *S. chrysophrii* stages ($\leq$ 6 pairs of clamps) were predominant and later on, at 32 dpe (T1) and 28 dpe (T2), adult *S. chrysophrii* stages appeared and became predominant over juveniles (Fig. 2). Furthermore, the infection prevalence in both trials was 100% for R fish in all sampling points. Juveniles were mostly found in a proximal plane, closer to the gill arch cartilage. In contrast, adults were located in a distal plane within the gill arches, closer to the gill filament apices (Fig. 3A, B). In both trials, Hb values of R dropped dramatically compared to C around 40 dpe, (Fig. 4A, B), after the increase of *S. chrysophrii* adult counts in gills. In both trials, the haemoglobin drop was sustained after 40 dpe, though more severe in T2, where significant differences were more robust. A concurrent drop of Hct at 42 dpe was observed only in T2, and became significant at 50 dpe. In T1, a non-significant decrease of haematocrit was found from 32 dpe onwards, and became significant only at 61 dpe (Fig. 4C, D). Three mortalities were registered in T2 at 38, 42, 47 dpe, respectively.

### 3.2. Egg shedding monitoring

The egg collectors proved to be very convenient for the evaluation of *S. chrysophrii* eggs circulating in the RAS. The peak of *S. chrysophrii* egg counts was observed at 35 dpe in T2, 14 days before eggs peaked in T1 (Fig. 5).

### 3.3. Correlation analysis

The correlation analysis showed that adult parasitic infection intensity and total parasite load significantly correlated with most biotic and abiotic factors. In particular, adult parasite load was positively correlated with exposure time (dpe), average water temperature, fish biometric parameters and biomass in tanks. It also negatively correlated with the haematological parameters (Fig. 6). Juvenile parasitic infection intensity, on the other hand, presented fewer significant correlations with any of the examined variables.

### 3.4. Histopathological response

In T2, from 28 dpe on, R gills showed a significantly higher abundance of EGCs infiltrating the gill lamellae than C gills (Fig. 7C-H; Fig. 8A). These granulocytes were also observed along the gill filament, in/around blood vessels and sometimes degranulated. Occasionally, melanomacrophages were found in R gills (Fig. 7E). Goblet cell abundance in R gills was also significantly higher than in C from 42 dpe on (Fig. 7I-J; Fig. 8B). Some filaments of R gills presented signs of mucosal inflammation or lamellar clubbing and synechiae, as shown in Fig. 3C, E and Fig. 7C, F, G. Goblet cell and EGC presence, though significantly increased upon infection, was dispersed throughout the gills and could not be specifically related with *S. chrysophrii* attachment or *S. chrysophrii*-inflicted lesions (Fig. 3). Epitheliocystis, a disease caused by secondary bacterial infections, appeared in R gills in both trials: from 61 dpe (T1) and from 42 dpe (T2) on (Supplementary Fig. 2). The signs consisted of cysts in gill epithelia, some surrounded by epithelial proliferation with squamous metaplasia, which promoted the fusion of secondary lamellae. Besides the parasite's target organ, lipid storage in the liver significantly decreased in R along the course of the trials, but was especially remarkable in T2 from 28 dpe on (Fig. 7K, M; Fig. 8C), whereas glycogen storage did not significantly vary (Fig. 7L, N). Furthermore, abundance of splenic MMCs was significantly higher in R (Fig. 7P, R; Fig. 8D). Details shown in Fig. 7O and Q evidence smaller and less dense splenic MMCs with dispersed melanin granules in C. When data from all samplings were grouped regardless of the exposure time to the parasite, the summary histopathological differences between C and R GSB were: R presented significantly more EGCs (R: 0.52 $\pm$ 0.08; C: 0.10 $\pm$ 0.05; mean $\pm$ SEM); more goblet cells (R: 1.54 $\pm$ 0.12; C: 0.76 $\pm$ 0.07; mean $\pm$ SEM); less fat depots in the liver (R: 2.40 $\pm$ 0.12; C: 1.14

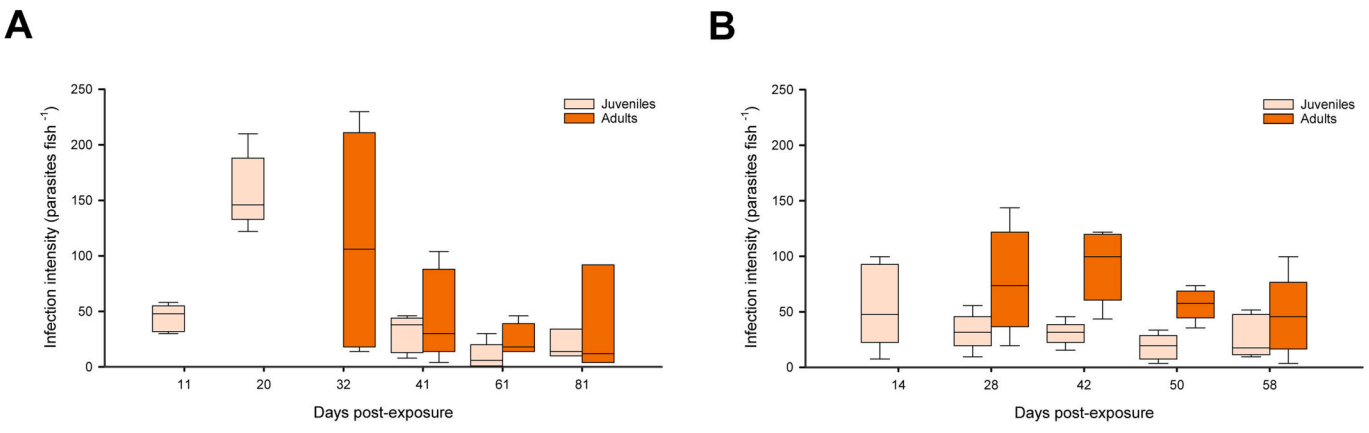


**Fig. 2.** *Sparicotyle chrysophrii* infection intensity (median + IQR) for Trial 1 (A) and Trial 2 (B). Infection intensity is represented for juveniles and adults separately.

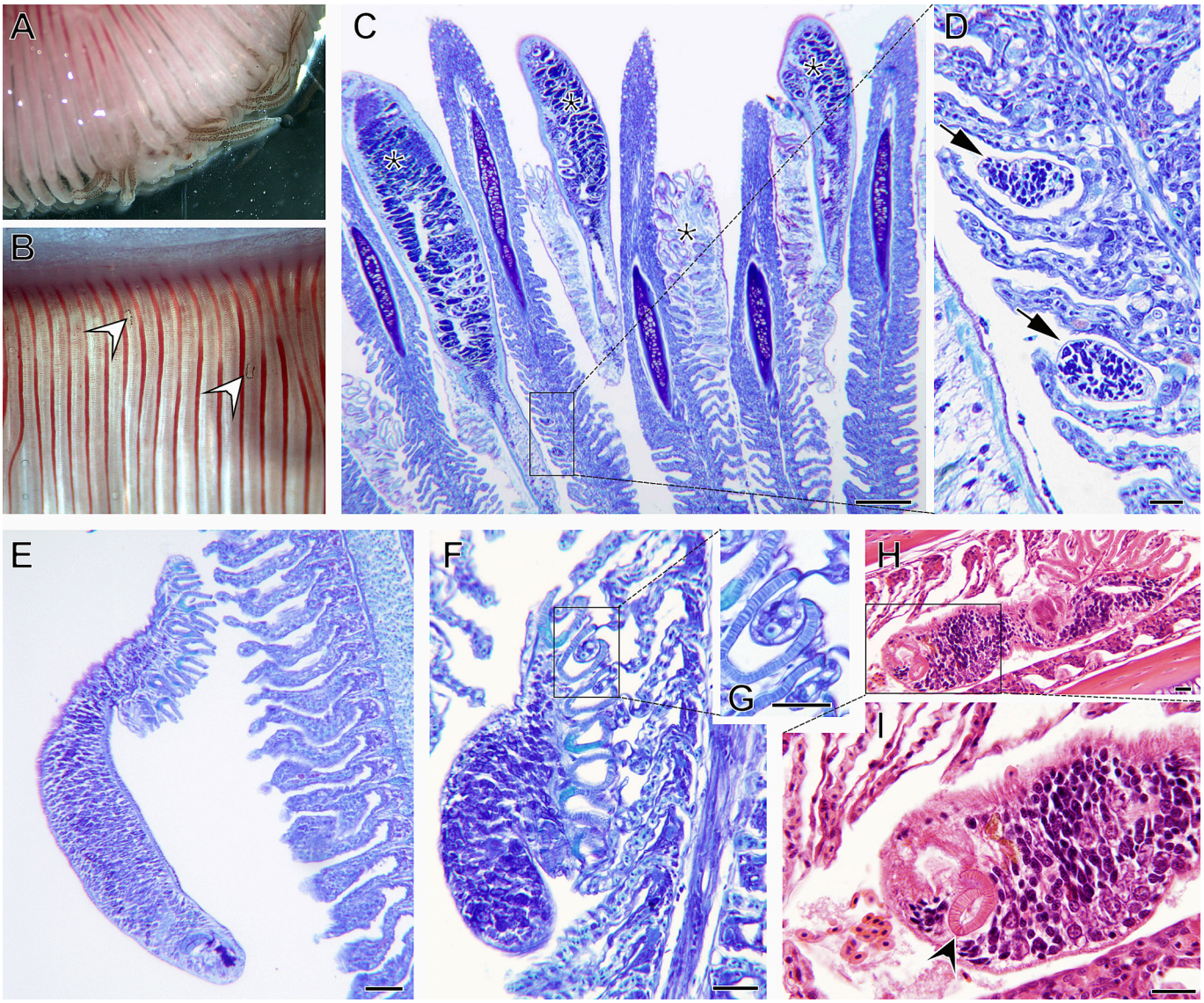


**Fig. 3.** *Sparicotyle chrysophrii* microphotographs. Fresh gills under the stereomicroscope showing adult parasite stages close to the filament apices (A) and post-larvae and juveniles close the arch cartilage (white arrow heads) (B). Histological section of adult specimens (asterisks) close to filament apices (C). Note the presence of two juveniles magnified in (D) (black arrows). Adult specimen close to gill lamellae presenting some clubbing (E). Section of the parasite haptor attached to gill lamellae (F). Note how clamps pinch secondary lamellar tips, which appear abraded and weakened, magnified in (G). Longitudinal section of an adult specimen between gill filaments including its head segment (H). Note the details of the oral suckers (black arrowhead) and the proximity of red blood cells, magnified in (I). Giemsa-stained sections (C-G) and H/*E*-stained sections (H—I). Scale bars = 20 μm, except in (C) = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

± 0.12; mean ± SEM); and more splenic MMCs (R: 1.98 ± 0.11; C: 1.55 ± 0.15; mean ± SEM) than C (Supplementary Fig. 3). No significant alterations were found in the head kidney.

## 4. Discussion

The current study has shown the successful experimental *in vivo* infection model, replicating the signs associated with clinical sparicotylosis in farmed GSB in a RAS environment. Prevalence of infection in experimental fish was 100% at all sampling times in both trials. Along the trials, several variables were monitored. The correlation analysis showed that one cluster of variables comprised of water temperature, exposure time, biomass, length and weight had a positive correlation with adult *S. chrysophrii* infection intensity, highlighting that the parasitic transmission was favoured by increasing temperature, higher biomass in R tanks and larger R fish (Fig. 2B). A second cluster related to the pathogenic effect of the parasite, including the condition factor, and haemoglobin and haematocrit variables, had a negative correlation with adult *S. chrysophrii* infection intensity, reflecting the signs of sparicotylosis described for diseased farmed GSB, namely anaemia and emaciation (Riera-Ferrer et al., 2022; Sitjà-Bobadilla et al., 2006; Sitjà-Bobadilla and Álvarez-Pellitero, 2009).

The establishment of an *in vivo* model for parasite maintenance provides researchers with an unlimited source of parasites for further studies regarding the parasites' biology, opening possibilities to develop *in vitro* culture methods. Moreover, *in vivo* models permit not only the study of the pathogenesis of the disease but also host-parasite interactions, immune response and *in vivo* assays on the efficacy of therapeutic candidates, including functional feeds, under controlled conditions (Ahmed, 2014; Hutson et al., 2018).

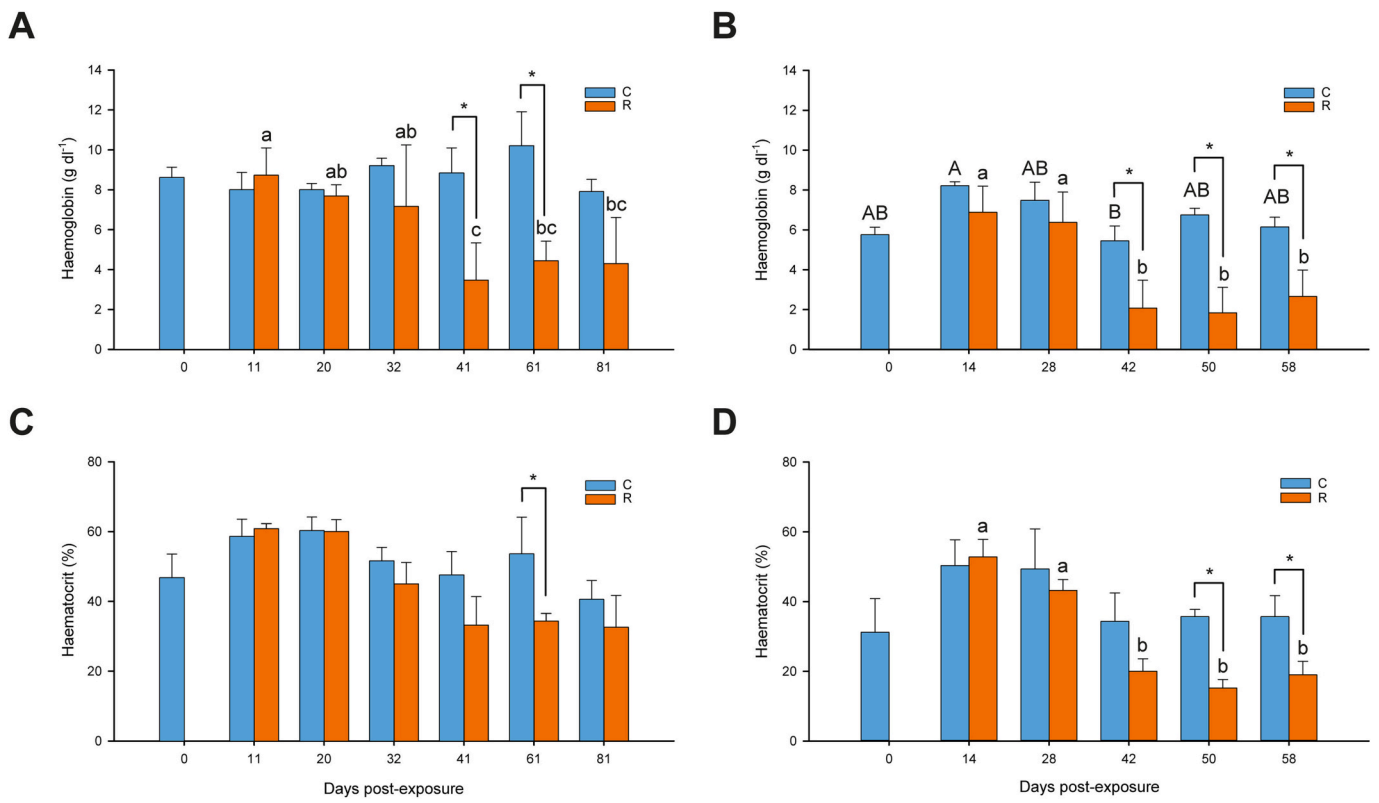


**Fig. 4.** Haemoglobin and haematocrit (mean + SEM) in control (C) and recipient (R) gilthead seabream in Trial 1 (A, C) and Trial 2 (B, D) throughout the experimental infection. Different uppercase and lowercase letters stand for statistically significant differences within C or R groups, respectively. Asterisks (*) indicate statistically significant differences between C and R groups within each sampling point ($P < 0.05$).

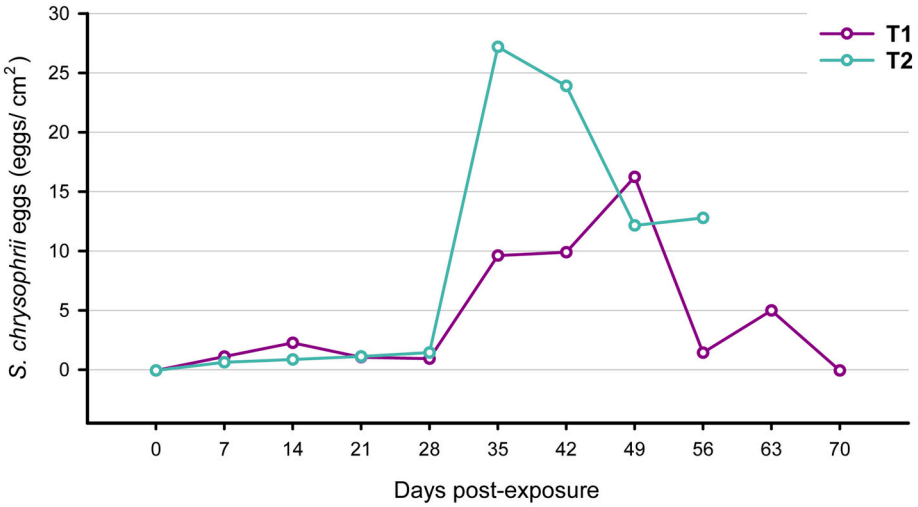


**Fig. 5.** Monitoring of *Sparicotyle chrysophrii* eggs (eggs/cm$^2$) in the egg collector throughout experimental Trials 1 and 2.

### *4.1. Constraint factors for sparicotylosis transmission*

Salinity, pH and water temperature are abiotic factors with an impact on the development of monogenean parasites. However, the latter is the most influencing factor (Ernst et al., 2005; Hoai, 2020; Lackenby et al., 2007; Repullés-Albelda et al., 2013; Valles-Vega et al., 2019; Villar-Torres et al., 2018; Zhang et al., 2022). In the past years, specific optimal temperatures regarding the embryonic development, hatching rate, oncomiracidial swimming behaviour and survival, and the development of the infective stages, as well as its thermal limits for survival, have been set for *S. chrysophrii* (Repullés-Albelda et al., 2012, 2011; Villar-Torres et al., 2018).

*Sparicotyle chrysophrii* prolificacy and egg viability studies are lacking. However, the current study gives some insights into the possible effects of temperature on the prolificacy of this parasite. Although neither the adult parasitic burdens nor the overall egg counts (Fig. 5, Fig. 2) statistically differ between T1 and T2, slight differences in egg shedding patterns were noticed. Egg counts in the colder T1 initially increased at 35 dpe and sustained until they peaked at 49 dpe, whereas this occurred around 14 dpe earlier in the warmer T2, with a unique peak at 35 dpe (Fig. 5). Apparently, warmer water temperature hastening the monogeneans' metabolism and development, including

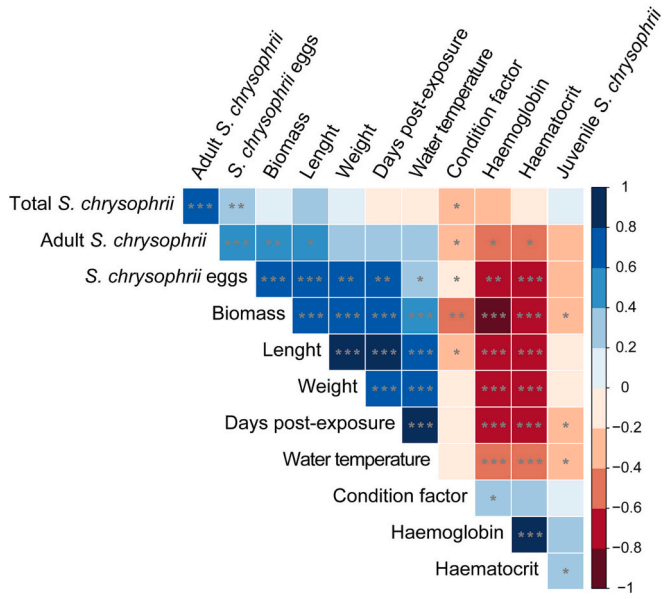


**Fig. 6.** Correlation matrix of parameters affecting *Sparicotyle chrysophrii* experimental transmission in Trial 2. The colour gradient represents the correlation coefficient between variables. *P* values are shown by asterisks (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

*S. chrysophrii*'s reproductive maturity (Lackenby et al., 2007) could have had an impact on the egg counts. This hypothesis would be supported by the fact that the number of *S. chrysophrii* eggs in the collectors was significantly and positively correlated with water temperature. In addition, the significant positive correlation between egg counts and *S. chrysophrii* adults (Fig. 6) showed that collector monitoring was an optimal non-lethal tool for the survey of the infection in GSB, besides the role of the collector as a way to retain eggs in the R tanks, favouring oncomiracidia-fish encounters.

Biomass (animal density) has been regarded as a key factor for parasite establishment in host communities (Hechinger and Lafferty, 2005). In T2, as the population size was kept constant, fish grew up along the trial, and the biomass in the tanks increased. The current transmission model seems to be highly dependent on biomass in the system (Fig. 6), as adult *S. chrysophrii* burden and the egg counts were highly and significantly correlated with the biomass (Fig. 6). This effect could be explained by an increase in the chances of oncomiracidia-fish encounters, as available gill surface of fish increases. Similarly, longer exposure times correlated positively with egg counts as chances for reproduction and egg entangling increased over time, favouring parasite transmission.

Surprisingly, the correlation outcome of juvenile and adult counts vastly differed. In the current experiment, numbers of *S. chrysophrii* juveniles correlated negatively with both water temperature and biomass (Fig. 6). Future experimental infections under same conditions will evidence if these findings, are in fact, a common trend. However, increasing temperature favours embryonic development and egg hatching rates and these observed negative correlations might be explained by the observations from Villar-Torres et al. (2018), where a decrease in the swimming activity and survival rate in *S. chrysophrii* oncomiracidia was observed with increasing temperatures from 10 °C to 26 °C, resulting in a lesser ability to infect hosts. However, this negative correlation could also be explained by a type II error in the diagnosis procedure, due to excessive gill mucus discharge and the poor and friable conditions of gills from diseased GSB, which hampered their detection under the stereomicroscope and/or favoured their detachment, but further evidences are needed. In any case, the results of juvenile *S. chrysophrii* burden certainly altered the expected results for the total *S. chrysophrii* counts correlations. These results stress out the importance of adult and egg counts for the monitoring of this disease, with the current knowledge and available techniques.

### 4.2. *Pathogenic effect of Sparicotyle chrysophrii*

Polyopisthocoltylean monogeneans have been described as haematophagous parasites (Halton and Jennings, 1965; Llewellyn, 1954). In the past years, a negative impact of *S. chrysophrii* on its hosts' haemostasis has been observed under experimental conditions (Henry et al., 2015; Riera-Ferrer et al., 2022; Sitjà-Bobadilla and Álvarez-Pellitero, 2009), and it has been suggested that the parasite causes anaemia by branchial tissue disruption inflicted by the parasite's haptoral clamps (Antonelli et al., 2010). Recently, the haematophagous nature of *S. chrysophrii* has been experimentally demonstrated (Riera-Ferrer et al., 2021b), and it has been suggested that this parasite could cause an haemolytic anaemia in its host (Riera-Ferrer et al., 2022). Either way, anaemia caused by haemorrhages and/or by haemolytic events would group into regenerative anaemia, as supported by the findings in previous studies (Sitjà-Bobadilla and Álvarez-Pellitero, 2009) where an increase in reticulocyte counts in diseased GSB was observed after 8 weeks of parasite exposure.

In the present study, Hb and Hct were significantly and negatively correlated with counts of *S. chrysophrii* adults (Fig. 6). Regardless of the trial conditions, a significant drop in haemoglobin was registered in R at 41 and 42 dpe (Fig. 4A, B), 9 to 14 days after the detection of adult *S. chrysophrii* stages (Fig. 2), and a sustained significant drop in Hct was also registered in T2 (Fig. 4C, D).

The head kidney is the main erythropoietic organ of teleost, while the spleen mainly acts as an erythrocytic reservoir (Verde et al., 2011; Witeska, 2013) supplying erythrocytes to the circulation after a splenic contraction when oxygen-deficient conditions take place (Valenzuela et al., 2005; Yamamoto, 1988; Yamamoto et al., 1980). Additionally, erythropoietic activity has been described as a physiological process strongly modulated by hypoxic events such as high-water temperature and low dissolved oxygen (strongly correlated parameters) (Boyd, 2020; Witeska, 2013). Severe damage to the gills due to the parasite, such as lamellar synechia, clubbing, shortening and/or secondary lamellar fusion, as evidenced in this work and in previous studies (Sitjà-Bobadilla and Álvarez-Pellitero, 2009) certainly compromise oxygen exchange and could, therefore, mask anaemia identification in diseased GSB when Hct is the only haematological parameter being monitored. Moreover, the impact of adult *S. chrysophrii* on the larger T1 fish was lower than on the smaller juveniles of T2 (Figs. 4–5), which coincides with field observations in affected farms (authors' observations). The importance of fish size/age probably not only relies on the difference in total blood volume, being greater in adults, and on the potential erythrocytic reservoir held in the adult spleen, as well as on the ability to enable a faster erythropoietic response (Yamamoto, 1988; Yamamoto et al., 1980), but also on the higher susceptibility to hypoxia in smaller sized fish, worsening the clinical outcome (Verberk et al., 2022).

Juvenile *S. chrysophrii* burden, contrary to adult burden, had a positive correlation trend with haematological parameters (Fig. 6), evidencing that these stages have a minor impact on their host's haemostasis, if any. In any case, post-larval and juvenile histophagous feeding strategies proposed by other authors (Antonelli et al., 2010) cannot be disregarded.

The negative significant correlation of the *CF* with adults, eggs and even total parasite counts further evidenced the emaciative syndrome provoked by the disease and the poor health status of parasitised GSB under the anaemic condition previously described.

### 4.3. *Histopathological findings*

Eosinophilic granular cells (EGCs) are a cell type described to be involved, among others, in host defence against gill parasites (Dezfuli and Giari, 2008; Reite and Evensen, 2006) as part of the innate immune

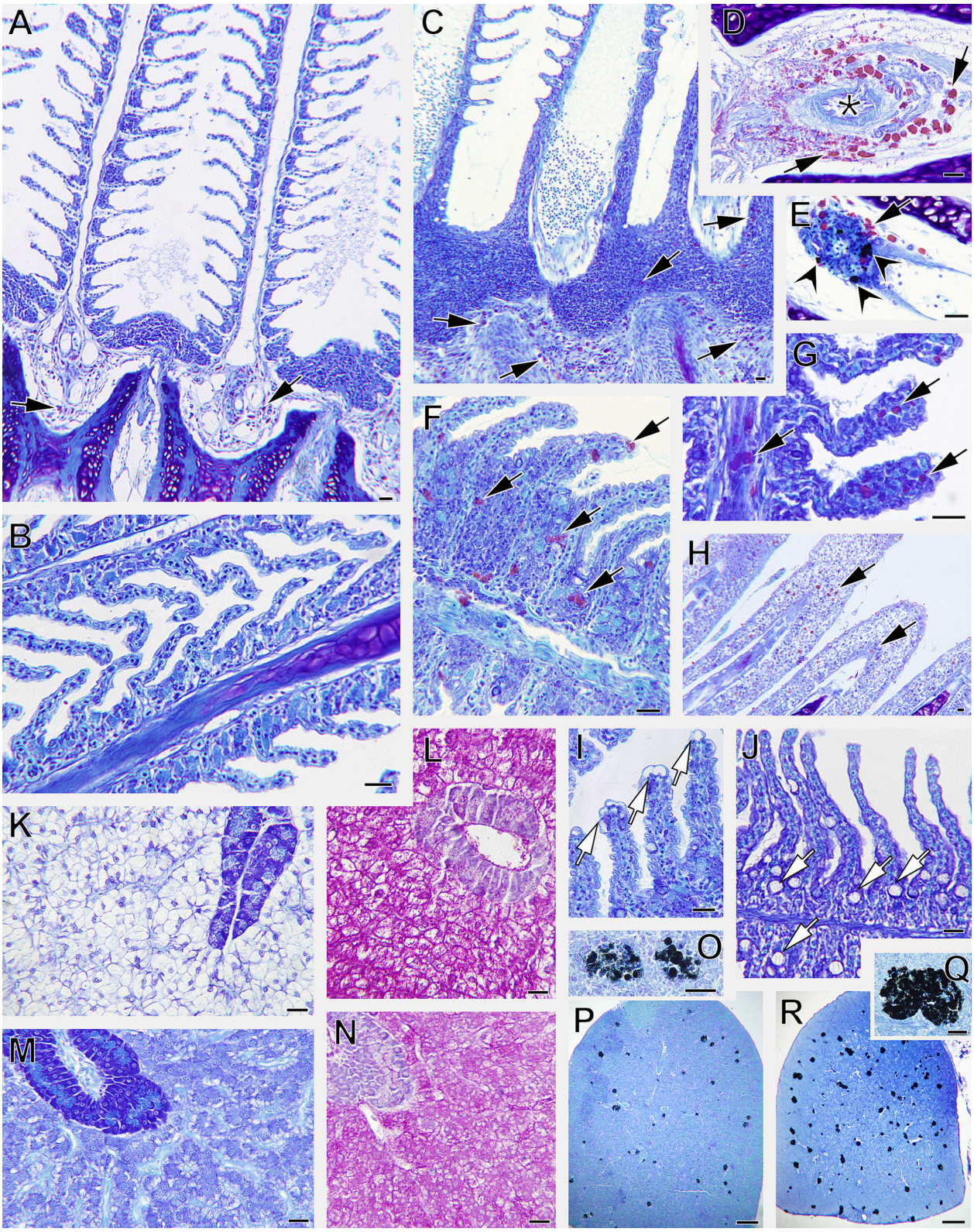


**Fig. 7.** Histological alterations found in GSB experimentally exposed to *Sparicotyle chrysophrii* in Trial 1 and Trial 2. Control gills (A, B), liver (*K,L*) and spleen (O, P) sections are shown for comparison. Higher abundance of eosinophilic granular cells (black arrows) is observed in infected gills, among inflammatory infiltrates at the base of the filaments (C), infiltrated around efferent arteries (asterisk) with apparent degranulation (D) or together with melanomacrophages (black arrowheads) (E), infiltrated in the epithelium of primary and secondary lamellae (F, G) and in filament tips (H). Goblet cell (white arrows) hyperplasia was observed in the tips (I) and base of secondary lamellae (J) in infected fish. Liver sections of control fish present the usual fat (K) and glycogen deposits (L) in hepatocytes, colourless and magenta, respectively. Note the severe lipid absence in hepatocytes of infected fish (M), while glycogen remains unchanged (N). The spleen section of control fish (O, P) presents small melanomacrophage centres (MMCs) with dispersed melanin granules. Note the higher abundance of MMCs in the spleen of infected fish (Q, R) with densely packed melanin granules. All sections are stained with Giemsa, except (L) and (N) stained with PAS. All scale bars = 20 μm, except in (O) and (Q) scale bars = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

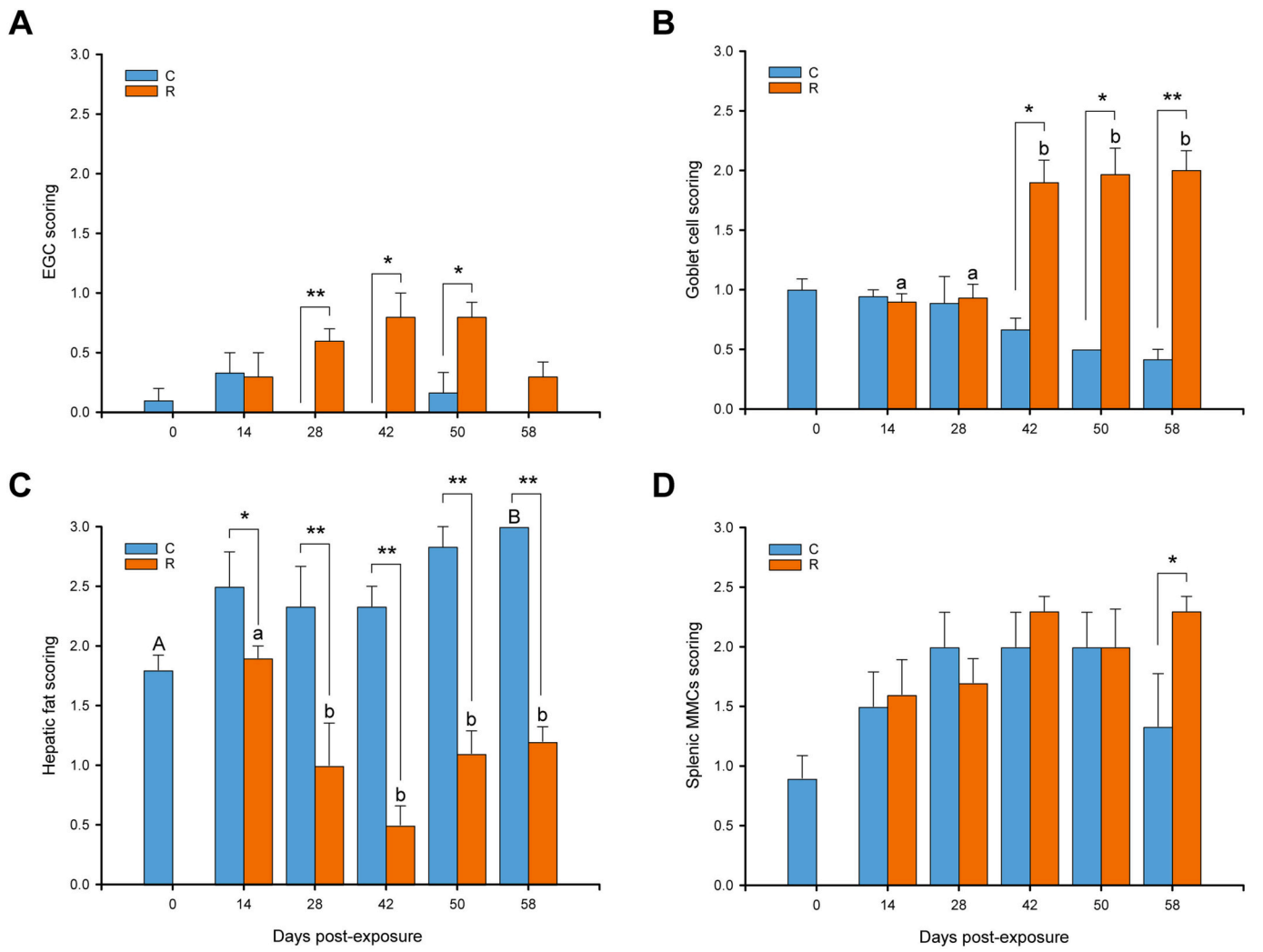


**Fig. 8.** Scoring of histological alterations in control (C) and recipient (R) gilthead seabream through the experimental infection in Trial 2. Mean semiquantitative scoring (mean + SEM) from 0 (absence) to 3 (very abundant) is shown for eosinophilic granular cells (EGC) in gill filaments (A), goblet cells in gill filaments (B), hepatic fat storage (C) and melanomacrophage centres (MMCs) in spleen (D). Different uppercase o lowercase letters stand for statistically significant differences within C or R groups, respectively, $P < 0.05$. Asterisks indicate statistically significant differences between C and R groups within the same sampling point, (*) $P < 0.05$ and (**) $P < 0.001$.

response in teleost fish, which is involved in inflammatory induction, vasodilation, neutrophil recruitment and macrophage activation (Reite and Evensen, 2006). Despite it has been suggested that GSB has a resident population of EGCs throughout the loose connective tissue of the gill arch and filaments (Noya and Lamas, 1996), the present study shows a significant increase of this cell type in R gills at 28 dpe (Fig. 8A), when adult *S. chrysophrii* worms first appeared in R, and most probably induced by the gill tissue disruption provoked by the parasites' haptoral clamps (Fig. 3F). Similar observations have been noted in GSB infected by other class gill parasites (Dezfuli et al., 2011), and also in other monogenean infections affecting different host species (Dezfuli et al., 2010). Among EGC populations of GSB, the acidophilic granulocytes are considered functional equivalents of the mammalian neutrophils, being the predominant cell type recruited during immune responses (Mulero et al., 2007; Sepulcre et al., 2002) in accordance to the authors' observations. The triggering of a local pro-inflammatory immune response arbitrated by acidophilic granulocyte degranulation during sparicotylosis was previously highlighted by transcriptomic data (Firmino et al., 2020).

Goblet cell hyperplasia was observed in the gills of infected fish from 42 dpe on, coinciding with the prevailing adult parasitic stages. The protective mucus layer of mucosal epithelia is synthesised by goblet cells. Gill mucus hypersecretion is common upon monopisthocotylean infections in maricultured fish (Ogawa, 2015), and goblet cell hyperplasia in response to gill parasitosis has been associated with an increased mucus secretion for preservation of the epithelial integrity, prevention of secondary infections and osmoregulatory failure after tissue damage (Castrillo et al., 2021). Dietary immunostimulation against *Neobenedenia girellae* in greater amberjack (*Seriola dumerili*) and against *S. chrysophrii* in GSB demonstrated that more immunocompetent fish relied on up-regulation of mucin genes, the main components of the mucus secretion, and increase of goblet cell number with shift in the glycosylation profile of their mucins, respectively (Fernández-Montero et al., 2019; Firmino et al., 2020). Involvement of goblet cell and mucin modulation in the local mucosal response of GSB against *S. chrysophrii* seems evident in the light of the current results, and could have worm-trapping and expulsion purposes as described for intestinal helminthiases in teleosts (Dezfuli et al., 2010). Interestingly, goblet cell hyperplasia became evident after the first month of parasite exposure, apparently triggered by the more pathogenic adult specimens.

Epitheliocystis, was found in the gills of R from 61 dpe on (T1) and from 42 dpe on (T2). This is a disease caused by pathogenic intracellular bacteria in a wide range of fish species, whose pathogenic agents are species-specific and opportunistic, and develops and progresses under

environmental or pathological stress conditions (Blandford et al., 2018). Co-infection of *S. chrysophrii* and epitheliocystis has been previously reported (Padrós and Crespo, 1995; Sitjà-Bobadilla and Álvarez-Pellitero, 2009), but attempts to characterise the causative agents point to complex bacterial associations of $\beta$-proteobacteria and chlamydial agents (Seth-Smith et al., 2017; Seth-Smith et al., 2016). In the current study, epithelial cysts with and without a proliferative tissue response around them were found and lead to lamellar fusion (Supplementary Fig. 2), in agreement to a previous description in GSB (Crespo et al., 1999). The effect of *S. chrysophrii* on the gill microbiota through clamp erosion and lesions on the mucosal surface or through the excretory/secretory products of the parasite is far from being understood, and further research efforts should focus on the interaction between this parasite and secondary infections, in particular, how the mucosal microbiota in GSB gills is modulated during sparicotylosis.

Melanomacrophage centres (MMCs) correspond to melanomacrophage (MMs) aggregations, phagocytes with high melanin, haemosiderin and lipofuscin content located in the stroma of haematopoietic and lymphoid tissues, mainly in the kidney and spleen of teleost fish (Agius, 1981; Agius and Roberts, 2003; Steinel and Bolnick, 2017; Uribe et al., 2011; Wolke, 1992). Multiple non-immunological and immune roles have been ascribed to MMCs (Steinel and Bolnick, 2017). The primary functions of MMs have been suggested to be phagocytosis, detoxification/ debris clearance (catabolism of erythrocytes), iron recycling, and long-term storage of either endogenous or exogenous highly indigestible or toxic material (Agius, 1979; Agius and Agbede, 1984; Agius and Roberts, 2003; Fulop and McMillan, 1984; Steinel and Bolnick, 2017). Recently, MMCs have been suggested to be the evolutionary precursors of mammalian germinal centres, key in the differentiation and clonal expansion of memory B cells (Buchmann, 2022; Steinel and Bolnick, 2017; Stosik et al., 2019). In this study, MMCs abundance was significantly higher in R than in C (Supplementary Fig. 3D), especially at 58 dpe (Fig. 8D). Furthermore, MMCs in R appeared to be larger in size and more dense (Fig. 7O-R), results which are in line with those obtained by De Vico et al., 2008. Moreover, the high catabolism of erythrocytes damaged by haemolytic anaemia, as previously suggested by Riera-Ferrer et al., 2022 would explain the higher haemosiderin-iron content in splenic MMCs reported by De Vico et al., 2008 during sparicotylosis. Even more interesting is the suggested role of MMCs in the onset of adaptive immune responses (Vigliano et al., 2006), which would explain the increase in MMCs abundance observed in this study in R. In this context, *S. chrysophrii* triggered activation of B cells upon infection in a previous study (Piazzon et al., 2019) suggesting a specific immune response with a probable shift of the immunoglobulin repertoire described by Riera-Ferrer et al. (2022).

The emaciation provoked by sparicotylosis was evidenced by a reduction of lipid deposits in the liver (Fig. 7K-N; Fig. 8C; Supplementary Fig. 3C), and likely reflects compensation for the disease's high energy costs with depletion of hepatic lipid storage as an energy source. Similar observations were previously registered during the course of a parasite-induced enteritis in GSB, which impaired nutrient absorption (Picard-Sánchez et al., 2020). Thus, lipid storage in the liver of GSB seems a good indicator of health condition. In addition, *S. chrysophrii* feeding on its host's lipid reservoir is plausible, as suggested by the hypocholesterolaemia and plasmatic depletion of very low-density and low-density lipoproteins (Riera-Ferrer et al., 2022) and by the fact that platyhelminths are unable to synthesise fatty acids *de novo* (Barrett, 1981). Other studies have reported dependence on the host lipid content for immune evasion by the digenean trematode *Schistosoma mansoni* (Bennett and Caulfield, 1991) or for modulation of the host's immune system by *E. nippocum* (Lombardo et al., 2022; Vorel et al., 2021). Hepatic fat depletion has been related with deriving low-density and high-density lipoproteins for the maintenance of erythrocytic membrane stability (Van Der Stoep et al., 2014), which might also be affected in *S. chrysophrii*-infected GSB. The normal presence of hepatic glycogen in the samples (Fig. 7M-N) suggests that *S. chrysophrii* is not reliant on glycogen as a source of energy, as previously suggested for the monogenean *Diclidophora merlangi*, which would obtain its energy from aerobic respiration rather than from glycogen reservoirs from their hosts (Arme and Fox, 1974).

*Sparicotyle chrysophrii*'s demand for oxygen would, in turn, explain the location of adult *S. chrysophrii* specimens within the gills, close to the gill filament apex and downstream in the ventilating water flow (Fig. 3A, C), and could additionally be supplemented by oxygen in the ingested blood meal, as suggested by Houlihan and Macdonald, 1979 in *D. merlangi*. Post-larval and juvenile *S. chrysophrii* specimens are, however, placed close to the branchial arches in the secondary interlamellar spaces (Fig. 3B, D), most probably due to an underdeveloped opisthaptor or lower numbers of clamps as this location may provide better shelter from water currents and detachment.

## 5. Conclusions

The proposed *in vivo* transmission model enables the maintenance of *S. chrysophrii* under experimental conditions, being temperature and biomass the most critical parameters. This model allows to achieve those pathological effects and parasite loads described in farmed GSB, and therefore opens the door to test treatment alternatives under experimental conditions. In addition, the use of egg collectors proved to be a reliable non-lethal tool to monitor the progression of the parasitosis without the need of handling or euthanising fish, therefore contributing to the 3R strategy in experimental animal procedures.

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## Author statement

Details of each author with their contribution in this paper.

## CRediT authorship contribution statement

**Enrique Riera-Ferrer:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Raquel del Pozo:** Methodology, Investigation, Writing – review & editing. **M. Carla Piazzon:** Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft. **Ariadna Sitjà-Bobadilla:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft. **Itziar Estensoro:** Methodology, Formal analysis, Data curation, Investigation, Supervision, Visualization, Writing – original draft. **Oswaldo Palenzuela:** Conceptualization, Funding acquisition, Formal analysis, Methodology, Resources, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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