Water temperature, time of exposure and population density are key parameters in *Enteromyxum leei* fish-to-fish experimental transmission

Running title: Time, temperature and myxozoan infections.

Amparo Picard-Sánchez; Itziar Estensoro; Raquel Del Pozo; Oswaldo Palenzuela; M. Carla Piazzon[#]; Ariadna Sitjà-Bobadilla

Instituto de Acuicultura Torre de la Sal, Consejo Superior de Investigaciones Científicas, Fish Pathology Group, Ribera de Cabanes, Castellón, Spain.

[#]**Correspondence**: M. Carla Piazzon, carla.piazzon@csic.es, (+34) 964319500.

Acknowledgments

The authors thank J. Monfort and L. Rodríguez for histological processing. This work has been carried out with financial support from European Commission, Horizon 2020 Framework Programme H2020, Societal Challenges under grant agreement 634429 (ParaFishControl). This publication reflects only the authors' view and the European Union cannot be held responsible for any use that may be made of the information contained therein. IE was contracted under APOSTD/2016/037 grant by the "Generalitat Valenciana", and MCP under Consejo Superior de Investigaciones Científicas CSIC PIE project no. 201740E013.

Data Availability Statement. All data related to this study is included in the manuscript or in the supplementary material.

Conflict of interests. The authors declare that they have no conflict of interest.

Abstract

Enteromyxum leei is a myxozoan histozoic parasite that infects the intestine of several teleost fish species. In gilthead sea bream (*Sparus aurata*), it provokes a chronic disease, entailing anorexia, delayed growth, reduced marketability and mortality. Direct fish-to-fish transmission, relevant in aquaculture conditions, has been demonstrated for *E. leei* via effluent, cohabitation, oral and anal routes. However, the minimum time of exposure for infection has not been established, nor the possible effect on the fish immune response. Two effluent trials were performed at different temperatures (high: average of 25.6°C, and low: constant at 18°C), different times of exposure to the effluent (1, 3, 5, and 7 weeks), and different population densities. The results showed that one week was enough to infect 100% of fish at high temperature, and 58.3% at low temperature. High temperature increased the prevalence of infection in posterior intestine, but also induced a higher production of specific antibodies, limiting the progression of the infection along the intestine. Longer time of exposure to the parasite and higher fish densities facilitated *E. leei* infection. These results show that effective diagnosis, lowering animal density and removal of infected fish are key aspects to manage this disease in aquaculture facilities.

Keywords: Host-parasite interaction, Growth delay, Time of exposure, Temperature, Antibody.

1 INTRODUCTION

Myxozoa are multicellular and spore-forming endoparasites that infect mainly marine and freshwater fish. Commonly, their life cycle includes two hosts; an invertebrate definitive host and a vertebrate intermediate host (Yokoyama, 2003). Definitive hosts (annelids) have only been described for a small number of myxozoan species (Fontes, Hallett & Mo, 2015), such as *Tubifex tubifex* for *Myxobolus cerebralis* (Markiw & Wolf, 1983) or *Manayunkia speciosa* for *Ceratonova shasta*, both parasites of salmonids (Bartholomew, Whipple, Stevens & Fryer, 1997). The study of myxozoans has been hampered mainly by the unavailability of *in vitro* cultures, and just few of them can be experimentally maintained *in vivo* (Abd-Elfattah et al., 2014; Eszterbauer et al., 2015; Korytář et al., 2019), including *Enteromyxum leei.*

E. leei, infects gilthead sea bream (GSB, *Sparus aurata*) among other sparids (Golomazou et al., 2006; Yanagida et al., 2008) and up to more than 60 different species, and its definitive host remains unknown. This parasite invades the intestinal epithelium, provoking chronic intestinal inflammation, causing poor food conversion rates, cachexia and eventually death (Sitjà-Bobadilla & Palenzuela, 2012). *E. leei* induces a slow-progressing disease in GSB that starts in the posterior intestine, spreading to the anterior and finally to the middle segment of the intestine (Sitjà-Bobadilla & Palenzuela, 2012). Experimental transmission of *E. leei* has been described for gilthead sea bream by effluent and cohabitation (Diamant, 1997; Diamant & Wajsbrot, 1997; Sitjà-Bobadilla, Diamant, Palenzuela & Álvarez-Pellitero, 2007), and anal or oral intubation of intestinal scrapings from infected fish (Diamant & Wajsbrot, 1997; Estensoro, Redondo, Álvarez-Pellitero & Sitjà-Bobadilla, 2010). Effluent transmission involves two sources of parasite, the stages in the water from the donor tank, and the stages released from the newly infected recipient fish that become

cohabitant donors. Anal intubation is a fast and homogenous transmission method, since the inoculated amount of parasites is similar in each set of experimental fish, and parasites are inoculated directly to the target tissue, the posterior intestine (Estensoro et al., 2010). However, this method could be too aggressive to test some *in vivo* interventions that could have a subtle effect slowing down the propagation of the parasite, such as some nutraceuticals, vaccines or passive immunization trials. Mimicking the most common route of infection in farmed fish, experimental transmission was also achieved for other fish-*Enteromyxum* spp. models through cohabitation/effluent of sharpsnout sea bream (*Diplodus puntazzo*) (Muñoz et al., 2007), tiger puffer (*Takifugu rubripes*) (Yasuda et al., 2002), and turbot (*Scophthalmus maximus*) (Redondo, Palenzuela, Riaza, Macías & Álvarez-Pellitero, 2002), or feeding infected gut tissue to Japanese flounder (*Paralichthys olivaceus*) (Yasuda et al., 2005).

Several *in vivo* studies with myxozoans have demonstrated that temperature, fish density, time of exposure and parasitic load are key parameters for the onset and development of infection (Fontes et al., 2015). Temperature is an important factor in the progression of myxozoan infections. Increased temperatures have been correlated with higher prevalence and severity of infection (de Buron et al., 2017; Udey, Fryer & Pilcher, 1975). However, extreme temperatures can also supress myxozoan proliferation, either at low temperatures (Estensoro et al., 2010; Palikova et al., 2017; Yanagida, Sameshima, Nasu, Yokoyama & Ogawa, 2006), or high temperatures (China et al., 2014).

Parasite load can be measured in degree days, which are correlated with the time that the fish have been exposed to infective stages of the parasite and the temperature during this period (Braden et al., 2018; Jones & Long, 2019; Thompson, Snekvik & Vincent, 2010). These infective stages can be either spores from the definitive

invertebrate host (Feist, Longshaw, Canning & Okamura, 2001; Markiw, 1989), or proliferative stages from already infected fishes (de Buron et al., 2017; Estensoro et al., 2010). In some cases, myxozoan infections have been described to occur after very short times of exposure, ranging from minutes to days, like *M. cerebralis* (Markiw, 1989), *Tetracapsuloides bryosalmonae* (Feist et al., 2001), or *Kudoa inornata* (de Buron et al., 2017). However, no short term exposure trials have been conducted with *E. leei* yet.

In vivo experimental infection models present more contributing variables than *in vitro* systems, as they depend on many biotic and abiotic factors. The lack of information of how these factors interact in a given fish-parasite model challenges the standardization of experimental infections *in vivo*. The contribution of the different factors needs be studied in order to achieve controlled infections, and to avoid severe infections induced by extreme doses or conditions. In addition, this information will be very useful for the management of fish farms with recurrent *E. leei* infections.

Therefore, the aim of the present work was to determine a minimum time that GSB needs to be exposed to *E. leei*-contaminated water to get infected, and show the differences in terms of prevalence and intensity of infection caused by the variables fish density and temperature. This information will help to minimize the time of exposure, resources and animal use underpinning the three R's principles of refinement, reduction and replacement (Flecknell, 2002).

2 MATERIAL AND METHODS

2.1 Fish and experimental infections

Clinically healthy GSB juveniles from a commercial fish farm were kept at the facilities of IATS-CSIC (Castellón, Spain) in 5 µm-filtered and UV irradiated sea water (salinity 37.5 g/L) at temperatures ≥ 18°C, with natural photoperiod at our latitude (40º5'N; 0º10'E). The absence of *E. leei* in the stock was confirmed by qPCR according to the protocol described by Picard-Sánchez et al. (2019). Fish were fed *ad libitum* a commercial diet (BioMar, Palencia, Spain). Animals were kept according to the Guidelines of the European Union Council (Directive 2010/63/EU), the Spanish RD 53/2013, and the CSIC National Committee on Bioethics under approval number 2018/VSC/PEA/0240. Trial 1 (T1) started in July 2018 and lasted 92 days (average water temperature: 25.6ºC, range 22.1ºC - 27.5ºC). Trial 2 (T2) started in March 2019 and lasted 92 days (constant water temperature: 18 ± 0.5ºC).

The challenges were performed by effluent exposure as previously described (Sitjà-Bobadilla et al., 2007). A schematic representation of the experimental setup can be found in Figure 1. Briefly, the trials consisted of two recipient (R) 500 L tanks, named R1 and R2, with 50 (T1) or 48 (T2) naïve fish each, receiving the water from a donor (D) tank holding *E. leei*-infected GSB. R fish in both trials belonged to the same stock (initial mean body weight: 68.7 g ± SD 21.9 for T1 and 102.1 g ± SD 22.2 for T2). In both trials, D fish consisted of a group of 50 infected fish, positive for *E. leei* by qPCR (average Ct values of 24.59 SD 2.58, and 25.10 SD 3.62 for T1 and T2, respectively). The experimental setup was similar in both trials: A constant number of fish was kept in tanks holding group R1 during the entire experiment. In contrast, from tanks holding group R2, 10 (T1) or 12 (T2) fish were removed at different weeks post-

exposure (wpe): 1, 3, 5, and 7 wpe in T1, and 1, 3, and 5 wpe in T2. The removed groups of each trial were kept in separate 170 L tanks receiving parasite-free water, and were named: R2-1, R2-3, R2-5, and R2-7. The parasite load was quantified in degree-days (DD), calculated as the sum of the recorded daily temperature (measured at the same time of the day) during the exposure to the *E. leei* effluent, as previously used in other myxozoan studies (Braden et al., 2018; Jones & Long, 2019). The renewal of the water in the D tank, was kept to a minimum to ensure exposure to the parasite, but without affecting water quality. In all tanks (including effluent recipient tanks), the water flow was adjusted to reach three renovations per day. In tanks R1 and R2, the effluent was not interrupted until the final sampling.

2.2 Samplings and parasite diagnosis

In each trial, parasite infection was evaluated by molecular diagnosis and histology in two samplings, a non-lethal intermediate sampling at 9 wpe, and the final lethal sampling at 13 wpe. In the non-lethal samplings (9 wpe), only molecular diagnosis was performed, and at the final samplings (13 wpe), the diagnosis was performed by both methods. Fish were starved for two days before samplings, and biometric parameters were measured. The non-lethal sampling consisted of a probe of the rectal mucosa with a cotton swab and a diagnostic qPCR carried out with specific primers for *E. leei* 18S rRNA gene, as previously described (Piazzon et al., 2017). At the final sampling (13 wpe), fish were killed by overexposure to anaesthetic (MS-222, 0.1 g/L; Sigma), and blood and intestinal tissue samples were obtained. Blood samples (1 ml) were taken from the caudal vessels by puncture with heparinized sterile needles. After overnight clotting at 4°C and centrifugation at 3,000 ×*g* for 30 min, sera were recovered and maintained at -80°C until further use. Tissue samples were processed for molecular and histological diagnostics, as previously described (Picard-Sánchez et al., 2019). For

histology, small portions (3-4 mm) of anterior (AI), middle (MI) and posterior (PI) intestine from all fish were fixed in 10% buffered formalin for standard histological procedures. DNA from the remaining intestine was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) following the manufacturer's instructions. Infection intensity was semiquantitatively evaluated on Giemsa stained histological sections using a scale from 1 (lowest) to 6 (highest), as previously described (Estensoro et al., 2010). A fish was considered positive for the infection, when the parasite was found at least in one intestinal segment.

2.3 Detection of parasite-specific IgM in serum

Specific IgM against *E. leei* in serum was measured by immunohistochemistry (IHC), as previously described (Picard-Sánchez et al., 2019). Briefly, paraffin embedded sections of an *E. leei*-infected GSB intestine obtained from a previous infection were used as an antigen sample, and the sera from the current study as a primary antibody. Each serum sample was tested in duplicate. Negative immunoreactive controls were carried out using serum of fish not exposed to the parasite. Negative controls were carried out omitting the fish serum, the primary and secondary antibodies or the avidin-biotin-complex. No signal was detected in any of these controls. Intensity of immunoreactivity of each fish serum against the parasite was evaluated by microscopic examination of the immunolabelled tissue sections according to a semiquantitative scale ranging from 0 to 6 as described in Picard-Sánchez et al. (2019).

2.4 Data analysis and statistics

Differences in biometrical parameters, parasite infection values, and specific circulating antibodies (IgM), among groups were evaluated within each sampling (9 wpe and 13 wpe), and each trial (T1 and T2). Continuous variables were analysed by one-way

analyses of variance (ANOVA-I) followed by Student–Newman–Keuls test. Data which failed the normality or equal variance test were analysed with Kruskal–Wallis ANOVA-I on ranks followed by Dunn's method. Two-way ANOVA was used to compare levels of specific antibodies between trials. Chi-squared test was used to evaluate differences in proportions (prevalence). Statistical significance was assumed in all tests when $P < 0.05$. All the data were analysed with GraphPad Prism 8 and SigmaPlot 14.0. Hierarchical clustering (*hclust*) and correlations were performed using R statistical software (v3.5.2). For these analyses, the mean values of all the parameters measured were included (Supplementary Table 1). For the correlation analyses, the *corrplot* package was used and the *cor.test* function was applied to compute significant correlation coefficients with a confidence level of 0.95. Visualizations were constructed using the *factoextra* (v.1.0.5) and *ggplot2* (3.2.0) R packages.

3 RESULTS

3.1 Abiotic factors

R1 and R2 were continuously exposed to the effluent in both trials, but each trial with a different temperature regime, which was translated into higher degree days (DD) in T1 (natural summer temperatures) than in T2 (constant 18°C). DD in T2 at the final sampling (13 wpe) were almost equal to those of T1 at the intermediate sampling (9 wpe) (1638 *vs* 1680 DD) (Table 1). However, subgroups resulting after R2 removals at the different time points received different DD (205.9, 585.9, 942.6, 1317 in T1; and 126, 378, 630 in T2) according to the time of exposure (1, 3, 5 or 7 (only in T1) weeks, respectively) (Table 1). The number of fish in R1 groups was kept constant (n = 50 for T1; n = 48 for T2), whereas R2 groups ended up with 10 (T1) or 12 (T2) fish. Thus,

their population density was also different and affected the course of the infection (see below) (Table 1).

3.2 Growth parameters

In T1, there were no statistically significant differences in weight, size and condition factor (CF: (100 × body weight)/fork length^3) among groups in either of the two sampling points. In T2, at both sampling points, the weight of R1 (the group that remained with a constant number of fish) was significantly lower than R2 and R2-5 groups. CF of R1 was statistically lower than all the other groups (R2, R2-1, R2-3 and R2-5) at 9 wpe. Those differences remained at 13 wpe except for R2-3. No differences were detected in size in either of the samplings (Table 1).

3.3 Parasite diagnosis

Molecular diagnosis showed high prevalence of *E. leei* infection in T1, particularly at the final sampling point, when all the groups reached at least 80% of prevalence of infection (Table 2). There were no differences in prevalence or in intensity of infection among groups in either of the sampling points. In T2, however, significant differences were observed in prevalence of infection at the intermediate sampling, showing R2-1 (only exposed for 1 week) the lowest value. This trend was maintained at the final sampling, but the statistical significance was lost. However, at the final sampling, R1 group (the group with higher density and continuously exposed to the parasite-contaminated effluent), showed a significantly higher intensity of infection than R2-1 group (fish exposed only for one week) (Table 2). When comparing both trials, a slower progression of the infection was observed in T2, with lower prevalence of infection than in T1 in all the groups except T2R1, particularly at the first sampling point. The

intensity of infection in T2R1 was strikingly higher than that of T1R1 (>10 Ct values), even though they showed the same prevalence of infection.

The histological diagnosis performed at the final sampling added valuable information on the progression of the infection along the intestine. In T1, no differences in prevalence, intensity and progression of the infection were detected among groups (Table 2). Of note, none of the fish exposed for only 1 or 3 weeks, acquired the infection at the middle intestine, which is the last intestinal segment invaded by the parasite (Figure 2a, c; Table 2). In T2, the contingency test revealed a significant relationship between the prevalence of infection and the time of exposure in all the intestinal segments (Table 2). In addition, the progression of the infection also differed from that in T1, as the percentage of fish with all the intestinal segments infected was higher in T2, especially in R1 and R2-5 (Figure 2c, d). Furthermore, a clear significant increase of the prevalence of infection with the time of exposure was noticed in both, when analysing the percentage of fish with more than one, and more than two infected intestinal segments (Figure 2b, d). Again, fish exposed only for one week showed the lowest prevalence and progression of the infection, whereas the highest were found in R1 group (Table 2 and Figure 2b, d).

3.4 Parasite-specific antibodies in serum

The intensity of response of parasite-specific IgM in serum at the final sampling point, measured by IHC, was significantly higher in T1 than in T2 (two-way ANOVA $P < 0.0001$). Within T1, no differences were found in antibody levels among groups (Figure 3a), whereas in T2, the group exposed for 1 week (R2-1) showed significantly lower antibody levels than R1 (constant number of fish during the experiment) (Figure 3b).

No differences were detected when comparing the percentage of fish with specific antibodies among groups within each trial (Figure 3c, d).

3.5 Global analysis

The cluster analysis of all the results obtained during the study showed a clear separation of groups by trial, as well as by their time of exposure (Figure 4). Within T1 (high water temperature), groups exposed during shorter times (1 and 3 wpe: R2-1 and R2-3) were more similar, and 5 and 7 wpe (R2-5 and R2-7) clustered closer to the continuously exposed groups (R1 and R2). At lower temperature (T2), R2-3 group was more similar to R2-5 group, and R1 group clustered closer to the high temperature groups than to the other T2 groups, due to its high prevalence of infection.

3.6 Infection status, abiotic, biotic and immune factors are correlated

Correlations among all the different variables in the study, merging the results from both trials, showed interesting links (Figure 5). The time of exposure and DD at the intermediate sampling were negatively correlated with the condition factor, whereas DD at the final sampling showed a negative correlation with the final weight and size of the fish. The final population density in the tanks showed a negative correlation with the specific growth rate and a positive correlation with the percentage of fish with all the intestinal segments parasitized. Most of the biometric parameters showed negative correlations with infection diagnostic variables, which also drove a negative correlation with the presence of parasite-specific antibodies in serum. On average, the percentage of fish with specific antibodies was positively correlated with the percentage of animals with more than one intestinal segment parasitized.

4 DISCUSSION

Host and parasites live in a constant struggle, and evolve together over time, both trying to win each battle. In the current host-parasite model, the speed of parasite transmission and progression, the final prevalence of infection, and the host immune status are influenced by three key aspects: water temperature, time of exposure (parasite load) and fish density (fish body mass per water volume in a tank). This study showed once more how water temperature directly influenced parasite proliferation and the immune response of GSB. In T1, the higher temperature induced two main effects: a faster infection speed (particularly observed in groups exposed for short times) and a higher production of specific antibodies, acting as an opposing force, yielding a lower progression of the infection along the intestinal tract in T1 than in T2. Myxozoan outbreaks tend to occur from spring to autumn, when temperature starts to be warmer (15ºC to 20ºC) (Fontes et al., 2015). Indeed, there is evidence that at cold temperatures, for the Mediterranean Sea lower than 15ºC, *E. leei* development and disease are suppressed (Estensoro et al., 2010; Yanagida et al., 2006). Similar effects of faster spreading due to higher temperature have already been described for other myxozoans. *C. shasta* in spring/summer induced higher mortalities and decreased mean days to death in chinook (*Oncorhynchus tshawytscha*) and coho (*Oncorhynchus kisutch*) salmon in the Klamath River (Ray, Holt & Bartholomew, 2012). *Myxobolus holzerae* caused severe gill disease in Indian major carp (*Labeo rohita*) in spring (Gupta & Kaur, 2017). More severe infections due to warmer water have also been described for *T. bryosalmonae* infecting *Fredericella sultana* (bryozoan host) (Tops, Lockwood & Okamura, 2006), and rainbow trout (fish host) (Clifton-Hadley, Bucke & Richards, 1984). However, there is also an upper limit, and *E. leei* showed to be inhibited at temperatures higher than 30ºC (China et al., 2014).

In fish, as ectotherms, temperature influences greatly the type, speed, and intensity of the immune response (Abram, Dixon & Katzenback, 2017; Le Morvan, Troutaud & Deschaux, 1998). GSB is a sparid fish mostly farmed in the Mediterranean Sea, with optimal growing temperatures between 18ºC and 26ºC (Colloca & Cerasi, 2009). Thus, at 18ºC (T2) the immune response of GSB is not expected to be compromised, but was probably functioning at a slower pace than at 25.6ºC (T1). This would explain the higher intensity of the humoral immune response in T1 fish (Figure 3). At the same time, the faster proliferation of the parasite (induced by the higher temperature) would account for a quicker invasion of the intestine and interaction with immune cells, and therefore, for inducing an earlier immune reaction. Parasite dose (antigen dose), together with location and extent of antigen presentation, are of key importance for the outcome of the immune response (Zinkernagel et al., 1997). Thus, in T1, the parasite could have been proliferating faster and fish may have developed a more intense immune response. In T1, the incipient differences (at the posterior intestine) in prevalence of infection among groups found at 9 wpe disappeared at 13 wpe, as all experimental groups showed very high levels (by qPCR: 80-100%). Thus, differences among groups due to the time of exposure and population density disappeared, probably due to the fast spreading of *E. leei* at high temperatures. A similar effect was also found in other experiments with short exposures or low parasite loads, such as in rainbow trout exposed for 10 min to *M. cerebralis* actinospores released by experimentally-infected tubificids showing a 100% prevalence after 4 - 5 months (Markiw, 1989), or the 100% of prevalence detected in rainbow trout when exposed to five *C. shasta* spores (Bjork & Bartholomew, 2009). By contrast, in T2, the lower temperature allowed detection of differences in prevalence of infection among groups due to the time of exposure and fish density by qPCR at 9 wpe, and also by histology at

13 wpe. Interestingly, despite the higher parasite prevalence at the posterior intestine in T1, there was a slower progression of the infection along the intestinal tract than in T2, which could be due to the higher levels of specific antibodies observed at high temperatures. Previous studies have described the important role of IgM in *E. leei*-GSB infections, with an overexpression of *IgM* in posterior intestine and head kidney in infected GSB (Piazzon et al., 2016). Another study showed that GSB that survived an infection with *E. leei* showed high levels of parasite-specific IgM in serum, even several months after eliminating the parasite (Picard-Sánchez et al., 2019). Thus, the higher specific IgM present in serum in T1 (Figure 3), could have slowed the spreading of the parasite down, explaining why, even though T1 fish had higher prevalence of infection, the progression and intensity of the infection was higher in infected fish from T2. The different prevalence of infection between the different diagnostic methods (histology and qPCR) may be caused by the *E. leei* patchy distribution when intensity of infection was low or medium, as previously described in Estensoro et al. 2010, and the size of the tissue samples that were taken to do the molecular test (qPCR). Indeed, this occurred on in T2-R2 group, in which one fish was scored with 1+ in histology and negative by qPCR.

In this study we have shown that the shortest time of exposure tested (one week) is enough to infect all the experimental fish after 13 wpe with high water temperature (100%, T1), whereas it only yields half this value at lower temperature (58.3%, T2). Similar to this, *K. inornata* was also able to be transmitted after 7 days of exposure in spotted seatrout (*Cynoscion nebulosus*) (de Buron et al., 2017). Moreover, a parasite from the same genus, *E. scophthalmi*, afecting turbot was detected in intestine by cohabitation at 48 dpe, and about 8 days when it was infected *per os* (Redondo, Palenzuela, Riaza, Macías & Álvarez-Pellitero, 2002). Thus, myxozoan infections could

be happening within minutes to hours of exposure. For instance, in rainbow trout (*Oncorhynchus mykiss*), *C. shasta* can be transmitted after 3 hours of exposure (Ray & Bartholomew, 2013), *T. bryosalmonae* after 90 min (Feist et al., 2001), or *M. cerebralis* in only 10 min (Markiw, 1989). Further studies will define whether a shorter time window is feasible for *E. leei* infection. As a general trend, intestinal infection spread was lower with shorter exposure times. Both trials showed the same time-dependent progression of the infection along the intestine, and the middle segment was less parasitized at the shortest times of exposure. However, when analyzing the infection levels in all intestinal segments, there was no time effect at high temperature (T1) in terms of prevalence of infection, whereas at low temperature (T2), the effect of time was clear. Time of exposure was also negatively correlated with growth performance, according to the emaciative effect induced by *E. leei* infections on GSB and olive flounder (Shin, Sohn, Jin, Kang & Lee, 2018; Sitjà-Bobadilla & Palenzuela, 2012). Consequently, T1R2-1 fish reached the highest weight, and T2R2-1 fish, the best condition factor at the end of each trial.

Presently, there are no treatments or vaccines available against myxozoan infections (Yokoyama, Grabner & Shirakashi, 2012), and control strategies are mostly preventive and are related to fish living conditions, such as good management techniques, reducing stock densities or improving water quality (Fontes et al., 2015). In this work, infection due to cohabitation within recipient tanks seems crucial for the speed of parasite establishment and spreading. R1 tanks had five and four times higher fish density and fish number than R2 tanks in T1 and T2, respectively. Moreover, R1 tanks, regardless of the temperature, had the same prevalence of infection at 9 wpe (70%), and reached the highest infection values for all the variables studied at 13 wpe. This was particularly highlighted by the progression of the infection along the intestinal

tract, since prevalence of infection at the middle intestine (the last segment being infected) was higher in R1 groups than in R2, and furthermore, in T2 this was also applicable to the anterior intestine. The main factor that could explain this phenomenon is the different probability of finding an infected fish comparing R1 and R2. In other words, in the tanks where fish were periodically removed (R2), the likelihood of parasite and host encounter decreased with the lowering animal density and, at the same time, the random removal of infected fish lowered the probability of infections by cohabitation within the tank. This work evidenced two sources of parasite in an effluent transmission model. The first source is the water from the donor tank (effluent transmission), and the second source, as a consequence of the first, are the infected cohabitant fish. Cohabitation effects can be stronger than the effluent transmission, since there is direct contact between hosts, although the exact time after infection when the parasite starts to be shed cannot be calculated. In experimental transmissions of *E. leei* from tiger puffer to red sea bream (*Pagrus major*), higher prevalence of infection was achieved by cohabitation (42%) than by effluent exposure (25%) (Yanagida et al., 2008). Similarly, the spreading of the infection by *E. scophthalmi*, was faster by cohabitation than by effluent transmission, once the infection was established (Redondo, Palenzuela & Álvarez-Pellitero, 2004). Although it is not possible to quantitatively differentiate the effects of effluent from those of cohabitation, both infection methods must be acting at the same time because at 9 wpe, all the R2 subgroups, exposed to the effluent less time than R2 tanks, but with higher density, reached the same prevalence of infection or even higher (except T1R2-3 and T2R2-1) than R2 tanks.

In conclusion, we have shown the relevance of time of exposure, temperature, and fish density on the prevalence, intensity and progression of infection in an effluent

transmission model with the parasite *E. leei* in GSB. We have also shown, for the first time, that one week of exposure to a parasite-contaminated effluent is enough to effectively transmit this disease (from 18ºC to 25.6ºC). In general, higher temperatures yielded higher prevalence of infection. Nevertheless, high temperatures also boosted the antibody response leading to lower intensity of infection. In any case, with long times of exposure and high fish density, temperature (at the tested ranges) is not the determining factor, as evidenced by the same prevalence of infection in R1 groups in both trials. Consequently, at moderate water temperature, other factors, such as time of exposure and fish density arose as relevant. For myxozoan infections, the main control measure is prevention, thus an effective diagnosis that allows the early removal of moribund or infected fish could dramatically change the scenario in a fish farm. Since *E. leei* infection in GSB can be easily transmitted fish-to-fish without any intermediate, other abiotic variables such as water flow or sediment that would compromise the establishment of the parasite in the system (Arndt, Wagner, Cannon & Smith, 2002; Fontes et al., 2015; Hallet & Bartholomew, 2008) could be new interesting topics to study in the future.

References

Abd-Elfattah, A., Fontes, I., Kumar, G., Soliman, H., Hartikainen, H., Okamura, B. & El-Matbouli, M. (2014) Vertical transmission of *Tetracapsuloides bryosalmonae* (Myxozoa), the causative agent of salmonid proliferative kidney disease. *Parasitology*, 141, 482-490. doi: 10.1017/s0031182013001650

Abram, Q. H., Dixon, B. & Katzenback, B. A. (2017) Impacts of low temperature on the teleost immune system. *Biology*, 6, 39. doi: 10.3390/biology6040039

Arndt, R. E., Wagner, E. J., Cannon, Q. & Smith, M. (2002) *Triactinomyxon* production as related to rearing substrate and diel light cycle. In: American Fisheries Society Symposium, 2002. *American Fishes Society*, 87-92.

Bartholomew, J. L., Whipple, M. J., Stevens, D. G. & Fryer, J. L. (1997) The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaete as an alternate host. *Journal of Parasitology*, 83, 859-868. doi: 10.2307/3284281

Bjork., S. J. & Bartholomew, J. (2009) Effects of *Ceratomyxa shasta* dose on a susceptible strain of rainbow trout and comparatively resistant Chinook and coho salmon. *Diseases of Aquatic Organisms*, 86, 29-37. doi: 10.3354/dao02092

Braden, L. M., Rasmussen, K. J., Purcell, S. L., Ellis, L., Mahony, A., Cho, S., Whyte, S. K., Jones, S. R. M. & Fast, M. D. (2018) Acquired protective immunity in Atlantic salmon *Salmo salar* against the myxozoan *Kudoa thyrsites* involves induction of MHII beta(+) CD83(+) antigen-presenting cells. *Infection and Immunity*, 86, e00556-17. doi: 10.1128/iai.00556-17

China, M., Nakamura, H., Hamakawa, K., Tamaki, E., Yokoyama, H., Masuoka, S. & Ogawa, K. (2014) Efficacy of high water temperature treatment of

myxosporean emaciation disease caused by *Enteromyxum leei* (Myxozoa). *Fish Pathology*, 49, 137-140.

Clifton-Hadley, R. S., Bucke, D. & Richards, R. H. (1984) Proliferative kidney disease of salmonid fish: a review. *Journal of Fish Diseases*, 7, 14. doi: 10.1111/j.13652761.1984.tb01201.x

Colloca, F. & Cerasi, S. (2019, November 13). *Sparus aurata* [Internet report]. *Cultured aquatic species fact sheets* (FAO, 2009). Retrieved from http://www.fao.org/tempref/FI/DOCUMENT/aquaculture/CulturedSpecies/file/en/en_giltheadseabr.htm

de Buron, I., Hill-Spanik, K. M., Haselden, L., Atkinson, S. D., Hallett, S. L. & Arnott, S. A. (2017) Infection dynamics of *Kudoa inornata* (Cnidaria: Myxosporea) in spotted seatrout *Cynoscion nebulosus* (Teleostei: Sciaenidae). *Diseases of Aquatic Organisms*, 127, 29-40. doi: 10.3354/dao03174

Diamant, A. (1997) Fish-to-fish transmission of a marine myxosporean. *Diseases of Aquatic Organisms*, 30, 99-105. doi: 10.3354/dao030099

Diamant, A. & Wajsbrot, N. (1997) Experimental transmission of *Myxidium leei* in gilthead sea bream *Sparus aurata*. *Bulletin of the European Association of Fish Pathologists*, 17, 99-103.

Estensoro, I., Redondo, M. J., Álvarez-Pellitero, P. & Sitjà-Bobadilla, A. (2010) Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata*. *Diseases of Aquatic Organisms*, 92, 51-58. doi: 10.3354/dao02267

Eszterbauer, E., Atkinson, S., Diamant, A., Morris, D., El-Matbouli, M. & Hartikainen, H. (2015) Myxozoan life cycles: practical approaches and insights. In B.

Okamura, A. Gruhl & J. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development* (pp. 175-198). Switzerland: Springer International Publishing.

Feist, S. W., Longshaw, M., Canning, E. U. & Okamura, B. (2001) Induction of proliferative kidney disease (PKD) in rainbow trout *Oncorhynchus mykiss* via the bryozoan *Fredericella sultana* infected with *Tetracapsula bryosalmonae*. *Diseases of Aquatic Organisms*, 45, 61-68. doi: 10.3354/dao045061

Flecknell, P. (2002) Replacement, reduction and refinement. *ALTEX*, 19, 73-78.

Fontes, I., Hallett, S. & Mo, T. (2015) Comparative epidemiology of myxozoan diseases. In B. Okamura, A. Gruhl & J. Bartholomew (Eds.), *Myxozoan Evolution, Ecology and Development* (pp. 317-341). Switzerland: Springer International Publishing.

Golomazou, E., Athanassopoulou, F., Karagouni, E., Tsagozis, P., Tsantilas, H. & Vagianou, S. (2006) Experimental transmission of *Enteromyxum leei* Diamant, Lom and Dyková, 1994 in sharpsnout sea bream, *Diplodus puntazzo* C. and the effect on some innate immune parameters. *Aquaculture*, 260, 44-53. doi: 10.1016/j.aquaculture.2006.06.013

Gupta, A. & Kaur, H. (2017) A new pathogen, *Myxobolus holzerae* (Myxosporea: Myxozoa) causing severe gill disease in an Indian major carp *Labeo rohita* in a cold water wetland, Punjab (India). *Microbial Pathogenesis*, 111, 244-251. doi: 10.1016/j.micpath.2017.08.044

Hallett, S., & Bartholomew, J. (2008). Effects of water flow on the infection dynamics of Myxobolus cerebralis. *Parasitology*, 135, 371-384. doi:10.1017/S0031182007003976

493 Jones, S. & Long, A. (2019) Host size influences prevalence and severity of *Kudoa*
494 *thyrsites* (Cnidaria: Myxosporea) infection in Atlantic salmon *Salmo salar*.
495 *Diseases of Aquatic Organisms*, 133, 99-105. doi: 10.3354/dao03335
496 Korytář, T., Wiegertjes, G. F., Zusková, E., Tomanová, A. L., M., Patra, S., Sieranski,
497 V., Šíma, R., Born-Torrijos, A., Wentzel, A. S., Blasco-Monleon, S., Yanes-
498 Roca, C., Policar, T. & Holzer, A. S. (2019) The kinetics of cellular and humoral
499 immune responses of common carp to presporogonic development of the
500 myxozoan *Sphaerospora molnari*. *Parasites & Vectors*, 12, 208-208. doi:
501 10.1186/s13071-019-3462-3
502 Le Morvan, C., Troutaud, D. & Deschaux, P. (1998) Differential effects of temperature
503 on specific and nonspecific immune defences in fish. *The Journal of*
504 *Experimental Biology*, 201, 165-168.
505 Markiw, M. E. (1989) Portals of entry for salmonid whirling disease in rainbow trout.
506 *Diseases of Aquatic Organisms*, 6, 7-10.
507 Markiw, M. E. & Wolf, K. (1983) *Myxosoma cerebralis* (Myxozoa: Myxosporea)
508 etiologic agent of salmonid whirling disease requires tubificid worm (Annelida:
509 Oligochaeta) in its life cycle. *The Journal of Protozoology*, 30, 561-564. doi:
510 10.1111/j.1550-7408.1983.tb01422.x
511 Muñoz, P., Cuesta, A., Athanassopoulou, F., Golomazou, H., Crespo, S., Padrós, F.,
512 Sitjà-Bobadilla, A., Albiñana, G., Esteban, M. A., Álvarez-Pellitero, P. &
513 Meseguer, J. (2007) Sharpsnout sea bream (*Diplodus puntazzo*) humoral
514 immune response against the parasite *Enteromyxum leei* (Myxozoa). *Fish &*
515 *Shellfish Immunology*, 23, 636-645. doi: 10.1016/j.fsi.2007.01.014
516 Palikova, M., Papezikova, I., Markova, Z., Navratil, S., Mares, J., Mares, L., Vojtek, L.,
517 Hyrsl, P., Jelinkova, E. & Schmidt-Posthaus, H. (2017) Proliferative kidney

disease in rainbow trout (*Oncorhynchus mykiss*) under intensive breeding conditions: Pathogenesis and haematological and immune parameters. *Veterinary Parasitology*, 238, 5-16. doi: 10.1016/j.vetpar.2017.03.003

Piazzon, M. C., Calduch-Giner, J. A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitjà-Bobadilla, A., Pérez-Sánchez J. (2017) Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome*, 5, 164. doi: 10.1186/s40168-017-0390-3

Piazzon, M. C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gómez-Casado, E., Novoa, B., Mulero, V., Sitjà-Bobadilla, A. & Pérez-Sánchez, J. (2016) Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a perciform fish. *Frontiers in Immunology*, 7, 637. doi: 10.3389/fimmu.2016.00637

Picard-Sánchez, A., Estensoro, I., Del Pozo, R., Piazzon, M. C., Palenzuela, O. & Sitjà-Bobadilla, A. (2019) Acquired protective immune response in a fish-myxozoan model encompasses specific antibodies and inflammation resolution. *Fish & Shellfish Immunology*, 90, 349-362. doi: 10.1016/j.fsi.2019.04.300

Ray, R. A., & Bartholomew, J. L. (2013). Estimation of transmission dynamics of the *Ceratomyxa shasta* actinospore to the salmonid host. *Parasitology*, 140, 907–916. doi:10.1017/s0031182013000127

Ray, R. A., Holt, R. A. & Bartholomew, J. L. (2012) Relationship between temperature and *Ceratomyxa shasta*–induced mortality in Klamath River salmonids. *Journal of Parasitology*, 98, 520-526. doi: 10.1645/JP-GE-2737.1

Redondo, M. J., Palenzuela, O., & Álvarez-Pellitero, P. (2004) Studies on transmission and life cycle of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of

turbot *Scophthalmus maximus*. *Folia Parasitologica*, 51, 188-198. doi: 10.14411/fp.2004.022

Redondo, M. J., Palenzuela, O., Riaza, A., Macías, A., Alvarez-Pellitero, P. (2002) Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus*. *Journal of Parasitology*, 88, 482-488. doi: 10.1645/0022-3395(2002)088[0482:ETOESM]2.0.CO;2

Shin, S. P., Sohn, H. C., Jin, C. N., Kang, B. J. & Lee, J. (2018) Molecular diagnostics for verifying an etiological agent of emaciation disease in cultured olive flounder *Paralichthys olivaceus* in Korea. *Aquaculture*, 493, 18-25. doi: 10.1016/j.aquaculture.2018.04.041

Sitjà-Bobadilla, A., Diamant, A., Palenzuela, O. & Álvarez-Pellitero, P. (2007) Effect of host factors and experimental conditions on the horizontal transmission of *Enteromyxum leei* (Myxozoa) to gilthead sea bream, *Sparus aurata* L., and European sea bass, *Dicentrarchus labrax* (L.). *Journal of Fish Diseases*, 30, 243-250. doi: 10.1111/j.1365-2761.2007.00804.x

Sitjà-Bobadilla, A. & Palenzuela, O. (2012) *Enteromyxum* Species. In P.T.K. Woo & K. Buchmann (Eds.), *Fish Parasites: Pathology and Protection* (pp. 163-176). Denmark: CAB International, Oxfordshire UK.

Thompson, J. B., Snekvik, K. R. & Vincent, E. R. (2010) The Effects of *Myxobolus cerebralis* on apache and gila trout in laboratory exposures. *Journal of Aquatic Animal Health*, 22, 87-91. doi: 10.1577/H09-007.1

Tops, S., Lockwood, W. & Okamura, B. (2006) Temperature-driven proliferation of *Tetracapsuloides bryosalmonae* in bryozoan hosts portends salmonid declines. *Diseases of Aquatic Organisms*, 70, 227-236. doi: 10.3354/dao070227

Udey, L. R., Fryer, J. L. & Pilcher, K. S. (1975) Relation of water temperature to ceratomyxosis in rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). *Journal of the Fisheries Research Board of Canada*, 32, 1545-1551. doi: 10.1139/f75-181

Yanagida, T., Palenzuela, O., Hirae, T., Tanaka, S., Yokoyama, H. & Ogawa, K. (2008) Myxosporean emaciation disease of cultured red sea bream *Pagrus major* and spotted knifejaw *Oplegnathus punctatus*. *Fish Pathology*, 43, 45-48. doi: 10.3147/jsfp.43.45

Yanagida, T., Sameshima, M., Nasu, H., Yokoyama, H. & Ogawa, K. (2006) Temperature effects on the development of *Enteromyxum* spp. (Myxozoa) in experimentally infected tiger puffer, *Takifugu rubripes* (Temminck & Schlegel). *Journal of Fish Diseases*, 29, 561-567. doi: 10.1111/j.1365-2761.2006.00752.x

Yasuda, H., Ooyama, T., Iwata, K., Tun, T., Yokoyama, H. & Ogawa, K. (2002) Fish-to-fish transmission of *Myxidium* spp. (Myxozoa) in cultured tiger puffer suffering from emaciation disease. *Fish Pathology*, 37, 29-33. doi: 10.3147/jsfp.37.29

Yasuda, H., Ooyama, T., Nakamura, A., Iwata, K., Palenzuela, O. & Yokoyama, H. (2005) Occurrence of the myxosporean emaciation disease caused by *Enteromyxum leei* in cultured Japanese flounder *Paralichthys olivaceus*. *Fish Pathology*, 40, 175-180. doi: 10.3147/jsfp.40.175

Yokoyama, H. (2003). A Review: Gaps in our knowledge on Myxozoan parasites of fishes. *Fish Pathology*, 38, 125-136. doi: 10.3147/jsfp.38.125

Yokoyama, H., Grabner, D. & Shirakashi, S. (2012) Transmission biology of the Myxozoa. In E. D. Carvalho, G. Silva & R.J. Silva (Eds.), *Health and Environment in Aquaculture* (pp. 1-42). Rijeka: IntechOpen. doi: 10.5772/29571

592 Zinkernagel, R. M., Ehl, S., Aichele, P., Oehen, S., Kündig, T. & Hengartner, H. (1997)
593 Antigen localisation regulates immune responses in a dose- and time-dependent
594 fashion: a geographical view of immune reactivity. *Immunological Reviews*,
595 156, 199-209. doi: 10.1111/j.1600-065X.1997.tb00969.x

596

Tables

Table 1. **Biometric data from trials 1 and 2 at 9 and 13 weeks post-exposure (wpe) to *Enteromyxum leei***. R1 was the tank from where no fish were removed during the experiment, whereas R2 represents the tank from which subgroups of fish were removed at 1 (R2-1), 3 (R2-3), 5 (R2-5) and 7 (R2-7, only in T1) wpe. CF: Condition factor. DD: Degree days. Density: Fish body mass per water volume of each tank. Data are presented as mean ± SEM of n = 10 (T1) or n = 12 (T2) individuals. Comparisons were made for each sampling point among individuals belonging to the same trial (one-way ANOVA + SNK test). Different letters in bold numbers indicate statistically significant differences within each group ($P < 0.05$).

607

		9 wpe					13 wpe				
Trial	Group	Weight (g)	Size (cm)	CF	DD	Density (kg/L)	Weight (g)	Size (cm)	CF	DD	Density (kg/L)
T1 (25.6ºC)	T1R1	114.7 ±9.0	16.7 ±0.4	2.42 ±0.10	1680.5	0.0115	112.3 ±10.9	16.2 ±0.4	2.58 ±0.10	2348.3	0.1120
	T1R2	98.0 ±8.4	15.9 ±0.4	2.41 ±0.03	1680.5	0.0019	119.5 ±10.7	16.7 ±0.5	2.54 ±0.05	2348.3	0.0024
	T1R2-1	116.8 ±7.9	16.8 ±0.4	2.41 ±0.05	205.9	0.0058	141.0 ±13.2	17.8 ±0.6	2.44 ±0.03	205.9	0.0071
	T1R2-3	106.5 ±7.0	16.1 ±0.4	2.51 ±0.04	585.9	0.0053	130.3 ±8.5	17.2 ±0.4	2.53 ±0.10	585.9	0.0065
	T1R2-5	104.0 ±9.5	16.1 ±0.5	2.41 ±0.04	942.6	0.0052	120.6 ±12.5	16.9 ±0.5	2.42 ±0.10	942.6	0.0060
	T1R2-7	96.9 ±7.3	15.9 ±0.4	2.38 ±0.04	1317.0	0.0048	109.9 ±12.4	16.3 ±0.5	2.47 ±0.10	1317.0	0.0055
T2 (18ºC)	T2R1	**117.8 ±3.7^{a}**	17.2 ±0.2	**2.28 ±0.02^{a}**	1134.0	0.0113	**127.7 ±4.0^{a}**	17.8 ±0.2	**2.27 ±0.04^{a}**	1638.0	0.0123
	T2R2	**141.5 ±7.4^{b}**	18.0 ±0.4	**2.45 ±0.05^{b}**	1134.0	0.0034	**161.9 ±11.0^{b}**	18.6 ±0.4	**2.49 ±0.05^{b}**	1638.0	0.0039
	T2R2-1	**131.5 ±7.2^{ab}**	17.3 ±0.2	**2.55 ±0.04^{b}**	126.0	0.0079	**153.3 ±6.7^{ab}**	18.2 ±0.2	**2.55 ±0.04^{b}**	126.0	0.0092
	T2R2-3	**136.3 ±7.3^{ab}**	17.6 ±0.3	**2.48 ±0.05^{b}**	378.0	0.0082	**152.0 ±7.8^{ab}**	18.4 ±0.3	**2.43 ±0.05^{ab}**	378.0	0.0091
	T2R2-5	**141.1 ±6.1^{b}**	17.6 ±0.3	**2.56 ±0.05^{b}**	630.0	0.0078	**161.8 ±7.7^{b}**	18.6 ±0.3	**2.51 ±0.04^{b}**	630.0	0.0089

608

609 **Table 2. *Enteromyxum leei* infection**. Molecular diagnosis was performed by qPCR at
610 9 (non-lethal) and 13 weeks post-exposure (wpe). Histological diagnosis was performed
611 by examination of anterior (AI), middle (MI) and posterior intestine (PI), at the final
612 sampling (13 wpe). R1 was the tank from where no fish were removed during the
613 experiment, whereas R2 represents the tank from which subgroups of fish were
614 removed at 1 (R2-1), 3 (R2-3), 5 (R2-5) and 7 (R2-7, only in T1) wpe. Values for
615 prevalence of infection are presented as percentage of infected fish from sampled fish.
616 Values for intensity of infection are presented as the mean ± SEM. Comparisons were
617 made for each sampling point among individuals of the same trial. Asterisks indicate a
618 statistically significant correlation within a sampling point (Chi-squared test, $P < 0.05$).
619 Different letters indicate statistically significant differences within each group (one-way
620 ANOVA on ranks + Dunn's test $P < 0.05$).

621

Trial	Group	Molecular diagnosis (qPCR)				Histology (13 wpe)					
		Prevalence (%)		Intensity (Ct)		Prevalence (%)			Intensity (0-6)		
		9 wpe	13 wpe	9 wpe	13 wpe	AI	MI	PI	AI	MI	PI
T1 (25.6ºC)	T1R1	70	90	28.1 ±1.8	32.9 ±1.6	30	20	50	3.3 ±1.2	1.0 ±0.0	4.0 ±1.2
	T1R2	50	90	28.0 ±1.9	31.0 ±2.4	33	11	67	3.7 ±1.2	6.0 ±0.0	4.0 ±1.2
	T1R2-1	50	100	26.6 ±0.6	27.7 ±1.5	30	0	70	2.3 ±0.3	0.0 ±0.0	5.1 ±0.6
	T1R2-3	30	80	31.9 ±2.8	28.4 ±1.5	10	0	30	5.0 ±0.0	0.0 ±0.0	4.0 ±1.5
	T1R2-5	70	90	26.9 ±1.2	30.0 ±1.9	40	10	60	3.0 ±1.2	1.0 ±0.0	3.7 ±1.1
	T1R2-7	80	100	27.2 ±1.3	29.5 ±1.6	40	20	60	4.8 ±1.3	5.5 ±0.5	4.3 ±1.1
T2 (18ºC)	T2R1	**72***	100	27.7 ±0.8	**20.5 ±0.7[a]**	**100***	**67***	**100***	4.8 ±0.6	5.3 ±0.4	5.8 ±0.1
	T2R2	**17***	83	24.8 ±0.7	**27.2 ±2.4[ab]**	**25***	**0***	**100***	2.3 ±1.3	0.0 ±0.0	3.0 ±0.6
	T2R2-1	**8***	58	24.1 ±0.0	**31.9 ±2.7[b]**	**8***	**0***	**58***	2.0 ±0.0	0.0 ±0.0	3.1 ±0.8
	T2R2-3	**33***	75	24.2 ±0.8	**25.8 ±2.4[ab]**	**33***	**17***	**75***	3.5 ±1.2	4.0 ±2.0	4.0 ±0.7
	T2R2-5	**25***	64	29.1 ±1.7	**26.5 ±3.6[ab]**	**36***	**27***	**45***	2.8 ±0.8	3.3 ±1.2	3.8 ±1.0

622

Figure legends

Figure 1: Schematic representation of the *Enteromyxum leei* infection trials. N_i and N_f stand for initial and final number of fish, respectively. V is the volume of each tank. T_m is the mean temperature for trial 1, whereas T_c is the constant temperature held in trial 2. wpe: weeks post-exposure.

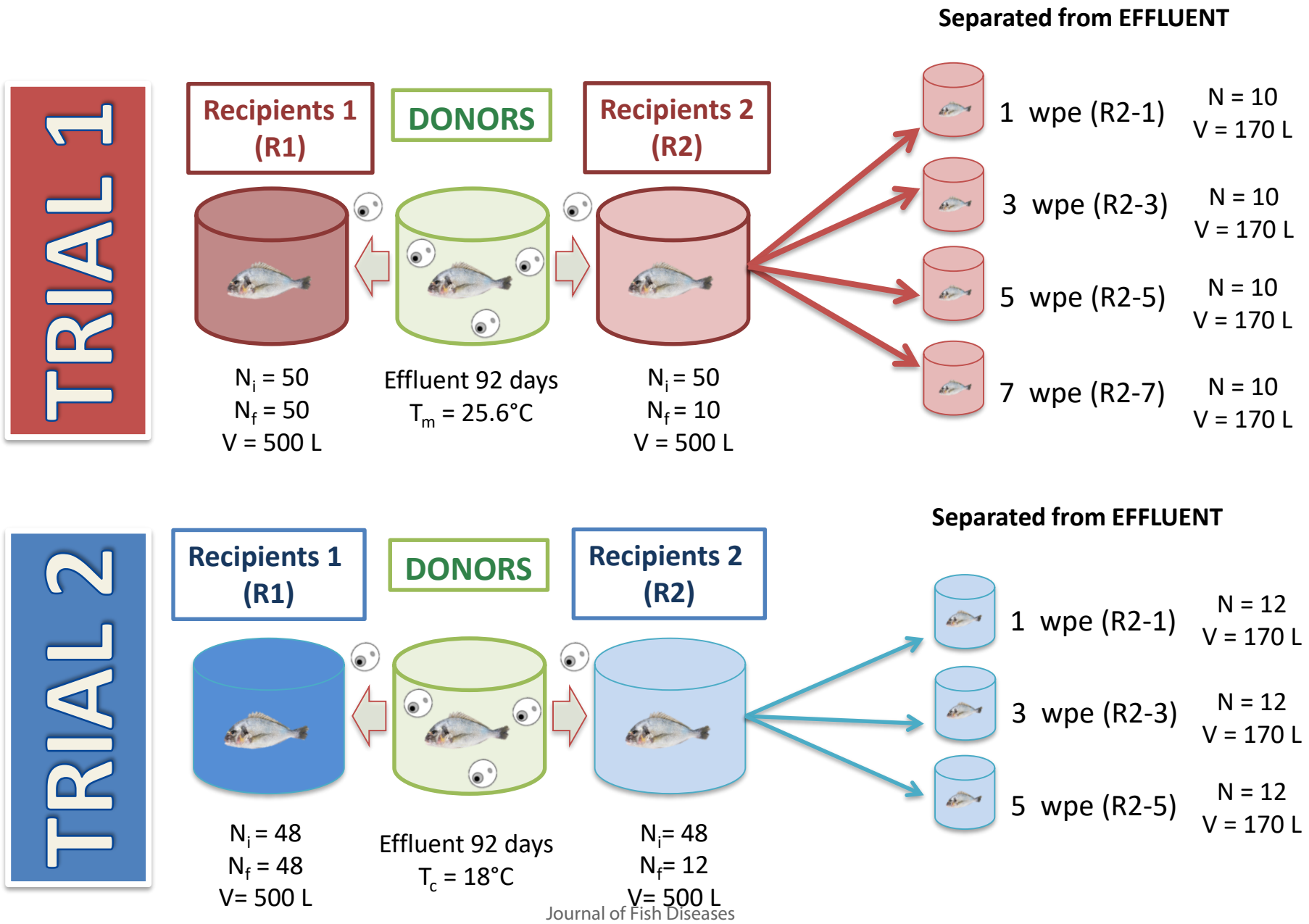
Figure 2: Intensity of *Enteromyxum leei* infection is related to time of exposure at low temperatures. The intensity of infection was evaluated by histology as the percentage of fish with more than one intestinal segment parasitized in trial 1 (T1) with mean temperature of 25.6ºC (a) and T2 with constant temperature of 18ºC (b); or three intestinal segments parasitized in T1 (c), and T2 (d). R1 was the tank from where no fish were removed during the experiments, whereas R2 represents the tank from which subgroups of fish were removed at 1 (R2-1), 3 (R2-3), 5 (R2-5) and 7 (R2-7) weeks post-exposure. *P* values result from a chi-square test to compare proportions and were considered significant when $P < 0.05$ (bold).

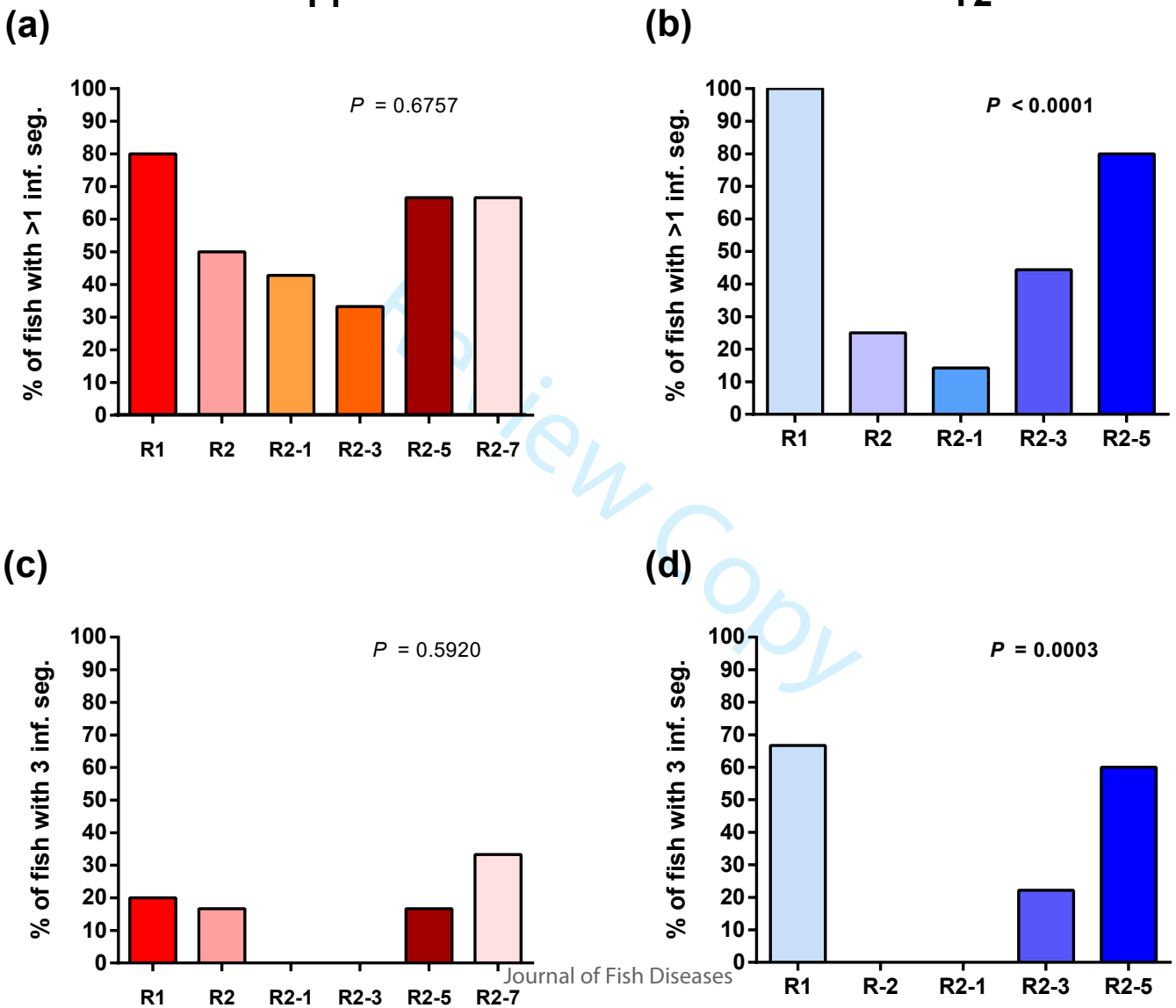
Figure 3: Production of specific antibodies is related to higher temperatures. Mean intensity of serum IgM immunoreactivity against *Enteromyxum leei*, measured by immunohistochemistry, for each group from trials T1 ((a), mean temperature 25.6ºC) and T2 ((b), constant temperature 18ºC). One-way ANOVA was used to determine statistical differences ($P < 0.05$) represented by different lower case letters. Overall, in T1 there was a higher intensity immunoreactivity than in T2 (two-way ANOVA $P < 0.0001$). Percentage of fish with parasite-specific IgM in T1 (c) and T2 (d). *P* values result from a chi-square test to compare proportions. R1 was the tank from where no fish were removed during the experiments, whereas R2 represents the tank from which

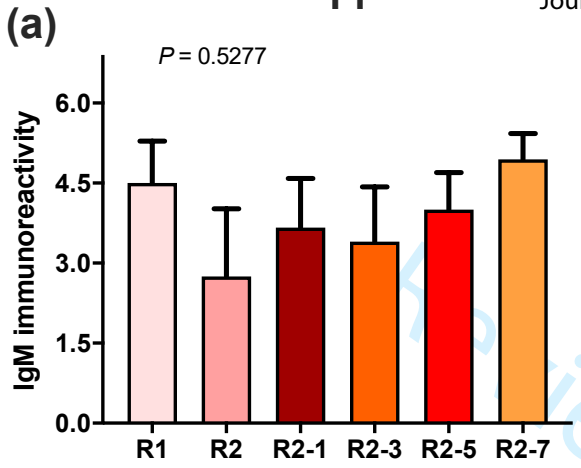
subgroups of fish were removed at 1 (R2-1), 3 (R2-3), 5 (R2-5) and 7 (R2-7, only in T1) weeks post-exposure.

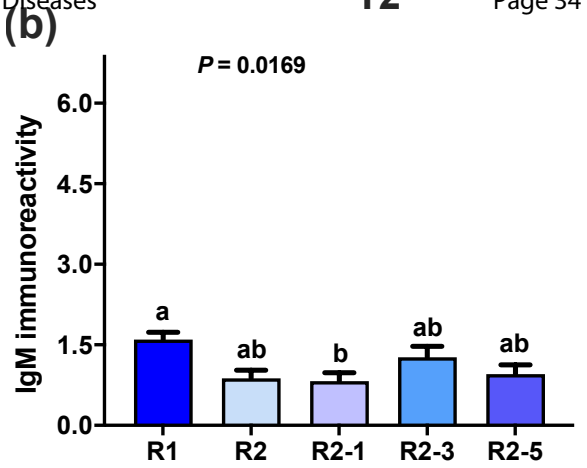
Figure 4: Temperature and time of exposure are key variables in the overall clustering of groups in *Enteromyxum leei* experimental infections. Hierarchical cluster analysis performed using all the variables measured in T1 (mean temperature 25.6ºC) and T2 (constant temperature 18ºC). R1 was the tank from where no fish were removed during the experiments, whereas R2 represents the tank from which subgroups of fish were removed at 1 (R2-1), 3 (R2-3), 5 (R2-5) and 7 (R2-7, only in T1) weeks post-exposure.

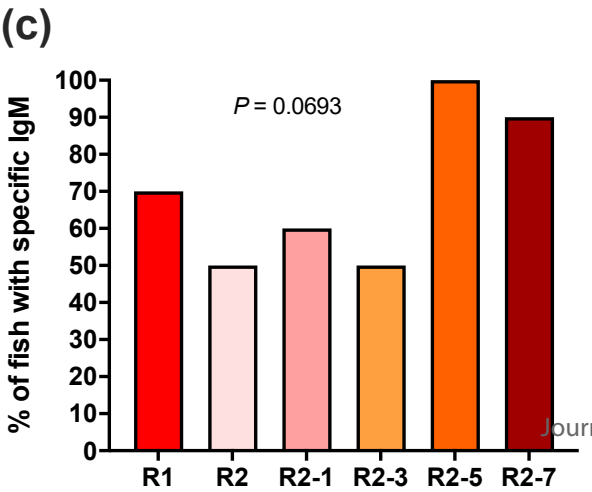
Figure 5: Correlogram highlighting the most correlated variables in the *Enteromyxum leei* study. The color and the size of the circles represent the correlation coefficients among variables. Only statistically significant correlations are shown (Pearson's product moment correlation coefficient, $P < 0.05$). Abiotic, biotic, diagnostic and immune variables are represented in blue, brown, violet and black, respectively. Dens: density in kg of fish per liter; CF: condition factor; SGR: specific growth rate; Prev: prevalence of infection; NL diag: non-lethal molecular diagnosis measured in Ct values; Molec diag: lethal molecular diagnosis measured in Ct values; Histol score PI, MI, AI: histological scoring for posterior, middle and anterior intestine, respectively; Prev 3InfSeg: prevalence of fish with the 3 intestinal segments parasitized; Prev >1InfSeg: prevalence of fish with more than one intestinal segment parasitized; IgM Immunoreact: immunolabeling intensity measuring the amount of parasite-specific IgM in serum; %Fish with SpIgM: percentage of fish with parasite-specific IgM in serum; wpe: weeks post-exposure.

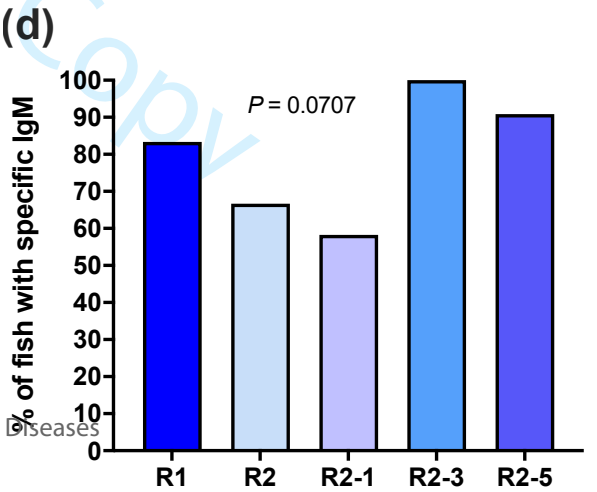

TRIAL 1
Recipients 1 (R1)
DONORS
Recipients 2 (R2)
Separated from EFFLUENT
N_i = 50
N_f = 50
V = 500 L
Effluent 92 days
T_m = 25.6°C
N_i = 50
N_f = 10
V = 500 L
1 wpe (R2-1)
N = 10
V = 170 L
3 wpe (R2-3)
N = 10
V = 170 L
5 wpe (R2-5)
N = 10
V = 170 L
7 wpe (R2-7)
N = 10
V = 170 L
TRIAL 2
Recipients 1 (R1)
DONORS
Recipients 2 (R2)
Separated from EFFLUENT
N_i = 48
N_f = 48
V= 500 L
Effluent 92 days
T_c = 18°C
N_i= 48
N_f= 12
V= 500 L
1 wpe (R2-1)
N = 12
V = 170 L
3 wpe (R2-3)
N = 12
V = 170 L
5 wpe (R2-5)
N = 12
V = 170 L

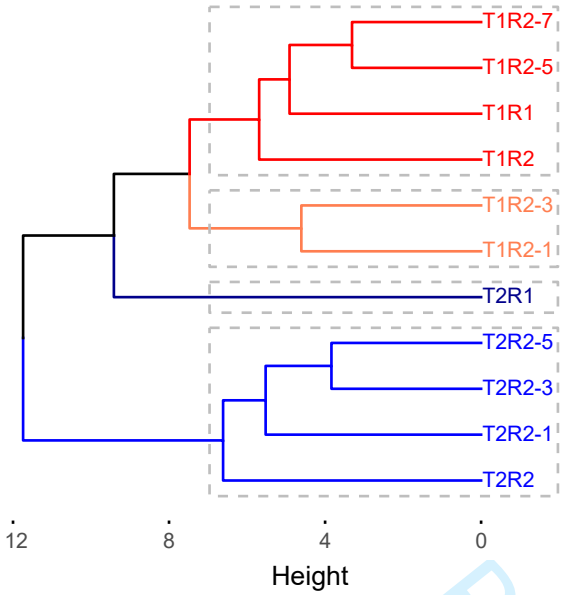


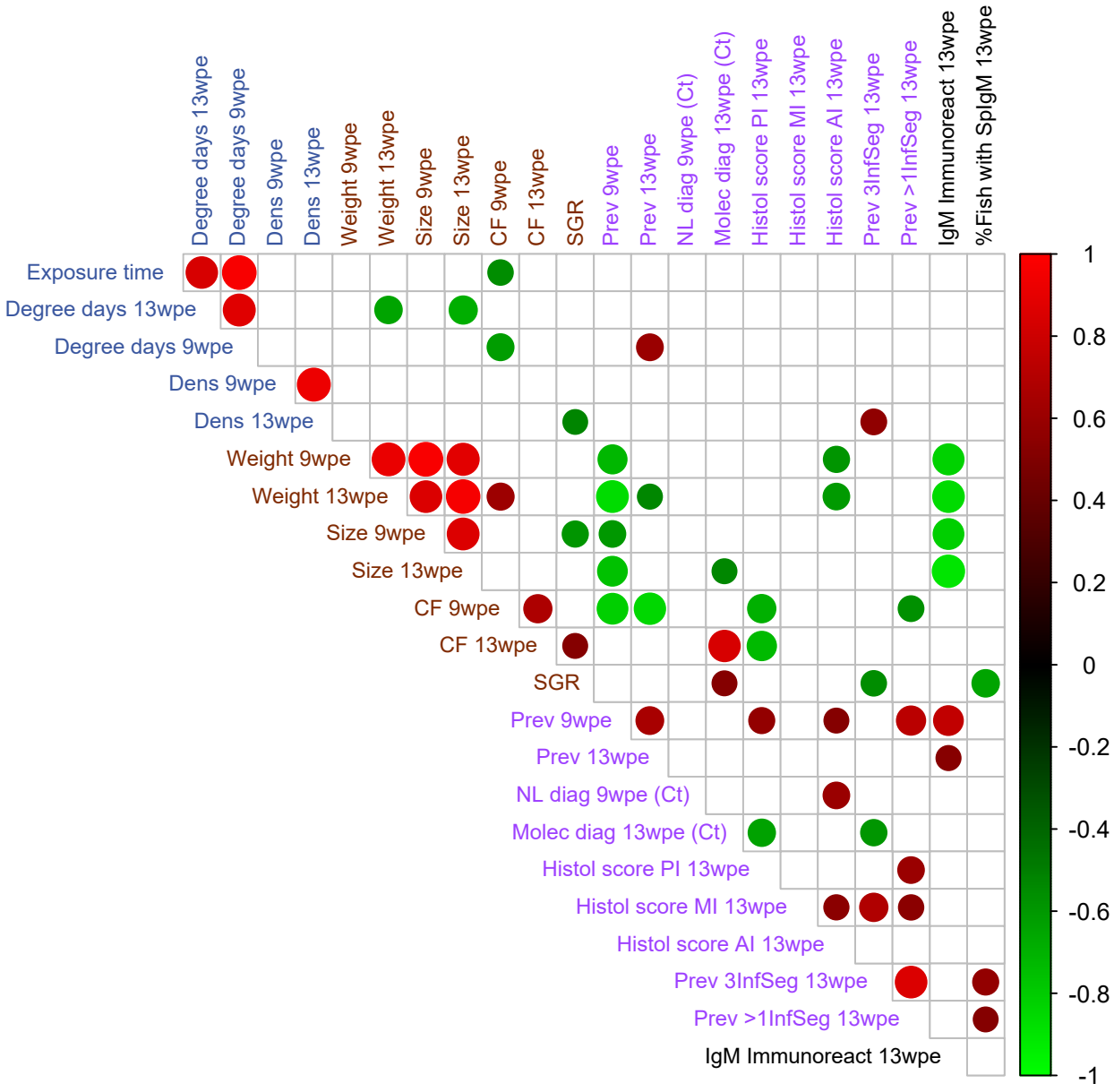









T1R2-7
T1R2-5
T1R1
T1R2
T1R2-3
T1R2-1
T2R1
T2R2-5
T2R2-3
T2R2-1
T2R2
12
8
4
0
Height


Degree days 13wpe
Degree days 9wpe
Dens 9wpe
Dens 13wpe
Weight 9wpe
Weight 13wpe
Size 9wpe
Size 13wpe
CF 9wpe
CF 13wpe
SGR
Prev 9wpe
Prev 13wpe
NL diag 9wpe (Ct)
Molec diag 13wpe (Ct)
Histol score PI 13wpe
Histol score MI 13wpe
Histol score AI 13wpe
Prev 3InfSeg 13wpe
Prev >1InfSeg 13wpe
IgM Immunoreact 13wpe
%Fish with SpIgM 13wpe
Exposure time
Degree days 13wpe
Degree days 9wpe
Dens 9wpe
Dens 13wpe
Weight 9wpe
Weight 13wpe
Size 9wpe
Size 13wpe
CF 9wpe
CF 13wpe
SGR
Prev 9wpe
Prev 13wpe
NL diag 9wpe (Ct)
Molec diag 13wpe (Ct)
Histol score PI 13wpe
Histol score MI 13wpe
Histol score AI 13wpe
Prev 3InfSeg 13wpe
Prev >1InfSeg 13wpe
IgM Immunoreact 13wpe
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Supplementary Table 1

Group	n	Exposure time	Degree days 13wpe	Degree days 9wpe	Density 9wpe	Density 13wpe	Condition Factor 9wpe	Condition Factor 13wpe	SGR	Prev 9wpe
T1R1	50	13	2348.30	1680.45	0.0115	0.0112	2.42	2.58	0.46	70.00
T1R2	10	13	2348.30	1680.45	0.0020	0.0024	2.41	2.54	0.71	50.00
T1R2-1	10	1	205.90	205.90	0.0058	0.0071	2.41	2.44	0.67	50.00
T1R2-3	10	3	585.90	585.90	0.0053	0.0065	2.51	2.53	0.72	30.00
T1R2-5	10	5	942.60	942.60	0.0052	0.0060	2.41	2.42	0.53	70.00
T1R2-7	10	7	1317.00	1317.00	0.0048	0.0055	2.38	2.47	0.45	80.00
T2R1	48	13	1638.00	1134.00	0.0113	0.0123	2.28	2.27	0.29	71.74
T2R2	12	13	1638.00	1134.00	0.0034	0.0039	2.45	2.49	0.37	16.67
T2R2-1	12	1	126.00	126.00	0.0079	0.0092	2.55	2.55	0.55	8.33
T2R2-3	12	3	378.00	378.00	0.0082	0.0091	2.48	2.43	0.39	33.33
T2R2-5	11	5	630.00	630.00	0.0078	0.0089	2.56	2.51	0.49	25.00

Group	Prev 13wpe	NL diag 9wpe(Ct)	molec diag 13wpe(Ct)	Histol score PI 13wpe	Histol score MI 13wpe	Histol score AI 13wpe	Prev 3InfSeg 13wpe	Prev >1InfSeg 13wpe	Immunoreact IgM 13wpe	%Fish with SpIgM 13wpe
T1R1	90.00	28.09	32.85	4.00	1.00	3.30	20.00	80.00	4.50	70.00
T1R2	90.00	27.95	31.02	3.50	6.00	3.70	16.67	50.00	2.75	50.00
T1R2-1	100.00	26.61	27.71	5.14	0.00	2.33	0.00	42.86	3.67	60.00
T1R2-3	80.00	31.91	28.36	4.00	0.00	5.00	0.00	33.33	3.40	50.00
T1R2-5	90.00	26.88	30.03	3.70	1.00	3.00	16.67	66.67	3.90	100.00
T1R2-7	100.00	27.21	29.51	4.30	5.50	4.75	33.33	66.67	4.94	90.00
T2R1	100.00	27.72	20.51	5.83	5.25	4.83	66.67	100.00	1.60	83.33
T2R2	100.00	24.75	27.18	3.00	0.00	2.33	0.00	25.00	0.88	66.67
T2R2-1	50.00	24.05	31.85	3.14	0.00	2.00	0.00	14.28	0.82	58.33
T2R2-3	75.00	24.19	25.80	4.00	4.00	3.50	22.22	44.44	1.27	100.00
T2R2-5	54.50	29.06	26.53	3.80	3.33	2.75	60.00	80.00	0.95	90.91

LEGEND:
Exposure time: in weeks post-exposure (wpe), Density: Fish density in kg/L, SGR: Specific growth rate between intermediate and final sampling, Prev: Prevalence of infection (% of infected fish), NL diag: non-lethal molecular diagnostic in Ct values, molec diag: lethal molecular diagnostic in Ct values, Histol score: mean histological score (range 0 - 6) of infected fish in anterior (AI), middle (MI) and posterior (PI) intestine, Prev 3InfSeg: percentage of fish with the 3 intestinal segments parasitized, Prev >1InfSeg: percentage of fish with <1 intestinal segment parasitized, Immunoreact IgM: IgM immunoreactivity. Mean intensity (range 0 - 6) for the presence of parasite specific antibodies (IgM) among fish with specific antibodies, %Fish with SpIgM: percentage of fish with parasite specific antibodies (IgM)