Effects of parasite exposure concentrations on infection dynamics and proliferative kidney disease pathogenesis in brown trout (*Salmo trutta*)

Running Title: Parasite exposure concentrations and infection dynamics

Nicole Strepparava[1], Albert Ros[1, 2], Hanna Hartikainen[3, 4], Heike Schmidt-Posthaus[1], Thomas Wahli[1], Helmut Segner[1*], Christyn Bailey[5]

[1] Centre for Fish and Wildlife Health, Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122, 3012 Bern, Switzerland
[2] LAZBW, Fischereiforschungsstelle, Argenweg 50/1, 88085 Langenargen, Germany
[3] ETH and Eawag, Institute for Integrative Biology, Ueberlandstrasse 133, 8600 Duebendorf, Switzerland
[4] School of Biological Sciences, University of Nottingham, NG7 5RD, Nottingham, United Kingdom
[5] Fish Immunology and Pathology Laboratory, Animal Health Research Centre (CISA-INIA), Madrid, Spain

*__Corresponding Author__: Helmut Segner, Centre for Fish and Wildlife Health, Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122, 3012 Bern, Switzerland, +41 (0)31 631 2441 , helmut.segner@vetsuisse.unibe.ch

source: https://doi.org/10.7892/boris.145496 | downloaded: 26.4.2024

Summary

Proliferative kidney disease (PKD) is an emerging disease of salmonids exacerbating with increasing water temperature. Its causative agent, the myxozoan parasite *Tetracapsuloides bryosalmonae*, exploits freshwater bryozoans as primary hosts and salmonids as intermediate hosts. Our experimental approach showed that the manipulation of exposure concentrations of infective malacospores had relatively minor impacts for the disease outcomes in the fish host. In this study brown trout (*Salmo trutta*) were exposed to three different exposure concentrations of *T. bryosalmonae* malacospores: 1) a single low parasite concentration (LC), 2) a single high parasite concentration (HC) and 3) three times a low concentration (repeat exposure, RE). Parasite dynamics in the fish host and release of fish malacospores were quantified and fish kidney histopathology was evaluated to determine PKD pathogenesis. Infection prevalence was always lower in the LC group than in the other groups over the course of the study. While the parasite proliferation phase was slower in the LC group, the maximum parasite burden did not differ significantly between all treatments. The onset of fish malacospores release (day 45 post-exposure), indicated by detection of *T. bryosalmonae* DNA in the tank water, occurred at the same time point for all groups. Reduced intensity of kidney pathological alterations was observed in the LC treatment indicating lower disease severity. While the LC treatment also resulted in reduced outcomes across several infection parameters (infection prevalence, parasite proliferation, total fish malacospores released), the overall differences were small. The RE and HC treatment outcomes were for most parameters comparable. Our results suggested that repeated exposure, as it is likely to occur in the wild during the summer months, might play a more important role in the dynamics of PKD as an emerging infectious disease than the actual parasite concentration itself.

Keywords

Host-parasite interaction, Disease transmission, Disease ecology, Proliferative kidney disease, Salmonids, *Tetracapsuloides bryosalmonae*

1. Introduction

Pathogen and parasite transmission is the driving force in the dynamics of any infectious disease (McCallum, Barlow, & Hone, 2001). Transmission rate depends on the frequency of viable parasite encounters with susceptible hosts and is further shaped by variation in the parasite virulence and host susceptibility factors, often including behavioural, physiological or immunological responses of the host (Ashworth, Kennedy, & Blanc, 1996; Jarungsriapisit et al., 2016). During waterborne transmission, the environmental concentration of infectious agents has a strong influence on encounter rates and thus the infection prevalence (Regoes, Hottinger, Sygnarski, & Ebert, 2003), which further depends on specific host-parasite genotype combinations (Ben-Ami, Regoes, & Ebert, 2008). Concentrations of waterborne pathogens in the environment depend on several interacting factors, including hydrological conditions and connectivity, survival time of the pathogen in the water and the source of the infectious agent, including the abundance, condition and infection status, and the presence of secondary hosts or vectors (El-Matbouli, McDowell, Antonio, Andree, & Hedrick, 1999; Murray, 2009; Ray & Bartholomew, 2013; Ray, Holt, & Bartholomew, 2012). Interactions amongst these processes could complicate the assessment of exposure dependency of infection prevalence in natural systems.

Understanding the interaction between parasite exposure, infection prevalence and disease severity is particularly important for emerging diseases that are provoked by changing environmental conditions. For example, proliferative kidney disease (PKD) of salmonids (caused by the myxozoan, *Tetracapsuloides bryosalmonae* (Myxozoa, Malacospora, Cnidaria, Canning) (Canning, Curry, Feist, Longshaw, & Okamura, 2000) is only mildly pathogenic under low water temperatures (Bailey, Segner, Casanova-Nakayama, & Wahli, 2017; Bettge, Wahli, Segner, & Schmidt-Posthaus, 2009a; de Kinkelin & Loriot, 2001; Ferguson, 1981). When water temperatures remain below 15°C, hosts tolerate the infection and show little or no mortalities, while with increasing water temperatures, mortalities occur in infected fish (Okamura, Hartikainen, Schmidt-Posthaus, & Wahli, 2011). Fish are infected upon contact with malacospores released from infected bryozoans, the invertebrate host of the parasite (Feist, Longshaw, Canning, & Okamura, 2001; Hartikainen & Okamura, 2015). However, there is no horizontal transmission of the parasite between fish. Therefore, the concentration of bryozoan malacospores in the water column may be a driving factor for infection prevalence in fish populations and partly determine the magnitude of an otherwise temperature-driven disease outbreak. The effects of exposure concentration are not known for PKD and higher infectious spore concentrations may also promote faster proliferation and earlier parasite

maturation, resulting in earlier onset, longer duration of disease symptoms and elevated mortality.

Temperature is known to play a major role in PKD development and may also indirectly modulate the concentration of infectious malacospores in the water column. Warmer water temperatures promote the growth of the invertebrate host (most commonly *Fredericella sultana* a freshwater bryozoan), and a higher number of malacospores are released during warm periods (Hartikainen, Fontes, & Okamura, 2013; Tops, Hartikainen, & Okamura, 2009; Tops, Lockwood, & Okamura, 2006; Tops & Okamura, 2003). Thus, the documented influence of water temperature on PKD may be driven not only through an influence on the parasite-fish interaction, but also through temperature mediated effects on the concentration of bryozoan malacospores. To better understand the main drivers of PKD and interactions with seasonally fluctuating temperatures and exposure concentrations, it is important to determine if different malacospore concentrations yield different disease outcomes.

The present study elucidated whether different *T. bryosalmonae* exposure concentrations in the water influenced (i) infection prevalence in the exposed fish, (ii) the subsequent parasite dynamics in the fish host including proliferation rate, maximum parasite load and the timing of fish malacospore release, and, related to the dynamics, (iii) disease severity in the fish host. To exclude potential effects of varying temperatures, a controlled laboratory experiment with a constant temperature (15°C) was used. Our results suggest that prevalence is positively related to parasite exposure concentration and that repeated exposure to low concentrations can result in high prevalence. However, the majority of infection read-outs were highly comparable.

2. Material and methods

2.1 Fish material

Young of the year brown trout (n=2110) weighing on average 2 ± 0.5g (Mean ± Standard error) were obtained from a hatchery with no history of PKD in southern Switzerland (Pescicoltura di Maggia, Maggia, Switzerland). Upon arrival, ten fish were tested for the presence of external and intestinal parasites by direct microscopical examination of wet mounts of skin, gills and intestinal content. For the presence of bacteria by culture on blood agar plates (Biomerieux, Switzerland), on bromthymol blue-lactose-agar plates (Merck, Germany) supplemented with 0.5% sucrose for 48 hours, and on special agar plates to favour growth of flavobacteria for five days (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli. 2018).

Screening for viruses was performed by inoculating samples from the central nervous system (CNS), spleen, kidney, heart and pyloric caeca on bluegill fry (BF) and epithelioma papulosum cyprini (EPC) cell cultures. DNA was extracted from the kidney and tested for the presence of *T. bryosalmonae* sequences as per a previous study (see 2.6 DNA extraction and qPCR for parasite DNA quantification) (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli. 2018). No infectious agents were found. The remaining 2100 brown trout were acclimatized to a temperature of 15°C for two weeks prior to the experiment in the future experimental tanks using the same conditions as in the main experiment (see next paragraph).

2.2 Parasite source for exposure trial

To obtain infective malacospores to create the LC, HC and RE treatments, samples of *T. bryosalmonae* infected bryozoans (*Fredericella sultana* only, identified based on morphology, Wood & Okamura, 2005) were collected from four Swiss rivers on June 29th, 2015 (as in Strepparava and colleagues 2018). The sites encompassed the rivers Furtbach (47°26′51″N 8°22′35″E) (two sites were visited around 500 m apart), Surb (47°31′58″N 8°17′51″E), Alte Aare (47°06′40″N 7°19′01″E) and Lyssbach (47°02′59″N 7°21′18″E). Bryozoans from each source were kept separately in 2.5 L water buckets at room temperature. After 12 h, qPCR was carried out on two 100 ml replicate samples of water from each bucket to confirm that malacospores were released from the bryozoans into the water (see section 2.6 for detailed qPCR description).

2.3 Experimental setup and exposure

The experiment comprised a control and three experimental treatments, each replicated twice (n=8 tanks). The treatment tanks contained 300 fish per tank, and two tanks were setup as controls with 150 fish per tank (Fig. 1). The experimental treatments were 1) an exposure to a single low parasite concentration (LC), 2) an exposure to a single high parasite concentration (HC, in magnitude 10xLC) and 3) an exposure to repeated low parasite concentrations (RE, consisting of LC applied once per day for three consecutive days. The eight tanks were all separate flow-through glass tanks with a volume of 130 L housed in a single room. The tanks were supplied with tap water (flow rate approx. 2 L/min established by fixed water taps with no appreciable variation in flow rates between the tanks) and continuous aeration. Water temperature was maintained at 15 ± 1°C (Mean ± standard error (S.E)) throughout the experiment.

Water from the bryozoan buckets, containing released parasite spores, was added to the fish tanks over three days using the following amounts and procedures. To create a mix of

parasites from different origins, equal amount of water was removed from each bryozoan bucket (n=4, each with 2.5L of water) and mixed thoroughly in a 10L bucket. The exposures were completed over three days and the water in the bryozoan buckets was replaced with tap water on days one and two. Prior to each experimental exposure with spores, the water flow in the fish tanks was stopped and the water level reduced to 30 L. On day one, 2 L of water was removed from each bryozoan bucket, mixed and 2L were added to the LC and RE tanks. On day two, 1L was removed from the bryozoan buckets, mixed and 2L/tank was added to the RE tanks only. On day three, same procedure was followed for the RE tanks, concluding the repeated exposure. The HC treatment was also implemented on day three, using all the remaining "infective water" from the bryozoan buckets (6L). To ensure higher spore concentrations in the HC, all the bryozoans were mechanically disrupted by rupturing coelomic cavities with forceps to release spores and spore sacs (which were further disrupted by gentle squeezing to encourage spore release). The resulting mixture was added to the 6L of "infective water", mixed thoroughly and divided into the two HC treatments.

To confirm that the exposure concentration differed between the low and high concentration (LC, HC) treatments, and to quantify the spore doses added to the RE treatment, water samples (2x100 ml) were taken from each dosed tank 20 min post initial exposure on every exposure time point. The samples were filtered using a 0.25 µm nitrocellulose filter (Merck-Millipore, Switzerland) and the filters were stored in 1.5 ml Eppendorf tubes at -20°C. The parasite DNA was extracted from the filter papers and measured by real-time quantitative PCR (qPCR) and back calculated via the exposure volume in the tanks using the same methodology as described below (section 2.6) for determination of parasite DNA in the fish kidney (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli, 2018). The qPCR-based estimation of parasite DNA concentration in the aquarium water 20 minutes after start of exposure confirmed a near 10-fold difference in parasite concentration between the low exposure concentration (LC) group ($5.4 * 10^6 \pm 20\%$ parasite DNA copies and the high exposure concentration (HC) group ($42* 10^6 \pm 20\%$ parasite DNA copy number). In the RE treatment, the average DNA concentration of the sum of the three exposures was $10* 10^6 \pm 20\%$ parasite DNA copy numbers. Thus, there were clear differences in the parasite exposure concentrations between the different experimental groups.

2.4 Fish sampling

The first day of parasite exposure within each treatment was assigned as day 0 for that experimental group. In all groups, fish sampling was carried out at 3, 5, 7, 10, 15, 20, 25, 30,

40, 50, 70- and 90-days post-exposure (dpe). Afterwards, monthly samplings were performed until 360 dpe. At every time point, from each treatment group ten fish per replicate were sampled and analysed, whereas only five fish were taken from the control per replicate as neither any infection nor pathological alterations had been expected, which would have justified to take more fish in accordance with the 3R principles (Fig.1) (Sneddon, Halsey, & Bury, 2017). In addition, mortalities were recorded daily, and fish dying between the sampling days were necropsied and investigated for the presence of parasites and other infectious agents.

Sampled fish were euthanized using 100mg/l bicarbonate-buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories). Length (L) and weight (W) were recorded and the body condition index (K) was calculated with the formula: $K = W \times L^{-3} \times 100$ where W is weight in grams, and L is total length in cm. The kidney was cut in two halves longitudinally: one part was removed, weighed (kidney weight, KW) and homogenized in 1.5 ml Tri-solution (Sigma, Switzerland) for future DNA extraction, while the other half was placed in 10% buffered formalin for histology. Presence and intensity of the parasite was determined using qPCR on DNA extracted from the kidney preserved in Tri-solution. Infection prevalence was calculated as the mean percent of parasite positive fish detected per each sampling date and treatment group while parasite intensity was calculated for each parasite positive individual fish and standardized using the KW.

2.5 Determination of fish malacospores release

To evaluate when infected fish malacospores were first released, water samples were collected from each tank over the course of the experiment. The presence of parasite DNA was used as an estimate of the parasite material released from the fish, and this indicates release of fish malacospores (Morris & Adams, 2006). Two 1 L water samples from each tank and time point were filtered using a 3.0 µm nitrocellulose filter (Merck-Millipore, Switzerland). Samples were taken every ten days starting at 20 dpe until day 130, then monthly until 360 dpe. Each filter was stored at -20°C until further processing for DNA extraction and qPCR assay.

2.6 DNA extraction and qPCR for parasite DNA quantification

Genomic DNA was extracted as previously described (Harun, Wang, & Secombes, 2011). DNA was finally eluted in 30 µl of EB buffer (QIAGEN, Switzerland) and stored at -20°C until qPCR was performed.

Filter papers from water samples were cut into small pieces using sterilised scissors. DNA was then directly extracted from these small pieces using the Blood & Tissue DNA extraction kit (QIAGEN, Switzerland) following the manufacturer instructions. DNA was eluted with 100µl EB buffer and stored at -20°C.

qPCR was performed targeting the *T. bryosalmonae* 18S rRNA gene (Acc. N.: AF190669) using the specific TaqMan method as reported by Bettge et al. (2009a). Briefly, each reaction was carried out in a final volume of 20 µl containing 1X TaqMan universal Master Mix (Applied Biosystems, Switzerland), 0.5 µM of each primer (PKDtaqf1: 5′–GCGAGATTTGTTGCATTTAAAAAG– 3′and PKDtaqr1: 5′ –GCACATGCAGTGTCCAA TCG–3′), 0.2 µM of the probe PKD (5′–CAAAATTGTGGAACCGTCCGACTACGA–3′), 1X of internal control Exo IPC Mix, 1X of IC DNA (TaqMan Univ. MMix w Exog IntPostC, Applied Biosystems), and 2 µl of template DNA. All reactions were carried out in duplicate. A cut-off CT-value of 32 was used under which amplification was considered specific to *T. bryosalmonae* DNA (Bettge, Segner, Burki, Schmidt-Posthaus, & Wahli, 2009a). Parasite intensities expressed as DNA copy numbers/g of kidney, were estimated from the qPCR reactions per duplicate of each sample by comparing the CT-value results with the linear relationship derived from the standard curve between CT values and log DNA copy numbers as per our previous study (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli. 2018)

2.7 Histological assessment of kidney pathology score (KPS) to determine disease severity

Histopathological assessment of the kidney pathology score (KPS) was used to assess disease severity. Formalin-fixed kidney samples were paraffin-embedded and processed for histological examination using routine protocols. One section of 3 µm thickness was prepared for histopathology (haematoxylin-eosin stain, H&E). H&E stained slides were examined by light microscopy (Eclipse E400, Nikon). Histopathological changes of the whole kidney section were classified from 0 (no alterations) to 6 (severe proliferation of the hematopoietic tissue, multiple areas of haemorrhage, widespread necrosis, multiple thrombi, severe multifocal infiltration) (Bettge, Wahli, Segner, & Schmidt-Posthaus, 2009b). For each time point, the mean value of histological changes for all examined fish (positive and negative animals for *T. bryosalmonae* DNA tested by qPCR) per replicate and per treatment was calculated and determined as the KPS.

2.8. Immunohistochemistry assessment of *T. bryosalmonae* maturation stages

Infected fish determined by histology at dpe 40 (5 days before fish malacospore release started) were evaluated using immunohistochemistry (IHC) to examine possible differences amongst treatments of *T. bryosalmonae* maturation in terms of number of nuclei per parasite, as well as time of first appearance and number of intratubular fish malacospores. The examination of *T. bryosalmonae* maturation (via number of nuclei per parasite) was based on the finding by Morris and Adams (2008) that the main replicative phase of *T. bryosalmonae* within vertebrates is via direct replication of cell doublets. One slide each was used for each fish sampled (n=20) for IHC with a monoclonal anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics). Staining was performed as previously reported (Adams, Richards, & De Mateo, 1992). Kidney tissue of a brown trout known to be infected with *T. bryosalmonae* from a previous exposure performed in our lab was used as a positive control (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli, 2018) . Slides incubated without the primary antibody were used as negative controls. The slides were screened for the presence of histozoic extrasporogonic stages with 1, 2 or 3 nuclei per parasite and for presence of intratubular coelozoic sporogonic stages. All visible parasites on the slide were counted, one slide was counted per fish consisting of one section of kidney.

2.9 Data management and statistical analysis

Cumulative counts of mortality over the testing period were compared between exposure concentration treatments using the Chi-squared test. Parasite intensity in the kidney (DNA copy number / g of kidney) was calculated as the average parasite quantification (over the duplicate qPCR reactions) in the DNA final elution (standardized by the dilution through the extraction), divided by the extracted KW in grams (g). Statistical analyses and graphical presentations were carried out using R (version 3.1.0)(de Micheaux, Drouilhet, & Liquet, 2013; Team, 2014). Mixed models were fitted with package "lme4"(Bates, Machler, Bolker, & Walker, 2015), and p-values were estimated by bootstrapping (P notation: P_b, 500 simulations, using PBmodcomp of "pbkrtest") or by estimating the denominator degrees of freedom (df). For data visualization the packages "ggplot2", "scales", and "cowplot" were used. A parametric approach was required in order to test the multi-factorial design (time [polynomial, 2] and treatment [mostly 3 levels: LC, HC and RE]) while correcting test outcomes for replications by adding "tank" as random effect (Bates, Machler, Bolker, & Walker, 2015). Normality was validated on residuals retrieved from the mixed models, by evaluating Q-Q plots and the K-S Lilliefors test ("nortest" package). Post-hoc comparisons of treatments following mixed models were calculated using Tukey contrasts from the package "multcomp" (Bolker, 2019). The differences between groups fish malacospore release were tested for with T-tests. We did not adjust for the declining tank population during fish malacospore release analysis (due to sampling) because the low exposures resulted in different infection prevalence. Therefore,

any calculation to obtain “per-fish” shedding rates would be biased between the low and high/repeated exposure treatments. Kidney pathology was scored as an ordered factor and was therefore analysed with proportional odds logistic regression using the function “polr” from the package “MASS”. The anova table was extracted using the “car” package. In both number of infected fish and infection intensity analyses, interactions between variables were excluded from the final model when not statistically significant. The microscopy counts using immunohistochemistry detection of the parasite stages at 40 dpe in the kidney were tested statistically applying the non-parametric Kruskal–Wallis anova on ranks, and Dunn’s non-parametric multiple comparison tests to reveal differences between treatments.

3. Results

3.1 Mortality and condition index

There were no significant differences in cumulative mortalities over the course of the experiment (mean ± SD) between groups: 6.8 ± 0.4 % in the LC treatment; 4.2 ± 0.7 % in the HC treatment; 6.2 ± 1.7 % in the RE treatment, and 5.6 ± 1.5 % in the control group (Chi-squared test: $X^2 = 48$, df = 42, p-value = 0.24). In addition, no significant differences between groups were found in growth (mean growth = 0.066 ± 0.080 gram/day; linear model, F(3,51) = 0.03, p=0.99.

3.2 Parasite infection dynamics

3.2.1 Infection prevalence

None of the kidney samples taken from control fish tested positive for the *T. bryosalmonae* 18S rRNA gene. Infection prevalence curves in each treatment group are shown in Fig. 2a. The parasite DNA was detectable in kidneys from the RE group at 3 and 5 dpe (5% of the examined fish). In the HC group, parasite DNA was first detected at 7 dpe (35%), and in the LC group at 10 dpe (30%). From 15 dpe until 160 dpe, prevalence remained stable at 100 % in the HC and RE group, and in the LC group prevalence fluctuated around a stable average (90%). Statistical analysis over the first 2 months showed a significant time and treatment effect (binomial GLM, time: deviance = 474.35, df = 2, $p_b < 0.01$, effect treatment: deviance = 71.25, df = 2, $p_b < 0.01$). Post-hoc Tukey contrasts indicated that the LC group had significantly lower prevalence in this time period than the RE and HC groups (HC, RE vs LC: $p < 0.001$; HC vs RE: $p = 0.58$).

Infection prevalence detectable by the applied method significantly decreased at the end of the sampling period (binomial GLM from 90 dpe, time: deviance = 77.47, df = 2, $p_b < 0.01$).

Post hoc calculated contrasts for time indicated that infection prevalence started to significantly decrease relative to previous infection prevalence values at 260 dpe in all groups (contrasts vs 320, 360: $p < 0.01$). As found for the initial period, prevalence values in the LC group were also significantly lower than those in the HC and RE groups (binomial GLM from 90 dpe, treatment: deviance = 21.81, df = 2, $p_b < 0.01$, post-hoc Tukey contrasts: HC, ME vs LC: $p < 0.01$; HC vs RE: $p = 0.21$). At the end of the experiment the percent of infected fish sampled in RE and HC groups was at 60 – 80%, whereas in the LC treatment only 20 % of the fish sampled were still infected.

3.2.2 Parasite intensity

Only *T. bryosalmonae*-positive fish were included to calculate parasite intensity. In all treatments, parasite intensity increased rapidly following exposure (Fig. 2b, glmm, random factor = tank; time: $F(2,398) = 2193$, $p_e < 0.001$). During the initial phase of increasing parasite intensity, treatment with different parasite concentrations showed a significant effect (Fig. 1b, Linear mixed model, glmm, random factor = tank; treatment: $F(2, 3) = 66.96$, $p_e = 0.003$; interaction with time: $F(2,398) = 0.18$, $p_e = 0.95$). The LC treatment fish showed significantly lower parasite intensity during the proliferation phase than fish of the other two treatments (post-hoc Tukey contrasts: HC, RE vs LC: $p < 0.001$; HC vs RE: $p = 0.41$).

After 50 dpe, the parasite intensity in the kidney of all treatments entered to a plateau phase. At the plateau phase the maximum burden reached was similar for all experimental treatments and at the same time point (160 dpe) (Fig. 1b, glmm, random factor = tank, treatment: $F(2, 3) = 0.17$, $p_e = 0.85$; interaction with time: $F(2,252) = 1.96$, $p_e = 0.10$).

Parasite intensity in the kidney showed a slightly declining trend in all treatments ~ 200 dpe (Fig. 1b, glmm, random factor = tank, time: $F(2,252) = 3.44$, $p_e = 0.034$). However, the extent of this decline was not significantly different between any of the experimental groups ($p_e = 0.10$).

3.2.3 Release of fish malacospores

The onset of fish malacospore release was deduced from the detection of parasite DNA in the tank water. The statistical analysis of fish malacospore release was complicated as the time pattern showed strong fluctuating dynamics. In order to simplify the interpretation of the statistical outcome, residual differences of the mean value were calculated per sampling date and these were expressed as percentages from the mean. Parasite DNA was never detected in samples taken from tank water of the control group. Parasite DNA was detected from 45 dpe onwards for all treatment groups. After an initial peak in fish malacospore release at 90

dpe, parasite DNA in the tank water first declined and then slightly increased again until the end of the sampling period (Fig. 2c). Overall the LC group had the lowest amount of fish malacospores detected and the RE group had a significantly greater amount of fish malacospores detected relative to the HC and LC groups over the course of the experiment (residual differences of the Mean ± S.E (n = 38 samples): LC: -26% ± 58%, HC: -15% ± 53%, RE: 41 ± 63%, T-tests: LC, HC vs RE: $p < 0.001$, LC vs HC: $p = 0.39$).

3.3 Histopathology confirmation of PKD and assessment of kidney pathology score (KPS) to determine disease severity

Microscopic assessment of the kidneys of control fish did not show any pathological alterations. This was clearly different with kidneys of *T. bryosalmonae* infected fish from all experimental groups in which clear pathological alterations were found consistent with those previously described for *T. bryosalmonae*-infected brown trout (Schmidt-Posthaus, Ros, Hirschi, & Schneider, 2017). These alterations must be attributed to the infection by *T. bryosalmonae* exclusively, as no other pathogenic agents have been detected.

To semi-quantitatively evaluate the differences in disease severity between the groups we assessed the kidney pathology score (KPS) to characterise kidney lesion severity as per previous studies (Bettge, Wahli, Segner, & Schmidt-Posthaus, 2009b) (Fig. 2). All scores for control fish were 0. Concerning the comparisons between the infected groups ordered logistic regression of the KPS score showed a significant treatment effect of parasite concentration (polr, $\chi^2 = 40.8$, $df = 2$, $p < 0.0001$). The score increased significantly over time (polr, $\chi^2 = 177$, $df = 2$, $p < 0.0001$) while this increase over time did not differ significantly between parasite concentrations (polr, $\chi^2 = 2.55$, $df = 4$, $p = 0.64$). Post-hoc tests showed that KPS was lowest in fish treated with the low parasite concentration (HC and RE vs LC: polr, $p < 0.0001$), while no significant difference was detected between the HC and RE groups (polr, $p = 0.46$).

3.4 Immunohistochemistry assessment of *T. bryosalmonae* maturation stages

Statistical analysis of microscopy counts of parasite maturation stages at 40 dpe in the kidney revealed significantly greater number of single nucleated parasites in the RE group in comparison to the HC and LC ($df = 2$, $p = 0.002$; post-hoc Dunn's methods RE vs HC and LC: $p < 0.05$). Applying this analysis across all the exposure groups we found mostly single and di-nucleated cells. However, there were no significant differences when comparing counts of the di and tri-nucleated cells between the treatments. Moreover, the ratios of single, di- and tri-nucleated cells found were similar in the three treatments (Table 1).

4. Discussion

The experimental treatment of young-of-the-year brown trout with varying *T. bryosalmonae* exposure concentrations resulted in significant differences in prevalence, corroborating expectations from mass-balance dynamics and previous experiments modulating exposure concentrations in fish host-myxozoan parasite systems (Ray & Bartholomew, 2013; Ray, Holt & Bartholomew, 2012; Ryce, Zale, & MacConnell, 2004). The LC treatment conferred lower infection prevalence than the HC and RE treatments. The exposure treatments also influenced the initial parasite proliferation in the kidney and kidney pathological alterations. Again, the LC treatment differed from the HC and RE treatments, exhibiting delayed parasite detection and reduced kidney pathology, although the overall differences between treatments were small. Our results provide evidence that the cumulative effects of repeated exposure to low parasite concentrations may result in similar responses as single exposures to high concentrations. Importantly, the maximum parasite intensity in the fish kidney, as well as the time point when fish malacospores were first released, were not affected by exposure concentration.

The development of parasite intensity in the fish kidney showed an initial exponential increase in parasite numbers, followed by a plateau phase as earlier described for brown trout (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli, 2018) and rainbow trout (Bettge, Segner, Burki, Schmidt-Posthaus, & Wahli, 2009a). During the exponential phase, parasite intensity increased faster in the HC and RE treatments compared to the LC treatment. While the cumulative parasite exposure concentration of the RE group was slightly lower than that of the HC group ($4.2 * 10^7 \pm 20\%$ parasite DNA copy number versus $1.0* 10^7 \pm 20\%$ parasite DNA copy numbers) yet the majority of measured disease read-outs did not differ statistically between the treatments. For example, fish malacospore release began in brown trout constantly held at 15 °C at 45 dpe in all treatments. While, the immunohistochemistry results showed a greater number of single nucleated parasites in the RE group still the ratios of single, di- and tri-nucleated cells were comparative in the three treatments, however this analysis was only performed at a single timepoint. In addition, at 50 dpe parasite intensity reached a plateau in all experimental groups, thus suggesting that fish malacospore release might be influenced by a maturation period, which was not dose dependent, but related to carrying capacity within the host (Strepparava, Segner, Ros, Hartikainen, Schmidt-Posthaus, & Wahli, 2018).

It needs to be emphasized that the initial increase in infection prevalence seen in the LC, HC and RE treatments is an artefact due to the limit of detection of the PCR for *T. bryosalmonae* DNA. Given the fact that the parasite exposure of the fish was restricted to 1.5 hours (see Methods 2.3 Parasite exposure) and that fish malacospores are not infective to fish, a new

infection of the fish subsequent to this defined exposure period was not possible. Instead, all infections must have taken place during the time window of exposure (day 0). Despite this, when we analysed fish one day after the parasite exposure, not a single fish was tested positive by the PCR. As described above, positive fish were detected for the first time at 3 dpe (which corresponds to 3 days after the first exposure in the case of the RE group), and then the prevalence continuously increased over the next 10-15 days to a stable plateau level. Interestingly, the time period it needs until the parasite has proliferated to an intensity level that can be detected by the PCR varied with the exposure conditions: the earliest detection was possible in the RE group, followed by the HC group, while the LC group displayed the longest “lag period” until parasite DNA could be detected.

The cumulative estimate of fish malacospores release was significantly greater in the RE treatment relative to both the HC and the LC treatments as was the amount of single nucleated parasites. This result might indicate that repeated exposure, as it is likely to occur in the natural environment, might play an important role in explaining high infection prevalence in many natural systems. Low concentrations of infective densities can appear, for example, in areas where parasites have newly invaded (low infection prevalence in bryozoan populations), or the parasites are at their distribution range margins. Variation in environmental conditions can amplify such edge effects and strongly affect the incidence and severity of disease by altering rates of infection and disease development (Fagan, Cantrell, & Cosner, 1999; Jarosz & Burdon, 1988; Johnson & Haddad, 2011). Low exposure concentrations can regardless result in high prevalence as the infectivity of *T. bryosalmonae* spores is high (McGurk, Morris, Auchinachie, & Adams, 2006). In this context, it is notable that our LC did not achieve a 100% infection prevalence. This result indicates that cumulative exposure is an important factor in promoting high prevalence’s in many natural systems, especially as in this study we exposed fish to infective densities that likely exceeded those encountered in most natural river systems, including our LC treatment. As a caveat we note that we are not able to directly compare the exposure concentrations in our treatments to measures obtained from the field (e.g. in environmental DNA obtained from water samples) due to the technical issues in discriminating between fish malacospores and malacospores from bryozoans during field sampling. Nevertheless, our premise that repeated exposure, as it occurs in the wild, might play a more important role in the dynamics of PKD than the prevailing parasite exposure concentration itself is still supported by almost all the parameters measured here (as many results were comparative in the RE and HC treatments). Thus, emergence and severity of PKD outbreaks may result from landscape level variation in exposure concentrations in the abundance and infection status of bryozoans, as invertebrate hosts of *T. bryosalmonae.* For example, warmer temperatures in lower altitude reaches of rivers, and those experiencing nutrient enrichment,

likely promote bryozoan growth and higher infected host biomass (Tops, Hartikainen & Okamura, 2009). This may drive the concentration of malacospores in the water body. Additionally, *T. bryosalmonae* itself may also develop more frequently into overt infections (during which the parasites are released into the water), resulting in elevated exposure concentrations for the fish (Tops Lockwood, & Okamura, 2006). In fact, recent studies have correlated high bryozoan abundance with increasing PKD occurrence (Carraro et al., 2017) and the increased disease severity resulting from prolonged, repeated low concentration exposure may expand the influence of infection hotspots especially in interconnected rivers.

The fish exposed to a greater parasite load (HC and RE) had increased disease severity indicated by greater kidney pathology score (KPS). The increased KPS could indicate that a more intense immunopathology occurred in those treatments in contrast to the LC treatment. Therefore, it may be that the initial rate of parasite proliferation influences immunomodulation and downstream immunopathology resulting in some alterations in the host immune response when tolerating the different number of initial parasites. However, in our previous work in brown trout an intense immune response was not seen until burdens were close to carrying capacity (Bailey, Strepparava, Wahli, & Segner, 2019) . In the current experiment parasite intensity plateaued at the same time point, therefore the differences in the host immune response when managing different parasite concentrations may also be subtle in line with the rest of our data. This would also contrast studies that have looked at the impact of temperature upon the immune response of *T. bryosalmonae* infected rainbow trout in which a difference of 3 °C was shown to result in massive differences in infection outcome and immune response (Bailey, Segner, Casanova-Nakayama, & Wahli, 2017). However, as we did not measure the immune response or the impact of temperature in the present study this can only be speculated upon.

5. Conclusion

This study investigated if different *T. bryosalmonae* exposure concentrations in the water influenced (i) infection prevalence in the exposed fish, (ii) the subsequent parasite dynamics in the fish host including proliferation rate, maximum parasite load and the timing of fish malacospore release, and, (iii) disease severity in the fish host. Our study highlighted a small but significant effect of exposure concentration on disease severity. In summary, our laboratory based results suggest a minor influence of the parasite transmission stage concentration on the disease dynamics in brown trout: higher exposure concentrations lead to a significantly higher infection prevalence – but the difference is small, from 90 % in the low concentration to 100 % in the high concentration. Even when exposure concentrations are low in e.g. newly invaded areas, the repeated exposure can drive high infection

prevalence and could partly explain the wide distribution and emergence of PKD. Higher spore concentrations and repeated exposures resulted in a faster initial proliferation of the parasite in the fish host. Yet, the maximum parasite burden, as well as the time point for the onset of fish malacospore release, were not significantly modified by exposure concentrations. This finding suggests that repeated exposure, as it is likely to occur in the natural environment might play a more important role in the dynamics of PKD as an emerging infectious disease than the actual parasite concentration itself. Thus, in this regard PKD should be considered an emerging infectious disease for the upcoming years in several habitats.

Our investigated temperature was 15 °C, which is the widely reported threshold for the clinical form of the disease to develop (Hedrick, MacConnell, & De Kinkelin, 1993). However, to better understand what the effect of temperature on different parasite exposure concentration is, a range of temperatures or fluctuating temperature regimes should be investigated. Further studies should also focus on assessing the conditions that modulate the patterns of recovery and fish malacospore production under fluctuating and varied temperature regimes.

Acknowledgements

We thank Bruno Polli (Ufficio Caccia e Pesca, Ticino) and the Fish farm of Maggia for providing us brown trout. Additionally, we would like to thank Barbara Müller and Regula Hirschi for taking care of the fish and all those that assisted in sampling. This research was supported by the Swiss National Science Foundation (SNSF) Sinergia grant CRSII3_147649_1. CB was funded by SNF Post Doc Mobility Fellowship number P400PB_183824.

Ethics Statement

Approval for animal experiments was obtained from the cantonal veterinary Office (Bern, Switzerland) (Authorization BE13/15) and guidelines for the Care and Use of Laboratory Animals were followed.

Conflict of interests

The authors declare no conflict of interests.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Adams, A., Richards, R. H., & De Mateo, M. M. (1992). Development of monoclonal antibodies to PK'X', the causative agent of proliferative kidney disease. *Journal of Fish Diseases, 15*(6), 515-521. doi:10.1111/j.1365-2761.1992.tb00683.x

Ashworth, S., Kennedy, C., & Blanc, G. (1996). Density-dependent effects of Anguillicola crassus (Nematoda) within and on its copepod intermediate hosts. *Parasitology, 113*(3), 303-309. doi:10.1017/S003118200008207X

Bailey, C., Segner, H., Casanova-Nakayama, A., & Wahli, T. (2017). Who needs the hotspot? The effect of temperature on the fish host immune response to Tetracapsuloides bryosalmonae the causative agent of proliferative kidney disease. *Fish & shellfish immunology, 63*, 424-437. doi:10.1016/j.fsi.2017.02.039

Bailey, C., Strepparava, N., Wahli, T., & Segner, H. (2019). Exploring the immune response, tolerance and resistance in proliferative kidney disease of salmonids. *Developmental & Comparative Immunology*, *90*, 165-175. doi: 10.1016/j.dci.2018.09.015

Bates, D., Machler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software, 67*(1), 1-48.

Ben-Ami, F., Regoes, R. R., & Ebert, D. (2008). A quantitative test of the relationship between parasite dose and infection probability across different host–parasite combinations. *Proceedings of the Royal Society B: Biological Sciences, 275*(1636), 853-859. doi:10.1098/rspb.2007.1544

Bettge, K., Segner, H., Burki, R., Schmidt-Posthaus, H., & Wahli, T. (2009a). Proliferative kidney disease (PKD) of rainbow trout: temperature-and time-related changes of Tetracapsuloides bryosalmonae DNA in the kidney. *Parasitology, 136*(6), 615-625. doi:10.1017/S0031182009005800

Bettge, K., Wahli, T., Segner, H., & Schmidt-Posthaus, H. (2009b). Proliferative kidney disease in rainbow trout: time-and temperature-related renal pathology and parasite distribution. *Diseases of Aquatic Organisms, 83*(1), 67-76.

Bolker, B. (2019, 28/5/2019). GLMM FAQ. Retrieved from https://bbolker.github.io/mixedmodels-misc/glmmFAQ.html

Canning, E.U., Curry, A., Feist, S.W., Longshaw, M. and Okamura, B., 2000. A new class and order of myxozoans to accommodate parasites of bryozoans with ultrastructural observations on Tetracapsula bryosalmonae (PKX organism). *Journal of Eukaryotic Microbiology*, *47*(5), pp.456-468. doi: 10.1111/j.1550-7408.2000.tb00075

Carraro, L., Bertuzzo, E., Mari, L., Fontes, I., Hartikainen, H., Strepparava, N., . . . Gatto, M. (2017). Integrated field, laboratory, and theoretical study of PKD spread in a Swiss prealpine river. *Proceedings of the National Academy of Sciences, 114*(45), 11992-11997. doi: 10.1073/pnas.1713691114

de Kinkelin, P., & Loriot, B. (2001). A water temperature regime which prevents the occurrence of proliferative kidney disease (PKD) in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Disease, 24*(8), 489-493. doi:10.1046/j.1365-2761.2001.00312.x

de Micheaux, P., Drouilhet, R., & Liquet, B. (2013). Programming in R. In *The R Software* (Vol. 40, pp. 193-282): Springer New York.

El-Matbouli, M., McDowell, T., Antonio, D., Andree, K., & Hedrick, R. (1999). Effect of water temperature on the development, release and survival of the triactinomyxon stage of

593 Myxobolus cerebralis in its oligochaete host. *International Journal of Parasitology,*
594 *29*(4), 627-641. doi:10.1016/S0020-7519(99)00009-0
595 Fagan, W. F., Cantrell, R. S., & Cosner, C. (1999). How habitat edges change species
596 interactions. *The American Naturalist, 153*(2), 165-182. doi:10.1086/303162
597 Feist, S. W., Longshaw, M., Canning, E. U., & Okamura, B. (2001). Induction of proliferative
598 kidney disease (PKD) in rainbow trout Oncorhynchus mykiss via the bryozoan
599 Fredericella sultana infected with Tetracapsula bryosalmonae. *Diseases of Aquatic*
600 *Organisms, 45*(1), 61-68. doi:10.3354/dao045061
601 Ferguson, H. W. (1981). The effects of water temperature on the development of proliferative
602 kidney disease in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish*
603 *Diseases, 4*, 175-177. doi:10.1111/j.1365-2761.1981.tb01122.x
604 Hartikainen, H., Fontes, I., & Okamura, B. (2013). Parasitism and phenotypic change in
605 colonial hosts. *Parasitology, 140*(11), 1403-1412. doi:10.1017/S0031182013000899
606 Hartikainen, H., & Okamura, B. (2015). Ecology and evolution of malacosporean-bryozoan
607 interactions. In B. Okamura, A. Gruhl, & J. L. Bartholomew (Eds.), *Myxozoan evolution,*
608 *ecology and development* (pp. 201-216). Cham: Springer.
609 Harun, N. O., Wang, T., & Secombes, C. J. (2011). Gene expression profiling in naive and
610 vaccinated rainbow trout after Yersinia ruckeri infection: insights into the mechanisms
611 of protection seen in vaccinated fish. *Vaccine, 29*(26), 4388-4399.
612 doi:10.1016/j.vaccine.2011.04.003
613 Hedrick, R., MacConnell, E., & De Kinkelin, P. (1993). Proliferative kidney disease of salmonid
614 fish. *Annual review of Fish diseases, 3*, 277-290. doi:10.1016/0959-8030(93)90039-E
615 Jarosz, A., & Burdon, J. (1988). The effect of small-scale environmental changes on disease
616 incidence and severity in a natural plant-pathogen interaction. *Oecologia, 75*(2), 278-
617 281. doi:10.1007/BF00378609
618 Jarungsriapisit, J., Moore, L. J., Mæhle, S., Skår, C., Einen, A. C., Fiksdal, I. U., . . . Patel, S.
619 (2016). Relationship between viral dose and outcome of infection in Atlantic salmon,
620 Salmo salar L., post-smolts bath-challenged with salmonid alphavirus subtype 3.
621 *Veterinary Research, 47*(1), 102. doi:10.1186/s13567-016-0385-2
622 Johnson, B. L., & Haddad, N. M. (2011). Edge effects, not connectivity, determine the
623 incidence and development of a foliar fungal plant disease. *Ecology, 92*(8), 1551-1558.
624 doi:10.1890/10-1072.1
625 McCallum, H., Barlow, N., & Hone, J. (2001). How should pathogen transmission be
626 modelled? *Trends in ecology & evolution, 16*(6), 295-300. doi:10.1016/S0169-
627 5347(01)02144-9
628 McGurk, C., Morris, D. J., Auchinachie, N. A., & Adams, A. (2006). Development of
629 Tetracapsuloides bryosalmonae (Myxozoa: Malacosporea) in bryozoan hosts (as
630 examined by light microscopy) and quantitation of infective dose to rainbow trout
631 (Oncorhynchus mykiss). *Veterinary parasitology, 135*(3-4), 249-257.
632 doi:10.1016/j.vetpar.2005.07.022
633 Morris, D., & Adams, A. (2008). Sporogony of Tetracapsuloides bryosalmonae in the brown
634 trout Salmo trutta and the role of the tertiary cell during the vertebrate phase of
635 myxozoan life cycles. *Parasitology, 135*(9), 1075-1092.
636 doi:10.1017/S0031182008004605
637 Morris, D. J., & Adams, A. (2006). Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa:
638 Malacosporea), the causative organism of salmonid proliferative kidney disease, to the
639 freshwater bryozoan *Fredericella sultana*. *Parasitology, 133*(6), 701-709.
640 doi:10.1017/S003118200600093X
641 Murray, A. G. (2009). Using simple models to review the application and implications of
642 different approaches used to simulate transmission of pathogens among aquatic
643 animals. *Preventive veterinary medicine, 88*(3), 167-177.
644 doi:10.1016/j.prevetmed.2008.09.006
645 Okamura, B., Hartikainen, H., Schmidt-Posthaus, H., & Wahli, T. (2011). Life cycle complexity,
646 environmental change and the emerging status of salmonid proliferative kidney
647 disease. *Freshwater Biology, 56*(4), 735-753. doi:10.1111/j.1365-2427.2010.02465.x

Ray, R. A., & Bartholomew, J. L. (2013). Estimation of transmission dynamics of the Ceratomyxa shasta actinospore to the salmonid host. *Parasitology, 140*(7), 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*(3), 520-526. doi:10.1645/JP-GE-2737.1

Ryce, E. K., Zale, A. V., & MacConnell, E. (2004). Effects of fish age and parasite dose on the development of whirling disease in rainbow trout. *Diseases of Aquatic Organisms, 59*(3), 225-233. doi:10.3354/dao059225

Schmidt-Posthaus, H., Ros, A., Hirschi, R., & Schneider, E. (2017). Comparative study of proliferative kidney disease in grayling *Thymallus thymallus* and brown trout *Salmo trutta fario*: an exposure experiment. *Diseases of Aquatic Organisms, 123*(3), 193-203. doi:10.3354/dao03102

Sneddon, L.U., Halsey, L.G. and Bury, N.R., 2017. Considering aspects of the 3Rs principles within experimental animal biology. *Journal of Experimental Biology*, *220*(17), pp.3007-3016. doi: 10.1242/jeb.147058

Strepparava, N., Segner, H., Ros, A., Hartikainen, H., Schmidt-Posthaus, H., & Wahli, T. (2018). Temperature-related parasite infection dynamics: the case of proliferative kidney disease of brown trout. *Parasitology, 145*(3), 281-291. doi:10.1017/S0031182017001482

Team, R. C. (2014). *R: A language and environment for statistical computing.* Vienna, Austria.

Tops, S., Hartikainen, H. L., & Okamura, B. (2009). The effects of infection by Tetracapsuloides bryosalmonae (Myxozoa) and temperature on Fredericella sultana (Bryozoa). *International Journal of Parasitology, 39*(9), 1003-1010. doi:10.1016/j.ijpara.2009.01.007

Tops, S., Lockwood, W., & Okamura, B. (2006). Temperature-driven proliferation of Tetracapsuloides bryosalmonae in bryozoan hosts portends salmonid declines. *Diseases of Aquatic Organisms, 70*(3), 227-236. doi:10.3354/dao070227

Tops, S., & Okamura, B. (2003). Infection of bryozoans by Tetracapsuloides bryosalmonae at sites endemic for salmonid proliferative kidney disease. *Diseases of Aquatic Organisms, 57*(3), 221-226. doi:10.3354/dao057221

Wood, T., & Okamura, B. (2005). A key to the British and European freshwater bryozoans with ecological notes. *Freshwater Biological Association, Ambleside*.

Tables

Table 1. Immunohistochemistry assessment of *T. bryosalmonae* maturation stages showing percent of single and multinucleated parasites observed in the posterior kidney of brown trout exposed to different concentration of infectious malacospores. N = 20 fish assessed per treatment.

Concentration	**Single nuclei**	**Di-nuclei**	**Tri-nuclei**
LC	59%	41%	0%
HC	57%	39%	4%
RE	59%	37%	4%

Figure legends

Fig 1. Schematic of experimental design, detailing establishment of exposure concentration groups and sample collection.

Fig 2. Parasite detection dynamics after brown trout exposure to different concentrations of infectious *Tetracapsuloides bryosalmonae* malacospores. A) Infection prevalence. B) Parasite intensity in fish tested positive for *T. bryosalmonae*, (n fluctuates between 1-10 fish/replicate at each sample point). C) Parasite intensity per L of water (averaged between duplicate treatment tanks).

Fig 3. Kidney pathology score (n=20 fish per sample per group) in brown trout held at 15°C, Severity of the histological changes was measured on a scale from 0 to 6. Box plots give median scores, 25 and 75 quantiles and the range of these scores. Control animals showed no pathology, thus always scoring a 0 and were therefore not included in the graph. Parasite exposure treatment was at day 0.