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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Host (*Salmo trutta*) age influences resistance to infestation by freshwater pearl mussel (*Margaritifera margaritifera*) glochidia

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Abstract

The freshwater pearl mussel (*Margaritifera margaritifera*) is an endangered bivalve with an obligate parasitic stage on salmonids. Host suitability studies have shown that glochidial growth and load vary significantly between host strains as well as among individuals of a suitable strain. Variation in host suitability has been linked to environmental conditions, host age and/or size, genetic composition of the host and parasite, or a combination of these factors. In our study, we wanted to investigate if brown trout (*Salmo trutta*) displayed an age-dependent response to glochidial infestation. We hypothesised that 1+ naive brown trout hosts tolerate glochidial infestation better than 0+ hosts. In order to test our hypothesis, we infested 0+ and 1+ hatchery reared brown trout with glochidia from closely related mothers and kept them under common garden conditions. This allowed us to observe a pure age dependent host response to infestation, as we eliminated the confounding effect of genotype-specific host interactions. We analysed the interaction between glochidial load and host condition, weight and length, and observed a significant age-dependent relationship. Glochidial load was negatively correlated to host condition in 0+ fish hosts and positively correlated in 1+ hosts. These contradictory findings can be explained by a change in host response strategy, from resistance in young to a higher tolerance in older fish. In addition, we also examined the relationship between glochidial load and haematocrit values in the 1+ hosts and observed that haematocrit values were significantly higher in heavily infested hosts. Our results have important conservation implications for the management of wild pearl mussel populations, as well as for captive breeding programmes.

Keywords *Margaritifera* · Glochidia · Host-parasite · Condition factor · Haematocrit · Tolerance · Host response

Introduction

The freshwater pearl mussel (FPM) *Margaritifera margaritifera* (also referred to as the pearlshell mussel in North America) is an endangered bivalve (Mollusc Specialist Group 1996; Araujo and Ramos 2000; Strayer et al. 2004), which has had a serious decline across its Holarctic range (Araujo and Ramos 2000; Machordom et al. 2003; Strayer et al. 2004; Geist 2010). This has made it the focus of several national and international conservation programmes (Araujo and Ramos 2000; Lopes-Lima et al. 2017). The FPM has a complex life cycle with an obligate parasitic stage on salmonids (Meyers and Milleman 1977; Young and Williams 1984; Larsen 2005; Geist 2010; Taeubert et al. 2010; Taeubert and Geist 2017). Infective glochidia, released by gravid mothers, passively attach to a suitable fish host and become encysted on gills (Young and Williams 1984; Wächtler et al. 2001; Taeubert et al. 2010; Taeubert et al. 2013) as parasites that depend on nutrient

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transfer from the host (Denic et al. 2015). In addition, they also reduce host swimming performance (Taeubert and Geist 2013). After 9–11 months (Larsen 2005), juvenile mussels excyst (May to June) and spend the next 5 years buried in the river sediment, after which they rise up to the substratum surface and develop into adults (Young and Williams 1984).

The FPM is a specialist parasite, which successfully metamorphoses only on the gills of Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta f. trutta*), and brown trout (*S. trutta f. fario*) in its European distribution (Young and Williams 1984; Larsen 2005; Geist et al. 2006; Taeubert et al. 2010; Salonen et al. 2016; Taeubert and Geist 2017). Furthermore, some FPM populations were found to sometimes exclusively infest either *S. salar* or *S. trutta* even though both species are present in the river (Hastie and Young 2001; Karlsson et al. 2014; Österling and Wengström 2015; Ieshko et al. 2016; Salonen et al. 2017). It is assumed that *M.margaritifera* populations are best adapted to (historically) sympatric hosts as suggested from infestation experiments (Taeubert et al. 2010; Salonen et al. 2017) as well as from similar genetic differentiation patterns among FPM and their hosts (Geist and Kuehn 2008); however, contradictory evidence has also been reported regionally (Österling and Larson 2013). Local adaptation of FPM has not yet been clearly demonstrated.

The parasitic glochidia are not selective in their attachment, and they passively attach to all objects (even wood, plastic, or paper) (Kat 1984; Dodd et al. 2005). Once attached to the gills of a suitable host, they induce an immune response and become encysted by gill epithelial cells (Nezlin et al. 1994). Glochidia that are unable to induce an immune response from their host will be shed off (Nezlin et al. 1994). On an unsuitable host, the cyst formed will be abnormal, causing the glochidia to die or be shed (Fustish and Millemann 1978; Kat 1984; Rogers-Lowery and Dimock Jr 2006). Encystment is essential for the metamorphosis of glochidia into juvenile mussels (Haag 2012). It has been demonstrated that the cyst provides nutrition and mechanical protection to the glochidium (Arey 1932a, 1932b; Ziuganov et al. 1994; Wächtler et al. 2001; Denic et al. 2015). The host immune response is clearly essential for the glochidial metamorphosis into free living-juveniles (Taeubert et al. 2010; Haag 2012; Taeubert and Geist 2017). In addition, the duration of the parasitic phase also influences size and post-parasitic fitness of juvenile mussels (Marwaha et al. 2017). Juvenile mussels, which had the longest parasitic phase, had a size, growth rate, and survival advantage compared with those with a short parasitic phase (Marwaha et al. 2017). Parasite-host compatibility is an important factor, influencing glochidial load (glochidia per fish), growth, and post-parasitic performance of juvenile mussels.

Host suitability studies, wherein the most suitable hosts are identified, are an important focus in several conservation programmes. FPM-host suitability studies have shown that the most suitable host strains result in higher glochidial growth and glochidial load (Taeubert et al. 2010; Österling and Larson 2013). Moreover, large individual host differences were observed with respect to glochidial growth and load among the suitable strains (Taeubert et al. 2010). The reason for individual differences among suitable hosts is not clearly understood. Bauer and Vogel (1987) observed that glochidial development was related to their mortality on the host: glochidia developed faster on hosts with low glochidial loss. They proposed that these individual differences could be related to host immune response. In addition, individual host suitability could also be related to genetic composition of the host, host age, host condition, host size (length or weight), environmental conditions, or a combination of several factors (Bauer and Vogel 1987; Taeubert 2014). Previous studies that have examined the relationship between host size (measured as either host weight or length) and age with FPM glochidial loads yielded several contradictory results.

Studies that have investigated the relationship between host size and glochidial loads have found positive correlations between host size and glochidial load (Young and Williams 1984; Bauer and Vogel 1987; Hastie and Young 2001; Thomas 2011), negative correlations (Bauer 1987; Hastie and Young 2001), and no correlations at all (Cunjak and McGladdery 1991; Beasley 1996; Treasurer and Turnbull 2000; Hastie and Young 2001; Treasurer et al. 2006). The positive relationship between host size and glochidial load is believed to be transitory, becoming insignificant over time (Young and Williams 1984; Bauer and Vogel 1987; Hastie and Young 2001; Thomas 2011). Larger fish initially have higher glochidial loads compared with smaller ones, probably as a result of larger gill surface area and higher ventilation rates (Young and Williams 1984; Bauer and Vogel 1987; Hastie and Young 2001; Thomas 2011). However, a significant number of glochidia are lost in the first few months post infestation (Bauer and Vogel 1987; Hastie and Young 2001), and the positive correlation between host size and load becomes insignificant after the first week post-infestation (Bauer and Vogel 1987). The decrease in glochidial loads in the following weeks is believed to be a result of the host mounting an immune response, and no correlation between host size and initial glochidial loads is observed thereafter (Meyers et al. 1980; Bauer and Vogel 1987; O'Connell and Neves 1999; Hastie and Young 2001). Most of these studies did not differentiate between the different host age classes, and the observed results were mostly based on the relationship between host size and glochidial loads. Different host age classes can provide variable resources as well as differing immune responses to parasites (Izhar and Ben-Ami 2015).

Host age has generally been observed to be negatively related to glochidial load, and a decrease in glochidial loads with increasing host age has been observed in both wild (naturally infested) and hatchery-reared (artificially infested) hosts (Bauer 1987; Hastie and Young 2001). Typically in the FPM rivers, young wild salmonids are found to have the highest

glochidial infestations (Awakura 1968; Karna and Millemann 1978; Bauer 1979, 1987; Young and Williams 1984; Bauer and Vogel 1987), although older and larger host fish seem to be important in some Northern European populations (Geist et al. 2006). Bauer (1987) observed a host age dependent relationship with glochidial mortalities; mortalities were higher in experimentally infested 1+ hosts compared with 0+ hosts, but this relationship was inversely density dependent in 1+ hosts. Age-related differences, especially in wild hosts, were believed to be a result of (a) reduced exposure of older hosts to glochidia due to behavioural differences (Hastie and Young 2001) and (b) acquired immunity in older hosts as a result of previous glochidial infestations (Karna and Millemann 1978; Meyers et al. 1980; Bauer 1987; Bauer and Vogel 1987; Bauer et al. 1991; Ziuganov et al. 1994). In experimentally infested naive fish, as well as in wild hosts, age-related differences could also be due to an age-related immune response (Bauer 1987; Ziuganov et al. 1994; Hastie and Young 2001).

Although the relationship between host size, age, and glochidial load has been investigated in several studies, it is difficult to disentangle the exact nature of the relationship between host size and age with glochidial loads. Moreover, some of the studies have used naturally infested wild fish, which could lead to biased results due to the effects of acquired immunity from previous infestations (Bauer and Vogel 1987; O'Connell and Neves 1999; Rogers-Lowery et al. 2007; Thomas 2011; Chowdhury et al. 2017). In addition, it is likely that most of these previous studies have used glochidia with differing genotypes. Normally, in any host-parasite interaction, parasite success will depend on both the parasite and host genotypes, and their interaction (Carius et al. 2001; Schmid-Hempel 2011; Lambrechts et al. 2005; Barribeau et al. 2014). The presence of two or more parasitic genotypes could lead to competition for resources and hence higher levels of virulence on different host genotypes (Taylor et al. 2005; Lagrue et al. 2011; Råberg 2014). These conditions would result in some glochidial genotypes being more successful compared with others on a single host, giving confounding results. Therefore, we believe that infesting naive fish hosts of two different age classes using glochidia with very similar genotypes will minimise the confounding effects of genotype-specific interactions. This will allow us to observe a host age-dependent response to glochidial infestation under common garden conditions.

The main objective of our study was to evaluate the difference in host response to glochidial infestation among 0+ and 1+ naive fish hosts. We hypothesised that the host response to parasite infestation is dependent on the host's age, and the 1+ group will tolerate infestation better. In order to test our hypothesis, we used glochidia from closely related mothers to infest hatchery raised naive 0+ and 1+ fish. This allowed us to analyse the host response to infestation among the two age groups. We evaluated the relationship between glochidial load and host size (measured as weight, length, and Fulton's condition factor) in our two host age groups, in order to identify a host age-related difference in host response. In addition, we also recorded haematocrit (Hct) values in our 1+ hosts. Hct values (% red blood cells in blood volume) are positively related to glochidial infestations and are often used as a measure of respiratory stress caused by glochidial infestations (Meyers et al. 1980; Thomas et al. 2014; Filipsson et al. 2017).

Materials and methods

All experiments were carried out at the FPM rearing facility at Austevoll, Norway. The main water source for the rearing station comes from Lake Kvernavatnet, an oligothrophic lake with a size of 0.125 km^2 and a mean depth of 17.5 m. This water was used for maintaining the fish and adult mussels. It has a pH of 6.6, alkalinity of 0.108 mmol/l, and the concentration of aluminium, iron, calcium, magnesium, and nitrate as follows: Al—180 μg/l; Fe—200 μg/l, Ca—4.2 mg/l, Mg—1.8 mg/l, Na—12 mg/l, and Nitrat-N—0.15 mg/l. The water was UV-treated and filtered through 30-μm mesh before use. The water temperature followed the natural fluctuation of the lake and was between 5 and 17 °C. Glochidial release and infestation of hosts occurred at an average water temperature of 16.2 °C.

Glochidial collection and DNA extraction

Adult mussels ($n = 50$) from the river Raudsjøbekken (Akershus County, Norway) were transferred to the FPM breeding station in June 2014. In a pre-screening, this mussel population was identified as one with very little genetic variation among individuals as revealed by analyses of nine microsatellites (data not shown). The mussels were kept in artificial rivers, with flowing water and fed with a diet of Shellfish® 1800 (Reed Mariculture Inc., Campbell, CA, USA) and Nanno 3600 (Reed Mariculture Inc.). Once the mussels started spatting in August, glochidial strings were collected and checked for maturation and viability (< 90%) using methods described by Watters and O'Dee (1999), before infesting the fish. Furthermore, 24 glochidia from each of the six randomly selected gravid mothers were analysed to confirm that they were genetically closely related.

A phenol-chloroform extraction was performed as described by Geist et al. (2008). Single and multiple glochidial samples were transferred into 1.5-ml Eppendorf tubes and manually ground. For cell lysis, we added 500-μl lysis buffer (20-mM Tris pH 8.0, 5-mM EDTA pH 8.0, 400-mM NaCl, 1% SDS) and 25-μl protinase K (10 mg/ml) to our samples and incubated them at 55 °C for 12 h. In order to separate the nucleic acids from the proteins and lipids, we added 600 μl (Roth) phenol/chloroform/isoamylalcohol (25:24:1) to our

samples and centrifuged it. In order to precipitate the DNA, 500 μl of isopropanol was added to samples, and they were centrifuged for 15 min. The DNA pellet was washed with 900 μl of 70% ethanol. Once the DNA pellet was dry, it was dissolved in 50 μl of 5-mM Tris pH 8.5 and incubated at 55 °C. Samples were then stored at − 20 °C for subsequent analyses.

We used nine microsatellite loci (MarMa2671, MarMa3050, MarMa3621, MarMa4143, MarMa4322, MarMa4726, MarMa5023, MarMa5167, MarMa5280) previously published by Geist et al. (2003) and Geist and Kuehn (2005). Analysis was carried out according to Geist and Kuehn (2005). Polymerase chain reactions were carried out in a final volume of 12.5 μl with the following components: 25–50 ng of genomic DNA, 200 nM of each primer, 0.2 mM of dNTP mix, 3-mM $MgCl_2$ (2-mM $MgCl_2$ for locus MarMa 5280), 1 × PCR buffer, and 0.25-U Taq (Solis Biodyne, Tartu, Estonia). The PCR was carried out on a gradient thermal cycler (Eppendorf Mastercycler, Eppendorf, Germany) under the following cycling conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 52–55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 3 min. PCR products were separated on 5% denaturing 19:1 acrylamide/bisacrylamide gels on an ALFexpressII DNA analyser and scored with ALLELELINKS 1.02 software (Amersham Pharmacia Biotech, Amersham, UK). Electrophoresis was carried out with two internal standards (70 and 300 bp) in each lane. Additionally, an external standard (50–500-bp ladder) and a previously genotyped reference sample were included on each gel to standardise allele scoring and to facilitate cross-referencing among gels.

Fish infestations

We used naive hatchery reared brown trout (Botsvannsørret, *S. trutta*) obtained from the Statkraft facility in Eidfjord, Norway. Juvenile trout (0+) were transferred to the rearing station in July 2015 and kept in aerated 90-L tanks and fed until satiated. The experiment was completed in two parts and ran over a period of 2 years (2015–2017). For the first part of the experiment, we used 400 of the naive 0+ hosts (weight 2.3 ± 0.78 g, standard length 7 ± 0.66 cm). The remaining 500 naive 0+ hosts from the same batch (fish weight 9.8 ± 3.5-g, standard length 10 ± 1.17 cm) were allowed to grow for 1 year before being used in the second part of the experiment. For each part of our experiment, our test fish were all kept in a single tank and had no contact with glochidia pre and post infestation. To infest the fish, water levels in the fish tank was lowered, and the fish were exposed to glochidia (500/L) for a period of 40 min with aeration. Glochidial strings collected from 16 mothers were used in the infestation baths. Fish samples ($n = 30$) were taken out 48 h post-infestation to ensure that the fish were infested. Post-infestation, all infested fish were kept under equal food and ambient temperature conditions, and fish mortalities were monitored. The temperature variation during the duration of our experiment did not vary significantly (Wilcoxon rank sum test: $W = 69.5$, p value = 0.9076).

For the first part of the experiment, we performed three controls over the infestation period, 60, 200, and 300 dpi. At each control, 30 fish (at 60 and 200 dpi) and 70 fish (at 300 dpi) were randomly sampled and sacrificed. Fish were euthanised with an overdose of benzocaine (Benzoak Vet, ACD Pharmaceuticals) (exposure period of 10 min). Fish length and weight measurements were recorded to the nearest 0.5 cm and 0.1 g. We also recorded their infestation status (infested or uninfested), and when infested, we counted the total number of glochidia (glochidial load) on one side of the fish. This was chosen randomly with a dice toss, with even and odd numbers deciding if all the left or right gill arches were used. In circumstances where no glochidia were found on one side of the fish, the other side was checked to confirm the infestation status of the fish. Glochidial load was estimated using the methods described by Dodd et al. (2005). Host gills were flushed thoroughly, and the numbers of mussels on all gill arches were counted. Juvenile mussel mean size was also recorded by measuring the length of the widest part of the mussels to the nearest 0.1 μm. For the second part of the experiment (1+ hosts), we only recorded fish length and weight measurements and glochidial load at 300 dpi. The Fulton's condition factor was calculated using the formula $CF = 10^5 * W / L^3$, where W is the weight in grams and L is the total length in centimetres (Morton and Routledge 2006; Davidson et al. 2009).

In addition, we measured the haematocrit values of the 1+ fish only, because of the difficulty involved in collecting adequate blood samples from the 0+ hosts. Haematocrit (Hct) values (% red blood cells in blood volume) can be used as a measure of the oxygen carrying capacity of blood in fish (Gallaugher and Farrell 1998). Blood samples (1 ml) were taken from the caudal vein using Venojec vacutainer 3-ml syringes coated with Li-heparin and Venojec multisample 20G fitted with 0.9 × 40-m needles. Blood samples were centrifuged in 100-μL microcapillary tubes at 13,000 rpm for 10 min in a Hettich haematocrit centrifuge, and Hct was calculated as the percentage of red blood cells of centrifuged samples.

Statistical analysis

We used the statistical package R, version 3.4.3 (R Core team 2017) for our analysis. We compared the difference in host Fulton's condition factor, weight and standard length (will be referred to as host traits), glochidial load, and juvenile mussel mean size between and within our host age groups. We also compared the difference in glochidial load between the two host age groups by standardising glochidial load by host weight (number of glochidia/gram fish weight). To do this,

we used either a Kruskal-Wallis test or ANOVA, depending on whether the data fulfilled conditions of normality. For Hct values, we subdivided the 1+ infested fish in three groups; high (200+ glochidia on one side), medium (1–199 glochidia on one side), and uninfested and then compared the Hct values among these groups. Correlation tests (Spearman's or Kendall Tau) were used to check correlations between all our test variables. We used a generalised linear mixed effect model (GLMM) with penalised quasi-likelihood approach and Gaussian as the family to examine the relationship between glochidial load and host traits. We used the glmmPQL function from the MASS library in R, with glochidial load as the predictor variable, host traits as the response variable, and individual hosts as random factor to bring in the heterogeneity among hosts. The same result was obtained if heterogeneity among hosts was not considered. We used a linear regression model to test the relationship between the glochidial load and juvenile mussel mean size and used mean size as the response variable and glochidial load, condition factor, and the interaction between them as covariates. We performed the above analysis using the R library leaps. Since only glochidial load was found to be significant, we performed a linear regression with mean size versus glochidial load. For Hct values, we used a generalised linear model with Gaussian as the family to examine the effect of glochidial load.

In order to verify whether the six adult pearl mussel mothers were closely related, glochidia of two randomly selected mothers were pooled, since the computational approach of pairwise analysis of genetic divergence requires more than four individuals per group. This grouping (three groups with eight glochidia each) was used for all subsequent population genetic computations. For each group, allele frequencies, average allele numbers per locus (A), and expected and observed heterozygosities (H_E, H_O) were calculated with GENEPOP 4.0 (Rousset 2008). The same software was used to test the loci for genotypic disequilibrium to test for significant population differentiation among all pairs of populations using 100,000 iterations and 1000 dememorisation steps (Raymond and Rousset 1995) and to test each locus in each population for conformance with Hardy–Weinberg (HW) expectations. Group pairwise analysis of genetic divergence (Jost's Dest), which measures the fraction of allelic variation among populations, was calculated with the software GENALEX (Peakall and Smouse 2006). Each microsatellite locus was assessed for the presence of null alleles and genotyping errors using MICROCHECKER v.2.2.3 (van Oosterhout et al. 2004).

Results

Glochidial load was significantly higher in the 1+ fish hosts (mean 212.79 glochidia/fish) compared with 0+ (4.47 glochidia/fish) (Kruskal test: chi-squared = 75.458, df = 1, p value ≤ 2.2e−16). This remained significant even when glochidial load was standardised by host weight (number of glochidia/gram fish weight) (Kruskal test: chi-squared = 15.899, df = 1, p value = 6.68e−05; Fig. 1). Mean juvenile mussel sizes did not vary between the two host age groups (ANOVA: Std. error = 0.0085, t value = 0.963, p value = 0.338).

0+ hosts

We did not observe any differences in Fulton's condition factor (Kruskal test: chi-squared = 0.0291, df = 1, p value = 0.865), host weight (Kruskal test: chi-squared = 0.7376, df = 1, p value = 0.514), or standard length (ANOVA: Std. error = 2.4949, t value = 1.201, p value = 0.232) between the infested and uninfested hosts at 300 dpi. However, a significant negative correlation was observed between glochidial load and host weight (Kendall tau: $\tau = -0.288$, p value = 0.0006), standard length (Kendall tau: $\tau = -0.256$, p value = 0.003), and a moderately significant one with Fulton's condition factor (Spearman's: $\rho = -0.2051$, p value = 0.0817) among the infested 0+ hosts. The GLMM model also showed a significant negative relation between glochidial load and Fulton's condition factor (glmmPQL: Estimate = −0.006, Std. error = 0.0032, t value = −1.891, p value = 0.0627; Fig. 2), host weight (glmmPQL: estimate = −0.281, Std. error = 0.094, t value = −2.995, p value = 0.0038), and host standard length (glmmPQL: estimate = −0.0806, Std. error = 0.033, t value = −2.44, p value = 0.0172. We did not observe any significant correlations between host traits and glochidial loads at 60 dpi (Kendall tau: Fulton's condition factors: $\tau = 0.0186$, p value = 0.887; weight: $\tau = -0.0238$, p value = 0.857, standard length: $\tau = -0.127$, p value = 0.334) and at 200 dpi (Spearman's: Fulton's condition factors: $\rho = -0.1337$, p value = 0.481; weight: $\rho = -0.01$, p value = 0.956; standard length: $\rho = 0.0599$, p value = 0.7531).

1+ hosts

There was a significant difference in Fulton's condition factor between the infested and uninfested hosts (ANOVA: Std. error = 0.018, t value = −4.038, p value = 0.0001, Fig. 3a). This became even more significant when comparing the highly infested and the uninfested groups (ANOVA: Std. error = 0.0189, t value = −6.039, p value = 2.73e−07, Fig. 3b). We observed a significant positive correlation between glochidial load and Fulton's condition factor (Spearman's: $\rho = 0.3054$ p value = 0.0368), and the generalised linear model also showed a significant positive relationship between these variables (glmmPQL: estimate = 1.251e−04, Std. error = 4.864e−05, t value = 2.572, p value = 0.0135, Fig. 4). In addition, a significant positive correlation between host weight (Spearman's: $\rho = 0.3968$, p value = 0.06) and standard length (Spearman's: $\rho = 0.4052$, p value = 0.055) was observed in the high infestation group. Juvenile mussels were larger on the high

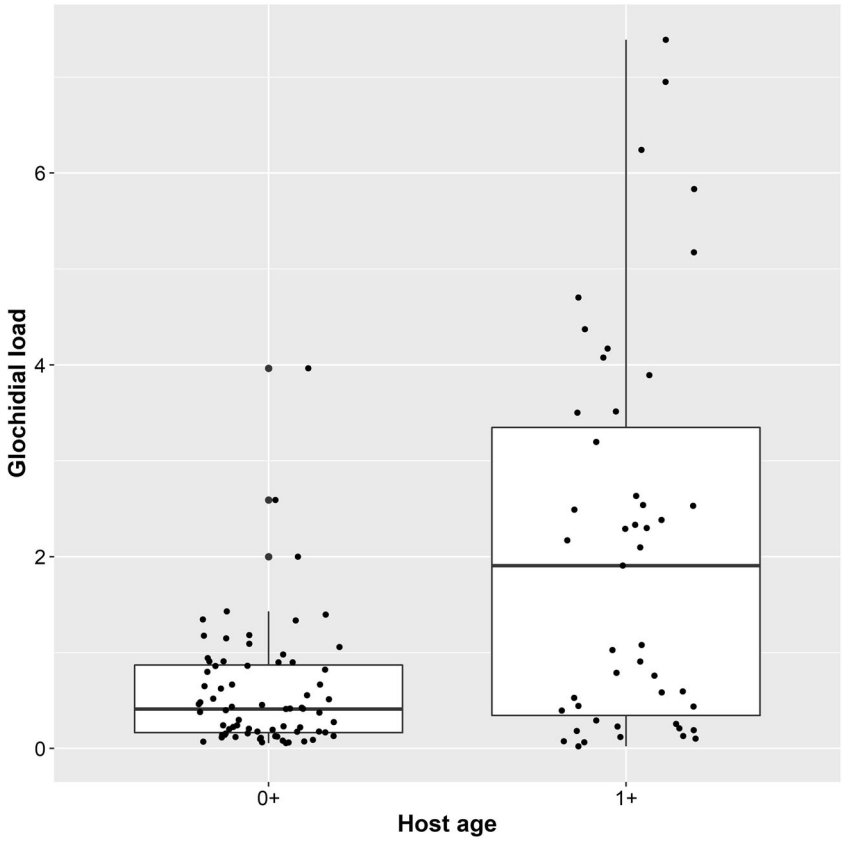

Fig. 1 A box plot showing the difference in glochidial abundance (normalised by fish weight) in the 0+ ($n = 72$) and 1+ ($n = 50$) fish hosts. The thick line displays the median, the boxes display the 25 and 75% quartiles, and the whiskers show the range of the dataset. The dots show individual data points

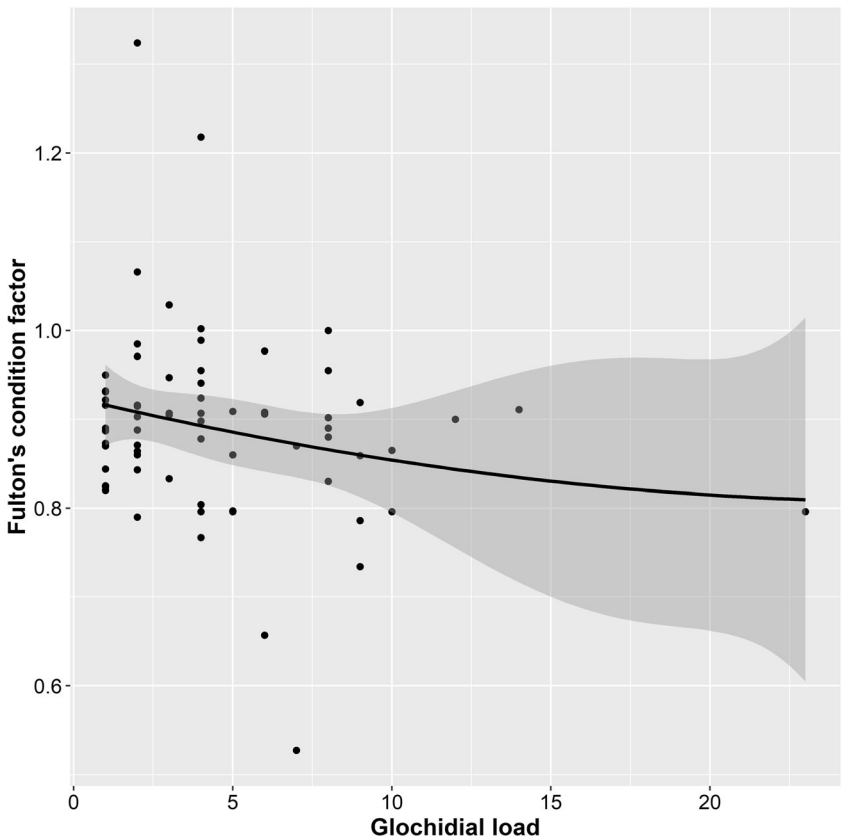

Fig. 2 Relationship between glochidial load and Fulton's condition factor in 0+ fish hosts. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey

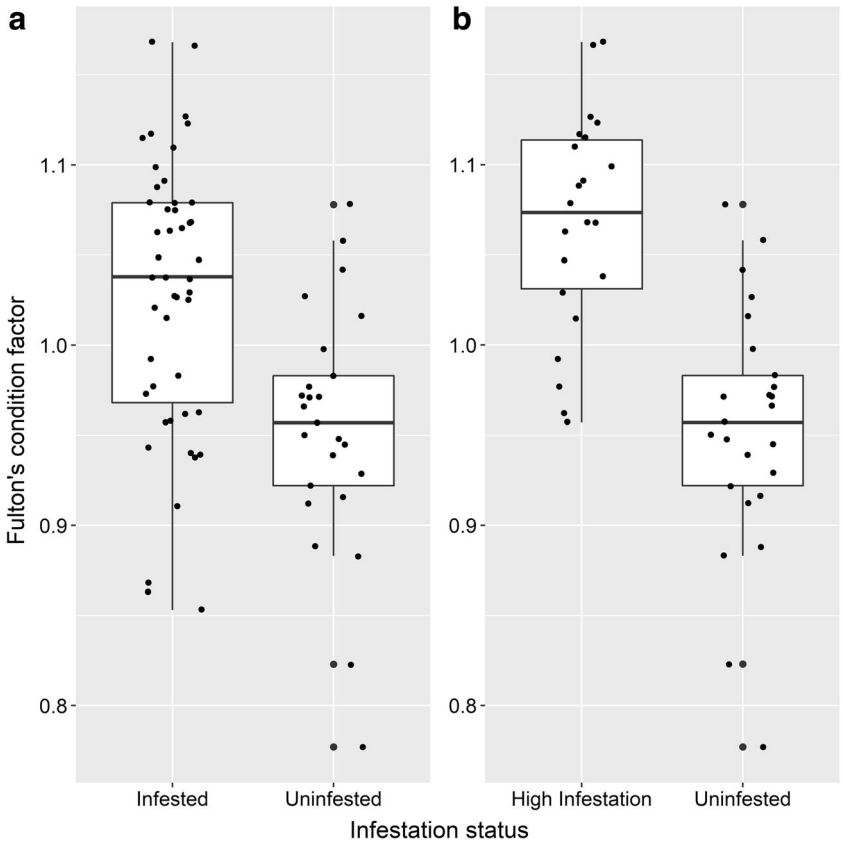

Fig. 3 Difference between the Fulton's condition factor between the **a** infested and uninfested 1+ hosts and **b** high infestation group (200+) and uninfested 1+ groups. The thick line represents the median, the boxes display the 25 and 75% quartiles, and the whiskers represent the range of the dataset. The dots represent individual data points

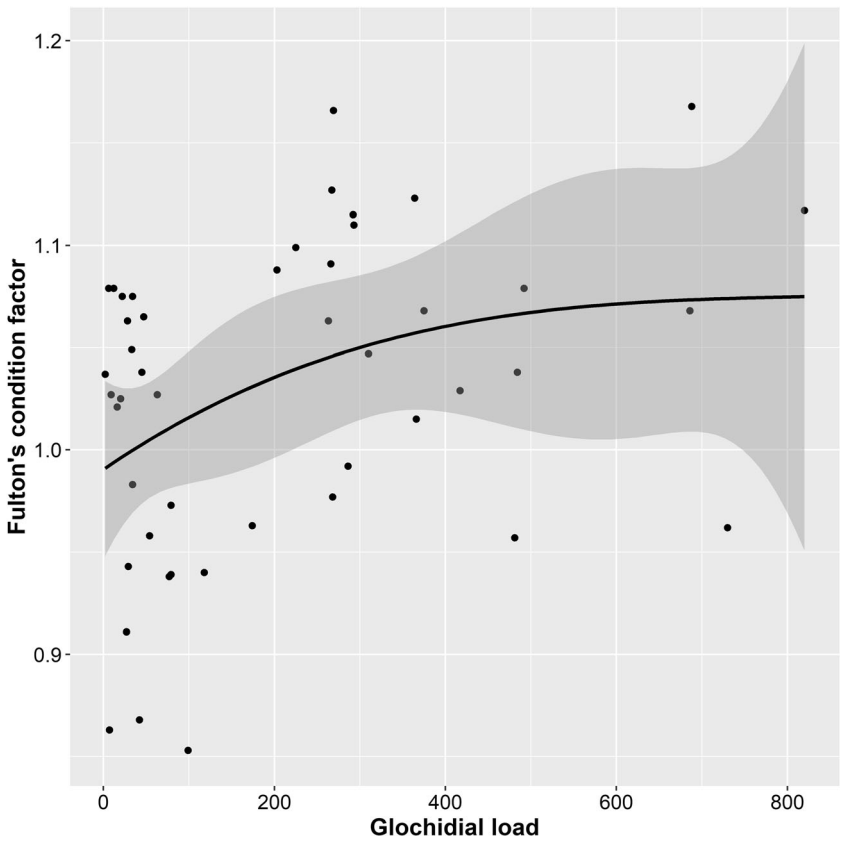

Fig. 4 Relationship between the glochidial load and Fulton's condition factor in 1+ hosts. The thick black line represents the cubic smoothing spline, and the 95% confidence intervals are in grey

infestation group compared with the medium group (ANOVA: estimate = − 0.0167, Std. error = 0.0085, t value = − 1.963, p value = 0.0559, Fig. 5a). A significant positive correlation was also observed between mean juvenile mussel size and glochidial load (Kendall tau: $\tau = 0.2893$, p value = 0.0043; LM: Std. error = 1.932e−05, t value = 2.408, p value = 0.0202, Fig. 5b).

Hct values did not differ between infested and uninfested fish groups (Kruskal-Wallis: chi-squared = 1.423, p value = 0.2329). However, Hct values of the high infestation group were significantly higher than the medium infested and uninfested groups (Medium: Kruskal-Wallis: chi-squared = 4.6055, df = 1, p value = 0.0318; uninfested: Kruskal-Wallis: chi-squared = 5.2263, df = 1, p value = 0.0223, Fig. 6a). The rank correlation test showed a significant positive correlation between glochidial load and Hct values (Spearman's: $\rho = 0.3312$, p value = 0.0299); however, the GLM model showed only a moderately significant relationship between these variables (GLM: estimate = 0.134e−05, Std. error = 5.291e−05, t value = 1.726, p value = 0.0912, Fig. 6b).

The test for the presence of null alleles and genotyping errors using MICROCHECKER v.2.2.3 (van Oosterhout et al. 2004) revealed null allele frequencies below 0.2. Since this level has been shown to have very little impact on population delineation and divergence estimates (Dakin and Avise 2004; Carlsson 2008), all loci were included. The test for genotypic disequilibrium for each pair of the nine polymorphic microsatellite loci over all populations gave no significant value, and no significant deviations from the expected HW proportions were observed after applying sequential Bonferroni correction.

Microsatellite diversity varied among loci, with one allele at MarMa2671, MarMa4322, MarMa5023, and five alleles at locus MarMa4143. Allelic richness (adjusted for sample size) ranged from 1.5 alleles per locus to 1.7 alleles per locus. Levels of observed and expected heterozygosity varied hardly between groups ranging from 0.100 to 0.167 and from 0.162 to 0.188, respectively. Glochidia of the three groups were closely related reflected by Josts's distance (Dest (mean) = 0.040; SD = 0.033).

Discussion

The results of our study show that host response to glochidial infestation was dependent on the host age under equal food and temperature conditions and under the condition that none of the hosts had experienced previous contact with glochidia. Host condition of the infested 0+ fish hosts had a negative correlation with glochidial load, whereas a strong positive one in the 1+ hosts was evident. In addition, the Hct values were significantly higher only in the heavily infested 1+ hosts. With minimal variation in the infecting glochidial genotypes, our results show a clear host age dependent response to glochidial loads.

Age-related differences in host susceptibility and immune response to glochidial infestation have been observed in some FPM glochidia-host studies (Bauer 1987; Bauer and Vogel 1987; Hastie and Young 2001). The reasons suggested for these differences were dissimilarities in gill morphology and the chemical composition of host gill mucus and blood (Young et al. 1987; Hastie and Young 2001). However, Karna and Millemann (1978) did not find any evidence of this. Nevertheless, evidence from several studies shows that FPM glochidia-host interaction, i.e. successful glochidial encystment, survival, and metamorphosis into juveniles, is highly dependent on the parasite-host compatibility, which in turn depends on host immune response (Nezlin et al. 1994; Haag 2012). In the first few weeks post-infestation, the host generally loses a significant number of glochidia. This loss occurs due to a tissue response in the first 7 days post-infestation and in the following weeks thereafter due to a humoral response

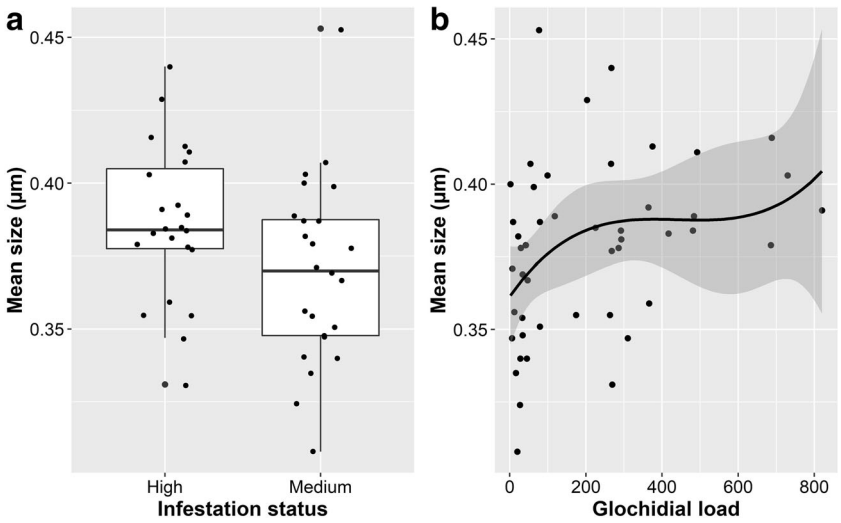

Fig. 5 **a** Differences in juvenile mussel mean size (µm) between the 1+ high infestation and medium infestation host groups. The thick black line represents the median, the boxes display the 25 and 75% quartiles, and the whiskers display the data range. The dots represent individual data points. **b** Relationship between glochidial load and juvenile mussel mean size in 1+ hosts. The thick black line represents the cubic smoothing spline, and the 95% confidence intervals are in grey

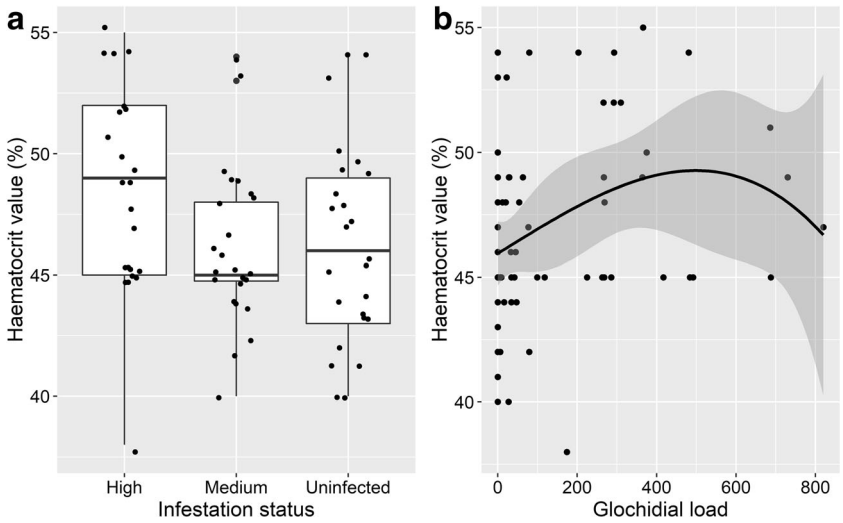

Fig. 6 **a** Differences between the Hct values between the high, medium, and uninfested 1+ hosts. The thick line represents the median, the boxes display the 25 and 75% quartiles, and the whiskers display the data range. The dots represent individual data points. **b** Relationship between glochidial load and Hct values. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey

(Bauer 1987; Bauer and Vogel 1987). As previously mentioned, Bauer (1987) observed an age-dependent difference in glochidial mortalities in *S. trutta* hosts and proposed that these were a result of age related differences in host immune response to glochidial infestation. He suggested that 0+ hosts had a weaker immune response to infestation, but this was density dependent, i.e. it increased with increasing glochidial density. The 1+ hosts displayed a stronger immune response which was inversely density dependent, i.e. the immune response was especially strong when the glochidial loads were low. In our experiment, we used host condition as a measure of host response to glochidial infestation, and we observed a positive relationship between glochidial load and host condition in the 1+ hosts and a negative one in the 0+ hosts. The 1+ hosts with the highest glochidial loads also had the best condition when compared with the uninfested and medium infested hosts. In accordance with Bauer's (1987) proposal, our results also indicate that the 1+ hosts mobilised a weaker immune response when glochidial loads were high. This probably resulted in enhanced growth in the heavily infested hosts. To the best of our knowledge, this is the first evidence of a positive effect of mussel glochidia on a fish host whose survival probability increases with size. The 0+ hosts with the highest glochidial loads had the lowest condition, suggesting a strong immune response which was density-dependent.

Bauer (1987) and Bauer and Vogel (1987) observed that glochidial development (size) was dependent on glochidial mortality on the hosts, i.e. glochidia were larger on fish with low glochidial mortalities and vice versa. The authors proposed that a weak host immune response would result in lower glochidial mortalities and would provide the developing glochidia with conditions conducive for glochidial development and growth. This would result in larger glochidia. Moreover, glochidial growth has also been reported to be positively related to host condition; i.e. hosts with a good condition provide higher energy resources for the developing larvae (Österling and Larsen 2013). In our study, we observed a significant positive relationship between the mean size of juvenile mussels and glochidial load in the 1+ hosts, i.e. heavily infested 1+ hosts with the best condition had larger juvenile mussels. However we did not observe any correlation between juvenile mussel size and glochidial load in the 0+ hosts. We propose that a weaker immune response in the heavily infested 1+ hosts provided the glochidia with conditions beneficial for their growth and development, resulting in larger juveniles on heavily infested 1+ hosts compared with medium infested ones. Our observations also support our proposal that the 1+ hosts mounted a weaker immune response compared with the 0+ hosts. In our experiment, we did not specifically examine the differences in immune response in our two age groups. Also, individual differences in growth of hosts could be a possible reason for observed differences in host condition at the end of the experiment. Nevertheless, based on evidence from previous studies (Bauer 1987; Bauer and Vogel 1987), in addition to the importance of host immune response in the FPM glochidia-host interaction, we believe that the difference in the relationship between glochidial load and condition factor we observed between the two host age groups is related to a difference in the immune strategy employed by them.

Two host defence strategies have been described in the literature: (i) resistance, which is the ability to prevent or reduce a given parasite and (ii) tolerance, which is the ability to limit the damage caused by a given parasite (Råberg et al. 2009; Best et al. 2014; Jackson et al. 2014; Råberg 2014; Klemme and Karvonen 2016; Kutzer and Armitage 2016; Adelman and Hawley 2017). An important prediction of the life-history of an organism is that optimal energy allocation is towards important traits, such as growth, maintenance, and survival (Sandland and Minchella 2003; Simkova et al. 2008). Under natural circumstances, hosts would typically have a limited access to resources, and resource allocation towards an optimally functioning immune system and/or an

effective immune response would be costly for the host (Sheldon and Verhulst 1996; Norris and Evans 2000; Martin II et al. 2003). Moreover, resource allocation towards an effective immune response can be influenced by the age, sex and life history stage of the host, and also by environmental or ecological factors that can have an effect on the physical condition of the host (Wilson et al. 2002; Sandland and Minchella 2003; Hämäläinen et al. 2015; Klemme and Karvonen 2016). Thus, the immune defence strategy employed by hosts can vary with age and younger hosts are generally expected to invest more in a stronger immune response (Poulin 1993; Thomas et al. 2000; Jackson et al. 2014). When a host is faced with the risk of parasitism, there could either be a higher investment in immune defence at the expense of other traits such as growth or reproduction, or a trade-off between resource allocation towards growth and an expensive immune response (Gustafsson et al. 1994; Nordling et al. 1998; Siva-Jothy et al. 1998; Veiga et al. 1998; Moreno et al. 1999; Ilmonen et al. 2000; Bonneaud et al. 2003; Soler et al. 2003; Brommer 2004; Jacot et al. 2004; Ahtiainen et al. 2005; Tschirren and Richner 2006; Lefèvre et al. 2008; Simkova et al. 2008). Sometimes, a trade-off between an expensive immune response and growth could be advantageous to the host, since an effective immune response can also lead to damage to host tissue (Klemme and Karvonen 2016). In most natural circumstances, host defence would be a combination of the two defence strategies (Jackson et al. 2014).

Host tolerance to parasitic infestation has been measured as the relationship between host condition and parasitic load (Jackson et al. 2014). There are several examples in the literature where this relationship was positive for parasite infested individuals. For example, an increase in growth and/or improved body condition has been observed in fish hosts infected by plerocercoids of *Schistocephalus solidus* (Milinski 1985; Arnott et al. 2000), *Ligula intestinalis* (Museth 2001; Loot et al. 2002), and *Posthodiplostomum cuticola* (Ondracková et al. 2004). The reason for an increase in host weight or an improved condition could be related to a change in fish foraging behaviour, food conversion efficiency and reduced activity, or a combination of these factors (Arnott et al. 2000). Fish infested with glochidial parasites have been reported to have reduced activity, and they also become less bold (Thomas 2011; Horky et al. 2014). This is believed to be a result of the physiological impact of glochidia on host gills leading to respiratory stress and thus reduced movement (Thomas 2011; Horky et al. 2014). We believe that reduced movement, which will conserve energy, in addition to ad libitum feeding will result in improved host condition. Moreover, the higher host condition observed in heavily infested 1+ hosts, compared with the medium and uninfested groups, despite all hosts being fed ad libitum, clearly indicates that heavily infested hosts invested more resources in growth due to high glochidial infestation.

In contrast to the 1+ hosts, we did not observe any difference in the host condition between infested and uninfested 0+ hosts. In addition, we observed a negative relationship between host traits and glochidial loads. We believe that the small size of the 0+ hosts and in turn less resources led to a resistance strategy. It is believed that younger hosts ideally invest more in fighting parasites to ensure future reproductive success, compared with older hosts (Poulin 1993). Host resistance or tolerance to infestation is believed to be influenced by host age and/or sex, genetic components of the immune system and environmental factors (Råberg 2014; Kutzer and Armitage 2016). Jackson et al. (2014) investigated the age-dependent physiological mechanisms influencing host tolerance to parasite infestations in male voles. They measured the expression of immunity genes (Gata3) in different age classes to observe if this explained variation in tolerance. Mature voles were observed to be less resistant to parasites compared with immature ones and a positive relationship was also observed between host age and parasite numbers. The age-dependent difference in tolerance was mirrored by an increase in the expression of Gata3, i.e. it increased with parasite load in adult voles and vice versa. The underlying genetic or physiological mechanisms that influence host age dependent tolerance or resistance are not yet clearly understood and further studies are required.

Haematocrit values, which represent respiratory stress as a result of glochidial infestation in host fish, were significantly higher in the 1+ hosts which were infested with 200+ glochidia (on one side) compared to those with moderate intensities (1–199, on one side) and uninfested hosts. We also observed a positive correlation between Hct values and glochidial loads. Although we were unable to measure Hct values in the 0+ hosts, nevertheless, our observations give a clear indication that glochidial loads exceeding 200 glochidia per fish (on one side) resulted in respiratory stress and hence a compensatory increase in Hct values. High glochidial loads are typically associated with reduced critical swimming speed in trout, which affects the oxygen requirements for a specific activity or reduces the oxygen uptake due to damaged gills (Taeubert and Geist 2013; Filipsson et al. 2017). Moreover, Filipsson et al. (2017) observed that glochidiosis affects host metabolic rates and oxygen carrying capacity, and the resulting compensatory increase in Hct levels was believed to enhance oxygen transport capacity of the host. The increase in Hct levels was explained by the increase in the mean corpuscular volume and decrease in the mean corpuscular haemoglobin concentration (Meyers et al. 1980; Thomas et al. 2014; Filipsson et al. 2017). Although low glochidial loads are not believed to have a harmful effect on salmonid performance (Treasurer et al. 2006; Taeubert and Geist 2013), Thomas et al. (2014) observed that fish with glochidial intensities of just 1–204 glochidia per fish took a longer time to reach the basal ventilation rate after a stressor. Glochidial

intensities in our experiment ranged between 200 and 820 glochidia (on one side) in our heavily infested hosts, and the elevated Hct values clearly indicate a compensatory response as a result of high glochidial infestation.

The results from our study show clear differences in host age-dependent response to glochidial infestation. This can be explained by a change in host response strategy from sensitivity in young to tolerance in older fish. We propose that the fish host is an important filter for glochidial attachment and metamorphosis. The results from our experiment are important in the context of developing optimal strategies for conserving endangered FPM populations and their host fish in the wild, as well as in captive breeding programmes. For instance, naive 1+ hosts were the most suitable hosts and should be preferentially used in captive breeding to minimise possible selection and drift effects, as well as to maximise the production of young mussels. Moreover, our observations also indicate that glochidial loads, which were within the recommended range on a host fish (5–100 per gram fish) (Taeubert and Geist 2013), resulted in respiratory stress, as indicated by the higher Hct values in heavily infested hosts. Since glochidial development and successful metamorphosis into juvenile mussels is highly dependent on good host condition and survival, conservation efforts should focus on methods that can guarantee this (Taeubert and Geist 2013; Filipsson et al. 2017). Artificial infestation programmes should ensure low infestation rates on hosts, as this can ensure the well-being and survival of infested fish that are released into streams, which in turn will promote successful release of juvenile mussels (Taeubert and Geist 2013, Filipsson et al. 2017). The pearl mussel salmonid parasite-host system is a unique system which involves the interaction between a very long-lived specialised parasite that can infest a host with a much shorter life span. This provides a particularly interesting system in which eco-evolutionary strategies can be identified.

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Compliance with ethical standards

Conflicts of interest The authors declare that there is no conflict of interest.

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