Master´s Thesis

Effect of Freshwater Pearl Mussel (*Margaritifera margaritifera*) infection on virulence of *Flavobacterium columnare* in Brown trout (*Salmo trutta*)

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Freshwater Pearl Mussel (FPM) *Margaritifera margaritifera* glochidia is an obligatory gill parasite to its salmonid host, brown trout (*Salmo trutta*). In general, it has been reported that parasiteic infections usually increase its host´s susceptibility to other secondary infections including bacterial infection. *Flavobacterium columnare* is a bacterium agent causing warm water disease (columnaris disease) in fish, which can result in a high mortality in fish populations at fish farms. Especially, in the northern Europe region, in the summer time with higher temperature this bacterium can be lethal to young salmonids. Therefore, in this study it was hypothesized that FPM glochidia will increase the mortality of its salmonid host during the co-infection with *F. columnare*. I exposed experimentally FPM-infected (glochidia attached and detached) and uninfected (control) brown trout (*Salmo trutta*) to a virulent *F. columnare* strain and measure survival time of fish. In an observation period of 29 hours, all the bacterium-exposed fish (n = 150) died while only one of the unexposed individuals (n =146) was dead. Among the bacterium-exposed fish, those were infected with FPM glochidia before the exposure survived statistically significantly longer—both shortly (two months) after the FPM infection (glochidia attached) and 14 months after the infection (glochidia detached). In addition, in the latter fish group, there was a statistically significant, positive correlation between FPM glochidia number and survival time of fish. The mechanism of lowered mortality among the FPM-infected fish, or whether the effect is FPM specific, is not known. However, these results suggest that the brown trout-*F. columnare* relationship can be modified by previous FPM infection, and possibly even decrease the virulence of *F. columnare* in brown trout.

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Amitav Roy: Jokihelmisimpukan (*Margaritifera margaritifera*) glokidium-infektion vaikutus taimenen (*Salmo trutta*) herkkyyteen kolumnaristaudille.

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Flavobacterium columnare on bakteeri, joka aiheuttaa kaloissa ns. lämpimän veden tautia (kolumnaris-tauti), mikä puolestaan aiheuttaa kuolleisuutta kalaisännissään. Erityisesti Pohjois-Euroopan alueella kesäaikana veden lämpötilan ollessa korkea tämä bakteeri voi olla hyvin haitallinen. Koska tautia vastaan ei ole toimivaa rokotetta, on antibioottien käyttö ainoa keino taudin haittojen vähentämiseksi kalanviljelylaitoksilla, mikä voi johtaa antibioottiresistenssin kehittymiseen. Kolumnaris-taudin kohteena olevat lohikalat ovat myös jokihelmisimpukan eli raakun isäntäkaloja, joten *F. columnare*n ja raakun glokidium-toukkien yhteisinfektio esimerkiksi taimenessa on mahdollinen. Yleensä aiempi loisinfektio lisää alttiutta uusille infektioille. Raakun glokidium-infektion on todettu haittaavan kalaisäntää esimerkiksi vaikeuttamalla hengitystä ja alentamalla kalan kasvua. Tutkin tässä työssä raakun glokidium-infektion vaikutusta taimenen alttiuteen kolumnaris.-taudille—tutkimushypoteesi ollessa, että raakkuinfektio lisää kalan alttiutta *F. columnare* -taudille. Raakun glokidium-toukilla laboratoriossa infektoidut (ja infektoimattomat kontrolliyksilöt) altistettiin virulentille *F. columnare* –kannalle ja seurattiin kalojen elossapysymistä. Kahdenkymmenenyhdeksän tunnin tarkkailujakson aikana kaikki bakteerille altistetut kalat (n = 150) kuolivat, kun altistamattomista vain yksi menehtyi (n = 146). Bakteeri-altistettujen kalojen joukossa glokidium-infektoitujen yksilöiden elinaika oli tilastollisesti merkitsevästi pidempi kun ei-infektoitujen kontrollikalojen elinaika. Bakteeri-altistuneista kaloista ne infektoitiin FPM-glokidialla ennen kuin altistus säilyi tilastollisesti merkittävästi pitempään - sekä pian (kaksi kuukautta) FPM-infektion (liitteenä glochidia) jälkeen että 14 kuukautta infektion jälkeen (glochidia irrotettu).Lisäksi jälkimmäisessä ryhmässä glokidium-toukkien lukumäärän ja elossapysymisajan välillä oli tilastollisesti merkitsevä, positiivinen korrelaatio. Mekanismia alentuneen kuolevuuden taustalla glokidium-infektoiduilla kaloilla ei tunneta, eikä sitä onko kyseessä pelkästää raakun toukille ominainen ilmiö. Tulos viittaa siihen, että jokihelmisimpukan glokidium-toukkainfektio muokkaa kalan alttiutta kolumnaris-taudille aikana ja jopa alentaa *F. columnare* -bakteerin haitallisuutta taimenessa.

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1 INTRODUCTION

Freshwater pearl mussel (*Margaritifera margaritifera*) (FPM), of order Unionida, is one of the most long-lived invertebrates with a top life span of 200 years (Helama and Valovirta 2008). Larval stage of FPM is known as glochidia. Like other Unionoida mussels, FPM glochidia has an obligatory parasitic stage in its life cycle with a suitable host for successful metamorphosis. FPM glochidia are highly host specific to the Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in Europe (Salonen et al. 2017; Taeubert and Geist 2017) although different strains of this species have different suitability range as a host (Österling and Larsen 2013). Exclusive host specificity of pearl mussel to salmonids can be the result of co-evolution for millions of years. aleontological data indicate that FPM and salmonids have co-existed in Europe for at least 8 million years (Ziuganov et al. 1994, 2005).

The relationship between glochidia and salmonids (brown trout) is parasitic (e.g., Filipsson et al. 2017). Glochidia have several impacts on its host´s growth, physical condition, swimming performance (Taeubert and Geist 2013; Österling et al. 2014; Filipsson et al. 2016, 2017; Taeubert and Geist 2017) as well as on their immune system (Chowdhury et al., 2017). Infected hosts are reported to develop acquired immunity against FPM (Chowdhury et al. 2017). Hence, because of memory phenomena and specific immune response, the death rate of glochidia is much higher during the second infection compared with the first one (Baur 1987). Several adverse impacts of FPM glochidia have been reported including hyperplasia and gill filament fusion, energetic cost, dysfunction of liver, kidneys and gills (Taeubert and Geist 2013; Österling et al. 2014; Thomas et al. 2014; Filipsson et al. 2016, 2017) change in behavior (Horky et al. 2014) and altered secondary sexual expressions and reduced sperm

quality (Kekäläinen et al. 2014). Glochidiosis also have been reported to have a negative impact on its salmonid host´s growth during infectivity and post infectivity stage (Treasurer et al. 2006, Ooue et al. 2017).

Generally, parasitic infections increase the susceptibility and virulence of co-infections, i.e. they can increase the risk of sub-sequent parasitic infections and can make the host more vulnerable to bacterial infections (Kotob et al. 2016, Gleischner et al. 2018). Ectoparasites have been shown to make their host more vulnerable to mortality in case of co-infection, for example, with a bacterial pathogen like *Flavobacterium columnare* (Bandilla et al. 2006; Dong et al. 2015). In line with these, it was recently demonstrated that brown trout infected with FPM increased its host's vulnerability to the secondary infection caused by the trematode *Diplostomum pseudospathaceum* (Gopko et al. 2018). FPM glochidia remain in the gills of the host up to 10 months (Bauer 1987; Ziuganov et al. 1994; Chowdhury et al. 2017; Salonen and Taskinen 2017). During this time period, the hosts can be encountered also by many bacterial pathogens along with glochidiosis. *Flavobacterium columnare* is the bacterial pathogen causing lethal columnaris disease mainly in fish farms (Declercq et al. 2015, Kunttu 2010). Thus, it can be expected that brown trout infected with FPM would be more susceptible to sub-sequent infection by *F. columnare* than the uninfected ones.

The objective of the study was to evaluate the effect of gill parasitic glochidia of FPM to virulence of *F. columnare* infection in brown trout, measured as survival rate of the fish during disease outbreak. Two age groups of fish were used: 'glochidia attached' group (0+ year fish) and 'glochidia detached' group (1+ year fish)—in order to evaluate the possible effect of FPM during the period when glochidia were attached to the gills of brown trout and when the glochidia had already detached from the gills, respectively. The hypothesis was that FPM infection will lower the survival of brow

trout (both when glochidia are attached and detached) and that the decreasing effect on survival would increase with the number of glochidia.

2 MATERIALS AND METHODS

2.1 ´Glochidia detached´ experiment

In the first experiment (glochidia detached experiment), total 310 young of the year (0+) brown trout (*Salmo trutta*) of Rautalampi strain were collected from Laukaa aquaculture unit of Luke (Natural Resources Institute Finland) and transported to the Konnevesi Research Station on the 25th of August 2016 and randomly distributed and moved into two 163 liter tanks. After that five individuals were randomly checked for glochidia presence to be confirmed that samples were free of previous glochidia infection. FPM glochidia was collected from the river Haukioja, northern Finland. After two weeks, in September, half of the fish were exposed to glochidia suspension of 5.0×10^5 while half of them were kept as control group, only exposed to water without glochidia. All the fish was then distributed randomly again into two 163 liter tank. Infection intensity (mean number of glochidia per infected fish) was checked 3 days after the infection by dissecting fish gills of three fish. The average infection intensity (± s.e.) was 1421±210 glochidia per fish. In July 2017, all fish was tagged with PIT tag. PIT (Passive Integrated Transponder) tag is a special type of tag that contain a specific code to recognize an individual fish with a digital PIT tag reader in July 2017. PIT tags were injected into the fish in between dorsal and adipose fins after being anesthetized with MS-222 with the concentration of 4.5 ml in 5 liters of water. Odd PIT tag numbers represented the glochidia control fish while even PIT tag numbers represented the glochidia infected fish. Length and weight of the fish were also measured along with the number of the glochidia which was counted using naked eye

counting method (Salonen and Taskinen 2017) in July 2017. The mean Length (± s.e.) of fish was 109.64±0.835 mm and the mean weight (±s.e.) was 14.54±0.32 g. After tagging the glochidia infected fish both infected and uninfected fish were then mixed together and transferred into two replicate tanks (tank 46 and 56 both contained 34 glochidia infected and 28 control fish each and 124 in total) (Figure 1) until the *F. columanre* challenge. Glochidia detachment began in July /August 2017. Before challenging with the *F. columnare* in November 2017, these fish had already become 1+ year old and glochidia infected fish already dropped off glochidia. So, the fish belonged to this experimental group were named as ´glochidia detached´ group.

2.2 ´Glochidia attached´ experiment

In the second experiment (glochidia attached experiment), 200 zero young of the year (0+) brown trout were collected, tested for glochidia (as described as above), randomly distributed and stored in two 163 liter tanks from the same source in August 2017. This experiment was also conducted in Konnevesi Research Station. Glochidia was collected from river Jukuanoja, northern Finland. After that they were infected with FPM glochidia in the same way as described above in September 2017 with a glochidia suspension of 4.0×10^5. After 3 days, fish was examined for glochidia infection intensity and the average infection intensity (± s.e.) was 1041±43 per fish. Later in September, all fish (181 fish in total) was marked with fin cut. Fish were anesthetized prior to marking using clove oil solution of 2 ml for 5 liters of water. Left fin cut represented the control group, whereas right fin cut represented the glochidia infected group and then was randomly distributed into two 163 liter tanks (tank 63 contained 47 glochidia infected and 43 control, tank 64 contained 47 glochidia infected and 44 control fish and 181 in total) (Figure 1) until they were exposed to *F. columnare* in November 2017. Two fish died from each tank before they were exposed to the bacterium. As the fish was

carrying glochidia during the bacteria exposure experiment, the group was named as ´glochidia attached´ group.

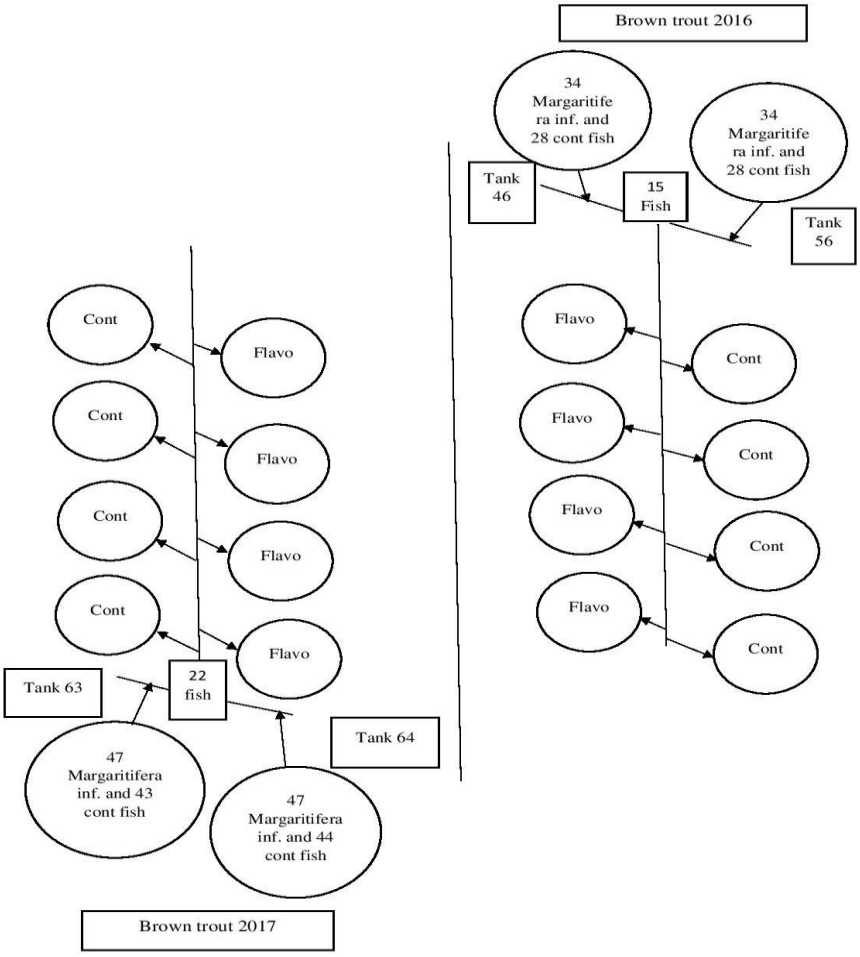

Figure 1: Experimental design; Here cont. = *Flavobacterium columnare* control bucket, Flavo. = *F. columnare* exposed buckets. *Margaritifera* inf.=FPM glochidia infected fish, *Margaritifera* cont.= FPM glochidia control fish. Brown trout 2016 depicts glochidia detached group experiment and brown trout 2017 depicts glochidia attached group experiment. Both circles and squares depict the experimental buckets, numbers depict the bucket number and number of experimental fish.

2.3 Exposing fish to *Flavobacterium columnare*

Both glochidia detached and attached group fish was exposed to *F. columnare* in November 2017. Fish from four replicate tanks (tank 63, 64, 46, 56) (Figure 1) were divided into total of 16 experimental buckets. In total 117 individual glochidia infected and uninfected fish of `glochidia detached` experiment were distributed into 8 buckets which were named from J to Q. Fish number in each bucket varied from 13 to 15 so that the number of FPM infected and control fish was in balance totally to equally distribute them per bucket as much possible. Again, all 179 individual glochidia infected and uninfected fish of `glochidia attached` group were distributed into 8 buckets which were named from A to H. Number of fishes in each bucket varied from 21 to 23.

16 dark colored buckets were chosen for the experiment. The capacity of the buckets were 80 liters, which was initially filled up to 20 liters and after adding the bacterial solution or Shieh media, additional 30 liters were added in each bucket. The water temperature was maintained at 18°C. All the buckets were supplied with oxygen through aerators.

F. columnare strain B549 used in this experiment was isolated from Lake Kuuhankavesi, central Finland in 2013 and stored at -80 ° C in a solution that contained 10% fetal calf serum and 10% glycerol. The strain was then revived by culturing in the Shieh medium. The revived culture was further sub-cultured in the same condition three times into larger medium volume in a ratio of 1-part bacterial culture to 10-parts of fresh medium to obtain enough concentration for the experiment. Shieh media was also prepared prior to the experiment and both solutions were transferred to Konnevesi Research Station. 500 ml of Shieh media was added in control buckets which were: N, O, P, Q for glochidia detached fish and E, F, G, H for glochidia attached fish. Density of the *Flavobacterium* solution used for infections was 5×10^8 CFU (cell

forming unit)/ml and was applied in an amount of 500 ml in the designated *Flavobacterium* buckets which were: J, K, L, M for glochidia detached fish and A, B, C, D for glochidia attached fish. The final concentration of bacteria in exposure buckets was 1×10^4 CFU/ml. The experiment continued until the last exposed fish died, which took 29 hours, and the mortality of the fish were recorded continuously throughout the experiment.

Experimental buckets were under hourly observations until the first fish died. After that the buckets were under continuous observation and whenever the fish turned upside down and completely stop moving, the time of the death was recorded on the data sheet. The dead fish was then collected using separate nets and trays and brought back to the observation platform where the bacteria was collected from the gills using inoculation loops and plated on petri dishes containing agar along with modified Shieh medium and tobramycin for selective isolation for *F. columnare.* Those plates were brought back to the University of Jyväskylä laboratory and were stored for 24 hours at the room temperature for examination of rhizoid, yellow *F. columnare* colony formation. Each of the plates were then observed against the light and clear visible colonies were observed.

2.4 Data collection and statistical analysis

Data were collected in three stages during the experiments: in July 2017 (marking fish, glochidia count and weight of `glochidia detached` group fish was recorded), September 2017 (marking with fin cut and glochidia intensity of `glochidia attached` group fish was recorded) and November 2017 after exposing the fish to *F. columnare* (mortality time was recorded). Results were analyzed for both glochidia detached and attached fish with two-way analysis of variance and regression analysis (see below). Furthermore, to illustrate the time-dependent mortality in each fish group, survival

curves were produced using Kaplan-Meier survival analysis for both glochidia attached and detached group experiments infected fish in eight buckets: A, B, C, D and J, K, L, M.

2.4.1 Statistical analysis for ´glochidia detached´ experiment:

In glochidia detached experiment, the effect of glochidia infection and possible tank effect were analyzed by using two-way analysis of covariance (2-ANCOVA) with glochidia infection as a fixed effect, tank as a random effect and fish weight as the covariate. This aspect was chosen to examine the effect of fish size (weight) to the survival time. The model assumptions (independence of observation and Homoscedasticity) were made in 2-ANCOVA with the additional assumptions were made as linearity of covariate and homogeneity of regression slopes. The independence of observations attained by randomizing every part of the experiment and by random division of both glochidia and *F. columnare* control and infected fish. Whether or not, all of the subpopulations have the same variance (homoscedasticity) was checked using Levene's test and was found to hold (Levene=0.917, df1=7, df2=52, p-value=0.501). The assumption of normality also met for all subpopulations (Shapiro-Wilk p-values ≥ 0.118) except for the glochidia infected fish sub population from bucket K. Due to the wide nature of ANOVA, this modest deviation from normal distribution in one subpopulation is not problematic for accuracy of the results but should be considered when interpreting the results (Leik, 1997). The assumptions of linearity of covariate and homogeneity of regression slopes were examined by both graphically and two-way ANCOVA. Here, instead of being full factorial, the model was checked for both all possible two way and three-way interactions (infection*weight, infection*bucket, bucket*weight, infection*bucket*weight). From the result ($p \geq 0.328$) it was found that both additional assumptions, linearity of covariate and homogeneity of regression slopes, were met. Thus, the results of 2-

ANCOVA can be considered valid. It was found that the effect of covariate (fish weight) and the glochidia infection x tank interaction both were insignificant and were, therefore, eliminated from the final ANOVA model (see results).

Multiple linear regression was performed to see the fish size (weight) and glochidia intensity was affecting the fish survival time. All the model assumptions (normality, homoscedasticity and linearity of residuals) were checked graphically by using Durbin-Watson statistics (=2.130). All assumptions were fulfilled. So, the result from the multiple linear regression can also be considered as valid.

2.4.2 Statistical analysis for 'glochidia attached' experiment

The effect of glochidia infection and tanks effects (tank: A, B, C, D) were analyzed using two-way analysis of variance (2-ANOVA) with glochidia infection as a fixed effect and tank as a random effect. Independence of observation was attained by randomizing every part of the experiment and division of both glochidia and *F. columnare* control and infected fish. Assumptions of ANOVA were checked before the analysis to ensure the validity of the results. Shapiro-Wilk was used to examine the normal distribution of survival times in each subpopulation from both glochidia control and infected fish from tank A to D as well they were analyzed graphically. The number of individuals from each subpopulation was between 10 and 13. Glochidia infected fish in tank D, found out not to be normally distributed both in Shapiro-Wilk test ($W=0.736$, $df=11$, p-value=0.001) and graphically. Except that rest of the subpopulations did appear to be normally distributed both graphically and by Shapiro-Wilk test (p-values ≥ 0.185). This slight deviation was also considered not violating the rather robust assumptions of ANOVA, as explained above for the glochidia detached group. Homoscedasticity of the subpopulations were examined using Levene's test. According to the test, there was no heteroscedasticity between the subpopulations (Levene=0.598, df1=7, df2=82, p-value=0.756). As all model

assumptions regarding 2-ANOVA were met, the results of the analysis can be considered reliable and valid. It was found that the glochidia infection and tank interaction was insignificant and thus, eliminated from the final ANOVA model (see results).

3 RESULTS

All the fish from both experiments died within 29 hours after exposing to *Flavobacterium columnare*. Only one fish died from the control group that was not exposed to bacterium.

3.1 Results for ´glochidia detached´ experiment

Form the ´glochidia detached´ experiment it was obtained that, glochidia infection effect was statistically significant and glochidia infected fish lived approximately 1 hour longer than the control fish.

The effect of covariate (weight of fish) on survival time was statistically insignificant ($F_{1, 51}$=1.282, p=0.263). In addition, the interaction between glochidia infection and bucket interaction was also statistically not significant ($F_{3, 51}$=1.330, p=0.275). Therefore, only the main effects 'glochidia infection' and 'bucket' were included in the final ANOVA model. Both the effect of glochidia infection (p = 0.038) and the bucket effect (p = 0.004) was significant (see Table 1), with a longer mean survival time in FPM-infected trout than in uninfected control trout (Figure 2). Using Tukey´s test it was found that fish from bucket K survived longer (p<0.029, compared with the rest of the buckets) than in the other buckets (Figure 3). However, as indicated by the lack of

interaction, the effect of glochidia infection was equal in all buckets—glochidia-infected individuals living longer.

The average lifetime for the glochidia-infected fish was 1313 minutes (22 hours 28 minutes). For the fish in the control group, it was 1251 minutes (21 hours 25 minutes). Survival curves for each bucket, given in Figure 4, 5, 6 and 7, show that the fish started to die not before around 1000 minutes (16-17 h) but after that the mortality rate was high in all buckets in this experiment.

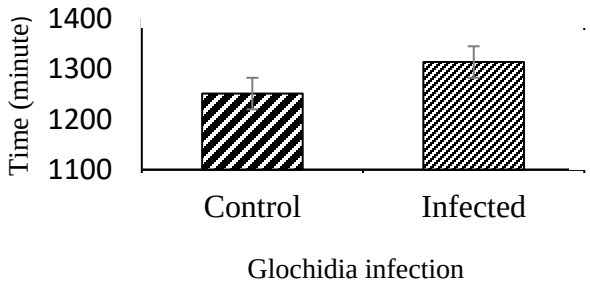

Figure 2: Comparative (average ± s.e.) survival time (minute) of *Margaritifera margaritifera* glochidia infected and control fish from 'glochidia detached' experiment (= glochidia already dropped off) where the fish was exposed to *Flavobacterium columnare* 14 months after the *M. margaritifera* glochidia infection.

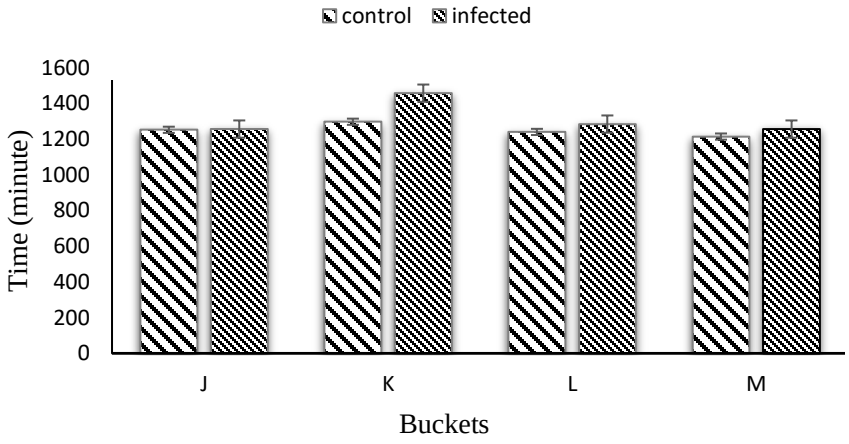

Figure 3: Bucket specific (bucket J, K, L and M) (average ± s.e.) survival time (minute) of *Margaritifera margaritifera* glochidia infected and control fish from 'glochidia

detached' experiment (= glochidia already dropped off) where the fish was exposed to *Flavobacterium columnare* 14 months after *M. margaritifera* glochidia infection.

Table 1. Results for two-way analysis of variance for ´glochidia detached´ group experiment.

Source		Type III sum of sqaures	df	Mean square	F	Significance (*p*)
Intercept	Hypothesis	30172352.49	1	30172352.49	1101.820	<0.001
	Error	230392.474	8.413	27384.111[a]		
Bucket	Hypothesis	224010.167	4	56002.542	4.310	0.004
	Error	714699.504	55	12994.536[b]		
FPM_infection	Hypothesis	58483.430	1	58483.430	4,501	0.038
	Error	714699.504	55	12994.536[b]		

a. 0.335 mean square (bucket)+0.665 mean square (error)
b. mean square (error)

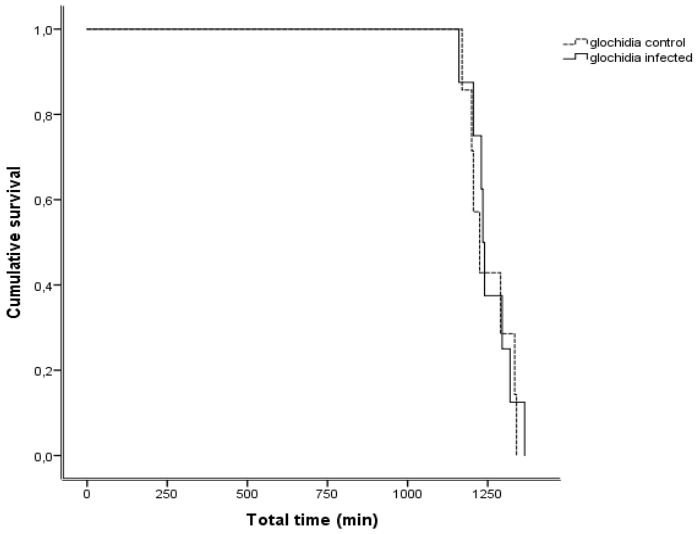

Figure 4. Comparative survival curves of ´glochidia detached´ experiment for control and glochidia infected fish in J bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

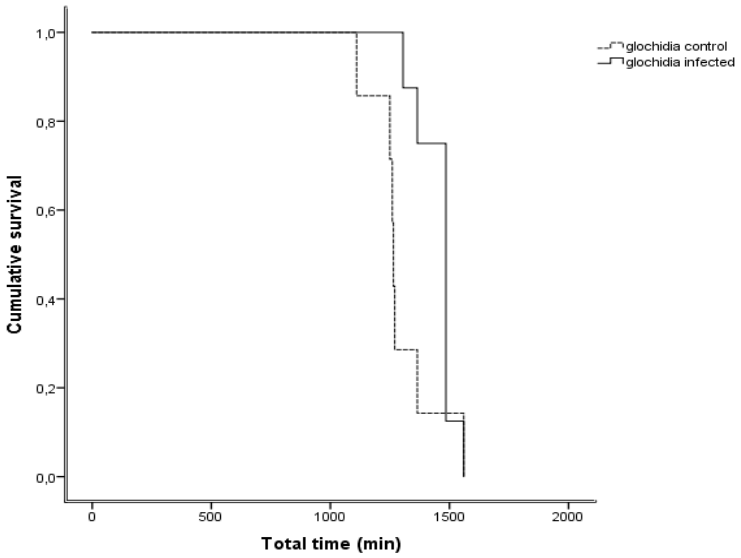

Figure 5. Comparative survival curves of ´glochidia detached´ experiment for control and glochidia infected fish in K bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

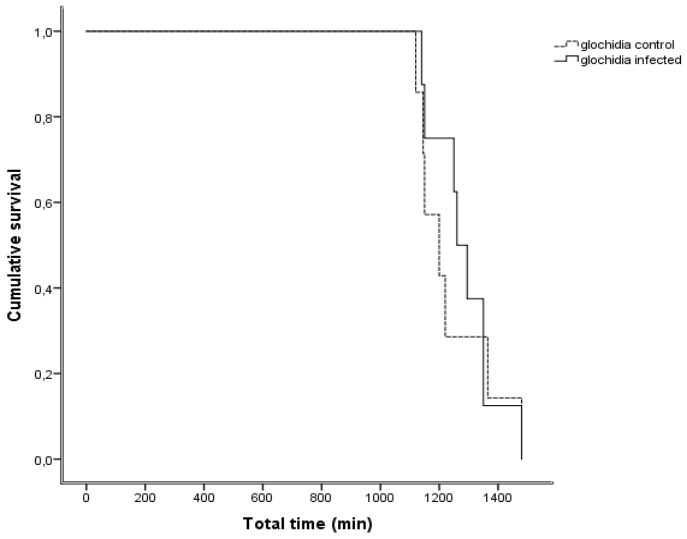

Figure 6. Comparative survival curves of ´glochidia detached´ experiment for control and glochidia infected fish in L bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

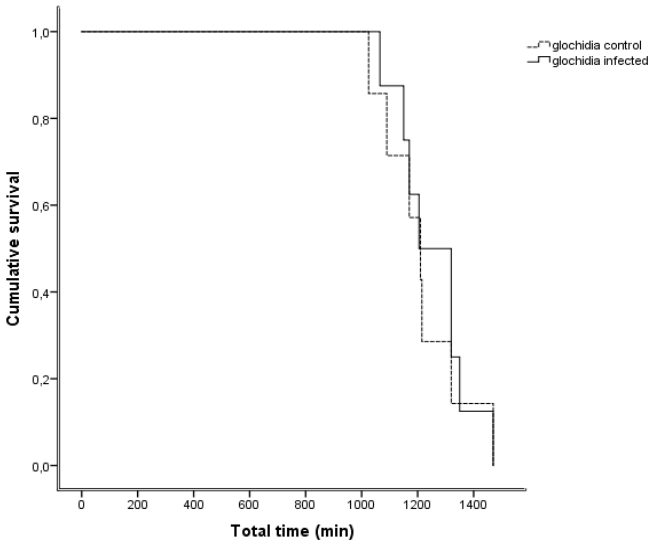

Figure 7. Comparative survival curves of ´glochidia detached´ experiment for control and glochidia infected fish in M bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

The effect of infection intensity and fish weight to the survival time was examined with multiple linear regression analysis for the 'glochidia detached' experiment. The resulting model was as:

Total survival time = 1094.382+0.097*glochidia intensity+12.173*fish weight(g)

(R^2 = 0.159) (Figure 8). It was found from the resulting model that the effect of fish weight was statistically not significant (p=0.105) while the effect of infection intensity was significant (p=0.045). An increasing number of glochidia provided better survival time in the experiment.

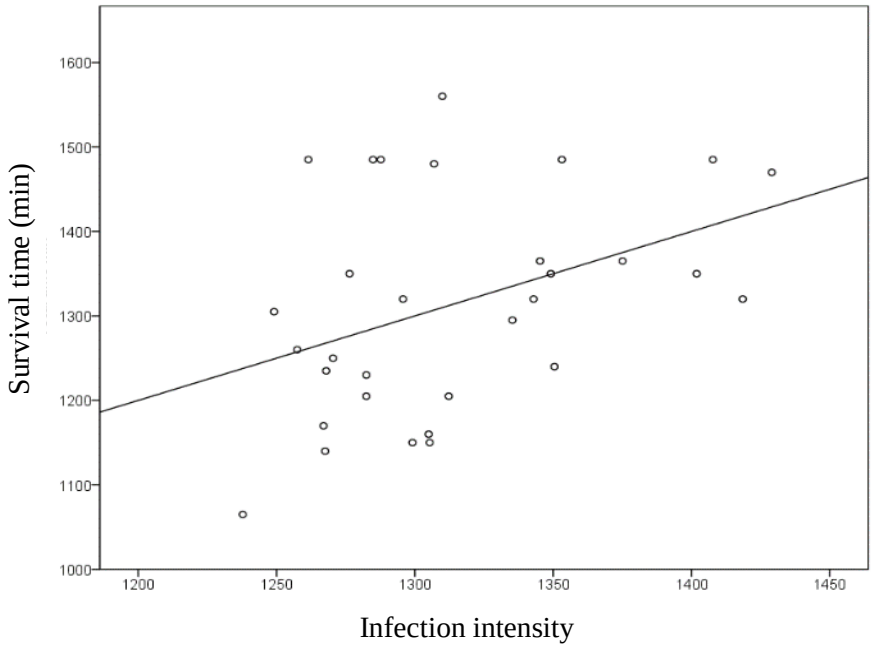

Figure 8: Relationship between the survival time and *Margaritifera margaritifea* glochidial intensity (number of glochidia per infected fish) for 'glochidia detached' experiment resulted from the multiple linear regression analysis. Glochidia intensity was recorded with naked eye counting method in July 2017, 4 months earlier the bacterial challenge with *Flavobacterium columnare* and the survival time was recorded.

3.2 Results for 'glochidia attached' experiment

Form the ´glochidia attached´ experiment it was obtained that, glochidia infected fish lived approximately 1 hour longer than the control fish.

Full factorial 2-ANOVA model indicated that the interaction term 'glochidia infection x bucket' was statistically not significant ($F_{3, 82}$=0.722, p=0.541). Therefore, the final ANOVA model, containing only the main effects, indicated statistically significant effects of both glochidia infection (p<0.001) and bucket (p<0.001) (Table 2). This shows that the *M. margaritifera* infected fish survived longer than the control fish (Figure 9). Differences between the buckets were analyzed using Tukey´s test and D bucket was found significantly different in terms of longer survival time (Figure 10) from rest of

the buckets: A, B and C (p<0.001). However, because it was found from the analysis that there was no interaction between the bucket and FPM infection indicate that the effect of FPM glochidia infection was equal in all buckets.

For 'glochidia attached' experiment the average survival time for the glochidia-infected fish was 1336 minutes (22 hours 27 minutes). The average lifetime for the fishes in the control group was 1277 minutes (21 hours 28 minutes). Survival curves for each bucket, given in Figure 11, 12, 13 and 14, show that the fish started to die off not until around 1000 min (16-17 h), except for bucket D where mortality started around 1300 min (21-22 h), but after that the mortality rate was high in all buckets in this experiment.

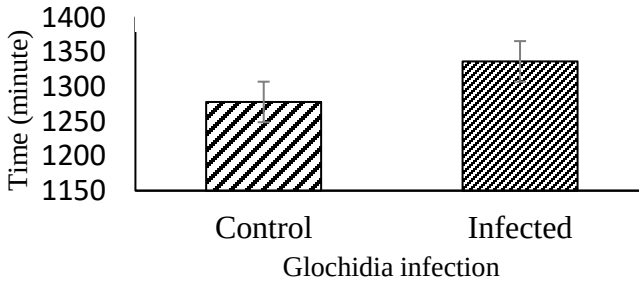

Figure 9. Comparative (average ± s.e.) survival time (minute) of *Margaritifera margaritifera* glochidia infected and control fish from 'glochidia attached' experiment (= glochidia present) where the fish was exposed to *Flavobacterium columnare* 2 months after *M. margaritifera* glochidia infection.

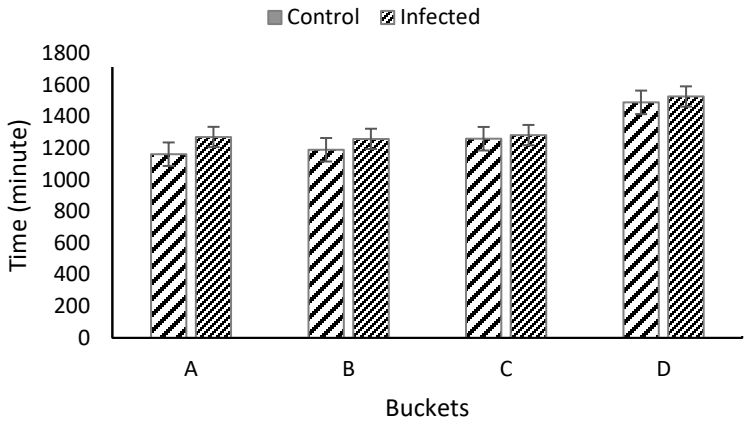

Figure 10. Tank specific (in tank A, B, C and D) (average ± s.e.) survival time (minute)of *Margaritifera margaritifera* glochidia infected and control fish from ´glochidia attached´ experiment where the fish was exposed to *Flavobacterium columnare* 2 months after *M. margaritifera* glochidia infection.

Table 2. Results for two-way analysis of variance for ´glochidia detached´ group experiment.

Source		Type III sum of squares	df	Mean square	F	Significance (*p*)
Intercept	Hypothesis	153099903.9	1	153099903.9	359.030	<0.001
	Error	1279365.090	3000	426427.033[a]		
Bucket	Hypothesis	1280858.228	3	426952.743	38.557	<0.001
	Error	941238.506	85	11073.394[b]		
FPM_infection	Hypothesis	80178.835	1	80178.835	7.241	0.009
	Error	941238.506	85	11073.394[b]		

a. 0.999 mean square (bucket)+0.001 mean square (error)
b. mean square (error)

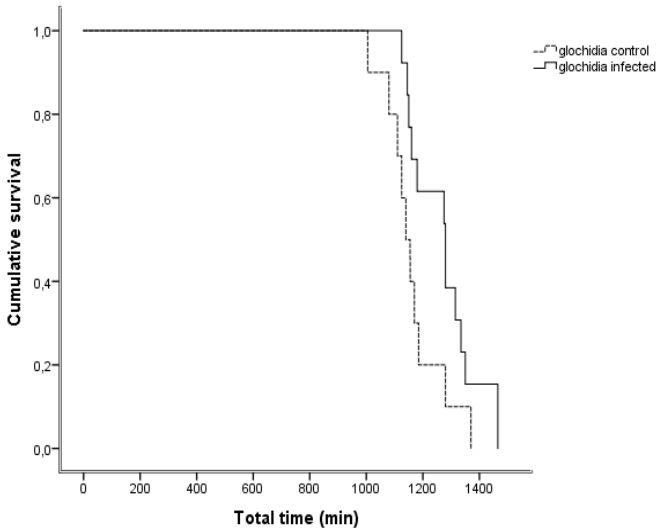

Figure 11. Comparative survival curves of ´glochidia attached´ experiment for control and glochidia infected fish in A bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

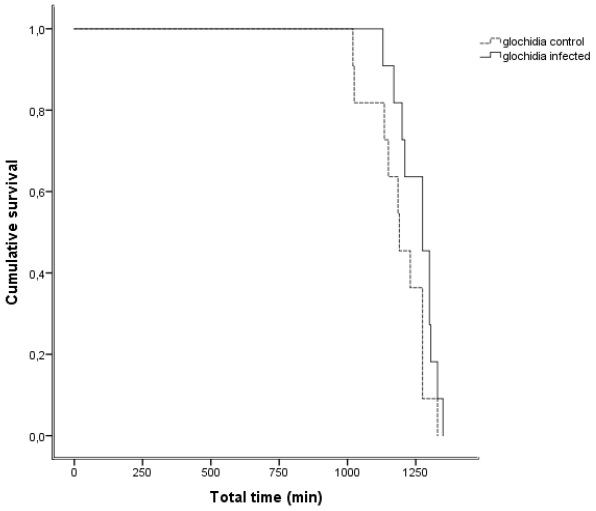

Figure 12. Comparative survival curves of ´glochidia attached´ experiment for control and glochidia infected fish in B bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

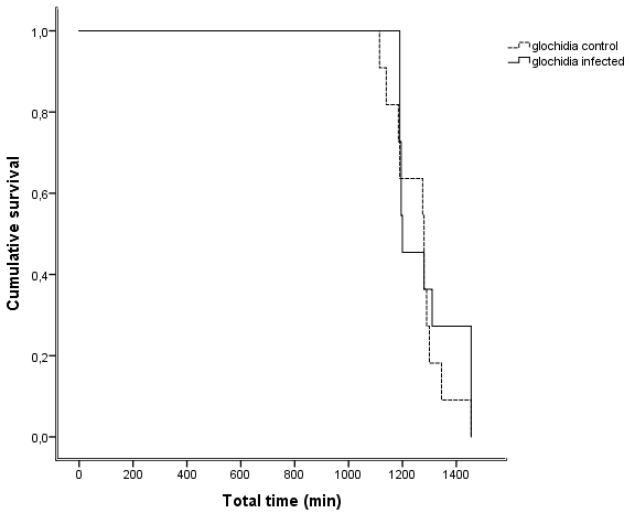

Figure 11. Comparative survival curves of ´glochidia attached´ experiment for control and glochidia infected fish in C bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

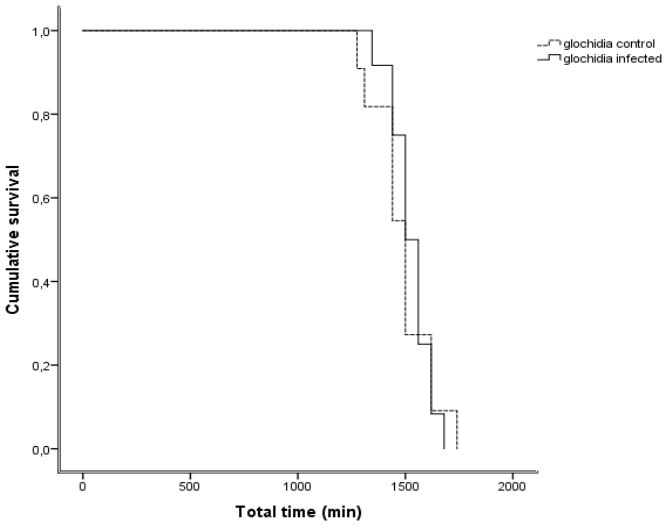

Figure 11. Comparative survival curves of ´glochidia attached´ experiment for control and glochidia infected fish in D bucket which were obtained during the *Flavobacterium columnare* exposure in November 2017.

3.4 Result of bacterial isolation

Yellow bacterial colony formation in the petri dishes from the collected gill mucus samples confirmed the presence of *F. columnare* in all the 150 *F. columnare* exposed fish. However, the gill mucus sample from the one dead fish individual that was not exposed to *F. columnare* did not show any bacterial colony formation, indicating that it did not die because of *F. columnare* infection.

4 DISCUSSION

In this study, I examined the impact of previously FPM infection on the survival time of brown trout when exposed to *Flavobacterium columnare*. In theory, parasitic infection should increase susceptibility to sub sequent infection (Kotob et al. 2016). But from the experiment it was found that, survival time of the previously-FPM-infected fish was longer than that of uninfected control individuals, both in the ´glochidia detached´ and ´glochidia attached´ experiment. Moreover, from the glochidia detached group it was found that the fish survival time was increasing with increasing number of glochidia per fish. This was also against the study hypothesis that FPM infection should increase susceptibility to secondary infections and survival rate will decrease with the increasing glochidia intensity.

The bacteria exposure experiment lasted for 29 hours till the death of the last *F. columnare* exposed fish. The mean survival time of previously FPM-infected fish was only about 1 hour longer than that of the control fish (in both experiments). It can be inferred from this result that, although FPM glochidia could not protect its host from the bacterial outbreak, it was providing some kind of protection. With a less virulent

bacteria, this protection effect may result in more pronounced difference between FPM glochidia infected and uninfected brown trout.

From the experiments, it was found that all the fish that were exposed to the bacterium was dead. Only one fish died from the control group that was not exposed to *F. columnare*, and this fish was found to be not infected by *F. columnare*. On contrary, all the *F. columnare*-exposed fish appeared to have *F. columnare* infection as indicated by the petri dish culture. Therefore, the mortality of brown trout in these experiments can be attributed to the *F. columnare*.

The mechanism of this protection phenomenon is not fully understood for this experiment but the immune system of the host, brown trout (*Salmo trutta*) may have an important role. Sequential infection with parasites of similar or different species can develop cross immunity which refers to the acquired immunity of fish to one parasite species can result in immunity also to the other species (Karvonen et al. 2009). Induction of acquired immunity against *F. columnare* by pre-infection with FPM glochidia is, though, questionable. Generally, fish host depends more on their non-specific immune system than the specific or acquired immune defense system (Anderson 1992, Kunttu 2010). Whenever a fish host is exposed to a new and unknown pathogen, its non-specific immune system is triggered. Different cell organs are responsible for the non-specific immune reaction like cell mucus. Cellular components like natural killer cells, phagocytic cells and humoral components like cytokines play important roles in the non-specific immune system of the teleost fish (Jørgensen 2014). So, it is possible that the FPM infection could have somehow boosted the non-specific immunity of the fish, acting as an immunostimulant, in this experiment.

Besides the immune system of the fish, alteration in the gill structure may have played an important role in the protection mechanism. As gills are considered as one of the most important entry points for *F. columnare* (Klesius et al. 2008), its entry can be

hindered due to the altered gill structure caused by the proliferation of gill cells and capsule formation around the glochidia (Ziuganov 1994). Also, gill filament fusion or less mucus formation (Thomas et al. 2014) can also lead to lower vulnerability to *F. columnare* as the ability to adhere is an important factor for the successful colonization in the host tissue for these bacteria (Figuieredo et al. 2005). During glochidia metamorphosis, gill structure also changes because of the rupture. It has been reported that, metamorphosed glochidia left the gill epithelium ruptured (Waller and Mitchel 1989) which should increase its host susceptibility to the secondary infection caused by *F. columnare*. However, it was found from the glochidia detached experiment that, the FPM glochidia infected fish was also better than the control fish even the metamorphosed glochidia left the gills effected as raptured and necrosis gills and hence more assumedly more vulnerable for subsequent infections. More intense study is needed to reveal this mechanism.

Co-infections are very common in fish farms (Madsen et al. 2005). During co-infections, sometimes one pathogen can alter the immune response of the host against the subsequent infections either by increasing or decreasing the immune system (Telfer et al. 2008). Rather than a single infection, co-infections with ectoparasites along with other pathogenic bacteria have been reported to accelerate the mortality rate of hosts (Bandilla 2006, Dong 2015, Roon 2015) as multiple infections favor faster host exploitation. It was recently demonstrated that brown trout infected with *M. margaritifera* increased its host vulnerability to the secondary infection caused by trematode *Diplostomum pseudospathaceum* (Gopko et al. 2018). This interactive effect has been explained as the result of the stress caused by the parasites reducing fish resistance to other secondary bacterial infection (Kotob et al. 2016). Unlike the subsequent infections with FPM glochidia which made brown trout host more vulnerable to the trematode infection, FPM glochidia was lengthening the survival

time of host in both attached and detached group. This aspect of co-infection needs to be furtherly investigated.

Temperature is one of the important factors for the bacterial infection severity, higher temperature causes more mortality (Suomalainen et al. 2005, Pulkkinen et al. 2010). The experiment was conducted at the room temperature of 18°C. It will be interesting to see the co-infection outcome in lower temperature. The strain used for the experiment was highly virulent. It can be expected that the survival time of the *F. columnare* exposed fish would be higher in natural columnaris outbreak conditions with less virulent bacterium strain. Moreover, the stress condition in the natural condition (e.g. density of both fish host and glochidia) would be much less. Furthermore, the glochidia density on host is also an important factor. There has been report of negative effect on the critical swimming speed of brown trout when the abundance of FPM glochidia exceeds approximately 900 glochidia per gram fish (Taeubert and Geist 2013). In the present study, glochidia average infection intensity (± s.e.) was 1041±43 per fish for glochidia attached group and for glochidia detached group the average infection intensity (± s.e.) was 1421±210 glochidia per fish. In natural condition, glochidia density barely exceeds 1000 glochidia per fish (Salonen and Taskinen 2017). This exceptional relationship of co-infection needs further investigation and will be interesting to see the outcome when glochidia infected host will be challenged under natural condition agains.t *F. columnare*.

FPM is an endangered species. Over the decades, the population of FPM is mainly declining due to e.g., anthropogenic contamination, overexploitation of both FPM and hosts, invasive species and lack of suitable hosts (Lopes-Lima et al. 2017). The invasive species e.g., brook trout is also responsible for this phenomenon in the European wild habitat (Salonen 2016). Proper understanding of the relationship between FPM and their host will not only help to increase public awareness to restore these two species

but also the aquatic environment itself. Freshwater pearl mussel and its larvae act as bioindicators (Ziuganov et al. 1994, 1998) and also serve other ecological services like biofiltration, nutrient cycling, habitat upgrade, water quality improvement etc. (Vaughn 2018). With its long-life span and host specificity it can serve as a biological tool to protect the salmonids host by upgrading the habitat and act as biological stimulant. Columnaris disease is a worldwide potential threat to the fish. There are a lot of approaches to treat this disease in Finland like chemical bathing or pro-biotics use, but the most effective measure is the application of antibiotics (Kunttu 2010). Antibiotic usage has been shown to lead to more drug-resistant bacterial strains (Miranda and Rojas 2007) including *F. columnare* (Declercq et al. 2013). In general, use of natural treatment like probiotics and bio-immunostimulants can be a sustainable approach for this problem although this approach needs a lot of expertise attention. In this experiment, it was clearly found that the glochidia failed to protect its host from the mortality, but infected fish survived longer period than the control fish. Further investigation requires to reveal the protection mechanism, and whether the protection is *M. margaritifera* specific or can be achieved with any kind of parasite pre-infection. Interestingly, the result was opposite the hypothesis and indicated that FPM glochidia may even decrease the virulence of *F. columnare*- a deadly pathogen harming the farmed fish.

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