JYU DISSERTATIONS 46

M. Motiur R. Chowdhury

Relationship between the Endangered Freshwater Pearl Mussel *Margaritifera margaritifera*, Its Salmonid Host and Co-infectants

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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ABSTRACT

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The relationship between the freshwater pearl mussel (hereafter FPM), *Margaritifera margaritifera,* and its salmonid host — pivotal for the conservation of this endangered bivalve — is characterized by a long parasitic stage (up to 11 months) and excessive host specificity. This thesis focuses on this relationship by experimentally studying the effect of FPM infection on the growth and resistance of salmonid fish and how the infection influences vulnerability of the host to other parasites and diseases, as well as how exposure of the host to other parasites affects vulnerability to FPM. Infection with FPM results in (i) reduced growth of the host, brown trout, during the parasitic period. Glochidia infecting fish gills cause a respiratory burden which may explain the negative growth-effect. In addition, (ii) infection induced dose-dependent acquired immunity in repeated exposures. Instead, FPM infection leads to (iii) increased vulnerability of brown trout to eye fluke *Diplostomum pseudospathaceum*. It maybe the respiratory burden of FPM infection that increases ventilation and exposes the fish to these gill-penetrating eye fluke. FPM infection also causes (iv) increased resistance of the host to *Flavobacterium columnare* (a pathogen that enters fish through the gills) — possibly due to enhanced nonspecific immunity or altered structure of the fish's gills. However, (v) pre-infection of trout with *Anodonta anatina* glochidia did not affect susceptibility to FPM, indicating that co-existing with *A. anatina*, is not a threat to FPM. Nevertheless, (vi) pre-infection with eye fluke cercariae within a short period (20 h) increased susceptibility of the host to FPM. Possibly, the tissue damage caused by recently penetrated eye fluke cercariae enhances attachment of FPM glochidia. These results shed light on complex, previously unstudied relationships between salmonid, FPM and other parasites/diseases and provide important new information that can be potentially utilized in conservation of this endangered species.

Keywords: Conservation; *Flavobacterium*; Immunity; Margaritiferidae; Parasitism; Salmonidae; Unionidae.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–IV.

The responsibilities of M. Motiur R. Chowdhury (MC) in the articles of this thesis are as follows: In I, the experiment was planned together by JT, JKS and MC. MC and JKS carried out the experiment. TM performed statistical analyses and MC wrote the article together with co-authors. In II, the experiment was planned by all authors. The experiment was conducted by MC and MG. Statistical analyses were done by MG and writing was done by MG with the help of the co-authors. In the case of III, the planning of the experiment was by JT and MC. MC performed the experiment. TM carried out analyses. MC wrote it with the help of the co-authors. IV was planned and performed by all authors except KA who was responsible for data analyses. MC and JT had the main responsibility of writing the manuscript.

I Chowdhury M.M.R., Salonen J.K., Marjomäki T.J. & Taskinen J. 2017. Interaction between the endangered freshwater pearl mussel *Margaritifera margaritifera*, the duck mussel *Anodonta anatina* and the fish host (*Salmo*): acquired and cross-immunity. *Hydrobiologia* 810: 273–281.

II Gopko M., Chowdhury M.M.R. & Taskinen J. 2018. Interactions between two parasites of brown trout (*Salmo trutta*): consequences of preinfection. *Ecology and Evolution* 8: 9986–9997.

III Chowdhury M.M.R., Marjomäki T.J. & Taskinen J. 2018. Effect of glochidia infection on growth of fish: freshwater pearl mussel *Margaritifera margaritifera* and brown trout *Salmo trutta*. Submitted manuscript.

IV Chowdhury M.M.R., Roy A., Auvinen K., Pulkkinen K., Suonia H. & Taskinen J. 2018. Glochidia of the endangered freshwater pearl mussel, *Margaritifera margaritifera*, lower virulence of a fish pathogen. Manuscript.

1 INTRODUCTION

1.1 Background, functions and decline of *Margaritifera margaritifera*

The freshwater pearl mussel (hereafter FPM) (*Margaritifera margaritifera*) is a long-living (more than 200 years) (Helama and Valovirta 2008), river-dwelling bivalve mollusc, which occurs in Europe and north-east North America but is now critically endangered throughout its range of occurrence (Geist 2010, Lopes-Lima *et al.* 2017). FPM has a larval stage, glochidium, which is parasitic on the Atlantic salmon (*Salmo salar*) and/or the brown trout (*Salmo trutta*) (e.g., Young and Williams 1984, Salonen *et al.* 2016). Some FPM populations exclusively develop on Atlantic salmon and others exclusively on brown trout (Geist *et al.* 2006, Karlsson *et al.* 2014, Salonen *et al.* 2017). Human impact in the form of the loss of habitats, loss of host fish, siltation, pollution, the introduction of invasive species and commercial exploitation have effected a considerable decline in freshwater mussels globally (Bauer 1988, Williams *et al.* 1993, Lydeard *et al.* 2004) – including FPM (Young and Williams 1983, Bauer 1986, 1988, Cosgrove *et al.* 2000, Oulasvirta 2011, Simon *et al.* 2015, Salonen *et al.* 2016). Currently, FPM is categorized as a critically endangered species in Europe (Anon. 2013).

Freshwater bivalves of the order Unionoida are effective ecosystem engineers and play key roles by burrowing and filtering activities (Howard and Cuffey 2006, Vaughn *et al.* 2008) in their ecosystems (Vaughn and Hakenkamp 2001, Gutierrez *et al.* 2004, Boeker *et al.* 2016, Lummer *et al.* 2016,) that link pelagic and benthic zones of a water body (Gutierrez *et al.* 2004, Lopes-Lima *et al.* 2017). These ecosystem functions may have a strong influence on primary and secondary production, biogeochemical cycles, sedimentation rates and clarification of water (Strayer 2014) along with facilitation of substrate to many other organisms by their shells (Vaughn and Hakenkamp 2001, Spooner *et al.* 2013). Humans also benefit directly from the mussels in many ways, e.g., water purification, prey to commercial fishes, environmental monitoring, direct

source of protein, shells, pearls and spiritual enhancement (Haag 2012, Vaughn 2018). Thus, freshwater mussels provide several long-term ecosystem services. FPM fulfils the criteria of indicator, flagship, key stone and umbrella species, and can thus be considered an ideal target species for the conservation of aquatic ecosystem functioning (Geist 2010).

There are two main factors contributing to the decline of FPM. First, decline and extinction of host salmonids in many rivers have critically impaired the recruitment of juvenile FPM. Second, human activities in the water shed (e.g. ditching and channel modifications) have decreased suitable habitats for FPM. *S*iltation and fine sediments have filled the interstitial space in the sediment and deteriorated the stream bed quality for juvenile mussels (Geist and Auerswald 2007). Many large Finnish rivers have been dammed for electric power production, preventing fish migrations, such as the River Iijoki since 1962. In the former salmon tributary of the River Iijoki, the River Livojoki, FPM use the Atlantic salmon as their primary host, while in brown trout tributaries, brown trout was clearly the best host for FPM (Salonen *et al.* 2017). Consequently, the River Livojoki pearl mussel population has been without a proper fish host for more than 50 years. It is imperative to build fish ways to restore the ascent of salmon to rivers such as the River Livojoki, but meanwhile, the stocking of *Margaritifera*-inoculated salmonids into rivers or captive breeding grounds where salmonid hosts are artificially infected with glochidia (Gum *et al.* 2011, Eybe *et al.* 2015) can be potential methods of restoration to increase recruitment of young mussels, not only in the dammed rivers but also in other suitable habitats.

Therefore, understanding the interactions between FPM and salmonids, a wider investigation of the effects of glochidiosis on the fish host is needed for the conservation of FPM. This understanding will be useful in the development of a successful restoration strategy to the stakeholders involved in the conservation of the endangered FPM.

1.2 Host–parasite relationship between salmonid and *M. margaritifera*

The endangered FPM has a complex life cycle (Fig. 1), including an obligate parasitic larval stage (glochidia) exclusively dependent on the salmonid fish host: Atlantic salmon and brown trout (Young and Williams 1984, Bauer and Vogel 1987, Geist *et al.* 2006, Dolmen and Kleiven 2008, Ieshko *et al.* 2016, Salonen *et al.* 2016, 2017). Generally, male mussels release sperm cells into the water in early summer, female mussels inhale them from the water and fertilize their eggs. These fertilized eggs develop into larvae in marsupial gills during the following weeks. Later, in the late summer or early autumn, gravid mussels release tiny parasitic glochidia into the water (Bauer and Wachtler 2001). In Finland this takes place in late August or September (Salonen and Taskinen

2017). These temporary gill parasites (glochidia) are attached and encapsulated by the epithelial cells of the host fish. There they undergo metamorphosis into juvenile mussels, in a process, that takes 8–12 months in Europe, from early autumn to the following summer (Young and Williams 1984, Bauer 1987, Hastie and Young 2001, Salonen and Taskinen 2017). Then metamorphosed juvenile mussels drop off from the host fish and start independent life in the river bed where they settle (Hastie and Young 2001). These juveniles reach maturity at approximately 20 years of age and reproduce until they die (Bauer 1987).

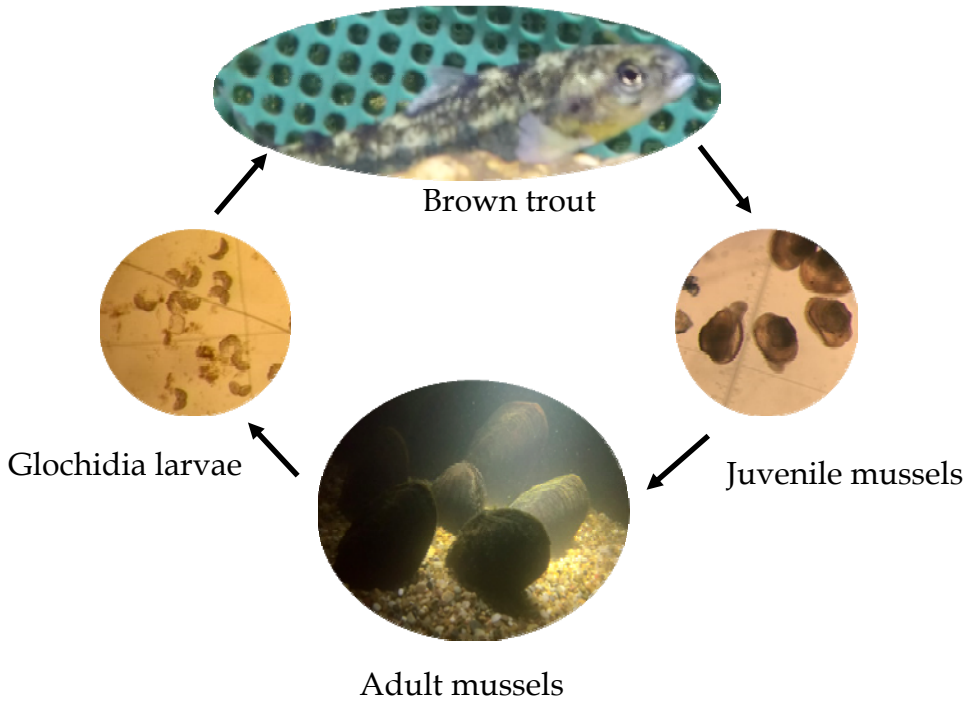

FIGURE 1 Life cycle of freshwater pearl mussel *Margaritifera margaritifera*, showing the connection between salmonid and *M. margaritifera*. Photos by Motiur Chowdhury and Jouni Taskinen.

1.3 Consequences of *M. margaritifera* on salmonid

Often FPM glochidia have been judged to have a weak negative, or even a positive, effect on the performance of the host (Ziuganov *et al.* 1994, Ziuganov 2005, Taeubert and Geist 2013). The former is more conceivably true since FPM glochidia exhibit more than seven-fold growth in length (from 60 µm to 400 µm) in the course of their 8–12 month development on the fish gills (Bauer and Vogel 1987, Young and Williams 1984, Salonen and Taskinen 2017) with a substantial nutrient transfer from fish to glochidia and a shift in the stable isotope composition during development of the mussel (Fritts *et al.* 2013, Denic

et al. 2015). In addition, FPM glochidia cause a noticeable immune response in their hosts, increasing their metabolic rates and hematocrit levels and hampering swimming and respiratory performance in brown trout (Taeubert and Geist 2013, Österling *et al.* 2014, Thomas *et al.* 2014, Filipsson *et al.* 2017).

These negative effects of glochidia can be expected to reduce the fitness of the fish host. However, till now, the possible negative impact of freshwater mussels on the growth rate of the host has not been clearly disclosed. Treasurer *et al.* (2006) observed no significant effect of FPM infestation on the growth of Atlantic salmon in the initial stage of the parasitic period but observed a negative effect at 15 weeks, which disappeared by the end of the 1-y monitoring period, perhaps due to therapeutic treatment as glochidial abundance steeply decreased. In the study on another margaritiferid, *M. laevis*, no effect on the growth of the salmonid host *Oncorhynchus masou masou* was found during the first 50 d of infection, but a negative impact was observed after the detachment of *M. laevis* glochidia at 70 d post-infection (Ooue *et al.* 2017) even though this was a relatively shorter parasitic period than that of the FPM. To my knowledge, these are the only papers focusing on the possible growth-effect of any freshwater mussels on their fish hosts.

Therefore, the exceptionally long parasitic period of FPM glochidia, which grow remarkably in size during their parasitic period, may have a negative effect on the growth of the salmonid host which should be assessed clearly here as compared to the mussel species with a short parasitic period.

1.4 Acquired immunity and cross-immunity

In nature, the larvae of two or more mussel species can utilize the same host either simultaneously or sequentially. For example, both mussel species FPM and *A. anatina* inhabit in the River Mustionjoki/Svartå (Finland) and the River Wye (UK) (Lopes-Lima *et al.* 2017). Thus they can live in sympatry and use the same host: brown trout (*Salmo trutta*). Sequential infection with parasites belonging to different (and often closely related) species can induce protection, which is known as cross-immunity (Dodd *et al.* 2005). Since the glochidia shedding of *A. anatina* takes place seasonally earlier (winter–spring, as late as May–June, Taskinen *et al.* 1997) than that of FPM (summer–autumn, e.g., Salonen and Taskinen 2016), it is possible that host fish can be consecutively exposed to the glochidia of *A. anatina* and FPM, which may consequently induce cross-immunity in their shared host. Bauer *et al.* (1991) conducted a short-term experiment by infecting brown trout first with *A. piscinalis* (= *A. anatina*) glochidia and then challenge infection with FPM glochidia. No evidence for cross-immunity was achieved. However, the length of the parasitic period of FPM can be more than 300 d (Young and Williams 1984), and a comprehensive monitoring covering the whole parasitic phase is required to assess the possibility and potency of this phenomenon.

Moreover, the exposure of an individual host fish to the glochidia of a parasite species can happen successively in consecutive years in nature. In this case, the host, which was previously exposed to glochidia, can develop an acquired immunity as a result of the previous exposure(s) to these glochidia (in unionoid mussels, e.g. Bauer and Vogel (1987), Rogers and Dimock (2003), Dodd *et al.* (2006), Treasurer *et al.* (2006)). In case of FPM, lower infection rates have been found in older brown trout in the field and after second infection in the laboratory, implying acquired immunity (e.g. Bauer 1987, Ziuganov *et al.* 1994, Hastie and Young 2001). Nevertheless, even a higher infection success in the second infection has been documented (Wächtler *et al.* 2001). In spite of these contradictory results, the acquired immunity in salmonid hosts against FPM glochidia has not drawn much attention by experimental studies. If there was a strong acquired immune response in the salmonid host, the most successful production of FPM recruits for conservation purposes would essentially require immunologically naïve one summer old host fish. Since the parasitic stage can largely contribute to the reproduction success of the endangered FPM, the role of acquired immunity in its conservation requires further attention.

1.5 Co-infection with trematodes

The eye fluke trematode *Diplostomum pseudospathaceum* is a common parasite of a wide array of fishes, for example various cyprinid and salmonid fishes including brown trout in its natural range of distribution (Betterton 1974, Valtonen and Gibson 1997, Rolbiecki *et al.* 2009). The eye fluke has three hosts in its life cycle (Seppälä *et al.* 2004): freshwater snails (often *Lymnaea stagnalis*) are the foremost intermediate hosts, freshwater fishes are the second intermediate hosts and fish-eating birds are the ultimate hosts. Infected snails generate thousands of cercariae which enter the epithelium of the fish primarily through gills (Mikheev *et al.* 2014). Therefore, the infectivity of the fluke strongly depends on the volume of water pumped through the gill chambers (Mikheev *et al.* 2014). After penetrating the epithelium, eye fluke moves towards the eye, the place of its final development into the metacercarial stage within the eye lens.

Höglund and Thuvander (1990) suggested that the eye fluke enhances the immunity of the host non-specifically. However, pre-infection may have no influence on challenge infection as revealed by Karvonen *et al.* (2009) in their study of two species of trematodes from the genus *Diplostomum* infecting rainbow trout. Nevertheless, according to my knowledge, the co-infections of FPM and *Diplostomum* eye fluke have not been studied in salmonid fish although they can co-occur in nature. Importantly, the eye fluke uses gills to penetrate their salmonid host (Whyte *et al.* 1991, Mikheev *et al.* 2014) – the same tissue that is occupied by FPM glochidia. Thus, it can be hypothesized that fish pre-infected with FPM glochidia are more vulnerable to the cercarial infection

of the eye fluke as the amount of water (and cercariae) pumped through the gill chamber increases in FPM-infected fish (Taeubert and Geist 2013, Thomas *et al.* 2014), thereby potentially also increasing exposure of fish to FPM glochidia. In contrast, fish pre-infected with the eye fluke may be less or similarly vulnerable to the challenge infection with FPM glochidia as the eye fluke use gills of the fish for quite a short time and settles in the eye. Therefore, an experimental study is needed to reveal the consequences, if any, of interaction between these parasites. This would be useful knowledge in the conservation activities of FPM.

1.6 Co-infection with bacterial disease

Flavobacterium columnare causes warm water disease (columnaris disease) in fish, including salmonids. This pathogen can cause dramatic economic losses in fish farming due to the high mortality associated with the disease (Wagner *et al.* 2002, Pulkkinen *et al.* 2010). *F. columnare* is an opportunistic fish pathogen that can grow outside the fish host as well (Kunttu *et al.* 2012). *F. columnare* strains differ in their virulence (Suomalainen *et al.* 2006, Pulkkinen *et al.* 2018), with some strains being capable of causing up to 100 % mortality in juvenile salmonids (Suomalainen *et al.* 2005). Since there is no effective vaccination available for young salmonids (Sundell *et al.* 2014), the only treatment against *F. columnare* is antibiotics (Rach *et al.* 2008).

Parasitic infections, in general, increase the risk of secondary infections and can act as a vehicle for transmission of bacteria to fish (Kotob *et al.* 2016). For example, the monogenean parasite *Dactylogyrus intermedius* increases susceptibility of gold fish, *Carassius auratus*, to *F. columnare*, resulting in a higher mortality when compared to non-parasitized fish (Zhang *et al.* 2015). For its part, FPM glochidia infection has many adverse effects on the host fish including increased susceptibility to challenge infection with the eye fluke (II). Significantly, the eye fluke and *F. columnare* use the same gateway (fish gills) to infect fish.

Importantly, co-infection of the salmonid host with FPM glochidia and a bacterial disease is possible during the months in which the glochidia-infested salmonids have to be maintained in captivity. However, the question of whether *Margaritifera* glochidia increase susceptibility of salmonids to bacterial diseases, i.e. the virulence of a pathogen, has not been studied. It can be hypothesized that the vulnerability of FPM glochidia-infected individuals to *F. columnare* would be higher than in uninfected fish since gills are the main and first site of infection in fish by *F. columnare* (Declercq *et al.* 2013, 2015). On the other hand, Ziuganov (2005) concluded from his field studies that FPM can stimulate nonspecific resistance in salmon towards dangerous diseases, e.g. epitheliomata or fungal lesions induced by Saprolegnia in salmon.

1.7 Aims

The general objective of this thesis was to investigate the multilateral relationships between FPM, its salmonid host fish and other co-infectants (parasites and pathogens) of the host. This relationship was sought from different perspectives to enrich knowledge and contribute to the conservation of the endangered FPM, *M. margaritifera*.

Specific aims of the study:

The first aim was to investigate the consequences of FPM glochidia infection to its host directly,

a) during the parasitic period, by affecting the growth of the host (III) and
b) after the parasitic period, by inducing acquired immunity in the host with varying doses of FPM glochidia (I).

The second aim was to study whether the FPM glochidia infection has consequences to the host indirectly by affecting its vulnerability to

a) infection by the trematode eye fluke, *D. pseudospathaceum* (II) and
b) a potentially fatal bacterial disease caused by *Flavobacterium columnare* (IV).

The third aim was to explore how the success of FPM glochidial infection depends on the sharing host with other co-infecting parasite species by testing with

a) glochidia of the duck mussel *A. anatina* (I) and
b) cercariae of the eye fluke *D. pseudospathaceum* (II).

2 MATERIALS AND METHODS

2.1 Study areas

All of the studies (I–IV) were conducted in the laboratory of Konnevesi research station (Central Finland) of the University of Jyväskylä. The fish used in different experiments (I–IV) were collected from the rearing station of Natural Resources Institute Finland (Luke) in Taivalkoski and Laukaa in northern and central Finland respectively. The FPM glochidia used in all studies (I–IV), were collected from the River Iijoki catchment of northern Finland whereas *Anodonta* glochidia (I) were collected from Lake Koijärvi in eastern Finland. The parasite *Diplostomum* cercariae (II) and the pathogen *Flavobacterium* strain (IV) were collected from Lake Konnevesi and Lake Kuuhankavesi respectively in central Finland. The experiments were conducted with the permission of the Centre for Economic Development, Transport and Environment of Southern Finland (license number ESAVI/6759/04.10.03/2011). The collection of FPM glochidia was performed with license POPELY/513/07.01/2011 from the North Ostrobothnia Regional Centre for Economic Development, Transport and the Environment (Oulu, Finland). Permission to conduct the experiment with fish, license ESAVI/10184/04.10.07/2014, was granted by the Animal Experiment Board of Finland (regional administration of Southern Finland).

2.2 Laboratory experiments

The fish used in these experiments were artificially exposed to FPM glochidia (I–IV), *Anodonta anatina* glochidia (I), *Diplostomum pseudospathaceum* cercariae (II) and *Flavobacterium* columnare pathogen (IV) (Table 1). During the exposures, fish were randomly allocated into 163 L flow-through tanks (except for 50 L tanks in the *Flavobacterium* experiment) with 2–8 replications. Dissection and examination of the gills of 5 individuals verified that the trout

were not previously infected by glochidia although fish in these rearing stations of Luke have no opportunity for FPM glochidia exposure.

The glochidia of FPM were collected by a non-destructive method, which was used earlier by Young and Williams (1984), Bauer (1987) and Salonen and Taskinen (2016). In brief, glochidia collection was performed by placing 30 adult FPM in plastic buckets in 5 L of river water for 30 min on the day of infection. Probably due to the stress of reduced oxygen concentration (Young and Williams 1984, Bauer 1987) or as a reaction to fluctuating water temperature (Hastie and Young 2001), the gravid mussels spawn their glochidia larvae within 30 min. After incubation, gravid mussels were restored to the river. Collected glochidia were quickly transported to the Konnevesi research station with aeration and additional water. This was done just before the natural spawning in early autumn so that fully matured glochidia could be obtained.

A. anatina glochidia were collected by dissecting marsupial gills from the gravid mussels. Lake water was added to the acquired glochidia mass and stirred to achieve glochidial suspension.

For collecting free swimming *Diplostomum* cercariae, first, intermediate host snail *Lymnaea stagnalis* were collected from Lake Konnevesi and kept at room temperature (+20 ^{o}C) in a separate plastic jar for 5 h. Then, the cercariae suspension was used during exposure to fish.

The *F. columnare* strain B549 was isolated from Lake Kuuhankavesi, in Central Finland, in 2013 and stored at −80 °C in solution containing 10 % fetal calf serum and 10 % glycerol. The strain was revived by culturing in modified Shieh medium (Song *et al.* 1988) at room temperature under agitation (120 rpm) overnight. The revived culture was further sub-cultured in the same conditions 3 times into a larger medium volume in a ratio of 1 part bacterial culture to 10 parts of fresh medium to obtain sufficient volume for fish infections.

During the exposure of fish to the glochidia (I–IV) or cercariae (II), water flow in the tank system was turned off and water volume was reduced to 70 L. After that, oxygenation was facilitated and the suspension of glochidia or cercariae was poured into the fish tank. Exposure time was 0.5–2 h while a plastic pipe was used to stir tank water to keep fish moving and to increase exposure of the fish to glochidia/cercariae. Simultaneously, control fish were handled similarly, except for the pouring of the suspension of glochidia/cercariae into their tanks. However, in the case of the *Flavobacterium* suspension, 80 L tanks were used with 50 L of water. Fish in 8 tanks were infected with 500 mL *F. columnare* suspension and the remaining 8 tanks of fish were exposed to 500 mL sterile modified Shieh medium without *F. columnare*.

In all the experiments, an effort was made to fulfil the key requirements for unbiased procedures for pre-infection and challenge infections of fish by mussel glochidia (see Taeubert *et al.* 2013). These included maintenance of experimental fish groups in identical conditions throughout the experiments, identical exposure of fish to glochidia, randomization and the use of a sufficient number of fish.

TABLE 1 The salmonid species and glochidia with their stocks used in the different experiments. First exposure of pathogens is referred to as pre-infection whereas the second exposure is referred to as challenge infection.

Article	Experiment	Fish & stock	Fish age	Pre-infection with glochidia (or other pathogens) & stocks	Challenge infection with glochidia (or other pathogens) & stocks
I	Cross-immunity	Brown trout; Iijoki stock	1+	*Anodonta*; Lake Koijärvi.	*Margaritifera*; Jukuanoja (the River Iijoki catchment)
I	Acquired immunity	Brown trout; Iijoki stock	0+	*Margaritifera*; River Jukuanoja (the River Iijoki catchment)	*Margaritifera*; Koivuoja (the River Iijoki catchment)
I	Acquired immunity	Atlantic Salmon; Iijoki stock	0+	*Margaritifera*; River Jukuanoja (the River Iijoki catchment)	*Margaritifera*; Luttojoki (the River Tuuloma catchment)
II	Pre-infected with glochidia	Brown trout; Iijoki and Rautalampi stock	0+	*Margaritifera*; Livojoki river	*Diplostomum*; Lake Konnevesi
II	Pre-infected with cercariae	Brown trout; Rautalampi stock	0+	*Diplostomum*; Lake Konnevesi	*Margaritifera*; Jukuanoja (the River Iijoki catchment)
III	Growth experiment	Brown trout; Rautalampi stock	1+	*Margaritifera*; River Jukuanoja (the River Iijoki catchment)	---
IV	Short experiment	Brown trout; Rautalampi stock	0+	*Margaritifera*; River Jukuanoja (the River Iijoki catchment)	*F. columnare* strain B549; Lake Kuuhankavesi
IV	Long experiment	Brown trout; Rautalampi stock	0+	*Margaritifera*; River Haukioja	*F. columnare* strain B549; Lake Kuuhankavesi

The success of infection was confirmed 3–5 d post-infection by dissecting and checking the gills (I–IV) or eyes (II) of the randomly collected fish using a

microscope except in IV after the *Flavobacterium* challenge, which was verified using plate culture. At the earliest opportunity, 2 weeks after infection, fish were marked using either fin clipping or PIT (Passive Integrated Transponder) tag and randomly re-allocated so that all the replicate tanks received an almost equal number of infected and control fish. Throughout the experiment, fish were fed commercial food pellets daily in the same amount in all cases except III, where two feeding levels were used to reveal the effects of feeding level.

2.3 Data collection

Data were collected in different time points in different experiments (I–III) based on the experimental design. Infected and control fish were randomly collected, killed either with overdose of MS–222 or with a sharp blow to the head and measured for the total length and fresh mass. Then, the gills were removed and glochidia were examined microscopically for the number and size (length from a subsample of 10 random glochidia per individual fish). In the case of the eye fluke (II), the number of eye fluke metacercariae was counted from the fish eyes.

In bacterial infection (IV), fish were monitored for signs of first morbidity at 1-h intervals, but after the first morbidity (upside down position of fish) was detected, the monitoring was continuous. Upon the detection of signs of morbidity, individual fish were anaesthetized with MS–222 and killed with a blow to the head. Bacterial samples were taken with a sterile loop from the gills of each dead fish and cultured on agar plates with modified Shieh medium and tobramycin for selective isolation of *F. columnare* (Decostere *et al.* 1997). To avoid contamination of the disease with the virulent *F. columnare* through the handling of diseased fish, all fish were immediately disposed of without length or weight measurements or examination of gills for glochidia. However, before the challenge infection, the abundance of FPM glochidia was checked with the naked eye before detaching from the gills as in Salonen and Taskinen (2017). In the case of short experiment, abundance and survival were not studied.

2.4 Data analysis

The effect of the previous infection (I) with either *A. anatina* (cross-immunity) or FPM (acquired immunity) on a mean number of glochidia in the gills and the mean size of glochidia was evaluated by ANOVA. In some cases, the hypothesis was also tested using more conservative non-parametric tests, e.g. Kruskall–Wallis and Mann–Whitney U test.

In fish pre-infected with glochidia, in the high dose treatment (II), the analysis began with a general linear model (GLM) where the eye fluke infection intensity was the response variable. Fisher's exact test was used to estimate

whether the prevalence of eye fluke infection in glochidia pre-infected fish was higher than in the control group. In the reciprocal experiment, where fish were pre-infected with the eye fluke in addition to GLM with a log link function and Gaussian error structure, post-hoc comparisons were carried out using a *glht* function in the 'multcomp' package (Hothorn *et al.* 2008). P-values were adjusted using Bonferroni corrections.

In the case of the effect of glochidia on the growth of the host (III), individual specific growth rate of fish was calculated in different periods from fresh weight of individual. The response variable was the tank- and period-specific difference in the average specific growth rate between FPM-infected and control fish individuals. A rough index of relative daily feeding per biomass unit was calculated for each tank and period as the daily feeding was constant throughout the experiment but the biomass in the tank changed due to growth and mortality.

In the bacterial experiment (IV), the effects of glochidial infection and possible tank effect were analysed using two-way ANCOVA to take into account the possible effect of fish size on survival time. The assumptions of linearity of the effect of covariate and homogeneity of regression slopes were also checked before using two-way ANCOVA. Multiple linear regression analysis was performed to assess how the number of glochidia (the intensity of glochidial infection) and fish size (weight) affect the survival time of the fish. The model assumptions – normality, homoscedasticity and independence of residuals – were met and no significant multicollinearity was found between these two independent variables.

3 RESULTS AND DISCUSSION

3.1 Salmonid–*Margaritifera* relationship

3.1.1 Effects on the growth of brown trout *Salmo trutta* (III)

The negative effect of FPM glochidia on the growth of brown trout was apparent even from the first monitoring period (from September to November 2015) and in every period separately, as well as for the entire experiment (Table 2). The tank-specific average specific growth rate was positively associated with the feeding regime (low/high food). However, neither the feeding regime nor the period had a significant effect on the difference in the growth between control and glochidia-infected fish. On average, the proportional increase in weight for control individuals was 11 % higher than that for infected fish during the experiment (III).

The negative effect of FPM infection on the growth of brown trout indicates the parasitic nature of FPM in terms of host growth as suggested before by Treasurer *et al.* (2006) and Ooue *et al.* (2017). Glochidia receive nutrients from the host fish (Fritts *et al.* 2013, Denic *et al.* 2015) and grow remarkably in size during the 11 month of the parasitic period (Hastie and Young 2001, Salonen and Taskinen 2017). These changes in the functionality of trout gills, as well as the reduced feeding efficiency revealed by Österling *et al.* (2014), may have contributed to the observed growth reduction caused by FPM.

Adverse growth-effect of FPM on the host fish was clearly revealed 11 weeks after the infection that lasted till the end of the experiment (10 months), indicating that the growth impairment began soon after the infestation of fish. Treasurer *et al.* (2006) found a negative growth-effect with Atlantic salmon, *S. salar* and FPM at 15 weeks post exposure but not later. They suggested that therapeutic treatment reduced glochidial abundance steeply. It probably also induced the disappearance of negative growth-effect after 15 weeks. In a study with masu salmon, *Oncorhynchus masou masou*, and *Margaritifera laevis* (Ooue *et al.* 2017), the weight of glochidia-infected fish was lower than that of control fish

after the parasitic period (70 d post-infection). The negative growth-effect was not visible during the parasitic period of *M. levis* that lasted up to 50 d. The reason of visibility of the reduced growth in infected fish found in the post-parasitic period may be due to the short parasitic period of *M. laevis*. Mean initial abundance of infection in studies by Treasurer at al. (2006) and Ooue *et al.* (2017) were close to 1,400 and 800 glochidia fish^{-1} respectively. These are clearly lower values than in III (beginning: 8,000 fish^{-1}; end: over 3,000 fish^{-1}), which may partly contribute to the less clear – although parallel – growth-effects of *Margaritifera* glochidia observed in those studies.

The feeding regime (60 and 36 g tank^{-1} day^{-1} in high and low food treatment respectively) of the host had an effect on the specific growth rate but did not have an effect on the level of growth-reduction induced by FPM glochidia on fish. As the fish grew, the relative feeding rate, in relation to the tank-specific biomass of the fish, decreased in both groups toward the end of the experiment. This may have intensified the competition for food and also caused a shortage of food in the high food group. Yet, the difference between glochidia-infected and control fish in the specific growth rate existed throughout the experiment, and, moreover, the study period had no significant effect on the difference in the growth between *Margaritifera*-infected and control fish, i.e. there was no observable interaction between the feeding rate and the negative growth-effect of FPM glochidia. However, there was a marginally significant difference in the abundance of FPM glochidia between the high food group and the low food group from the data of March 2016 (III). Thus, the low feeding rate may make glochidia detach prematurely, if the initial glochidial abundance is assumed equal in high and low food groups. However, this result should be considered preliminary and urges further studies. The sample size was low, and the experiment was not precisely designed to study the effect of the feeding regime on the growth or number of glochidia.

Ziuganov (2005) proposed that FPM glochidia could delay smoltification of the host salmonid. This would benefit the glochidia since, after smoltification, young salmon and trout migrate (with glochidia) to an unsuitable environment for juvenile FPM: the sea or a lake. Smoltification of brown trout and Atlantic salmon, the host fishes of FPM, is connected to the growth of fish so that within a population the fastest growing individuals smoltify earlier than slower-growing individuals (e.g. Økland *et al.* 1993). So, the negative growth-effect by FPM glochidia could result in delayed smoltification and postponed migration of the fish host from the river, thereby increasing the probability of FPM larvae to successfully develop and detach to a suitable habitat. Ziuganov (2005) provided evidence for a slower growth and higher age at smoltification in Atlantic salmon from a river (main channel of Varzuga River, Russia) with abundant FPM population compared to bayous of 5 rivers without mussels. In that sense, the negative effect on host growth could also be a sort of host manipulation by FPM, leading to a parasite-induced change in host behaviour in order to increase the success of the parasite (see e.g., Poulin 2010, Hughes *et al.* 2012).

TABLE 2 Different consequences from the relationship between *Margaritifera margaritifera* and its salmonid host fish.

M. margaritifera–salmonid relationship	Source
Negative effects on host fish	
1. Infection decreases growth of brown trout and Atlantic salmon.	Treasurer et al. 2006, III
2. Heavy infection impairs foraging, activity and dominance performance in juvenile brown trout.	Filipsson et al. 2016
3. Encystment of glochidia corresponds with the increasing haematocrit and metabolic rate in juvenile brown trout.	Filipsson et al. 2017
4. Heavy infection reduces swimming performance and causes mortality of brown trout.	Taeubert and Geist 2013
5. Infection causes spleen enlargement, thickening and lengthening of gill lamellae, reduction in mucous cells and respiratory burden to brown trout.	Thomas et al. 2014
6. Infection reduces the drift-feeding rate of juvenile brown trout.	Osterling et al. 2014
Immunity by fish host	
1. Brown trout and Atlantic salmon develop acquired immunity against *M. margaritifera* by lowering abundance and/or growth of glochidia.	Bauer 1987, Bauer & Vogel 1987, Treasurer et al. 2006, I

3.1.2 Acquired immunity in salmonid fish (*Salmo salar* and *S. trutta*) (I)

The results indicate that previous infection of brown trout with FPM had a significant effect on the number of glochidia but no effect on the size of glochidia when re-infected with FPM glochidia (I). Further, the previous infection of Atlantic salmon with FPM had a significant dose-dependent effect on the number and size of glochidia in the challenge infection (I). Salmon individuals exposed to a high dose of glochidia had significantly lower numbers and smaller sizes of glochidia than the low dose group or the control group but no significant difference was found between the low dose group and the control group (I) (Table 2).

In nature, it is highly probable that the same salmonid individual can be a host of FPM larvae in different years. It is even possible to happen more than

once in a salmonid individual before its smoltification. Thus, there might be potential adverse effects on the metamorphosis of FPM larvae due to the direct interference of antagonism as Dodd *et al.* (2006) found, even 12 months later in largemouth bass. In addition, Bauer and Vogel (1987) assumed an immunological memory from the serum factor with the decreased infection success in repeated infection in brown trout whereas, Treasurer *et al.* (2006) found a significant reduction of glochidia in previously infected Atlantic salmon at week 15. This result in (I) confirmed this finding as the number of FPM glochidia was lower 3 months after the challenge infection in the group that was previously (1 y earlier) pre-infected with FPM glochidia than in the control group that was not previously pre-infected with FPM. Similarly acquired immunity was found in Atlantic salmon, which is another suitable host (Salonen *et al.* 2016) of FPM.

A novel finding was the dose-dependency of acquired immunity in the FPM–salmonid host relationship (I): the higher the number of FPM glochidia in the pre-infection, the lower the number and the smaller the size of glochidia in the challenge infection with FPM glochidia. Therefore, the availability of 0+ aged (immunologically naïve) hosts is essential for the conservation of FPM.

3.2 Pre-infection with *M. margaritifera* glochidia

3.2.1 Vulnerability of brown trout to the eye fluke *D. pseudospathaceum* (II)

The pre-infection with FPM glochidia made fish more susceptible to the challenge infection with the eye fluke (high and low dose of cercariae), when compared with the control fish (II) (Table 3). This susceptibility of the pre-infected (with FPM glochidia) brown trout to the challenge infection with the eye-fluke was probably due to the increased respiration (Thomas *et al.* 2014) and ventilation rate. Taeubert and Geist (2013) showed that FPM infection induces a respiratory burden that presumably increases the ventilation of the host fish, thereby exposing them to the eye fluke cercariae (Mikheev *et al.* 2014) which use the gills of fish as their entrance into the body of the fish (Mikheev *et al.* 2014). In addition, the period when the challenge infections with the eye fluke cercariae were performed (June–July) was also the period when FPM detaches from the fish (Salonen *et al.* 2017) as juveniles. The rupturing of glochidial cysts typically damages gills. This may increase the vulnerability of fish to eye fluke cercariae. Earlier, the salmonid-*Margaritifera* relationship has been proposed to be neutral or mutualistic (e.g. Ziuganov *et al.* 1994, Ziuganov 2005), but this finding (II) provides evidence of a moderately antagonistic relationship. Hence, the results support the previous findings that brown trout infected with FPM glochidia perform generally poorer compared to uninfected fish (Taeubert and Geist 2013, Österling *et al.* 2014, Thomas *et al.* 2014, Filipsson *et al.* 2016, 2017).

Interestingly, the intensity of the glochidial infection did not correlate with the size of the fish, even though it usually does in parasites. However, this result agrees with a previous study on FPM (e.g. Thomas *et al.* 2014). Also, the intensity of the FPM glochidial infection did not make its host susceptible to the challenge infection by the eye fluke. This may result from uneven detaching of glochidia from the gills (Marwaha *et al.* 2017) and the damage to gill lamellae due to FPM pre-infection (Thomas *et al.* 2014) may vary between fish due to their different immune response. Therefore, pure glochidia numbers may not fully reflect the condition of the host respiratory system.

3.2.2 Resistance against *Flavobacterium* infection in brown trout (IV)

Notably, FPM glochidia provided protection against *F. columnare*, as the survival time of FPM glochidia-infected individuals was longer than that of the control fish, both in the short (fresh infections, glochidia of *Margaritifera*-attached to the gills of brown trout) and long (post-parasitic period when glochidia of *Margaritifera* had detached from the brown trout) experiments. In addition, in the long experiment, the higher the abundance of glochidia, the longer the brown trout survived. However, fish weight did not have any effect on the survival time or on the abundance of glochidia (IV) (Table 3).

All fish, both *Margaritifera*-infected and control, challenged with *F. columnare* died within 29 h, and the difference in the mean survival time between FPM infected and control fish was 1 h in both experiments (IV). Virulent *F. columnare* strains can cause 100 % mortality in juvenile salmonids within hours (Kunttu *et al.* 2009, 2012, Kinnula *et al.* 2017, Pulkkinen *et al.* 2018). Although the average survival time of FPM glochidia-infected fish was only 5 % longer than that of control fish in this experiment with a highly virulent bacterial strain, this can provide a substantial survival benefit with a less virulent pathogen or in less stressful (natural) conditions. Parasitic infections, in general, increase the risk of secondary infections by acting as a vehicle for the transmission of bacteria to the fish (Kotob *et al.* 2016) or by facilitating the challenge infection, e.g., in *Margaritifera*-infected fish to the eye fluke, trematodes (II). FPM glochidia have been shown to have a number of negative effects on salmonid fish hosts. Overall, the protective effect of FPM infection after the glochidia drop-off was unexpected, as the changes in gill structure caused by detaching glochidia can be assumed to increase vulnerability to secondary infections – especially to *F. columnare*, which enters the fish mainly through the gills (Declercq *et al.* 2013, 2015) – as metamorphosed glochidia rupture the gill epithelium when detaching (Waller and Mitchel 1989). Thus, it is safe to conclude at least that FPM glochidia infection does not increase the susceptibility of fish to *F. columnare*.

The mechanism leading to increased tolerance against *F. columnare* is not known. It could be enhancement of unspecific immunity of fish due to FPM infection. Alternatively, the structure of the gills may change due to FPM infection so that the entry of the bacterium through the gills or the establishment of the bacterium on the gills is decelerated. It is known that FPM

glochidia cause hyperplasia and fusion of gill filaments (Treasurer and Turnbull 2000, Thomas *et al.* 2014) and lessen the mucous cells of the gills (Thomas *et al.* 2014), but it is questionable whether these changes could increase resistance of brown trout to *F. columnare*. On the other hand, Kunttu *et al.* (2009) did not find any resistance against *F. columnare* in rainbow trout (*Oncorhynchus mykiss*) using immunostimulants, even though the applied treatments raised values of several parameters of innate immunity in fish. However, immunostimulation as an explanation for the current result cannot be rejected but requires further investigation. If the enhancement of unspecific immune defence is behind the effect, it means that the immunostimulating effect of FPM glochidia is long-lasting, as the exposure to *Flavobacterium* in the long-term (post-parasitic period) experiment took place 14 months after infection with glochidia and 3–4 months after detachment of glochidia from brown trout.

Ziuganov (2005) proposed that FPM can stimulate nonspecific resistance by exposure to air, thermal burn of gills and hook wounds and provide resistance against epitheliomata and cutaneous mycoses. Yet, Makhrov and Bolotov (2011) critically brought up the methodological shortcomings of Ziuganov's (2005) study, concluding that FPM infection does not affect the health of the *Salmo* host. Nevertheless, in theory, it is possible that the observed protective effect of glochidia is an adaptive feature of FPM to increase its own survival and fitness. Manipulation of the host's phenotype by parasites in order to increase their own fitness is a common phenomenon especially among trophically transmitted parasites (Poulin 2010, Hughes *et al.* 2012, Gopko *et al.* 2015, 2017). It is not unprecedented that parasites could enhance the immune defence of the host or in some other way impair the ability of a second parasite/microbe to enter the host (Ashby and King 2017), but why should the effect exist long after the glochidia have detached?

To conclude, the results (IV) agree with the Ziuganov's (2005) proposal of some beneficial effects of FPM infection on fish health. Resistance of the salmonid host against the harmful *Flavobacterium* pathogen by FPM can be added to the list of favourable ecosystem services provided by freshwater mussels. This should increase the attention and eagerness of commercial fish farmers and other stakeholders to contribute to conservation activities by infecting salmonids with FPM and turn public opinion in favour of FPM conservation since FPM may protect fish against a severe bacterial disease

It was shown (II) that FPM glochidia infection increases susceptibility of host fish to challenge infection with the eye fluke cercariae. Thus, it is evident that while FPM infection is protecting fish against one enemy (bacterium) it will expose the host to another enemy (trematode parasite). In addition, it remains unclear how deleterious these particular enemies are for the wellbeing of salmonids in their natural habitats. However, in fish farming, bacterial diseases, especially *F. columnare*, are clearly more harmful than diplostomiasis (Managing director Yrjö Lankinen, Savon Taimen Oy, personal communication).

TABLE 3 Different consequences from the relationship among *Margaritifera margaritifera*, its salmonid host fish and co-infectants.

Salmonid, *M. margaritifera* and co-infections	Source
Pre-infection with *M. margaritifera*	
1. Increases vulnerability of brown trout to *Diplostomum* infection.	II
2. Provides protection against *Flavobacteriun* infection in brown trout.	IV
Pre-infection with other parasites	
1. Recent exposure to *Diplostomum* (20 h ago) increases susceptibility of brown trout to *M. margarifera* glochidia.	II
2. Aged exposure to *Diplostomum* (2 weeks ago) does not increase susceptibility of brown trout to *M. margarifera* glochidia.	II
3. *A. anatina* infection does not influence susceptibility of brown trout to *M. margaritifera*.	Bauer 1991, I

3.3 Pre-infection with other parasites

3.3.1 Fish pre-infected with *Anodonta anatina* (I)

The results indicate that brown trout did not develop immunity against FPM due to the previous infection with *A. anatina* glochidia; neither the number nor the size of FPM glochidia was affected by previous *A. anatina* infection (I) (Table 3).

An acquired immune reaction that has developed against one parasite genotype may show cross-reaction and partially protect against other genotypes of the identical species (Rellstab *et al.* 2013) or even those of different species (e.g., Dodd *et al.* 2005, Karvonen *et al.* 2009). In challenge infection, only the first parasite relishes the slow activation of the adaptive immune system whereas the later arrival suffers the full costs of the acquired immunity (see Jackson *et al.* 2006, Hoverman *et al.* 2013, Klemme *et al.* 2016).

In nature, a potential adverse effect by *A. anatina* on FPM may occur due to cross-immunity as Dodd *et al.* (2005) found. However, the findings in I do not show any cross immunity between FPM and *A. anatina*. Moreover, experimentally pre-infecting brown trout by *A. anatina* did not lower the

number and size of glochidia significantly when challenged with FPM. This outcome was steady during the 9-month parasitic period of FPM. This is also in line with the findings of a short-term (35 d) experiment performed earlier by Bauer *et al.* 1991. It is conceivable that FPM (family Margaritiferidae) and *A. anatina* (family Unionidae) are immunologically so distinct that the antibodies generated against one species do not defend against the other species. Nevertheless, the acquired immunity is related to the dose of exposure (I). Thus, the prospect of cross-immunity can not be ruled completely out, for example, if brown trout is heavily exposed to *A. anatina* glochidia.

Thus, occurrence of *A. anatina* in the environment does not harm FPM in terms of increased resistance of the host fish against glochidia, even though these two mussel species can co-occur in the same river and can use the same host fish, brown trout.

3.3.2 Fish pre-infected with the eye fluke *D. pseudospathaceum* (II)

Fish pre-infected with the eye fluke differed in their susceptibility to the challenge infection with FPM glochidia depending on the length of time spent between pre-infection and the challenge infection (II). Fish pre-infected with the eye fluke just 20 h before the challenge infection with FPM glochidia was significantly more vulnerable to the FPM glochidia infection compared with the control fish. However, the fish that were pre-infected with the eye fluke 14 d prior did not differ in the success of the challenge infection with FPM glochidia. In this experiment, glochidial loads increased significantly with the increase of the fish weight, but fish weights did not differ between the treatments (II) (Table 3).

The higher level of FPM glochidial infectivity in fish with 20 h prior infection with eye fluke suggests that the eye fluke cercariae could induce temporary damage to gill epithelium or modify gill structure so that the succeeding challenge infection by FPM glochidia would be easier. Yet, this handicap seems to last only for a short period, being absent 14 d after exposure to the eye fluke. In addition, the eye fluke cercariae may need the first 24 h after the infection to move through fish host tissues to the eye lenses (Whyte *et al.* 1990), which can trigger an innate immune response (Wegner *et al.* 2007).

Therefore, organisms, which are defending themselves against two threats (the parasitic and predation threat) simultaneously, are able to allocate fewer resources to immune defence, when compared with organisms which are defending only against parasites (Rigby and Jokela 2000). Similarly, when two parasites (eye fluke and FPM glochidia) are infecting the same host, it is likely that both of them benefit, because the immune system of the host has to battle on two fronts simultaneously.

4 CONCLUSIONS

The success of restoration of the endangered FPM *M. margaritifera* is contributed by the success of glochidium larvae in the salmonid fish host. This study was able to show that exposure of host fish to glochidia of another mussel species, *A. anatina*, does not increase resistance against the glochidia of FPM. Thus, it can be concluded that *A. anatina* does not pose a threat to endangered FPM by cross-immunizing the fish, even though these two mussel species can co-occur and use the same host fish species. However, acquired immunity and its dose dependence were evident, emphasizing the significance of the availability of one-summer-old, immunologically naïve Atlantic salmon or brown trout for efficient conservation of FPM.

On the other hand, pre-infection with the eye fluke cercariae can make fish more susceptible to the challenge infection with FPM, but only shortly after cercarial infection. Thus, this knowledge could be used to enhance the success of captive glochidia infestation if the supply of either FPM glochidia or the host fish is insufficient and a higher glochidia infestation rate is needed.

In the study on the effects of FPM infection on the growth of the host, it was quantified, for the first time, that glochidia of freshwater mussels – at least FPM having a long parasitic period – can impair the growth of fish the host. This supports the view of the parasitic nature of FPM glochidia.

Finally, the contradicting results from the trematode (eye fluke) and bacterial (*F. columnare*) challenge increase our knowledge of the complex interactions in the multi-parasite system between mussels, their fish hosts and fish parasites/pathogens. The results indicate that FPM protects salmonids against *F. columnare* but makes it vulnerable to the eye fluke. As bacterial diseases are a challenge to fish farming, this finding should increase the interest and willingness of different stakeholders to participate in conservation activities involving the infection of salmonids with FPM (since it can defend fish against bacterial disease) and turn public opinion in favour of FPM conservation. However, if diplostomiasis is the problem in a given aquaculture unit or environment, the enhancing effect of FPM on eye flukes should be taken into account.

In a nut shell, this thesis enriches our knowledge on the *Salmo–Margaritifera* relationship from different angles, including infection and co-infection with different parasites and pathogens. This thesis provides evidence for the parasitic nature of FPM glochidia, but also supports the idea that FPM could increase survival of its fish host to increase its own success. The information provided in this thesis can potentially contribute to conservation measures for the endangered freshwater pearl mussel *M. margaritifera*.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Uhanalaisen jokihelmisimpukan, lohikalaisännän ja muiden loisten/patogeenien välinen suhde

Pisimmillään yli 200 vuotta elävä jokihelmisimpukka (raakku, *Margaritifera margaritifera*) kuuluu maailman pitkäikäisimpiin eläinlajeihin. Jokihelmisimpukan esiintymisalue kattaa Länsi-Euroopan Atlanttiin laskevat vesistöalueet Espanjasta Kuolan niemimaalle sekä Pohjois-Amerikan itärannikon pohjoisosat.

Kuten muutkin makean veden suursimpukat (Unionoida), jokihelmisimpukka suodattaa vedestä ravinnokseen kasviplanktonia ja eloperäisiä partikkeleita puhdistaen samalla vettä useita kymmeniä litroja vuorokaudessa. Suodattamastaan materiaalista se syö itse osan, ja loppu jää joen pohjalle ensin mikrobien ja sitten selkärangattomien käytettäväksi. Nämä selkärangattomat ovat puolestaan kalojen ravintokohteita. Simpukat myös mylläävät jalallaan joen pohjasedimenttiä, mikä lisää pohjasedimentin hapekkuutta.

Jokihelmisimpukka elää ensimmäiset kuukautensa loisena lohessa tai taimenessa. Syksyisin jokihelmisimpukan glokidium-toukat erittyvät veteen ja tarttuvat hengitysveden mukana lohen ja taimenen poikasten kiduksille, jossa ne kasvavat ja kehittyvät 10–11 kuukauden ajan. Alkukesästä ne pudottautuvat kalasta ja kaivautuvat syvälle joen pohjasoraan, jossa ne elävät ensimmäiset vuotensa. Tästä syystä jokiin huuhtoutuva hienojakoinen aines on merkittävä ongelma: liettyminen peittää sorapohjat, jolloin jokihelmisimpukan poikaset kuolevat. Jokihelmisimpukat saavuttavat sukukypsyyden 15–20 vuoden iässä.

Aikoinaan hyvin yleisenä ja runsaana esiintynyt jokihelmisimpukka on nykyisin erittäin uhanalainen. Vaikka esimerkiksi Suomi kuuluu yli sadalla raakkujoellaan maailman ”raakkurikkaimpiin” maihin, meilläkin lajin lisääntyminen on kestävällä tasolla alle kymmenessä joessa. Syitä uhanalaisuuteen (ja monien populaatioiden häviämiseen) ovat jokien perkaukset, metsä- ja suojitukset (liettyminen), lohikalakantojen romahtaminen patojen tai muiden vaellusesteiden rakentamisen tai kalastuksen seurauksena sekä rehevöityminen tai saastuminen (vaikutukset sekä raakkuihin että loheen ja taimeneen) kuten myös vieraslaji puronieriän leviäminen (ei sopiva kalaisäntä raakulle). Lajin rauhoittaminen vuonna 1955 lopetti helmenpyynnin, mutta se ei ole estänyt raakun kannalta vahingollisia elinympäristömuutoksia.

Vaikka raakku viettää vain murto-osan elämästään loisena kalan kiduksilla, on se kuitenkin tärkeä ja pakollinen vaihe lajin elämänkierrossa. Mitä paremmat olosuhteet glokidium-toukalla on kalaisännässään, sitä paremmat mahdollisuudet siitä kehittyvällä raakunpoikasella on selviytyä ja menestyä. Tästä syystä tässä väitöskirjassa tutkittiin kalaisännän ja raakun välistä suhdetta sekä vuorovaikutusta muiden kalaloisten/tautien kanssa.

Työn tavoitteena oli selvittää kokeellisin tutkimuksin, (i) mikä on raakkuinfektion vaikutus taimenen kasvuun ja syntyykö kalassa immuniteetti jokihelmisimpukan toukkia vastaan, (ii) vaikuttaako raakkuinfektio taimenen alttiuteen sairastua vaaralliseen, *Flavobacterium columnare* -bakteerin aiheuttamaan

lämpimänvedentautiin tai loiskaihia aiheuttavan *Diplostomum pseudospathaceum* -silmäloisen (Trematoda) tartuntaan sekä (iii) miten altistuminen toisen simpukkalajin (pikkujärvisimpukka *Anodonta anatina*) glokidium-toukille ja loiskaihin aiheuttajalle puolestaan vaikuttaa raakun toukkien infektiivisyyteen taimenessa.

Jokihelmisimpukkainfektio hidasti taimenten kasvunopeutta. Kymmenen kuukautta kestäneessä kokeessa, jossa glokidium-toukkien lukumäärä kalaa kohti oli alussa noin 8 000 toukkaa, raakkuloisittujen taimenten kasvu oli 11 prosenttia heikompaa kuin ei-loisittujen kontrollikalojen kasvu. Lisäksi jokihelmisimpukkainfektio indusoi taimenen ja lohen poikasissa immuunivasteen. Uudelleen infektoitaessa toukkamäärä jäi pienemmäksi aikaisemmin kertaalleen loisituiksi tulleissa kaloissa kuin ensikertalaisissa. Hankittu immuunivaste oli annosriippuvainen: mitä suuremmalla toukkamäärällä ensimmäinen infektointi oli tehty, sitä parempi oli kalan vastustuskyky raakun toukkia vastaan toisella infektointikerralla. Tämän tuloksen perusteella 0-vuotiaiden (eli immunologisesti naiivien) lohen/taimenen poikasten saatavuus on jokihelmisimpukan menestymisen kannalta ensiarvoisen tärkeää.

Raakun toukkien vaikutus alttiuteen saada muita loisia/tauteja oli vastakkainen altistettaessa taimenet bakteeritaudille ja loiskaihin aiheuttajalle. Raakkuinfektio suojasi kalaa *F. columnare* -bakteerilta ja vähensi bakteerin aiheuttamaa kuolevuutta taudinpurkauksen aikana – sekä akuutin glokidium-infektion aikana että vielä senkin jälkeen, kun jokihelmisimpukan toukat olivat jo irronneet kalan kiduksilta. Koska *F. columnare* -bakteerin reitti kalan elimistöön kulkee kidusten kautta, voivat niin ikään kiduksilla loisivat jokihelmisimpukan toukat heikentää bakteerin infektiivisyyttä joko suoraan tai muuttamalla kiduksen rakennetta bakteerin kannalta epäedulliseksi. Raakkuinfektio voi myös tehostaa taimenen epäspesifiä immuniteettia, mutta on epävarmaa, voisiko mahdollinen tehostava vaikutus näkyä kalassa vielä useita kuukausia sen jälkeen, kun glokidiot ovat irrottautuneet kalasta. Raakkuinfektio sen sijaan lisäsi taimenen alttiutta loiskaihin aiheuttajan, *D. pseudospathaceum* -silmäloisen kerkaria-toukille, jotka tunkeutuvat kalaan kidusten kautta. Koe tehtiin kesällä aikana, jolloin raakun toukat irrottautuvat taimenesta. Koska kalan kudos ympäröi glokidioita, niiden irrottautuminen aiheuttaa kudosvaurioita kiduksiin, mikä saattaa helpottaa loiskaihin aiheuttajien eli kerkaria-toukkien tunkeutumista kalaan. Vaikka jokihelmisimpukkainfektio lisäsi kalan alttiutta loiskaihin aiheuttajalle, *F. columnare* -bakteerin aiheuttama lämpimänvedentauti on esimerkiksi kalanviljelyssä merkittävä ongelma. Näin raakun suojaava vaikutus tätä patogeenia vastaan on myös vesiviljelyn kannalta mielenkiintoinen tulos. Bakteeritautia ehkäisevä vaikutus voi olla eduksi lajin hupenevien kantojen vahvistamisessa, jos lohen ja taimenen poikasistutuksiin käytettäisiin raakun glokidium-toukilla loisittuja istukkaita.

Toisen suursimpukkalajin, pikkujärvisimpukan toukille altistuminen ei lisännyt taimenen vastustuskykyä raakun toukkia vastaan, mikä viittaa siihen, että pikkujärvisimpukka ei aiheuta uhkaa jokihelmisimpukalle ainakaan taimenen immunisoitumisen muodossa. Altistuminen loiskaihin aiheuttajan toukille

ei myöskään lisännyt taimenen vastustuskykyä raakun toukkia vastaan, jos se tapahtui kaksi viikkoa ennen raakkuinfektiota. Jos loiskaihi-kerkarioille altistumisesta oli kulunut vain 20 tuntia, se lisäsi jokihelmisimpukan glokidiumtoukkien infektiivisyyttä. Vastikään tapahtunut loiskaihi-kerkarioiden invaasio taimenen kidusten läpi saattaa aiheuttaa kudosvaurioita ja lisätä raakun glokidioitten tarttumismahdollisuutta.

Väitöskirjan tulokset valaisevat jokihelmisimpukan, sen kalaisännän ja muiden loisten/tautien välisiä monimutkaisia vuorovaikutussuhteita. Koska jokihelmisimpukka on uhanalainen laji, kaikki tieto sen menestymiseen vaikuttavista tekijöistä voi olla avuksi lajin suojelussa. Vaikka tutkimuksessa ei tarkasteltu varsinaisia suojelusovellutuksia, voidaan tulosten perusteella arvioida ainakin 0-vuotiaiden (immunologisesti naiivien) lohikalojen saatavuuden olevan jokihelmisimpukan lisääntymisen kannalta kriittinen tekijä. Jokihelmisimpukan elinoloja voitaisiin parantaa monissa raakkujoissa lisäämällä lohikalojen poikasten määrää esimerkiksi kalojen vaellusesteitä poistamalla, kalateitä rakentamalla, lohikalojen kutu- ja elinalueita kunnostamalla tai istutuksin. Koska Suomessa lohikalakantoja hoidetaan paljon istutusten avulla, voisi raakun toukkien tartuttaminen istutettaviin lohen ja taimenen poikasiin olla vaihtoehto raakkukantojen vahvistamiselle alueilla, joissa raakku luonnostaan esiintyy. Tässä työssä havaittu raakkuinfektion suojaava vaikutus *F. columnare* -bakteeria vastaan voisi motivoida kalanviljelysektoria osallistumaan tämän kaltaiseen kokeiluun.

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ORIGINAL PAPERS

I

INTERACTION BETWEEN THE ENDANGERED FRESHWATER PEARL MUSSEL *MARGARITIFERA MARGARITIFERA*, THE DUCK MUSSEL *ANODONTA ANATINA* AND THE FISH HOST (*SALMO*): ACQUIRED AND CROSS-IMMUNITY

by

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Interaction between the endangered freshwater pearl mussel *Margaritifera margaritifera*, the duck mussel *Anodonta anatina* and the fish host (*Salmo*): acquired and cross immunity

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Abstract
The common duck mussel *Anodonta anatina* can live in sympatry with– and use the same host, brown trout (*Salmo trutta*) – as the endangered freshwater pearl mussel *Margaritifera margaritifera*. Since the glochidia release of *A. anatina* takes place seasonally earlier than that of *M. margaritifera*, brown trout can be sequentially exposed first to *A. anatina* and then to *M. margaritifera*. Cross immunity, an immune reaction induced in fish host against glochidia after the infection with glochidia of another mussel species, is possible. Thus, it was studied experimentally if brown trout can be cross immunized against *M. margaritifera* by earlier infection with *A. anatina*. In addition, the hypothesis that consecutive exposures of same glochidial species in different years in the same host may create acquired immunity was tested in brown trout against *M. margaritifera*. Furthermore, the dose dependence of acquired immunity against *M. margaritifera* glochidia in the Atlantic salmon (*S. salar*) was also studied. Cross immunity was not found; suggesting that occurrence of *A. anatina* does not pose a threat to *M. margaritifera*. Instead, acquired immunity and its dose dependence were evident, emphasizing the significance of availability of 0+ age group, immunologically naïve Atlantic salmon/brown trout for efficient conservation of *M. margaritifera*.

Keywords: Bivalves, Brown trout, Atlantic salmon, Conservation, Unionoida

Introduction

The freshwater pearl mussel (*Margaritifera margaritifera*) is a long-lived (Helama & Valovirta 2008), river dwelling bivalve mollusc, which occurs in Europe and North-East North America, but is now critically endangered throughout its range of occurrence (Geist 2010, Lopes-Lima et al. 2016). *M. margaritifera* has a larval stage, glochidium, which is parasitic on the Atlantic salmon (*Salmo salar*) and/or the brown trout (*S. trutta*) (e.g., Young & Williams 1984, Salonen et al. 2016). Some pearl mussel populations exclusively develop on Atlantic salmon (e.g., Ieshko et al. 2016) and others exclusively on brown trout (e.g. Geist et al. 2006). *M. margaritifera* fulfils the criteria of indicator, flagship, key stone and umbrella species, and can thus be considered an ideal target species for the conservation of aquatic ecosystem functioning (Geist 2010).

The duck mussel, *Anodonta anatina*, occurs commonly in lakes and rivers of Europe (Lopes-Lima et al. 2016). The glochidia of *A. anatina* are known to be able to complete their development on 15 fish species, including the brown trout (Bauer et al. 1991). Although the results by Bauer et al. (1991) suggest that the co-occurrence of both *M. margaritifera* and *A. anatina* in a river is not frequent, it is still possible. For example, the River Mustionjoki/Svartå (Finland) and the River Wye (UK) inhabit both mussel species (Lopes-Lima et al. 2016). As the glochidia shedding of *A. anatina* takes place seasonally earlier (winter-spring, as late as May–June, Taskinen et al. 1997) than that of *M. margaritifera* (summer–autumn, e.g., Salonen & Taskinen 2016), it is likely that brown trout can be sequentially exposed to the glochidia of *A. anatina* and *M. margaritifera*.

The immune defense system in vertebrates includes innate and acquired (adaptive, specific) components so that the acquired immunity is based on antibodies that bind to a specific antigen. In a repeated contact with the same parasite or pathogen, the immune reaction is quicker and stronger (memory) due to a faster antibody production and the antibody reserves left from the previous infection (Mutoloki et al. 2014). For example, fish hosts can develop an acquired immunity against the glochidia of unionoid mussels (Bauer and Vogel 1987, Rogers and Dimock 2003, Dodd et al. 2006, Treasurer et al. 2006). Sequential infection with parasites belonging to different (often closely related) species can also provide protection, which is called cross-immunity or cross-resistance. Thus, cross-immunity is a special type of acquired immunity. It has been shown, for instance, that infection with glochidia of one unionid mussel can result in immunity against glochidia of another unionid species (Dodd et al. 2005). As the parasitic stage can largely contribute to the reproduction success of the endangered *M. margaritifera*, the role of acquired immunity and cross immunity in conservation of *M. margaritifera* requires attention.

Bauer & Vogel (1987) showed that brown trout can eliminate *M. margaritifera* glochidia by both tissue and humoral reaction so that in repeated exposures to *M. margaritifera* glochidia the immunologic responses by brown trout against glochidia are strengthened, indicating an acquired immunity against *M. margaritifera*. In the case of *M. margaritifera*, lower infection rates have been found in older brown trout in the field or after second infection in the laboratory, suggesting acquired immunity (e.g. Bauer 1987, Ziuganov et al. 1994, Hastie & Young 2001). Nevertheless, even a higher infection success in the second infection has been recorded (see Wächtler et al. 2001). In spite of these contradictory results, the acquired immunity in salmonid hosts against *M. margaritifera* glochidia has not received much attention by experimental studies. For example, an acquired immune response would mean that the success of *M. margaritifera* would largely depend on immunologically naïve 0+ host fish in the environment – with important consequences for the conservation of the species.

The question remains whether the infection by *A. anatina* can cross-immunize brown trout against *M. margaritifera* glochidia such a phenomenon could pose a threat to declined *M. margaritifera* populations in rivers where these unionids live in sympatry. Nevertheless, this is still a widely under researched issue. Bauer et al. (1991) conducted an experiment by infecting brown trout first with *A. piscinalis* (= *A. anatina*) glochidia, and subsequently with *M. margaritifera* glochidia. No evidence for cross-immunity was achieved in this short-term (35 days) experiment. However, as the length of parasitic period of *M. margaritifera* can be even more than 300 days (Young & Williams 1984), a thorough evaluation of the likelihood and strength of this phenomenon should cover the whole parasitic period.

The aim of the present study is to investigate cross-immunity, i.e. whether the infection with glochidia of the duck mussel, *A. anatina*, will induce immunity against *M. margaritifera* in an experiment covering the whole parasitic period of *M. margaritifera*, as well as acquired immunity in salmonids hosts by *M. margaritifera*. The aim of the acquired immunity experiment is to examine the magnitude of acquired immunity in brown trout and the dose-dependent acquired immunity in Atlantic salmon against the glochidia of *M.*

margaritifera, i.e. whether the intensity of immunity depends on the number of glochidia to which fish are exposed. Our hypotheses are that (i) *Anodonta* infection induces cross immunity against *M. margaritifera* glochidia, (ii) Atlantic salmon and brown trout develop acquired immunity against *M. margaritifera* and that (iii) the acquired immunity is dose dependent.

Materials and Methods

In all the experiments, an effort was made to fulfil the key requirements for unbiased procedures for priming and challenge infections of fish by mussel glochidia (see Taeubert et al. 2013). These included e.g., maintenance of experimental fish groups in identical conditions throughout the experiments, identical exposure of fish to glochidia, randomization and sufficient number of replicate fish individuals.

Cross immunity experiment

A total of 300 brown trout fry (age group 1+, River Iijoki stock) were transported from the Taivalkoski fish farm of the Natural Resources Institute Finland (Luke) to Konnevesi research station, University of Jyväskylä, on May 23, 2012. Fish had not been exposed (hereafter 'exposed' and 'infected' are synonymous) to *M. margaritifera* glochidia in the fish farm. Dissection and examination of the gills of five individuals verified that the trout were not previously infected by glochidia. Fish were randomly allocated into four 163 L flow-through tanks with 100, 100, 50 and 50 individuals per tank. Two-hour exposure of trout with glochidia dissected from *A. anatina* (collected from Lake Koijärvi, eastern Finland), was performed on May 24, 2012, by decreasing water volume to 70 L and adding 12.3 x 10^4 and 7.1 x 10^4 glochidia to the two tanks with 100 and 50 trout, respectively. The two control tanks holding another 100 and 50 fish per tank received *A. anatina* gill extract suspension without glochidia. Water temperature during the priming infection with *A. anatina* was 7.7 °C.

Five brown trout from the 100-fish-tanks and three from the 50-fish-tanks from both primed and control groups were examined for glochidia five days post infection. All primed fish were infected and the number of *A. anatina* in primed fish varied from 90 to 232 glochidia $fish^{-1}$, indicating a successful priming infection. No glochidia were found from the control fish. On 15 August 2012, when the water temperature had increased to 15.7 °C (2.5 months post infection), one brown trout from each tank was examined and found uninfected, indicating that *A. anatina* glochidia had already excysted (see also Douda et al. 2013).

On 15 August 2012, fish were marked using fin clipping, and randomly re-allocated into four new 163 L flow-through tanks so that all tanks received both primed and control fish. In every other tank the primed fish were fin-clipped and control fish unclipped while in every other tank the primed fish were unclipped and control fish fin-clipped. The number of primed fish per tank varied from 21 to 36 whereas the number of control fish per tank varied from 21 to 62. Both the fin-clipped and unclipped fish were anesthetized using MS-222 before marking and handled similarly, except for the clipping.

Challenge infection with *M. margaritifera* glochidia was done two weeks after marking, on 28 August, 2012, with glochidia from the River Jukuanoja (the River Iijoki catchment), northern Finland. The 2-hour exposure was performed technically as in the priming infection above, by adding 3.0 x 10^5 glochidia to all the four tanks. Water temperature was 16.7 °C. Glochidia collection was performed by placing 30 adult *M. margaritifera* in plastic buckets in 5 L of river water for 30 min on the day of infection. The mussels were returned to the river after incubation. Timing of challenge infection was based on the previous knowledge that the River Jukuanoja *Margaritifera* release glochidia in the end of August (Salonen & Taskinen 2016).

Data were collected at four time points; September 2012 (3 weeks post infection), December 2012 (3 months), May 2013 (9 months) and June 2013 (10 months) (Table 1). Primed and control fish were randomly collected, killed with a sharp blow on the head, and measured for the total length and fresh mass. The gills were cut off and glochidia were examined microscopically for the number and size (length from a subsample of 10 random larvae), except for September sampling when only the right side gills were examined. Therefore, only the data for the right side gills were used in statistical analyses. Throughout the experiment, fish were daily fed with commercial food pellets. During this phase of the experiment the minimum and maximum temperatures were 1.1 °C and 16.8 °C, being the highest in September 2012 and June 2013.

Acquired immunity experiment

Testing of acquired immunity was performed for both of the salmonid host species of *M. margaritifera*, Atlantic salmon and brown trout. Both host species individuals (age group 0+) originated from the River Iijoki stock reared at Taivalkoski fish farm of the Natural Resources Institute Finland (Luke), from where they were moved to Konnevesi research station on August 21, 2012.

In the brown trout experiment, fish were first randomly allocated to primed vs. control groups in two separate 163 L flow-through tanks with 50 fish per tank. Priming infection of trout was performed on August 28, 2012, using similar methods and origin of *M. margaritifera* glochidia as in the cross immunity experiment, with 2.9 x 10^5 glochidia $tank^{-1}$. Control fish were not exposed to glochidia, but experienced otherwise the same treatment as the primed fish. Next year, in August 26, 2013, when age of the fish was 1+, adipose fins of the primed fish were cut, after which both the primed and control fish were put in one tank. On August 28, 2013, the fish were infected with *M. margaritifera* glochidia (2.3 x 10^5 glochidia $tank^{-1}$) collected from the River Koivuoja (the River Iijoki catchment), northern Finland. In November 25 (3 months post infection), all the fish were examined with the methods described above (Table 1).

Atlantic salmon were primed at the same time (August 28, 2012), with same origin of glochidia and with the same methods as brown trout mentioned above. Salmon were allocated to two tanks with three treatment groups in each, (1) primed with a high dose (8.8 x 10^5 *M. margaritifera* glochidia, tip of the right pectoral fin clipped), (2) primed with a low dose (1.7 x 10^5 *M. margaritifera* glochidia, tip of the left pectoral fin clipped), and (3) control group (not prime infected, adipose fin clipped) with 17, 14 and 13 fish per tank, respectively. After one year, in August 28, 2013, all salmon were infected with *M. margaritifera* glochidia collected from the River Luttojoki (the River Tuuloma catchment), northern Finland. The challenge infection was performed with 6.0 x 10^5 glochidia $tank^{-1}$. As brown trout above, all salmon were examined on November 25, 2013 (3 months post infection) (Table 1). Throughout the experiments, fish were daily fed with commercial food pellets. Due to lack of logistic supports, the dose-dependence experiment was not performed for brown trout.

Table 1. Different time points of infection and fish examination along with fish mortality throughout the experiments.

Experiment	Infection	Challenge Infection	Examination					Mortality
			Sep. 2012	Dec. 2012	May 2013	Jun. 2013	Nov. 2013	
Cross immunity in trout	May 2012	August 2012	20 Control + 20Infected	30 Control + 20Infected	29Control + 45Infected	61Control + 30Infected	--	20 fish in 13 month
Acquired immunity in trout	August 2012	August 2013	--	--	--	--	34 Control + 21Infected	25 fish in 16 month
Acquired immunity in salmon	August 2012	August 2013	--	--	--	--	33 high& 21low dose Infected + 22 control	6 fish in 16 month

Statistical analyses

The effect of the previous infection with either *A. anatina* (cross immunity) or *M. margaritifera* (acquired immunity) and other factors (month, dose, tank) on glochidia number in gills and the size of glochidia was analysed by ANOVA. If the tank effect was not significant, the analysis was reduced to the effect of other variables. If necessary, the response variables studied were transferred by Box–Cox-transformation {BCSN = $(N^{\lambda}-1)/\lambda$} to yield as normally distributed variable as possible within each treatment cell. In some cases the distribution within a treatment cell still deviated significantly from normal, which induced a tendency for incorrect rejection of H_0-hypothesis (bias for too low p-value). Therefore, if the H_0 was rejected ($p < 0.05$) the hypothesis was also tested using more conservative non-parametric tests (e.g. Kruskall–Wallis).Fish were not measured for length and weight at the time of exposure but when examined. Therefore, the number of *M. margaritifera* in the gills was not standardised based on the size of the fish as the individual growth rate after exposure, and consequently the size during exposure, was not known.

Results

Cross immunity experiment

Previous infection with *A. anatina* glochidia had no statistically significant effect (ANOVA) on the number of *M. margaritifera* glochidia (Fig. 1) or glochidium size (Fig. 2). Thus, brown trout does not develop any non-specific immunity that would decrease the success of *M. margaritifera* to parasitize them. Month had a significant effect (ANOVA and Kruskall–Wallis $p < 0.001$) on the response variables: the number of glochidia declined during the incubation period (Fig. 1) and their size (Fig. 2) increased.

Acquired immunity experiment

The previous infection of brown trout with *M. margaritifera* had a significant effect on the number of glochidia (Mann-Whitney U, $p < 0.001$) when re-infected with *M. margaritifera* glochidia (Fig. 3). Brown trout individuals earlier exposed to glochidia had significantly (Tukey and non-parametric pairwise test $p < 0.01$) less glochidia than the control (no exposure) group. No significant difference ($p > 0.05$) in the size of glochidia between the control and exposed group was found (Fig. 4).

Previous infection of Atlantic salmon with *M. margaritifera* had a significant dose-dependent effect on the number of glochidia (ANOVA and Kruskall-Wallis $p < 0.001$) when re-infected with *M. margaritifera* glochidia (Fig. 5). Salmon individuals exposed to high dose of glochidia had significantly (ANOVA with Tukey and Kruskall-Wallis with non-parametric pairwise test $p < 0.01$) less glochidia than the low dose group or the control group. The difference in the number of glochidia between the control and low dose treatment groups was not significant.

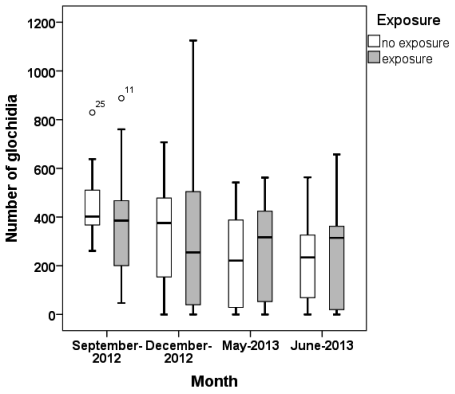

Figure 1. Box-plot showing the number of *M. margaritifera* in the gills of brown trout at different times after challenging with *M. margaritifera* on 28 August, 2012. No exposure = the fish were not exposed to *A. anatina* before challenging with *M. margaritifera*, exposure = the fish were exposed to *A. anatina* before challenging. The box indicates range between lower and higher quartile, the vertical line in the box is median and the whiskers indicate minimum and maximum values, excluding outliers (values deviating more than 1.5 interquartile ranges from the closest quartile).

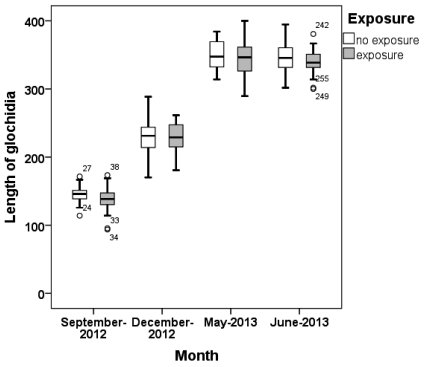

Figure 2. Box-plot showing the length (μm) of *M. margaritifera* glochidia at different times. No exposure = the fish were not exposed to *A. anatina* before challenging with *M. margaritifera*, exposure = the fish were exposed to *A. anatina* before challenging.

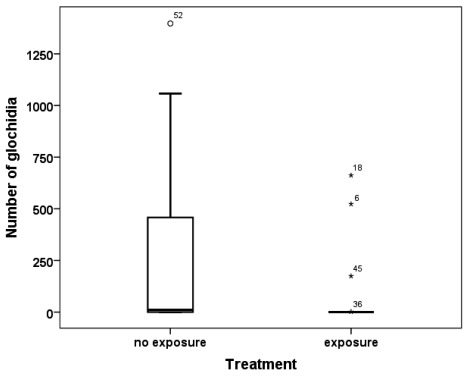

Figure 3. Box-plot showing the number (N) of *M. margaritifera* glochidia in the gills of brown trout in individuals previously not infected with *M. margaritifera* (no exposure) and infected with *M. margaritifera* glochidia (exposure).

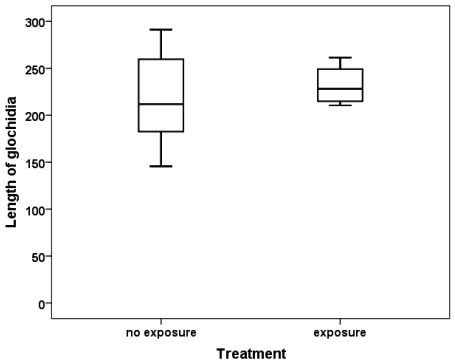

Figure 4. Box-plot showing the length (µm) of *M. margaritifera* glochidia in the gills of brown trout in individuals previously not infected with *M. margaritifera* (no exposure) and infected with *M. margaritifera* glochidia (exposure).

In addition, previous infection with *M. margaritifera* had a significant effect ($p < 0.05$) on the size of *M. margaritifera* glochidia when re-infected (Fig. 6). Glochidium size was significantly smaller in the high dose treatment than in the control group ($p < 0.05$). The tank effect was also significant with one tank having larger glochidia than the other of the two replicate tanks, presumably due to higher water temperature. Thus, previous infection with *M. margaritifera* led to both lower intensity of infection and poorer growth of *M. margaritifera* larvae when re-infected.

Discussion

In natural populations, individuals are usually infected not only by one but also with multiple parasitic species. Interaction between the co-infecting parasitic species within a host individual can be negative (antagonistic), leading – in extreme cases – to competitive exclusion (Holmes 1961). However, species can also be independent of each other or the interaction can be even positive (co-operation, facilitation) (Poulin 2001, Lello et al. 2004). Thus, the interaction between

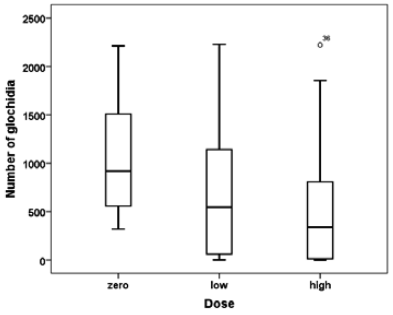

Figure 5. Box-plot showing the number (N) of *M. margaritifera* glochidia in the gills of Atlantic salmon in individuals previously not exposed to *M. margaritifera* (zero dose), exposed to a low number of *M. margaritifera* glochidia (low dose) and to a high number of *M. margaritifera* glochidia (high dose).

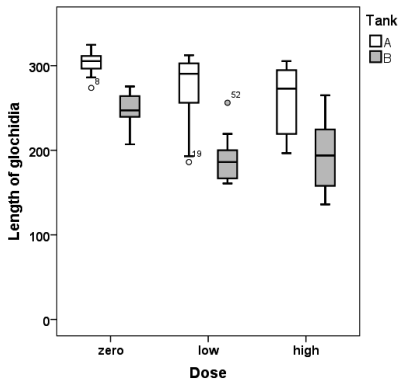

Figure 6. Box-plot showing the length (µm) of *M. margaritifera* glochidia in the gills of Atlantic salmon in individuals previously not infected with *M. margaritifera* (zero dose), infected with a low number of *M. margaritifera* glochidia (low dose) and with a high number of *M. margaritifera* glochidia (high dose).

A. anatina and *M. margaritifera* could also lead to one of these three possible outcomes. Because the earlier study by Dodd et al. (2005) showed that the previous infection of the host fish with the glochidia of *Lampsilis reeveiana* lowered the infection success of other unionid mussels, *L. abrupta*, *Villosa iris* and *Utterbackia imbecillis*, we hypothesised that the effect of *A. anatina* infection on *M. margaritifera* would be negative, or at most insignificant. We also hypothesised that if the effect of *A. anatina* infection on *M. margaritifer*a would be negative, it would be due to cross-immunity – representing so-called immune-mediated 'apparent competition' where one parasite species elicits an immune response which harms its competitors (see Read & Taylor 2001). In natural conditions, the possible negative impact of *A. anatina* on *M. margaritifera* could be also due to direct interference competition. *M. margaritifera* glochidia that occupy brown trout gills in autumn could be interfered by the glochidia of *A. anatina* in the spring when the glochidia shedding of the latter species takes place. However, in the present study, the exposure of brown trout was sequential so

that *A. anatin*a infection occurred earlier (in spring/early summer) and that of *M. margaritifera* started in autumn; glochidia of only one species was present in brown trout at a time.

The acquired (adaptive) immune system of vertebrates activates slowly, but brings a specific and long-lasting immunity against subsequent infections. However, the acquired immune response developed against one parasite genotype may be cross-reactive and provide protection against other genotypes of the same species (e.g., Rellstab et al. 2013), or even to those of different species (e.g., Dodd et al. 2005, Karvonen et al. 2009). From our point of view, in the parasite, two factors determine the importance of cross immunity in the case of multiple infections. First, probability of cross immunity decreases with the genetic distance between the infecting parasites strains/species (Read & Taylor 2001). Second, the sequence of infections influences on the relative benefits and costs of cross immunity. In sequential exposure, only the first parasite enjoys the slow activation of the adaptive immune system whereas the later arrival bears the full costs of the acquired immunity (see Jackson et al. 2006, Hoverman et al. 2013, Klemme et al. 2016).

The previous experimental study on the acquired immunity in brown trout fish host against the glochidia of *M. margaritifera* (Bauer & Vogel 1987) suggested acquired resistance; brown trout developed humoral immunological response and the infection success decreased in repeated infection. Our results verified this finding. Three months after the challenge infection the number of *M. margaritifera* glochidia was lower in individuals infected 1 y earlier with *M. margaritifera* glochidia than in the control group that were not previously exposed to *M. margaritifera*. Furthermore, evidence for acquired immunity was obtained not only in brown trout but also in the Atlantic salmon, indicating that both of the two suitable host fishes of *M. margaritifera* (see e.g., Salonen et al. 2016) are able to mount an acquired immune reaction against *M. margaritifera*.

In addition to the number of glochidia, the negative impact of previous infection on success of *M. margaritifera* was seen also in the growth rate of glochidia. When measured 3 months after the challenge infection, glochidia in previously infected fish were smaller than in control fish in the Atlantic salmon. The size of glochidium at the time of excystment from the fish host correlates with the survival rate of the juvenile *M. margaritifera* (Eybe et al. 2015). Thus, previously infected hosts produce less and lower quality juveniles than immunologically naïve hosts.

A novel finding in the present study was the dose dependence of acquired immunity in *M. margaritifera*-fish host relationship. The higher the number of *M. margaritifera* glochidia that the fish were exposed to in priming the lower the number of glochidia, and the smaller their size, after re-infection with *M. margaritifera* glochidia. Dose dependence was evident in Atlantic salmon but since the dose dependence has been earlier observed in immunization and vaccination of salmonids (Munag'andu et al. 2013, Ballesteros et al. 2015) it is reasonable to assume that the result can be extrapolated also to brown trout. Thus, the negative effect of the previous infection on both the number and the quality of *M. margaritifera* glochidia (and juveniles) depends on the density of glochidia in the previous exposure.

The acquired immunity could explain the previous contrasting findings of *M. margaritifera* infection rate with respect to host fish age. In some studies the infection rate has been lower in older host fish (Bauer 1987, Hastie & Young 2001) while in some studies the opposite was found (see Wächtler et al. 2001). For example, if production of glochidia does not take place every year in a particular *M. margaritifera* population, there can be years in which both the 0+ and 1+ age group fish are immunologically naïve with respect to *M. margaritifera* glochidia. In such a condition, the larger sized 1+ fish, due to their large gill area, are probably more intensively parasitized by *M. margaritifera* glochidia than the 0+ fish that is also supported by Geist et al. (2006). During the year that follows production of *M. margaritifera* glochidia, the negative effect of acquired immunity may override the positive effect of larger size among the 1+ age group fish, resulting in situation where the younger and smaller but immunologically naïve 0+ individuals are more heavily infected by *M. margaritifera* than the 1+ fish. The dose dependence of acquired immunity can strengthen this process.

Importantly, the acquired immunity emphasizes the importance of the availability of 0+ age group fish for *M. margaritifera* – and explains the association between the density of 0+ fish hosts and density of young *M. margaritifera* in the population (Bauer 1987). For the conservation of *M. margaritifera*, therefore, the availability of 0+ aged (immunologically naïve) hosts is essential. In other words, the acquired immunity would mean that the recruitment success to post-parasitic life stage of *M. margaritifera* could strongly depend on the abundance of immunologically naïve 0+ host fish in the environment.

Our results suggest that the cross immunity between *M. margaritifera* and *A. anatina* is not as important an impediment for the success and conservation of *M. margaritifera* as the acquired immunity. Brown trout primed with glochidia of the unionid mussel *A. anatina* did not harbor significantly lower number of glochidia when challenged with *M. margaritifera*. The pattern was consistent throughout the 9-month parasitic period of *M. margaritifera*. This is in line with the results of the short term experiment performed earlier: previous infection with *A. anatina* had no influence on the survival of *M. margaritifera* glochidia in brown trout within 35 d (Bauer et al. 1991). That study also showed that previous infection with another unionoid species, *Unio crassus*, had no influence on the survival of *M. margaritifera* glochidia in brown trout (Bauer et al. 1991). Together these results indicate that the exposure of host fish to glochidia of other mussel species would not pose a threat to the endangered freshwater pearl mussel, *M. margaritifera*. However, the present study shows that the immunity is related to the dose of exposure. Thus, possibility of such a cross immunity cannot be ruled completely out, for example, if brown trout is heavily exposed to *A. anatina* glochidia.

As hypothesized, acquired immunity and its dose dependence existed in *M. margaritifera*-host fish relationship. However, our experiment, as the previous study by Bauer et al. (1991) did not find evidence for cross immunity between *M. margaritifera* and *A. anatina* – contrasting the earlier study by Dodd et al. (2005) conducted between two Unionidae species. It is possible that *M. margaritifera* (family Margaritiferidae) and *A. anatina* (family Unionidae) are immunologically so distant that the antibodies produced for one species do not protect against the other species.

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II

INTERACTIONS BETWEEN TWO PARASITES OF BROWN TROUT (*SALMO TRUTTA*): CONSEQUENCES OF PREINFECTION

by

Mikhail Gopko, M. Motiur R. Chowdhury & Jouni Taskinen 2018

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Interactions between two parasites of brown trout (*Salmo trutta*): Consequences of preinfection

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Abstract

Preinfection by one parasitic species may facilitate or by contrast hamper the subsequent penetration and/or establishment of other parasites in a host. The biology of interacting species, timing of preinfection, and dosage of subsequent parasite exposure are likely important variables in this multiparasite dynamic infection process. The increased vulnerability to subsequent infection can be an important and often overlooked factor influencing parasite virulence. We investigated how the preinfection by freshwater pearl mussel *Margaritifera margaritifera* glochidia could influence the success of subsequent infection by the common trematode *Diplostomum pseudospathaceum* in brown trout *Salmo trutta* and *vice versa* whether preinfection by the trematode made fish more susceptible to glochidia infection. The first experiment was repeated twice with different (low and high) exposure doses to initiate the subsequent trematode infection, while in the second experiment we varied the timing of the preinfection with trematodes. The preinfection with glochidia made fish more vulnerable to subsequent infection with trematodes. Since the trematodes penetrate through the gills, we suggest that increased host vulnerability was most likely the result of increased respiration caused by the freshwater pearl mussel glochidia encysted on gills. In turn, brown trout preinfected with trematodes were more vulnerable to the subsequent glochidial infection, but only if they were preinfected shortly before the subsequent infection (20 hr). Fish preinfected with trematodes earlier (2 weeks before the subsequent infection) did not differ in their vulnerability to glochidia. These effects were observed at moderate intensities of infections similar to those that occur in nature. Our study demonstrates how the timing and sequence of exposure to parasitic species can influence infection success in a host–multiparasite system. It indicates that the negative influence of glochidia on host fitness is likely to be underestimated and that this should be taken into consideration when organizing freshwater pearl mussel restoration procedures.

KEYWORDS

community ecology, *Diplostomum*, experimental infection, freshwater pearl mussel, host–parasite interactions, multiple infections

1 | INTRODUCTION

Individuals of free-living organisms are usually infected by several parasite species. However, a majority of host–parasite studies are concentrated around single parasite and single host interactions (Rigaud, Perrot-Minnot, & Brown, 2010). This is partly because studies concerning multiple parasite species interactions are much harder to perform and interpret. Indeed, interactions between parasitic species within host organisms can take very different forms even in the simplest case when a host harboring one parasite is subsequently infected with another parasite (see Cox, 2001; Thomas, Adamo, & Moore, 2005; Cézilly, Perrot-Minnot, & Rigaud, 2014; Vaumourin, Vourc'h, Gasqui, & Vayssier-Taussat, 2015 for review and theoretical considerations).

Thus, the presence of certain parasitic species in a host can be more or less beneficial or deleterious to other parasites that subsequently attempt to infect that same host depending on the transmission ecology of the co-occurring parasite species and host-mediated effects (Cox, 2001; Thomas et al., 2005; Vaumourin et al., 2015). A parasite which has already entered a host organism, for example, can influence (directly or indirectly) the success of subsequent infections by other parasitic species (see Cox, 2001; Johnson, de Roode, & Fenton, 2015; Kotob, Menanteau-Ledouble, Kumar, Abdelzaher, & El-Matboulim, 2016; Pedersen & Fenton, 2007; Vaumourin et al., 2015). This influence may lead to a positive interaction, i.e., the presence of one parasite may facilitate subsequent infections by other parasites, or negative interaction between parasitic species (Hoverman, Hoye, & Johnson, 2013; Jackson, Pleass, Cable, Bradley, & Tinsley, 2006; Klemme & Karvonen, 2017; Vaumourin et al., 2015).

In general, mechanical damage and immunity are likely to be among the most important factors influencing the success of the infection in preinfected hosts (Pedersen & Fenton, 2007). Thus, in humans preinfection with herpes simplex virus type 2 causes damage to mucous membranes paving the way to HIV infection (Vaumourin et al., 2015 and references therein). In fish, similar mechanical lesions caused by the ectoparasitic crustacean *Argulus coregoni* increase host susceptibility to the pathogenic bacterium *Flavobacterium columnare* (Bandilla, Valtonen, Suomalainen, Aphalo, & Hakalahti, 2006). By contrast, the infection with one myxozoan species can prevent the subsequent invasion of other myxozoan species presumably due to cross-immunity (Kotob et al., 2016). Likewise, preinfection also can have no impact on subsequent infection as demonstrated by Karvonen, Seppälä, and Valtonen (2009) in their study of two species of trematodes from the genus *Diplostomum* infecting rainbow trout and by Chowdhury et al. (2018) in a freshwater pearl mussel study. Although the impact of preinfection by one parasite on the infection success of other parasites has been studied in fish (Kotob et al., 2016), these studies, mainly concerned interactions between microparasites. Preinfection studies with macroparasites have received far less attention. Kotob et al. (2016), for example, reviewed data on parasitic interactions in fish, but did not present any examples of macroparasite–macroparasite interactions (but see Karvonen et al., 2009).

Parasitic associations, i.e., positive or negative correlations between infection intensities, are rather commonly reported from wild hosts (Booth, 2006; Johnson & Buller, 2011; Karvonen et al., 2009; Pedersen & Fenton, 2007; Rigaud et al., 2010). In natural conditions, however, true interparasitic interactions are often masked by positively or negatively correlated coinfection (Johnson & Buller, 2011). Consequently, associations between two species can disappear or change from positive to negative, when a controlled experiment is performed (e.g., Johnson & Buller, 2011; Karvonen et al., 2009). However, it is interactions, rather than associations, among parasites that play an important role in structuring populations and communities of both hosts and parasites (Rigaud et al., 2010; Vaumourin et al., 2015). In addition, the order in which different parasitic species or genotypes attack and enter the host can influence the interaction between parasites within the host individual (Hoverman et al., 2013; Klemme & Karvonen, 2017; Read & Taylor, 2001; Telfer et al., 2010). Therefore, an experimental approach is needed to reveal, whether there is real interaction between parasites or, at least, evaluate the consequences of such interactions.

The freshwater pearl mussel, *Margaritifera margaritifera* is a freshwater bivalve critically endangered through its native range (Geist, 2010; Lopes-Lima et al., 2017). This bivalve has an obligate parasitic larval stage, the glochidium, which attaches to gills of salmonid fishes, such as Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) (Salonen, Marjomäki, & Taskinen, 2016; Salonen et al., 2017; Taeubert & Geist, 2017). The latter fish is the exclusive host for freshwater pearl mussel glochidia in many Central European populations (Geist, Porkka, & Kuehn, 2006; Taeubert & Geist, 2013). Often freshwater pearl mussel glochidia are considered to have a positive or, at most, a very weak negative effect on the host's health (Ziuganov, Zotin, Nezlin, & Tretiakov, 1994; Ziuganov, 2005; but see Taeubert & Geist, 2013). The latter is perhaps more likely to be true since freshwater pearl mussel glochidia demonstrate more than sevenfold growth in the course of 8–10-month development on the fish gills with a substantial nutrient transfer from fish to glochidia and a shift in the stable isotope composition during the mussel's development (Denic, Taeubert, & Geist, 2015). In addition, *M. margaritifera* glochidia cause a pronounced immune response in their hosts, increase metabolic rates and hematocrit levels and hamper swimming and respiratory performance in brown trout (Chowdhury, Salonen, Marjomäki, & Taskinen, 2018; Filipsson et al., 2017; Österling, Ferm, & Piccilo, 2014; Taeubert & Geist, 2013; Thomas, Taylor, & Garcia de Leaniz, 2014). Impairment of the respiratory capacity of host fish is especially important since gills can be a place where other infectious agents enter the host. It has been demonstrated that exposure of fish to pathogens and parasites increases with the ventilation rate (Mikheev, Pasternak, Valtonen, & Taskinen, 2014). Therefore, freshwater pearl mussel glochidia are likely to be a suitable predecessor for parasitic species infecting brown

trout through gills (i.e., increase their infection success). However, this assumption has never been tested experimentally. Moreover, to our knowledge, there were no previous studies, where the interaction between glochidia and other parasites was tested experimentally.

The eye fluke trematode *Diplostomum pseudospathaceum* is a ubiquitous species (Klemme & Karvonen, 2017) which is a common parasite of a wide array of fishes, such as many cyprinid and salmonid fishes including brown trout in natural environments (Betterton, 1974; Rolbiecki, Sciazko, & Schütz, 2009; Valtonen & Gibson, 1997). *D. pseudospathaceum* has three hosts in its life cycle (Seppälä, Karvonen, & Valtonen, 2004). Freshwater snails (often *Lymnaea stagnalis*) are the first intermediate hosts, freshwater fishes are the second intermediate hosts and fish-eating birds are the definitive hosts. Infected snails produce thousands of cercariae (the trematode's dispersal stage) which penetrate fish epithelium primarily through gills (Mikheev et al., 2014). Therefore, the fluke's infection success strongly depends on the amount of water pumped through fish gill chambers (Mikheev et al., 2014). After penetrating fish epithelium, *D. pseudospathaceum* moves toward the eye, the place of its final development into the metacercarial stage within the eye lens.

In contrast to *M. margaritifera*, *D. pseudospathaceum* is likely to be an unsuitable "predecessor" for other parasitic species. In the eye lens, flukes are unprocurable for the host immune system (Höglund & Thuvander, 1990; Wegner, Kalbe, & Reusch, 2007). Therefore, there is no need for them to suppress the host's immune system to avoid its negative influence on the parasite. Although such immunity regulation is known for numerous other parasitic species (Cox, 2001; Maizels & McSorley, 2016; McSorley, Hewitson, & Maizels, 2013), it is unlikely to be the case for *D. pseudospathaceum*. Moreover, Höglund and Thuvander (1990) suggested that *D. pseudospathaceum* nonspecifically enhances host immunity. Although specific antibody production was not recorded, they found that preinfection with *D. pseudospathaceum* induces some degree of protective immunity against subsequent infections with these parasites, suggesting some kind of nonspecific immunity enhancement caused by eye flukes (Höglund & Thuvander, 1990; see also Karvonen, Paukku, Seppälä, & Valtonen, 2005).

In our study, we sequentially infected brown trout with glochidia of the freshwater pearl mussel and cercariae of the eye fluke and vice versa. We hypothesized that fish preinfected with freshwater pearl mussel glochidia are more vulnerable to the cercarial infection by *D. pseudospathaceum* because glochidia can impair respiratory gas exchange and elevate resting respiratory rate of infected fish (Thomas et al., 2014), thereby increasing the amount of water (and cercariae) pumped through the gill chamber. To test this hypothesis we repeated the experiments twice using different (high and low) doses of cercariae during the infection. We also hypothesized that brown trout preinfected with *D. pseudospathaceum* would be less or similarly vulnerable to the subsequent freshwater pearl mussel glochidia infection because *D. pseudospathaceum* does not change or even enhances the performance of the host immune system. In this experiment we also manipulated the timing of preinfection with trematode (i.e., 20 hr vs. 2 weeks before the subsequent infection by glochidia). This took into account the possible host mediated effects because since an immune response may occur in fish soon after the infection *D. pseudospathaceum* is likely to cause in fish, whereas once it has migrated to the eye lens, it is "invisible" to the host's immune system (Höglund & Thuvander, 1990; Wegner et al., 2007).

TABLE 1 Experimental design. (a) Preinfection with *M. margaritifera* glochidia and (b) Preinfection with *D. pseudospathaceum*. Fish numbers (*n*) obtained after the exclusion of individuals with unclear infection state (see the text)

Preinfection with glochidia	Treatment	Exposure to cercariae	Fish mass, g, mean ± *SD*
(a) *M. margaritifera* → *D. pseudospathaceum* experiment			
Exposure dose = 5,000 glochidia/fish	Infected (*n* = 36)	High dose (300 cercariae/fish)	7.03 ± 2.17
	Control (*n* = 29)		
	Infected (*n* = 28)	Low dose (200 cercariae/fish)	20.10 ± 4.66
	Control (*n* = 55)		
	September, 2014	July, 2015	
Preinfection with cercariae	**Treatment**	**Exposure to glochidia**	**Fish mass, g, mean ± *SD***
(b) *D. pseudospathaceum* → *M. margaritifera* experiment			
Exposure dose = 250 cercariae/fish	Preinfected 20 hr before glochidial infection (*n* = 15)	Exposure dose = 1,500 glochidia/fish	21.58 ± 4.65
	Preinfected 2 weeks before glochidial infection (*n* = 48)		
	Control (*n* = 56)		
	August, 2015		

2 | MATERIAL AND METHODS

2.1 | Experimental infection procedures

2.1.1 | Fish preinfected with freshwater pearl mussel glochidia

Young-of-the-year brown trout (*Salmo trutta*) from the Iijoki (Finland) sea-run and Rautalampi (Finland) lake-run stock were collected from the Natural Resources Institute Finland (Luke) in Taivalkoski and the Luke in Laukaa (Finland), respectively, in the end of August 2014 and transported to the Konnevesi Research Station of the University of Jyväskylä, Finland. Fish from each stock were placed into four (altogether eight) 163-L flow-through tanks. Fish were acclimated in the laboratory for 3 weeks and then some were mass-infected with freshwater pearl mussel glochidia (exposure dose = 5,000 glochidia per fish) that originated from the Livojoki river (Table 1a). The procedure of the glochidia collection and exposure to fish was similar to Chowdhury et al. (2018). To collect glochidia we placed adult freshwater pearl mussels in plastic buckets filled with 5 L of river water for 30 min on the day of infection. The mussels were returned to the river after incubation and the glochidial suspension was transported to the Konnevesi research station.

Brown trout were mass-exposed to glochidia in maintenance tanks, where the water volume was reduced to 70 L and water-flow turned-off. A similar procedure (i.e. mass-exposure in 70 L of still water) was used in all experimental infections mentioned throughout the paper. Though individual exposure is sometimes recommended (Douda, 2013 and references therein), simultaneous exposure of the group of fish is commonly used in experimental parasitological practice (e.g., Gopko, Mikheev, & Taskinen, 2015, 2017; Seppälä, Karvonen, & Valtonen, 2008; Seppälä et al., 2004; Taeubert, Gum, & Geist, 2013; Taeubert, Martinez, Gum, & Geist, 2012). Such "mass-exposure" approach is especially logical when studying fish, which spend a substantial amount of time in shoals (Taeubert et al., 2013) as juvenile salmonids do (Brännäs, Jonsson, & Lundqvist, 2003; Hicks & Watson, 1985). The exposure time was 1 hr, average exposure dose ~5,000 glochidia per fish, and water temperature was 14.2°C. Control animals were treated identically to preinfected fish with the exception that instead of cercariae they were exposed only to water. These methods were used during all infection procedures.

After exposure, brown trout were marked using fin clipping and randomly reallocated according to their original stock so that all eight tanks received a similar amount of infected and control fish. In four tanks the right fin was notched in control fish, while the left fin was notched in fish exposed to glochidia. In four other tanks, opposite fins were notched in control and infected fish. Though notching can cause an increased immune response in fish, this effect is likely to be relatively short term. Henrich, Hafer, and Mobley (2014) found out that the effect of spine clipping on host immunity became indistinguishable 2 weeks after the procedure. In our study, we marked fish 3 weeks after the exposure to glochidia (i.e., 9 months before the exposure to *D. pseudospathaceum* cercariae). Therefore, it is unlikely that fin notching could have had a substantial effect on the success of trematode infection. In addition, fish were fin-notched both in infected and control groups.

Following Taeubert et al. (2012, 2013) five fish from each tank were sacrificed randomly 3 days postexposure to check for infection success. We found successful infection (ranging from 19 to 782 glochidia per fish with mean ± *SD* = 313.0 ± 239.3) in glochidia exposed fish, while fish from the control group had no glochidia. Fish were subsequently maintained for 10 months in four similar 163-L round flow-through tanks. Throughout the experiment control fish were treated identically to infected fish.

In June–July 2015 brown trout from two randomly chosen tanks with fish from each stock were exposed to *D. pseudospathaceum*. The fish were exposed in summer to make experimental conditions synchronous with their natural seasonal occurrence. In Finland detachment of *M. margaritifera* glochidia from fish gills happens in June–July (Salonen & Taskinen, 2017). Therefore, damage caused to fish gills by glochidial excystment and the increase in host vulnerability to subsequent infections is likely to be most pronounced in this period of the year. In addition, snails infected with *D. pseudospathaceum* are starting to produce cercariae actively when water temperatures are above 10°C (Lyholt & Buchmann, 1996; Voutilainen, Taskinen, & Huuskonen, 2010). Therefore, in boreal ecosystems of the northern hemisphere the probability of interactions between *M. margaritifera* glochidia and *D. pseudospathaceum* eye flukes within hosts is especially high in summer.

The exposure procedure was generally similar to Gopko et al. (2015). In brief, brown trout were exposed to the mixture of cercariae shed by 10 freshwater snails *Lymnaea stagnalis*. In our study, we did not identify parasites using molecular methods. However, previous studies showed that *L. stagnalis* snails are typically infected with *D. pseudospathaceum* (e.g. Rellstab, Louhi, Karvonen, & Jokela, 2011; Selbach, Soldánová, Georgieva, Kostadinova, & Sures, 2015). For instance, in Finland (including Lake Konnevesi) *L. stagnalis* is infected with *D. pseudospathaceum* (Louhi, Karvonen, Rellstab, & Jokela, 2010; Rellstab et al., 2011).

Cercariae used in our experiments were no older than 5 hr. The exposure time was 30 min which is similar to previous studies (e.g., Gopko et al., 2015, 2017; Klemme & Karvonen, 2016, 2017; Mikheev, Pasternak, Taskinen, & Valtonen, 2010; Mikheev et al., 2014; Seppälä et al., 2004). During the exposure water levels in each tank were decreased to 70 L and water flow was turned off. After the exposure, water flow was turned on and water volumes in the tanks were again increased to 163 L.

We repeated the experiment twice with a two-week interval. In first two (out of four) randomly chosen tanks, fish from Iijoki stock were exposed to the low infection dose (200 cercariae per fish) at 12.5°C. Such a dose, however, resulted in lower metacercariae numbers in fish eye lenses than we planned (some fish were uninfected) (see Section 3). Therefore, we repeated the experiment with a higher doses of cercariae during the exposure (300 cercariae per fish, 12.8°C). In this high dose experiment we used fish from the Rautalampi stock.

After the *D. pseudospathaceum* infection (on 7th and 12th days in low and high dose infection experiment, respectively), fish were killed with an overdose of 0.01% MS 222 (Sigma Chemical Co., St Louis, U.S.A.), weighed and dissected. It is improbable that a small difference in the periods between infections and dissections influenced the results of dissections. This is because the *Diplostomum* metacercarial mortality rate within host lenses is likely to be extremely low, while the life span is very long, with a maximum of 4 years (Klemme & Karvonen, 2017; Shigin, 1980). The number of *D. pseudospathaceum* metacercariae and freshwater pearl mussel glochidia were counted using a dissection microscope (32× magnification).

2.1.2 | Fish preinfected with *D. pseudospathaceum*

A reciprocal experiment (brown trout preinfected with eye flukes and then infected with glochidia at 14.2°C) was conducted in August 2015. One hundred and twenty young-of-the-year brown trout, "Rautalampi" stock, were obtained from the Natural Resources Research Institute (Luke) fish farm at Laukaa (Table 1b). Exposure procedures were similar to the described above. The exposure dose was 250 cercariae per fish. Fish were divided into three groups: (a) exposed to cercariae 2 weeks prior to the glochidia infection, (b) exposed 20 hr prior to the glochidia infection and (c) control fish exposed only to lake water). The 20 hr group was added because eye flukes usually need 24–48 hr to migrate to the host eye lens. Within-host migration is the only period when the parasite is vulnerable to the fish's immune system (Höglund & Thuvander, 1990). Then all fish were infected with the glochidia of the freshwater pearl mussel. The exposure dose of glochidia infection was 1,500 glochidia per fish and an exposure time of 1.5 hr. Glochidia were collected from Jukuanoja, a tributary of the Iijoki River and were transferred to Konnevesi research station on 31 of August 2015. Fish were dissected on the 13th day after the infection with trematodes. Metacercariae and glochidia were counted using a dissection microscope (32× magnification).

Throughout the experiments, fish were daily fed with commercial food pellets (1.5 mm size, Nutra Parr LB, Norway). The experiments were conducted with the permission of the Centre for Economic Development, Transport and Environment of South Finland (license number ESAVI/6759/04.10.03/2011).

2.2 | Statistical analysis

During dissections we found that not all fish marked as preinfected with freshwater pearl mussel really had glochidia on their gills. It could be a result of unsuccessful infection or active dropping off the host gill by the mature parasites. The latter is more likely because the freshwater pearl mussel glochidia prevalence in experimental infections usually is close to 100% (e.g., Taeubert & Geist, 2013). By contrast, the duration of glochidial development on the fish gills is highly variable (from 3 weeks to 10 months) and depends on several factors including parasites' compatibility with the host (Geist et al., 2006; Marwaha, Jensen, Jakobsen, & Geist, 2017). Since we could not estimate how much time passed since glochidia dropped off the gills, those fish that were marked as infected, but did not have any glochidia on their gills were considered as being of unknown status and were excluded from the subsequent analysis (23 and 44 fish in low and high dose trematode infection treatment, respectively). However, when these fish are included in the analyses, statistical effects remain significant.

R software was used for all statistical analyses (R Core Team, 2017). Plots were drawn using the "ggplot2" package (Wickham, 2009).

In fish preinfected with glochidia, we started with a general linear model (GLM) where the Box–Cox transformed *D. pseudospathaceum* infection intensity was a response variable, infection state (preinfected with glochidia or not) and experiment (low vs. high exposure dose) were factors and fish mass was a covariate. Response variable transformation was needed since the residuals' distribution of our model strongly violated the normality assumption (Shapiro–Wilk (S–W) test, $W = 0.928$, $p < 0.001$). After the transformation, data distribution became close to normal (S–W test, $W = 0.984$, $p = 0.09$). Since experiments with low and high exposure doses were conducted with a time lag and can be considered independent, we also fitted separate models for each experiment. In the high dose treatment, we started with a general linear model (GLM) where the *D. pseudospathaceum* infection intensity was the response variable, infection state was a factor and fish mass as a covariate. However, mass did not have a significant influence on the eye fluke infection intensity and was excluded from the final model. A residual values distribution did not differ from normal (S–W test, $W = 0.971$, $p = 0.13$).

In the low dose treatment, the Shapiro–Wilk test showed that even after transformation there was a violation of the normality assumption in our data due to several obvious outliers seen on the Q–Q plot (S–W test, $W = 0.944$, $p = 0.001$). Therefore, we had to fit a robust regression based on M-estimator with Huber's weights, with the tuning constant $k = 1.345\sigma$ using MASS package in R programming software (Venables & Ripley, 2002). The f.robtest function in the sfsmisc package was used to compute robust *F*-test and get *p*-values (Maechler, 2017). The square root transformed intensity of the *D. pseudospathaceum* infection was a response variable. The Fisher's exact test was used to estimate whether the probability of getting infected in preinfected fish was higher than in the control group.

In the reciprocal experiment (where fish were preinfected with *D. pseudospathaceum*), we used a generalized linear model with a log link function and Gaussian error structure, where the glochidial infection intensity was the dependent variable, infection state was a factor and fish mass was a continuous predictor. Post hoc comparisons were done using a *glht* function in the "multcomp" package (Hothorn, Bretz, & Westfall, 2008). *p*-Values were adjusted using Bonferroni corrections.

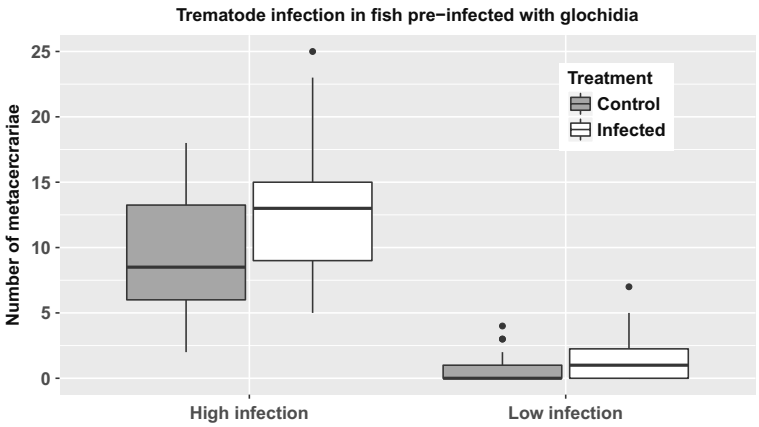

FIGURE 1 Both in the low and the high cercaria dose treatment, brown trout preinfected with freshwater pearl mussel glochidia were more vulnerable to infestation by *D. pseudospathaceum* trematode cercariae than control fish. The "box" represents the interquartile range (IQR) of the *D. pseudospathaceum* metacercaria infection intensities within groups with median (black line). Whiskers extend from the highest to lowest values within 1.5*IQR. Suspected outliers, i.e., all observations lying outside 1.5*IQR, are shown as dots

TABLE 2 The GLM demonstrated that there were significant effects of treatment (preinfection with glochidia), fish mass and exposure dose on the success of the subsequent *D. pseudospathaceum* infection (Box–Cox transformed values). Preinfected fish were more vulnerable to the infection compared with controls. In addition, bigger brown trout had lower eye fluke infection intensity than smaller ones. As expected lower exposure dose of trematode's cercariae result in lower infection intensities in brown trout

Source	Estimate	*SE*	*t*-Value	*p*-Value
Treatment (infected)	0.323	0.082	3.913	0.0001
Mass	−0.031	0.011	−2.874	0.0047
Experiment (low exposure dose)	−1.586	0.160	−9.944	<0.0001

3 | RESULTS

3.1 | Fish preinfected with freshwater pearl mussel glochidia

There was a significant effect of the preinfection with glochidia on the intensity of the subsequent eye-fluke infection (Table 2; Figure 1). As hypothesized, fish preinfected with glochidia were more vulnerable to the subsequent infection with trematodes than control fish (Figure 1). The effect of the covariate fish mass was statistically significant (Table 2), indicating that the intensity of the trematode infection decreased with host size. Finally, as hypothesized, higher exposure dose of cercariae resulted in higher infection intensities in brown trout (Table 2, Figure 1). The effects of interactions were nonsignificant and therefore interactions were excluded from the final model.

If low and high dose treatments were analyzed separately, the effect of the glochidial preinfection on the success on subsequent trematode infection was still significant. In the high dose treatment (fish infected with a high dose of cercariae) all brown trout ($N = 64$, fish mass mean ± *SD* = 7.03 ± 2.17 g) were successfully infected with *D. pseudospathaceum* metacercariae (range 2–25). In the high dose treatment, the mean intensity of *D. pseudospathaceum* infection in fish preinfected with freshwater pearl mussel glochidia was significantly higher compared with the control group (GLM ANOVA, $t = 2.7$, $p = 0.008$; Figure 1). When included in the model, fish mass did not significantly influence the *D. pseudospathaceum* infection intensity ($p > 0.6$). Weights of control and preinfected brown trout were similar (GLM ANOVA, $t = 0.03$; $p = 0.98$).

In the low dose treatment (fish infected with a low dose of cercariae) only 28 brown trout out of 83 ($N = 83$, fish mass mean ± *SD* = 20.10 ± 4.66 g) were infected with the eye fluke. In the low dose treatment, the probability of being infected was significantly higher in preinfected fish compared with the control group (Fisher's exact test, $p = 0.016$).

The robust regression based on M-estimation showed that in the low dose experiment both preinfection with glochidia and fish mass significantly influence the *D. pseudospathaceum* infection intensity. Fish preinfected with *M. margaritifera* glochidia were significantly more vulnerable to the subsequent infection with *D. pseudospathaceum* trematode compared with control fish (Robust $F = 7.113$, $p = 0.009$). The infection intensity of *D. pseudospathaceum* decreased with the fish size in the low dose experiment (Robust $F = 10.47$, $p = 0.002$). Fish mass (mean ± *SD* = 21.58 ± 4.65 g) was similar in control and preinfected fish (GLM ANOVA, $t = 1.25$; $p = 0.22$).

Surprisingly, in both treatments there was no significant relationship between the intensity of glochidia preinfection and the intensity of *D. pseudospathaceum* subsequent infection, when only preinfected fish were taken into consideration (Pearson's correlation coefficient, $r = -0.13$, $p = 0.53$, $N = 28$ and $r = 0.30$, $p = 0.12$, $N = 28$, high and low dose treatment, respectively). There was also no significant correlation between the fish mass and the glochidia infection intensity ($r = 0.19$, $p > 0.33$, $N = 28$ in the high dose treatment and $r = 0.15$, $p = 0.47$, $N = 28$ in the low dose treatment). When the two datasets (glochidia-infected fish from high and low dose treatment) were merged and possible confounding variables (fish mass and treatment) were taken into account, the result was similar (see Supporting Information Table S1). Intensities of the glochidial infection were (mean ± *SD*) 376.5 ± 275.3 and 438.7 ± 286.7 glochidia/fish in low and high dose treatment respectively.

TABLE 3 Results of the GLM and subsequent post hoc comparisons examining the effect of the preinfection with *D. pseudospathaceum* on brown trout vulnerability to subsequent freshwater pearl mussel glochidia infection in brown trout

Source	Estimate	*SE*	*t*-Value	*p*-Value
Results of generalized linear model				
Treatment 1 day	0.202	0.075	2.69	0.008
Treatment 14 days	0.030	0.058	0.52	0.602
Mass	0.116	0.023	5.10	<0.0001
Comparison	**Estimate**	***SE***	***z*-Value**	***p*-Value**
Multiple comparisons				
20 hr–14 days	−0.172	0.076	−2.25	0.073
Control–14 days	−0.030	0.058	−0.52	1.000
Control–20 hr	0.202	−0.075	−2.69	0.022

3.2 | Fish preinfected with *D. pseudospathaceum*

The GLM followed by post hoc comparisons showed that fish preinfected with *D. pseudospathaceum* 20 hr before the subsequent infection with *M. margaritifera* glochidia were significantly more vulnerable to the glochidial infection compared with control fish (Figure 2, Table 3). On the other hand, fish preinfected with *D. pseudospathaceum* cercariae14 days prior to the infection with glochidia had glochidial loads that did not differ significantly from those of the control fish. Glochidial loads significantly increase with the increase of the fish weight (Table 3). Fish weights did not differ between the treatments (ANOVA $F_{2,116} = 0.097$; $p = 0.91$). The intensities of glochidial infection were moderate (mean ± *SD* glochidia/fish was 138.9 ± 41.5 in 20 hr earlier preinfected, 119.4 ± 36.8 in 14 days earlier preinfected and 114.6 ± 38.9 in control fish respectively, Figure 2, see also Supporting Information Figure S1).

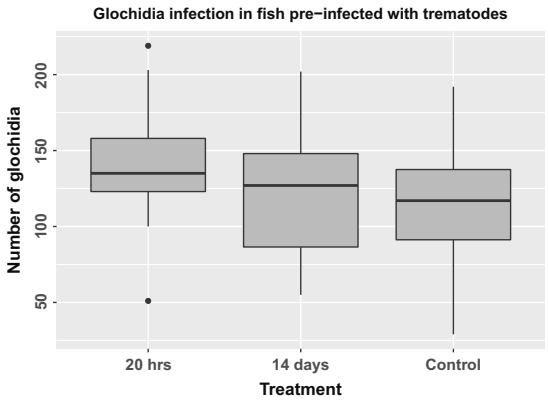

FIGURE 2 Fish preinfected with the *D. pseudospathaceum* were more vulnerable to the freshwater pearl mussel glochidia infection compared with control fish, when the preinfection took place 20 hr before the exposure to glochidia. Two weeks after preinfection, however, this pattern did not occur. For an explanation of the box, vertical line and whiskers, see Figure 1. Suspected outliers, i.e., all observations lying outside 1.5*IQR, are shown as dots. These points were included in the analysis, but when a robust regression was used, results of statistical tests remained similar (see the Supporting Information Table S2)

4 | DISCUSSION

The results of our study indicate that the infection with glochidia of the freshwater pearl mussel can predispose fish to a concomitant infection with other parasitic species. Brown trout preinfected with freshwater pearl mussel glochidia were infected with more *D. pseudospathaceum* during the subsequent experimental infection compared with control fish. We repeated the experiment with two different doses of cercariae during the subsequent infection and both times the result was similar. In addition, in the low dose treatment—i.e., when a low dose of cercariae was used in the subsequent infection—fish preinfected with glochidia were more likely to become infected with *D. pseudospathaceum* than the control fish. The numbers of freshwater pearl mussel glochidia in preinfected fish (~400 glochidia per fish on average) were within the range of glochidia numbers found in natural salmonid populations (e.g., Salonen & Taskinen, 2017; Ziuganov, 2005), thereby providing a close biological relevance to the experimental findings.

Previous studies have demonstrated that freshwater pearl mussel glochidia constitute a respiratory burden for the infected fish (Thomas et al., 2014). In turn, *D. pseudospathaceum* cercariae enter their hosts mainly through gills and increased ventilation rate also increases infection rate of *D. pseudospathaceum* infection in fish (Mikheev et al., 2014). Therefore, an enhanced ventilation rate in fish infected with glochidia is likely to be a plausible mechanism explaining their higher vulnerability to the *D. pseudospathaceum* infection. In addition, June–July, when subsequent infections with *D. pseudospathaceum* cercariae were performed, is the period when freshwater pearl mussel glochidia detach from fish (Salonen et al., 2017)—an event that typically damages gills as glochidial cysts rupture. This should further increase the vulnerability of fish to *D. pseudospathaceum* cercariae.

It has been proposed earlier that the relationship between *M. margaritifera* glochidia and fish host is neutral or mutualistic

(e.g., Ziuganov, 2005; Ziuganov et al., 1994), but our results provide evidence that the relationship at least moderately antagonistic. Therefore, our data is consistent with previous studies, which demonstrated that brown trout infected with freshwater pearl mussel glochidia perform generally worse compared to uninfected fish (Filipsson et al., 2017; Österling et al., 2014; Taeubert & Geist, 2013; Thomas et al., 2014). This is in line with the fact that freshwater pearl mussel glochidia grow intensively on the fish gills and obtain nutrients from their hosts (Denic et al., 2015). However, Ziuganov (2005) found lower prevalence of saprolegniosis in Atlantic salmon infected by freshwater pearl mussel glochidia than in noninfected salmon and suggested an increase in nonspecific resistance in fish host caused by freshwater pearl mussel glochidia infection. Our results do not rule out this possibility, but indicate that either the proposed immunity enhancement does not include macroparasites, such as trematode cercariae, or that negative effects of gill damage due to detaching glochidia override a possible positive effect of the immune enhancement. Therefore, it would be interesting to perform subsequent infections with fungal, bacterial and viral pathogens in addition to parasites and at different times after the preinfection with freshwater pearl mussel glochidia. Shortly after the preinfection and at the time of glochidia detachment, the negative effect of gill damage should prevail, but in the course of glochidia development on gills or after the detachment period the possible immunity enhancement should be observed.

A concomitant infection by trematodes caused by preinfection with freshwater pearl mussel glochidia may hamper the host directly, e.g., by utilizing additional host resources and/or loading its immune system. However, indirect effects of such concomitant infection can be even more severe. *Diplostomum* eye flukes, for example, can cause cataracts in fish eye lenses hampering fish vision and their ability to forage (Karvonen, Seppälä, & Valtonen, 2004; Owen, Barber, & Hart, 1993). More importantly, *D. pseudospathaceum* along with many other trophically transmitted parasite species (see Poulin, 2010 for the review) are able to manipulate host behavior predisposing fish to predation by the definitive host—fish-eating bird (e.g., Crowden & Broom, 1980; Gopko et al., 2015, 2017; Mikheev et al., 2010; Seppälä et al., 2004, 2008). Therefore, by increasing the probability of concomitant infection freshwater pearl mussel glochidia can hamper their hosts in more subtle ways than it was suggested earlier (Filipsson et al., 2017; Österling et al., 2014; Taeubert & Geist, 2013; Thomas et al., 2014) which are not easy to detect without conducting comprehensive experimentation.

Salmonids and freshwater pearl mussels inhabit running waters while the snail host of *D. pseudospathaceum*, *L. stagnalis*, prefers standing waters. Thus, co-occurrence of freshwater pearl mussel glochidia and *D. pseudospathaceum* in a salmonid host is probably infrequent, even though the geographic the distribution of the two species largely overlaps. However, many river systems have lentic sections or a lake as the source of the river. Because trematode cercariae can aggregate as dense clouds in the surface layer (Horák et al., 2015) and potentially can be carried over long distances by the wind, the flow of *D. pseudospathaceum* cercariae into freshwater pearl mussel rivers is possible. Indeed, today many freshwater pearl mussel rivers have been dammed for hydroelectric power, or modified for other purposes creating standing water reservoirs, ponds or stagnant water microhabitats favoring occurrence of *L. stagnalis*. Diplostomatids are masterful dispersers owing to their bird definitive host (see Louhi et al., 2010). Consequently, diplostomatid parasites are commonly recorded in brackish water and freshwater systems harboring fish and snails worldwide (Blasco-Costa & Locke, 2017; Louhi et al., 2010; Valtonen & Gibson, 1997). In addition, the nutrient input to rivers is also increasing, which should favor *L. stagnalis*. These anthropogenic changes, together with climate warming, which should also benefit snails, probably increase the probability of the freshwater pearl mussel glochidia—*Diplostomum* coinfection in the future.

When fish preinfected with freshwater pearl mussel glochidia were subsequently infected with a low dose of cercariae, the number of *D. pseudospathaceum* metacercariae declined with fish mass, whereas in the high dose treatment no significant relationship was found. A possible explanation could be related to fish size. In the low dose treatment fish were about three times bigger compared to the high dose treatment. Although in the place of their final localization (eye lens) *D. pseudospathaceum* metacercariae are unprocurable for the host immune system, they are attacked by innate immunity while moving to the lens through host tissues (Höglund & Thuvander, 1990; Karvonen et al., 2005; Wegner et al., 2007). The bigger the fish, the longer the distance for the parasite to migrate to the eye lens and the longer the period when the parasite is thus vulnerable to a host immune response. It is necessary to mention, however, that we are unaware about studies, where a correlation between the fish size and time taken by *D. pseudospathaceum* to reach eye lenses was demonstrated.

The difference in fish sizes between experiments may be explained by different origin of brown trout used in the high- and low-infection treatment. Since fish originated from different stocks, their tolerance to laboratory conditions (e.g., feeding regime, temperature, salinity etc.) can be slightly different, which in turn can lead to unequal growth rates during 10-month maintenance in the laboratory. This also refers to the different *D. pseudospathaceum* infection intensities in fish from low- and high-exposure experiments. Fish from different stocks (populations) may differ in their susceptibility to parasites (Bryan-Walker, Leung, & Poulin, 2007; Hasu, Benesh, & Valtonen, 2009; Scharsack & Kalbe, 2014). However such difference is often a result of the evolution in different environmental condition, e.g., in lake (high parasitic pressure) compared to rivers (low parasitic pressure) (Scharsack & Kalbe, 2014), while in our study we were dealing with two river populations. Therefore, difference in parasitic load in two experiments is likely to a result of different exposure doses. However interpopulation differences also may play some role.

The intensity of the glochidial infection also did not correlate with the fish size both in the high and the low dose treatment, which is in agreement with previous studies (e.g., Thomas et al., 2014). Though in early stages (few weeks after the infection) bigger fish

have more parasites, later this pattern disappears (Thomas et al., 2014). This is presumably because of uneven shedding of glochidia from different hosts due to differences in hosts' immunity and/or nutrition and host–parasite compatibility (Marwaha et al., 2017).

A similar explanation can be applied to lack of the correlation between the glochidia and trematode infection intensity. Since glochidia are unevenly detached from gills (Marwaha et al., 2017) and damage to gill lamellae caused by glochidia (Thomas et al., 2014) may vary between fish due to the different immune response, pure glochidia numbers may not fully mirror the condition of the host respiratory system. Thus, increased host's vulnerability to the subsequent infection can be connected with damage caused by freshwater pearl mussel glochidia during the excystment. In such case *D. pseudospathaceum* infection intensity would correlate with the number of glochidia recently shed rather than with number of glochidia on gills. Another constraint, which can potentially obscure the relationship between glochidia and trematode infection intensities, is hosts' individual differences in the innate immune response to the *Diplostomum* infection. Thus, Rauch, Kalbe, and Reusch (2006) found out that even within the same population fish can substantially differ in their vulnerability to *D. pseudospathaceum* and Kortet, Lautala, Kekäläinen, Taskinen, and Hirvonen (2017) observed between family differences in vulnerability to *D. pseudospathaceum* in fish.

Potentially, freshwater pearl mussel glochidia can also predispose their hosts to infectious agents other than eye flukes. For instance, a myxozoan endoparasite *Tetracapsuloides bryosalmonae* causes proliferative kidney disease (PKD) in salmonid fishes including brown trout (Vasemägi et al., 2017). PKD outbreaks have a strong economic and ecological importance (Okamura, 2016). Infection of the fish occurs via penetration of the gills by parasite spores (Vasemägi et al., 2017). Since at least in northern Europe freshwater pearl mussel and PKD are likely to be sympatric, increased respiratory burden connected with freshwater pearl mussel glochidia infection can also increase brown trout susceptibility to PKD, though, to our knowledge, this suggestion has never been tested explicitly.

Fish preinfected with *D. pseudospathaceum* differed in their vulnerability to the subsequent freshwater pearl mussel glochidia infection depending on when they were preinfected. Fish preinfected 2 weeks before the glochidia infection obtained a similar glochidia load to control fish, suggesting that the *Diplostomum* infection at least 2 weeks before exposure to freshwater pearl mussel glochidia does not increase host's vulnerability to glochidia. Generally, this result supports our initial hypothesis that *D. pseudospathaceum* metacercariae, which are unprocurable to the host's immune system within eye lenses, has no need to modulate or weaken the host's immune system as many other parasites do. By contrast, the intensity of freshwater pearl mussel glochidia infection in fish preinfected with *D. pseudospathaceum* just a 20 hr before the subsequent glochidia infection was higher than in control fish. During the first 24 hr after the infection eye flukes are moving through host tissues to eye lenses (Klemme & Karvonen, 2017) and therefore can cause an innate immune response (Wegner et al., 2007). According to the "optimal defense theory," resource allocation to defense is flexible and there is a trade-off between defense and other physiological functions related to the host (Stamp, 2003). For example, organisms, which are defending simultaneously from two threats (e.g., against the parasitic and predation threat), invest fewer resources towardsthe immune defense, when compared with organisms which are defending only against parasites (Rigby & Jokela, 2000). Similarly, when two unrelated parasites are entering the same host they can both benefit, because the host's immune system has to battle on two fronts simultaneously. Alternatively, it is possible that the penetration of *Diplostomum* cercariae could damage gill epithelium or change gill structure so that the subsequent infection by freshwater pearl mussel glochidia would be enhanced—but this effect lasts only for a short period. In theory, an exposure to *Diplostomum* cercariae shortly before the infestation by freshwater pearl mussel glochidia could be used in captive breeding programmes to facilitate infection success, for example if glochidia of this endangered mussel species are in short supply.

It is necessary to mention, that despite a clear positive ecological relationship between the two parasitic species demonstrated in our study, it is unlikely that both parasites can simultaneously increase their fitness due to this interaction. Since *Diplostomum* eye flukes and freshwater pearl mussels have very different life cycles, their notions about the future of the host's body are very different. For the eye fluke, a preferable scenario of the fish's future is predation by a fish-eating bird, while the freshwater pearl mussel glochidia are interested in maintaining its host alive until glochidia will be metamorphosed into the juvenile stage and detached from gills. In spite of their positive relationship in sequential infections of fish, these parasites cannot be regarded as collaborators in the host's body and for each of them the facilitation of the subsequent infection success is probably no more than a byproduct of their own virulence. After establishing in the host, they can potentially start to hamper each other, for instance, by influencing host behavior in opposite directions (reviewed in Hafer, 2016). Therefore, more prolonged studies are needed to understand how the simultaneous infection with eye flukes and glochidia influences fitness of both parasitic species and their host.

5 | CONCLUSIONS

In our study, we experimentally demonstrated how two common parasitic species of brown trout facilitate each other infection success by predisposing their host to subsequent infections. The infection with freshwater pearl mussel glochidia predisposes the fish host to the subsequent parasitic infection by the trematode entering the fish body trough gills during the respiration. Our findings demonstrate that our knowledge about the virulence of freshwater pearl mussel parasitic stages is still incomplete and that the negative influence of glochidia on host conditions is likely to be underestimated. In turn, the preinfection with *D. pseudospathaceum* can make fish more susceptible to the subsequent

glochidial infection, but only, when the second parasite enters the host shortly after the first one. However the mechanism of this phenomenon remains unclear.

These data provide new evidence of how the timing and sequence of parasite exposure can influence infection success in a host–multiparasite system.

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CONFLICTS OF INTEREST

Authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors conceived the study. MC and MG conducted experiments. MC was mainly responsible for FPM part of the experiments, while MG for the trematode part. MG wrote the major part of the manuscript, while MC and JT added minor passages and edited the manuscript. JT supervised the study.

DATA ACCESSIBILITY

Data are stored in the open figshare repository without any embargo and can be accessed following this link: https://doi.org/10.6084/m9.figshare.6063032.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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III

EFFECT OF GLOCHIDIA INFECTION ON GROWTH OF FISH: FRESHWATER PEARL MUSSEL *MARGARITIFERA MARGARITIFERA* AND BROWN TROUT *SALMO TRUTTA*

by

M. Motiur R. Chowdhury, Timo J. Marjomäki & Jouni Taskinen 2018

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Effect of glochidia infection on growth of fish: freshwater pearl mussel *Margaritifera margaritifera* and brown trout *Salmo trutta*

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ABSTRACT
Although increasing evidence has accumulated about the parasitic, rather than commensalistic or symbiotic relationship of freshwater mussels (Unionoida) glochidia in their fish hosts, effect of glochidia on the growth of host fish has remained poorly studied. We compared the specific growth rate of the juvenile, PIT-marked brown trout (*Salmo trutta*) between uninfected controls to those experimentally infected (average initial number of glochidia was 8,000 fish^{-1}) with *Margaritifera margaritifera* glochidia. Fish were monitored for ten months starting from September with two feeding regimes. Three tanks were allocated to high feeding while another three tanks to low feeding regime. Our hypothesis was that glochidiosis will impair the growth of fish. According to our hypothesis, infected fish gained statistically significantly less weight than control fish throughout the experiment. A proportional increase in weight of control individuals was 11 % higher than that of the infected fish. However, neither the feeding regime (high, low) nor the period (Sep–Nov, Nov–Mar, Mar–May), had a significant effect on the growth difference between control and infected fish. Results indicate that *M. margaritifera* glochidia, with a long parasitic period, can impair the growth of the fish host.
Keywords: Bivalvia, conservation, endangered species, host-parasite relationship, Salmonidae.

INTRODUCTION
Although the relationship between glochidium larvae of freshwater mussels Unionoida and their obligatory fish hosts has been frequently described as phoretic commensalism (glochidia benefit from transportation services by fish, with no harm to fish) or even symbiotic (both partners benefit; Ziuganov, 2005), increasing evidence has accumulated that the relationship is actually parasitic. During the course of attachment to fish, stable isotope signatures of glochidia approach those of fish host, indicating acquirement of nutrients from fish (Fritts et al., 2013; Denic et al., 2015). Heavy glochidia load can induce mortality of fish, indicating clear harm to the fish host (Teaubert and Geist, 2013). When infected with glochidia, host fish develop acquired immunity, indicating activation of the immune system as a result of the attached glochidia (Bauer and Vogel, 1987; Chowdhury et al., 2017). Consequently, several adverse effects of glochidiosis on fish host have been reported, including, for example, energetic cost and dysfunction of liver, kidneys and gills (Slavik et al, 2017), altered behavior (Horky et al., 2014, Terui et al., 2017) and reduced expression of secondary sexual traits and decreased sperm quality (Kekäläinen et al., 2013).

These negative effects of glochidia can be expected to reduce the fitness of the fish host, thereby suggesting the role of freshwater mussels (glochidia) as a selective force and emphasizing the possibility of mussel-fish coevolution. Indeed, the recent findings of population-specific adaptations of mussels to infect certain host species or local fish host (Salonen et al. 2017, Douda et al. 2017) support the view of (antagonistic) co-evolution between mussels and their fish hosts.

However, to date, the possible negative impact of freshwater mussels on one of the main fitness components, the growth of the host fish, has not been clearly shown. Treasurer et al. (2006) observed no significant effect of *M. margaritifera* infestation on the growth of Atlantic salmon (*Salmo salar*) in the initial stage of the parasitic period but a negative effect at 15 weeks, which, however, disappeared by the end of the 1-y monitoring period. In a study on another margaritiferid, *M. laevis*, no effect on growth of the salmonid host *Oncorhynchus masou masou* was found during the first 50 days of infection, but a negative impact was observed after the detachment of *M. laevis* glochidia at 70 days post infection (Ooue et al., 2017). To our knowledge, these are the only studies focusing on the possible effects of freshwater mussels on the growth of their fish hosts.

Therefore, the objective of the current study was to examine the effect of glochidia infection on the growth of fish host. Brown trout (*S. trutta*) and freshwater pearl mussel *M. margaritifera* were chosen because the exceptionally long parasitic period of *M. margaritifera*—up to 11 months (Hastie and Young, 2001; Salonen and Taskinen, 2017) — should enhance the detection of growth-effect as compared to mussel species with a short parasitic period. In addition, *M. margaritifera* is one of the few unionoids that grow remarkably in size during their parasitic period. Glochidia of *M. margaritifera* multifold their size while attached to the gills of the fish host (Bauer and Vogel, 1987; Young and Williams, 1984; Salonen and Taskinen, 2017), which should intensify the possible growth-effect of glochidia on the host. Moreover, this methodology was chosen since it was possible to have mussel–host association where the suitability of host has been verified and where the mussel population used is specialized to infect exactly the fish host used. Results of Salonen et al. (2016) show that brown trout is a suitable host for *M. margaritifera*, and the *M. margaritifera* population utilized in the present study, sourced from the River Jukuanoja, is adapted to use exclusively brown trout as the host fish species (Salonen et al. 2017).

M. margaritifera is an endangered species (e.g., Geist, 2010, Lopes-Lima et al., 2017; Oulasvirta 2017) whose glochidia are produced in captive breeding programmes using brown trout as host fish (e.g., Buddensiek, 1995; Thomas and Garcia de Leanis, 2010; Gum et al., 2011; Eybe et al., 2015; Moorkens, 2018). One way to strengthen declining *M. margaritifera* populations—or to restore the extinct populations—would be to stock juvenile brown trout infected with *M. margaritifera* glochidia to the target rivers (Wellmann, 1943). This is a realistic, though little utilised option in *M. margaritifera* areas where juvenile salmonids are stocked, either for recreational fishing purposes or to strengthen the salmonid populations, and where conditions for completion of the life cycle of *M. margaritifera* are otherwise favourable, like in northern Fennoscandia. If *M. margaritifera* glochidia do not impair the growth of brown trout, or if the growth-effects of *M. margaritifera* infestation are negligible, this might encourage landowners, fishing managers, fishing right owners' associations and salmonid conservation programmes to use glochidia-infected juvenile *Salmo* in their stockings. The question of whether dietary limitation upon the fish host could enhance the possible growth-effect of glochidia on fish is also interesting because the breeding programmes of *M. margaritifera* require feeding of the fish host in captivity, even though feeding opportunities of fish

hosts are variable in natural conditions. Therefore, the current study varied experimental conditions by applying two food levels, high and low feeding regimes, to study the interaction between the possible growth-effect of *M. margaritifera* and feeding of host fish.

A number of negative effects of *M. margaritifera* on its fish host have been described. *M. margaritifera* glochidia cause hyperplasia and fusion of gill filaments (Treasurer and Turnbull, 2000; Thomas et al., 2014), inhibit swimming capability and increase mortality (Taeubert and Geist, 2013), reduce foraging, activity and dominance success (Österling et al., 2014; Filipsson et al., 2016), induce spleen enlargement and lessen mucous cells of gills (Thomas et al., 2014) and increase metabolic rate and level of hematocrit (Filipsson et al., 2017). Therefore, our hypothesis was that *M. margaritifera* glochidia infection is harmful, decreasing growth of the host fish, brown trout. In addition, we also hypothesized that the negative growth-effect of glochidia would be more pronounced in fish receiving less food.

MATERIAL AND METHODS

The experiment was conducted at the Konnevesi Research Station, University of Jyväskylä. On 31th August 2015, brown trout (age group 1+ y, originating from Laukaa fish farm of the Natural Resources Institute Finland) were exposed to *M. margaritifera* glochidia, collected on the same day from the River Jukuanoja, a tributary of the River Iijoki, northern Finland (for map, see Salonen and Taskinen, 2017). Fish were transported to the research station two weeks before exposure and randomly allocated into two 163 L flow-through tanks. Exposure was performed in one of the tanks with 14.3 x 10^5 glochidia for 1.5 h, at a temperature of 16.8 °C. During the exposure, water volume was reduced to 70 L, water flow stopped, and aeration provided. Control fish in the other tank were treated in a similar way except they were exposed to lake water without glochidia. Fish had not been exposed earlier to *M. margaritifera* or any other glochidia in the fish farm.

A subsample of fish from both the infected and control group were euthanized in order to check the success of glochidial infection 3 days post-exposure. The infection was highly successful in the glochidia-exposed fish, among which the average (± s.e.) number of glochidia was 7889 ± 390 per $fish^{-1}$ individual (n = 3), or 138± 3 glochidia per $g^{-1}$of fish, whereas no glochidia were found in the control group (n = 3).

On 12th September2015, all fish were anesthetized using MS-222, measured for total length and weight and marked individually with PIT (Passive Integrated Transponder, 7x1.35 mm from Loligosystems) tags between adipose and dorsal fins. Then, fish were randomly allocated into six replicate tanks (163 L, flow-through), each tank containing mixed infected and control fish in approximately50:50 ratio, the total number of fish per $tank^{-1}$ varying from 45 to 69. Moreover, two different levels of feeding were applied so that half of the tanks (Tank 13, 14 and 15; high food group) were fed with 60 g and half (Tank 18, 19 and 20; low food group) were fed with 36 g of commercial food pellets per $tank^{-1}$ daily, except for Sunday. Total numbers of control and infected fish were 160 and 159, respectively. Total numbers of individuals in low food and high food groups were 175 and 144, respectively. Tank-specific numbers of infected and control fish varied from 24 to 28 and from 21 to 43, respectively, while the tank-specific numbers of fish in low food and high food groups varied, respectively, from 21 to 27 and from 25 to 43.After the initial size measurement in mid-September (2 weeks post-infection), fish were anesthetized and measured on 21st November 2015 (12 weeks), 19th March 2016 (29 weeks), 20th May 2016 (38 weeks) and 6th July 2016 (45 weeks post-infection).

Three to four fish were randomly netted and euthanized to check gills of fish microscopically for the abundance (number) of glochidia, at each monitoring period except for November 2015. Mean ± s.e. abundance of infection (with number of fish studied in parentheses) in September 2015, March 2016, May 2016 and July 2016 was 7889 ± 390 (n = 3), 5473 ± 445 (n = 18), 5202 ± 804 (n = 3) and 3125 ± 566 (n = 4) glochidia per $fish^{-1}$. In the March 2016 sampling period, three glochidia-infected fish were examined from each of the high food and the low food tanks (3 fish x 3 tanks x 2 food treatments = 18 fish). From this sampling, it was possible to compare abundance and size (growth) of glochidia between high and low food treatments, as the length of glochidia was microscopically measured from a random subsample of 25 glochidia per $fish^{-1}$. To study the number and size of glochidia, fish individual-specific averages were first calculated. Then those individual-specific values were used to calculate the mean tank-specific values (over the three fish individuals). Tank-specific glochidia abundance and size values were then used in analysis of covariance (ANCOVA) where tank was the statistical unit, feeding rate (low, high) was a fixed factor and the tank-specific average initial weight of fish was used as the covariate. The initial weight of fish, measured in September, two weeks after exposure to glochidia, was used as the covariate because the number of glochidia attached to fish depends on the size of fish at the time of infection, a higher number of glochidia being able to attach to larger fish (Thomas et al., 2014).

After May 2016, monitoring of growth was terminated in two tanks—one from the high food treatment (Tank 13) and one from the low food treatment (Tank 18), because the fish were used for collection of juvenile mussels for another study. Therefore, the complete growth data for all six tanks were available for three periods (1) September–November 2015, (2) November 2015–March 2016 and (3) March–May 2016.

During the experiment, water temperature varied from a maximum of 15.3°C in mid-September 2015 to a minimum of 1.1°C in mid-March 2016.Average temperatures during the periods (1) September–November 2015, (2) November 2015–March 2016, (3) March–May 2016 and (4) May–July 2016 were 9.3, 2.8, 3.7 and 10.4 °C, respectively.

Total fish mortality during the experiment from September 2015 to July 2016 was 19 and 16 % for glochidia-infected and control fish, respectively. Most of the mortality took place during the first period, September-November 2015, while there was no mortality among any of the fish from March 2016 to the end of the experiment in July2016.

The specific growth rate (G) was calculated for each individual (x) and period (P) by

$$G_{x,P} = 100 \ln(W_{x,P,E} / W_{x,P,B})/t_P$$

where W = fresh weight of individual, E = end of period, B = beginning of period and t_P = length of period in days

The response variable was the tank (T) and period (P) specific difference (D) of the average of specific growth rate between non-infected (control = C) and infected (I) individuals

$$D_{T,P} = \bar{G}_{T,P,C} - \bar{G}_{T,P,I}$$

The difference in specific growth rate between control and infected fish was calculated for the three periods (1) September–November 2015; 2) November 2015–March 2016;

3) March–May 2016) between consecutive sampling as well as for the whole experiment from September 2015 to May 2016. To take into account the fact that the growth of an individual can depend on its initial size, all G-values were corrected for the effect of initial weight at the beginning of the period and all statistics produced for those calibrated values. However, the results were not affected by this calibration and therefore only the non-calibrated results are shown here.

As the daily feeding within the tank was kept constant throughout the experiment, a rough index of relative daily feeding per biomass unit (FUB) was calculated for each tank and period. First, the average biomass (B) for every tank and period was estimated by simple linear interpolation

$\underline{B}_{T,P} = (B_{T,P,B} + B_{T,P,E}) / 2$

Then this was scaled to the minimum average biomass of all tanks and periods (tank 13, period 1) and feeding regime (FR)

$FUB_{T,P} = 100\ FR_T\ \min(\underline{B}) / \underline{B}_{T,P}$

where FR_T = 100 % for tanks 13, 14 and 15 and 60 % for tanks 18, 19 and 20.

The collection of *M. margaritifera* glochidia was performed with license POPELY/513/07.01/2011 from the North Ostrobothnia regional Centre for Economic Development, Transport and the Environment (Oulu, Finland). Permission to conduct the experiment with fish, license ESAVI/10184/04.10.07/2014, was granted by the Animal Experiment Board of Finland (regional administration of Southern Finland).

RESULTS

The body weight of fish increased over the course of the 45 week (10 months) experiment, both in glochidia-infected and control fish, and in low and high food treatments (Fig. 1).

The tank-specific difference in average specific growth rate between control and infected individuals was significantly greater than 0 (all $p < 0.1$) for every period separately as well as for the whole experiment ($p < 0.006$) (Table 1, Fig. 2). Thus, the negative effect of *M. margaritifera* glochidia on growth of brown trout was evident, and was already observed by the end of the 1st monitoring period, from September to November 2015). Although the tank-specific average specific growth rate was positively associated with the feeding regime (low/high food) (Fig. 2), the feeding regime or period had no significant effect on the difference in the growth between control and glochidia-infected fish (repeated measure ANOVA, $p > 0.4$). On average, the proportional increase in weight for control individuals was 11 % higher than that for infected individuals (exp (0,106) – 1) during the experiment.

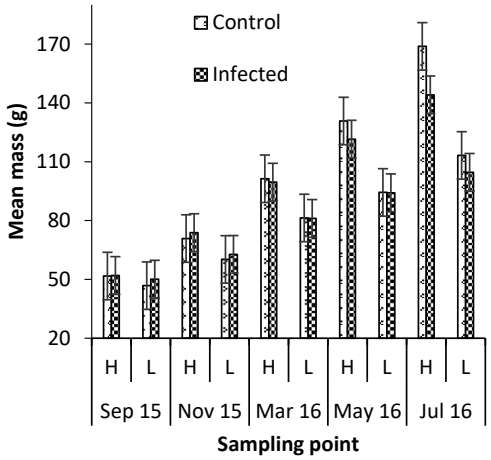

Figure 1. Mean (± s.e.) mass of control and infected brown trout in three replicate tanks of high (H) and three replicate tanks low (L) food treatments in five sampling points after the infection with *M. margaritifera* glochidia on 31th of August 2015; 12th of September 2015 (Sep 15), 21st of November 2015 (Nov 15), 19th of March 2016 (Mar 16), 20th of May 2016 (May 16) and 6th of July 2016 (July 16).

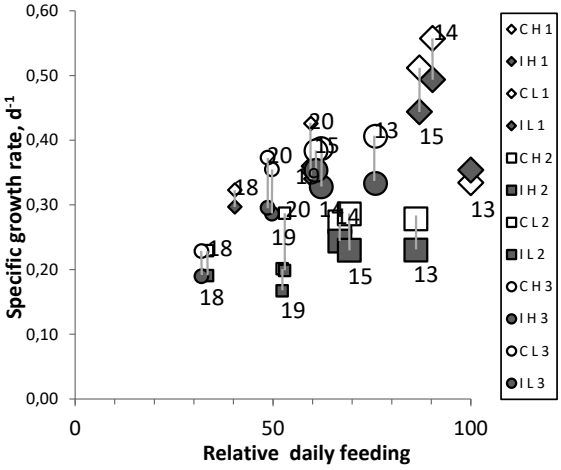

Figure 2. The tank-specific average specific growth rate for control (C; open symbols) and infected (I; filled symbols) fish in relation to daily feeding rate (H = high feeding, large symbols; L = low feeding, small symbols). The numbers 1, 2 and 3 (and the symbols diamond, square and circle, respectively) refer to monitoring periods (1, September–November 2015; 2, November 2015–March 2016; 3, March–May 2016). The vertical bars indicate the difference between control and infected individuals within a tank. The numbers refer to different tanks, 13–15 being high food tanks and 18–20 being low food tanks.

Table 1.The tank-specific difference in average specific growth rate between control and *Margaritifera* glochidia-infected brown trout by monitoring periods (1, September–November 2015; 2, November 2015–March 2016; 3, March–May 2016) and for the whole monitoring, in high food (60 g tank^{-1} day^{-1}) and low food (36 gtank^{-1} day^{-1}) treatments. Average difference, standard error (s.e.) and the p-value for the hypothesis that the average difference > 0 are also given.

Tank	Feeding	Difference for period			
		1	2	3	whole monitoring
13	high food	-0.019	0.048	0.073	0.048
14	high food	0.064	0.030	0.059	0.163
15	high food	0.068	0.056	0.030	0.156
18	low food	0.026	0.038	0.039	0.055
19	low food	-0.008	0.034	0.069	0.066
20	low food	0.085	0.089	0.077	0.150
Average		0.036	0.049	0.058	0.106
s.e.		0.018	0.009	0.008	0.022
p <		0.094	0.003	0.001	0.006

Neither the tank-specific mean number nor size of glochidia in March 2016 was affected by the covariate, initial weight of fish (ANOVA, $p > 0.175$). However, whilst the size of glochidia was not affected by the feeding regime (ANOVA, $p = 0.341$), there was a marginally significant feeding-effect on glochidia number (ANOVA, $p = 0.057$). Therefore, a trend was observed for higher glochidia abundance in high food treatment, as the tank-specific, fish weight-adjusted mean ± s.e. was 5,939 ± 218 and 5,007 ± 218 glochidia per fish^{-1} in high food and low food groups, respectively.

DISCUSSION

As hypothesized, *M. margaritifera* glochidia infection reduced the growth of the fish host, brown trout, causing an 11 % reduction in the proportional increase in weight during the 45 week experiment. This implies a parasitic nature of *M. margaritifera* glochidia. Results from this study, together with those by Treasurer et al. (2006) and Ooue et al. (2017), provide clear evidence for an adverse effect of glochidia of *Margaritifera* mussels on the growth of their fish hosts. Thus, freshwater mussels—at least those having a long parasitic period—can negatively affect the growth rate of their fish host. Negative growth-effect of *M. margaritifera* was evident by the end of the first monitoring period, suggesting that the growth impairment began upon infestation of fish. Indeed, it is not surprising that mussel species that attach to fish for periods up to 11 months and that grow remarkably in size during that time (Hastie and Young, 2001; Salonen and Taskinen, 2017; present study), intakes nutrients from the host (Denic et al. 2015), causes respiratory burden and damage to gills (Teaubert and Geist, 2013; Thomas et al. 2014) and may impair feeding efficiency (Österling et al., 2014) of fish host..Mussels that have short parasitic periods could also impair the growth of their fish hosts, but this has remained unstudied or unnoticed.

Adverse effects of parasites on a host are usually dependent on the abundance of infection. For example, a negative effect of *M. margaritifera* glochidia on critical swimming speed of brown trout, in an experiment conducted with similar sized fish as in the present study, increased with the abundance of glochidia and was evident only when abundance exceeded 10,000 glochidia per host (Taeubert and Geist, 2013). Initial mean number of glochidia per fish^{-1} in our study was almost 8,000 and numbers remained high at the end of the experiment in July 2016, with over 3,000 glochidia per fish^{-1}. In contemporary natural populations, mean abundance of *M. margaritifera* in salmonids only rarely exceeds 1,000 glochidia per fish^{-1}(Young & Williams, 1984; Cunjak and McGladdery, 1991; Hastie and Young, 2001; Salonen and Taskinen, 2017). Thus, the number of glochidia in our study was higher than one would expect to see in natural conditions. Yet, it is reasonable to extrapolate that a negative growth effect, although less prominent, of *M. margaritifera* glochidia on fish host also exists with lower glochidia abundances.

Feeding regime of fish host did not affect the level of growth-reduction induced by *M. margaritifera* glochidia on fish. Applied feeding regime included a constant amount of feed throughout the experiment, 60 and 36 g per tank^{-1} day^{-1} in high and low food treatment, respectively. As fish grew, the relative feeding rate, in relation to the size of fish, decreased in both groups towards the end of the experiment. This may have intensified the competition for food and caused a shortage of food in the high food group. In spite of this, an observed difference in the specific growth rate between glochidia-infected and control fish existed throughout the experiment, whilst, the study period had no significant effect on the difference in growth between *glochidia*-infected and control fish. This indicates that there is no observable interaction between feeding rate and the negative growth-effect of *M. margaritifera* glochidia.

In a study conducted with Atlantic salmon, *S. salar*, and *M. margaritifera* (Treasurer et al., 2006), the weight of glochidia infected fish was significantly lower than that of the control fish at 15 weeks post exposure, but not later. In the study performed with masu salmon, *Oncorhynchus masou masou*, and the freshwater pearl mussel *M. laevis* (Ooue et al, 2017), weight of glochidia infected fish was not reduced in the middle of the parasitic period but was significantly lower than

that of the control fish after the parasitic period (70 d post infection).Mean initial abundance of infection in studies by Treasurer at al. (2006) and Ooue et al. (2017) were close to 1,400 and 800 glochidia per fish^{-1}, respectively. These are clearly lower values than in the present study, which may partly contribute to the similar, but less obvious growth-effects of *Margaritifera* glochidia observed in those studies. Therapeutic treatment of fish at week 15, reducing high glochidia abundance, may have also influenced the disappearance of negative growth-effects after 15 weeks in the experiment by Treasurer et al. (2006).

Ziuganov (2005) proposed that *M. margaritifera* glochidia could delay smoltification of the host salmonid. This would benefit the glochidium larvae since, after smoltification, young salmon and trout migrate (with glochidia) to the sea or a lake, an unsuitable environment for juvenile *M. margaritifera*. Smoltification of brown trout and Atlantic salmon, the host fishes of *M. margaritifera*, is connected to size of fish so that within a population the faster-growing individuals smoltify earlier than slower-growing individuals (e.g. Økland et al., 1993). Ziuganov (2005) provided evidence for a slower growth and higher age at smoltification in Atlantic salmon from the main channel of the Varzuga River (Kola Peninsula, Russia) where a dense *M. margaritifera* population existed compared to populations in the tributaries of the Varzuga River without *M. margaritifera*.The negative growth-effect of *M. margaritifera* glochidia could, therefore, result in delayed smoltification and postponed migration of fish host from the river, thereby increasing the probability of *M. margaritifera* larvae successfully developing and detaching to a suitable habitat. If this is true, the negative growth-effect of *M. margaritifera* on the salmonid host would not just be an inevitable consequence of utilization of host resources for the larvae's own growth but would also serve as a mechanism to make the host individual stay longer in the favourable habitat from the glochidium's point of view. In that sense, the negative effect on host growth would also be a form of host manipulation by *M. margaritifera*, leading to a parasite-induced change in host behavior that would increase the success of the parasite (see e.g., Poulin, 2010; Hughes et al., 2012).

Our results from March 2016 sampling did not indicate a relationship between the feeding of fish and growth of glochidia, but there appeared to be a marginally significant trend for a higher abundance of *M. margaritifera* glochidia in the high food treatment than in the low food group. Thus, low feeding of fish may increase the number of glochidia detached prematurely, if the initial glochidia abundance is assumed to be equal in high and low food groups. However, this result should be considered cautiously and urges further studies. The sample size was low, and our experiment was not precisely designed to study the effect of feeding regime on the growth or number of glochidia.

Ideally, the fish should be kept in individual containers to enable the use of each fish as an independent observational unit. However, individual fish could not be used as an independent sampling unit in this experiment because the individuals in one tank can interfere with each other e.g. by food competition and dominance hierarchy between different sized individuals. If the control group grows faster and the food competition/hierarchy favours largest individuals, then the difference in growth between these groups (control vs. infected) can be larger than that in the case where the groups were kept separate. The results, therefore, may bias (exaggerate) the negative main effect of infection. Further, even in a separated group of only control or only infected individuals, there might be competition/hierarchy-based differences between individuals, which could at least affect the variance in growth between individuals if not the average. Thus, in order to measure the effect of parasitism on growth without bias, the individuals should be kept in separate tanks with controlled feeding and their parasite number should be monitored. It was not possible to keep the fish individually but we formed mixed, replicated groups of PIT-tagged fish. Fish used in this experiment were collected from the same age group and similar size to minimise the bias. In addition, having infected and control fish in the same tank ensured equivalent conditions for both groups in terms of temperature, oxygen, and water flow. The present design also enabled the establishment of natural social interactions between (and within) infected and control fish. Therefore, we decided not to try to keep fish in individual containers but formed mixed, replicated groups of PIT-tagged fish.

To avoid growth rate biasness, equal sized 1+ y old fish were used for this experiment. However, in the first sampling period, the infected fish had slightly higher mean mass than the control fish both in the high and low food treatment (Fig. 2). This could have contributed to their lower specific growth rate since the larger the initial size the less potential there is for growth, in principle, and the higher the initial biomass, the less feeding per weight unit with a fixed feeding rate. Nevertheless, the slightly larger size in the beginning of the experiment does not explain the clearly observed lower size of the infected fish, as compared to the control individuals, at the end of the experiment (Fig. 2).

The observed negative growth effect of *M. margaritifera* glochidia on growth of host fish could be at least partly attributed to the respiratory burden caused by glochidia (Teaubert and Geist, 2013; Thomas et al. 2014). Moreover, *M. margaritifera* glochidia induce acquired immunity response in fish (Chowdhury et al., 2017) that is presumably energetically costly, and glochidiosis, in general, can incur an energetic cost in host fish (Slavik et al, 2017)

Freshwater mussels are in decline all over the world (Lydeard et al., 2004; Bogan, 2008; Geist, 2010). Lopes-Lima et al., (2018) reported that 40% of the freshwater bivalves are near threatened, threatened or extinct worldwide. IUCN Red List included 75% (12 out 16) of European freshwater mussel species as threatened or near threatened (Lopes-Lima et al., 2017). For example, *M. margaritifera* has become widely extinct in central Europe during the last century (Bauer, 1986; Buddensiek, 1995) although the species used to be abundant previously in European salmonid fish rivers (Young and Williams, 1984; Cosgrove et al., 2000; Geist, 2010).

From a conservation point of view, the observed growth-effect of *M. margaritifera* on the salmonid host can be regarded as a disadvantage. If glochidia-infected stockings are used to strengthen or restore *M. margaritifera* populations (see Wellmann, 1943), it would be attractive to encourage landowners, fishery managers, fishing right owners' associations and salmonid conservation programmes to use glochidia-infected juvenile *Salmo* in their stockings.. However, , the observed 11 % reduction in the growth of brown trout due to *M. margaritifera* glochidia may not be an obstacle for the fish farming industry to participate in the production of glochidia-infected brown trout stockings. In some *M. margaritifera* regions in northern Fennoscandia, such stockings are being conducted on a regular basis anyway, so there is perhaps no reason to not use stock infected with *M. margaritifera* glochidia. An advantage of this method would

be that the declining salmonid host populations would strengthen, as the low density of host fish can be an important limiting factor for *M. margaritifera* recruitment e.g. in northern European rivers (Arvidsson et al. 2012; see also Bauer, 1988).

Another conservation implication of this study, studies, could be that low feeding of fish may decrease the number of glochidia during the parasitic period. If this is the case, low feeding of fish could expose the glochidia to detach prematurely. This would lead to a lower yield of *M. margaritifera* juveniles in captive breeding.

To conclude, this is the first unambiguous evidence for negative growth-effect of freshwater mussel glochidia on the fish host, thereby supporting the view of the parasitic nature of glochidia. Together with earlier results (Treasurer et al., 2006; Ooue et al., 2017), this suggests that at least mussel species with a long parasitic period can impair the growth of their fish hosts. The number of glochidia used was high, but the 11 % reduction in growth suggests that negative growth-effect probably takes place also with lower glochidia abundance. Results from this study add to the list of negative effects of *M. margaritifera* on its salmonid host (Treasurer and Turnbull, 2000; Taeubert and Geist, 2013; Thomas et al., 2014; Österling et al., 2014; Filipsson et al., 2016; Filipsson et al., 2017). However, as the growth-effect on brown trout can be expected to be low in natural abundances of less than 1,000 glochidia per fish^{-1}, this result should encourage testing the introduction of fish which are artificially infected (Wellmann, 1943) as a method of conservation of this endangered species. Finally, results indicate that there can be a risk of lowered number of parasitizing glochidia if the feeding rate of host fish is low, but this should be verified by further studies.

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IV

GLOCHIDIA OF THE ENDANGERED FRESHWATER PEARL MUSSEL, *MARGARITIFERA MARGARITIFERA*, LOWER VIRULENCE OF A FISH PATHOGEN

by

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Manuscript

Glochidia of the endangered freshwater pearl mussel, *Margaritifera margaritifera*, lower virulence of a fish pathogen

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Abstract

1. With declines of species, we may lose ecosystem functions, services and species' interactions that are important for natural communities and valuable for humans. The freshwater pearl mussel, *Margaritifera margaritifera* a keystone species threatened throughout its distribution range, is tightly associated with its specific fish hosts, Atlantic salmon (*Salmo salar*) and brown trout (*S. trutta*) via gill-parasitizing glochidium larvae.
2. During the months over which the glochidia develop in fish, salmonid hosts may also contact bacterial infections. Here we tested whether glochidia-infested salmonids are more susceptible to bacterial disease—a potentially important question also for captive breeding used in the conservation of *M. margaritifera.*
3. We compared mortality induced by a bacterial pathogen, *Flavobacterium columnare* (entering fish body mainly via gills and causing columnaris disease, one of the biggest problems in freshwater salmonid farming) between brown trout infected with *M. margaritifera* glochidia and uninfected control fish in a laboratory experiment. Our hypothesis was that glochidia would increase the mortality of brown trout during a disease outbreak with *F. columnare*.
4. Unexpectedly, glochidia provided protection against *F. columnare*, as revealed by a longer survival of *M. margaritifera* infected trout compared to the control fish, and a positive relationship between glochidia abundance and fish survival time. The protective effect was evident both during the parasitic period (fresh infections) and after the glochidia had detached (post-parasitic period).
5. The mechanism of protection is not known, but could be connected to an enhanced non-specific immunity or changed gill structures.
6. The result (i) increases our knowledge of the interactions between *M. margaritifera*, their fish host and fish pathogens, (ii) emphasizes how unknown interactions and services can be potentially associated with lost biodiversity, and (iii) should increase the interest and willingness of different stakeholders and aquaculture sector to participate in conservation activities of *M. margaritifera*.

Keywords: Bivalvia, brown trout, co-infection, conservation, ecosystem services, *Flavobacterium columnare*, glochidia, pathogen, resistance, virulence

INTRODUCTION

Biodiversity loss, i.e. decline or extinction of species can affect a number of associated taxa (e.g. Boast et al. 2018). For example, with the extirpation of species, we may lose ecosystem functions, services and species' interactions that are important for natural communities and valuable for humans (Ehrlich and Mooney, 1983; Valiente-Banuet et al. 2015). Freshwater mussels, including freshwater pearl mussel *Margaritifera margaritifera*, have declined worldwide due to habitat destruction, loss of host fish, siltation, pollution, invasive species and over exploitation (Young and Williams, 1983; Bauer, 1986, 1988; Williams *et al.*, 1993; Lydeard *et al.*, 2004; Cosgrove *et al.*, 2000; Oulasvirta, 2011; Simon *et al.*, 2015; Salonen et al. 2016). The life cycle of *M. margaritifera* in Europe includes an obligatory, host-specific parasitic period in the gills of Atlantic salmon *Salmo salar* or brown trout *S. trutta* (Salonen et al., 2016; Salonen et al., 2017), lasting 10-12 months (e.g. Hastie and Young, 2001; Salonen and Taskinen, 2017). When matured and metamorphosed, glochidia detach from the fish host, drop on the bottom of the river as juvenile mussels, where they start their benthic life that can be up to 200 years (Ziuganov *et al.*, 2000; Helama and Valovirta, 2008). Today, *M. margaritifera* is classified as critically endangered in Europe (IUCN Red List of Threatened Species, 2013). Since freshwater mussels—having glochidium larva parasitizing fish— are involved with many ecosysten functions and services (Vaughn and Hankenkamp, 2001; Vaughn et al., 2008; Strayer, 2014; Vaughn, 2017) and because *M. margaritifera* is categorized as a keystone and umbrella species (Geist, 2010), the decline of *M. margaritifera* can potentially induce changes in ecological interactions and services in aquatic ecosystems. However, the possible interaction between *M. margaritifera* (or any freshwater mussel), its fish hosts and other fish-infecting pathogens is not known.

Captive breeding of *M. margaritifera*, where salmonid hosts are artificially infected with glochidia, plays an important role in the conservation of the endangered freshwater pearl mussel (Buddensiek, 1995; Thomas and Garcia de Leanis, 2010; Gum et al., 2011; Eybe et al., 2015; Moorkens, 2018). Co-infection of the salmonid host with *M. margaritifera* glochidia and a bacterial disease is possible during the months over which the glochidia-infested salmonids have to be maintained in captive breeding. However, the question whether *Margaritifera* glochidia increase the susceptibility of salmonids to bacterial diseases, and consequently the virulence of such pathogens, has not been studied.

Flavobacterium columnare causes the warm water disease/columnaris disease in fish, including salmonids. This pathogen can result in remarkable economic losses in fish farming due to high mortality associated with the disease (Wagner et al., 2002; Pulkkinen et al., 2010). *F. columnare* is an opportunistic fish pathogen that can grow also outside the fish host (Kunttu et al., 2012). *F. columnare* strains differ in their virulence (Suomalainen et al., 2006; Pulkkinen et al., 2018), being capable of causing up to 100% mortality in juvenile salmonids (Suomalainen et al., 2005). Since there is no functioning vaccination available for young salmonids (Sundell et al. 2014) the only treatment against *F. columnare* is antibiotics (Rach et al., 2008).

Parasitic infections, in general, increase the risk of secondary infections and can act as a vehicle for transmission of bacteria to fish (Kotob et al., 2016). For example, the monogenean parasite *Dactylogyrus intermedius* increases the susceptibility of gold fish, *Carassius auratus*, to *F. columnare*, resulting in higher mortality when compared to non-parasitized fish (Zhang, et al. 2015). In addition, *M. margaritifera* glochidia infection has many adverse effects on the fish host, such as hyperplasia and fusion of gill filaments, reduced swimming capability, increased mortality, reduced foraging, reduced activity and lowered social dominance, as well as increase metabolic rate (Treasurer and Turnbull, 2000; Taeubert and Geist, 2013; Thomas et al., 2014; Österling et al., 2014; Filipsson et al., 2016; Filipsson et al., 2017). It was recently shown that infection of brown trout with *M. margaritifera* glochidia increased the host's susceptibility to subsequent infection with the trematode parasite *Diplostomum pseudospathaceum* (Trematoda) (Gopko et al., 2018). Finally, as infection by *M. margaritifera* glochidia is a respiratory burden, increasing operculum beat rate and the ventilation volume of the host (Taeubert and Geist, 2013; Thomas et al., 2014; Filipsson et al., 2017), it can be expected that exposure to *F. columnare* would be higher in glochidia-infected

individuals than in uninfected fish since gills are the main and first site of infection in fish by *F. columnare* (Declercq et al. 2013, 2015). On the other hand, Ziuganov (2005) proposed that *M. margaritifera* can stimulate nonspecific resistance in exposure to air, thermal burn of gills and hook wounds, and provide resistance against epitheliomata and cutaneous mycoses.

Our hypotheses were that (i) *M. margaritifera* glochidia would increase susceptibility to *F. columnare*, (ii) that the increase would be dose-dependent (depending on abundance of glochidia) and (iii) that the increase in susceptibility would be highest among fish from which the metamorphosed glochidia have dropped off, as glochidia detachment involves rupture of gill epithelium of fish, helping bacterium to enter the gill tissues. In other words, we expected the survival time of brown trout in our experiment to be shorter in glochidia-infected than among control fish and decrease by the number of glochidia, especially after the detachment of glochidia.

METHODS

Long experiment

To study the effect of *M. margaritifera* infection on the susceptibility of fish to *F. columnare* after the glochidia have detached (post-parasitic period), a total of 310 zero+ y old brown trout of Rautalampi strain were transported from the Laukaa fish farm of the Natural Resources Institute Finland to the Konnevesi Research Station, University of Jyväskylä, on the 25th of August 2016. Laukaa and Konnevesi are located in a watershed not inhabited by *M. margaritifera*, but five individuals were dissected and verified microscopically of having no glochidia. Rest of the fish were allocated randomly into two 163 L flow-through tanks. Two weeks later, half of the fish were exposed to 5.0 x 10^5 *M. margaritifera* glochidia that were collected on the same day from the River Haukioja, northern Finland, while rest of the fish (control group) in the other tank were exposed to an equal volume (1,5 L) of filtered glochidia suspension without glochidia (see, Chowdhury et al., 2017). Before the 1.5 h exposure at the temperature of 14.3°C, water volume in tanks was reduced to 70 L, water flow stopped, and aeration provided. Success of glochidia infection was checked by dissecting three fish individuals 3 days after the exposure. All primed fish were infected, the average ± S.E. abundance of infection being 1,421 ± 210 *M. margaritifera* glochidia per fish. In July 2017, nine months after the infection, fish were individually marked with PIT tags (Passive integrated transponder, 7 x 1.35 mm, Loligo Systems, Denmark) and examined for *M. margaritifera* glochidia with a naked eye (see Salonen and Taskinen, 2017) while anesthetized using MS-222. After marking, 123 fish were allocated randomly in two tanks (34 infected + 28 control fish; 34 infected + 27 control fish) to be maintained until the challenge with *F. columnare* in November 2017. Four fish from glochidia infected and two fish from the control group died before the challenge with *F. columnare.*

Short experiment

To study the effect of *M. margaritifera* infection on susceptibility of fish to *F. columnare* during the parasitic period, when glochidia are actually attached to gills of fish, a group of fish (200 brown trout, 0+, Rautalampi strain, from the Laukaa fish farm, Natural Resources Institute Finland) was established in Konnevesi Research Station in late August in 2017. Fish were randomly allocated into two 163 L flow-through tanks and were verified uninfected by glochidia by dissection of the gills of five individuals. Exposure to *M. margaritifera* glochidia was performed on the 2nd of September 2017 in water temperature of 14.4°C, using the same methods as above, but with suspension of 4.0 x 10^5 glochidia collected on the same day from River Jukuanoja, northern Finland. Three brown trout were examined for glochidia three days post exposure. All primed fish were infected, the average ± S.E. abundance of infection being 1,041 ± 43 glochidia per fish. Later, on the 19th of September 2017 all the fish were marked with fin clipping while anesthetized using MS-222. After that, they were reallocated randomly with replications similar to the long experiment into two 163 L flow- through tanks, containing both infected and control fish (47 infected + 43 control fish; 47 infected + 44 control fish). They were maintained under these conditions until the challenge with *F. columnare* in November 2017. One fish died from each group before the bacterial challenge.

Challenge of fish with *F. columnare*, survival monitoring and detection of bacterial infection

The *F. columnare* strain B549 used in the experiment was isolated from Lake Kuuhankavesi, Central Finland, in 2013 and stored at −80°C in solution containing 10% fetal calf serum and 10 % glycerol. The strain was revived by culturing in modified Shieh medium (Song et al. 1988) at room temperature under agitation (120 rpm) overnight. The revived culture was further sub-cultured in the same conditions three times into larger medium volume in ratio of 1-part bacterial culture to 10 parts of fresh medium to obtain sufficient concentration for the experimental exposures. The strain had been tested to be highly virulent in previous rainbow trout challenges (Aaltonen, Pulkkinen and Taskinen unpubl.).

During the week before the challenge exposure, water temperature in the fish tanks (short- and long experiments) was slowly increased from 4.5°C to 18 °C, which was the challenge temperature, as infection with *F. columnare* (warm water disease) is not effective in cold water (Pulkkinen et al. 2010). In both the long and the short experiment, challenge of fish with *F. columnare* was started on the 16th of November 2017. Fish were allocated into four randomly chosen replicate bacterium-challenge tanks and four replicate unchallenged control tanks in both experiments, a total of 16 x 80-L tanks with a water volume of 50 L in each. The number of fish per tank varied between 13 and 15 (long experiment) and 21 and 23 (short experiment) so that the total number of bacterium-challenged/control fish was 60/57 (long experiment) and 90/89 (short experiment). Within the bacterium-challenged fish, the total number of glochidia-infected/uninfected control fish was 32/28 (long experiment) and 47/43 (short experiment). Among the unchallenged fish the corresponding figures were 32/25 (long experiment) and 46/43 (short experiment).To start the challenge, 500 mL of the bacterial culture was added to each of the challenge tanks, so that the final bacterial cell concentration was 1.0×10^4 CFU ml^{-1} (continuous challenge method; Kinnula et al., 2015). An equal volume of sterile modified Shieh medium was added to control tanks.

Fish were first monitored for signs of bacterial infection and morbidity at one-hour intervals, but after the first morbid fish was detected, the monitoring was continuous. Upon detection of signs of morbidity, fish were anaesthetized with MS-222 and killed with a blow on the head. Bacterial samples were taken with a sterile loop from the gills of each dead fish and cultured on agar plates with modified Shieh medium and tobramycin for selective isolation of *F. columnare* (Decostere et al. 1997) and incubated for 48 h at room temperature. The plates were then checked for growth of yellow, rhizoid colonies characteristic for *F. columnare.* The experiment was ended after 29 hours, when all the fish from the challenge tanks had been removed as dead/moribund. After this the remaining (unchallenged) fish were killed with

overdose of MS-222. To avoid contamination of the research station facilities with the virulent *F. columnare* by handling of diseased fish, all fish were immediately disposed without length or weight measurements, or examination of gills for glochidia.

Statistical analyses

Only the *Flavobacterium*-challenged tanks were included in statistical analyses to investigate the dependence of brown trout mortality on *M. margaritifera* infection as there was only one fish died from the *Flavobacterium*-control tanks.

Long experiment

In the long experiment, the effects of glochidia infection and possible tank effect on the survival of the fish were analyzed by two-way analysis of covariance. The possible effect of fish size to the survival time was also checked. The model assumptions of two-way ANCOVA—independence of observations, normal distribution of the depended variable in all subpopulations, linearity of covariate and homogeneity of regression slopes and that all subpopulations have the same variance (homoscedasticity)—were checked before the analysis. The assumption homoscedasticity of the subpopulations— was checked using Levene's test—and met (Levene = 0.917, df1 = 7, df2 = 52, p = 0.501). The assumption of normality—examined graphically and by using Shapiro-Wilk test, as the number of individuals in each subpopulation was between 10 and 13—was met in all subpopulations (Shapiro-Wilk, $p \geq 0.118$) except one of the subpopulations (Shapiro-Wilk, p = 0.030). Due to the robust nature of two-way ANOVA, this slight deviation from normal distribution in one subpopulation is not problematic to the 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 checked graphically and by performing two-way ANCOVA, where the model included all possible two-way interactions and three-way interaction (infection*tank, infection*weight, tank*weight, infection*tank*weight), instead of full factorial. The results suggested that both assumptions were also met (p-values ≥ 0.328).

Multiple linear regression analysis was performed to see how the number of glochidia, i.e. intensity of glochidial infection, and fish size (weight) might affect fish survival times. The model assumptions (normality, homoscedasticity and independence of residuals) were checked graphically by examining the residual plots, and independence of residuals by using Durbin-Watson statistics (d=2.130). All model assumptions were met and there was not any multicollinearity between the two independent variables. Thus, results of the multiple linear regression analysis can be considered valid.

Short experiment

In the short experiment, the effects of glochidia infection and possible tank effect on the survival of the fish were analyzed by using two-way analysis of variance. Before conducting the analysis, all model assumptions were checked as above. One of the subpopulations did not appear to be normally distributed according to Shapiro-Wilk test (W = 0.736, df = 11, p = 0.001) nor when examined graphically. Rest of the subpopulations were normally distributed both graphically and by Shapiro-Wilk test (p-values ≥ 0.185). As before, this slight deviation from normal distribution is not jeopardizing the reliability of the results (Leik, 1997). The assumption of homoscedasticity of subpopulations was met (Levene = 0.598, df1 = 7, df2 = 82, p = 0.756).

RESULTS

Mortality among the *F. columnare* challenged tanks was 100 %, but none of the fish that were not exposed to *F. columnare* challenge infection (four tanks in long experiment and four tanks in short experiment) died, except for one individual. While *F. columnare* could be isolated from all individuals from the *F. columnare* challenged tanks, the bacterium was not isolated from the only unchallenged fish that died.

Long experiment

It was found that the effect of glochidia infection was statistically significant ($F_{1, 51}$ = 4.227, p = 0.044). Survival time of *Margaritifera*-infected individuals was longer than that of the control fish (Fig. 1). The covariate fish weight was not statistically significant ($F_{1, 51}$ = 1.282, p = 0.263), suggesting that survival time of fish was independent of fish size (weight) in this experiment. On the other hand, there was a significant tank-effect ($F_{3, 51}$ = 5.156, p = 0.003). Tukey's test in equivalent two-way ANOVA without the covariate fish weight indicated that one tank had a higher survival rate that differed from all other tanks (p < 0.029 in all comparisons between tanks) (Fig. 1). However, the interaction between glochidia infection and tank was not significant ($F_{3, 51}$ = 1.330, p = 0.275), indicating that the effect of glochidia infection was parallel, with increased survival time, in all tanks.

Multiple linear regression indicated statistically significant, positive associations between the number of glochidia (counted in July, before the challenge exposure) and survival time of fish in Flavobacterium challenges (t =, 2.103, p = 0.045), but not between glochidia number and fish weight (t = 1.677, p = 0.105). The resulting regression model was

$$Survival\ time = 1094.382 + 0.097 * Glochidia\ intensity + 12.173 * Fish\ weight_g$$

(R^2 = 0.159). Thus, the higher the abundance of glochidia, the longer was the survival of trout in this experiment (Fig. 2).

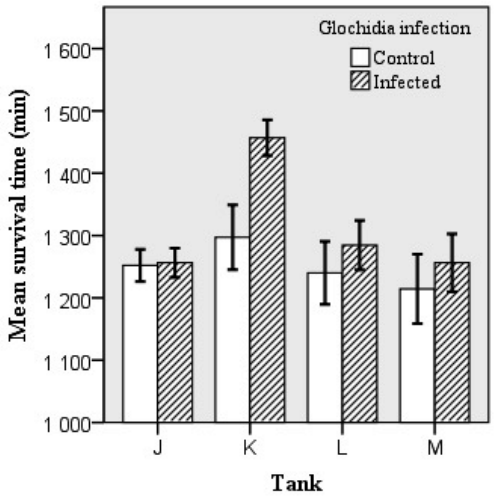

Figure 1. Tank specific mean ± S.E. survival times of brown trout previously infected with glochidia of *Margaritifera margaritifera* and those of uninfected control trout in the Long experiment where fish were challenged with *Flavobacterium columnare* 14 months after the exposure to *M. margaritifera*, i.e. when glochidia had already detached from the infected fish.

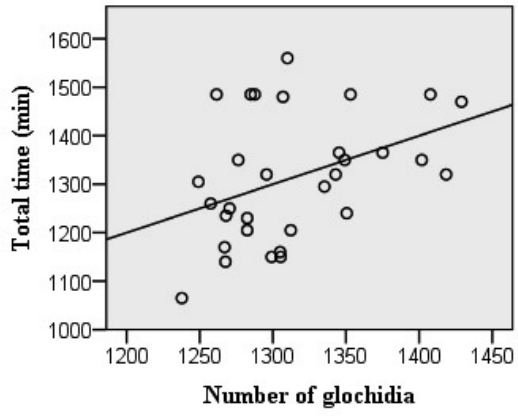

Figure 2. Survival time of brown trout previously infected with glochidia of *Margaritifera margaritifera* as plotted against the unstandardized predicted value of the number of glochidia in trout, according to results of the multiple linear regression analysis (line), in the long experiment where fish were challenged with *Flavobacterium columnare* 14 months after the exposure to *M. margaritifera*. In this experiment, numbers of glochidia were counted four months before the bacterial challenge, before their detachment.

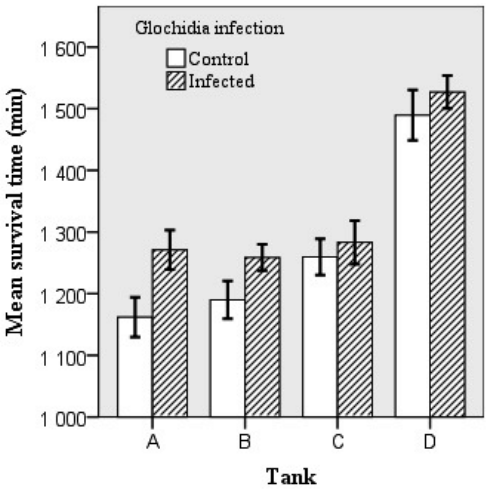

Figure 3. Tank specific mean ± S.E. survival times of brown trout previously infected with glochidia of *Margaritifera margaritifera* and those of uninfected control trout in the short experiment where fish were challenged with *Flavobacterium columnare* two months after the exposure to *M. margaritifera*, i.e. when glochidia had recently attached to (and not yet detached from) the infected fish.

Short experiment

Two-way ANOVA on survival time of fish, with glochidia infection (infected, uninfected) and tank (four tanks; *Flavobacterium*-challenged tanks, only) as fixed factors indicated that the effect of glochidia infection was statistically significant ($F_{1, 82} = 7.144$, $p = 0.009$). Survival time of *Margaritifera*-infected trout was longer than that of the control fish (Fig. 3). There was also a significant tank-effect ($F_{3, 82} = 38.557$, $p < 0.001$). Tukey's test indicated that tank D, having a high survival rate, differed from all other tanks by its mean survival time of ($p < 0.001$ in all comparisons between tanks) (Fig. 3). However, the interaction between glochidia infection and tank was not significant ($F_{3, 82} = 0.722$, $p = 0.541$), indicating that the effect of glochidia infection was parallel, increasing survival time, in all tanks.

DISCUSSION

Opposite to our hypothesis, survival time of fish previously infected with *M. margaritifera* glochidia was longer than survival time of uninfected control fish during the *F. columnare* disease outbreak, both in the short and long experiment. In addition, in the long experiment, survival time of fish increased with the number of *M. margaritifera* glochidia infecting trout (before the challenge with *F. columnare*). The bacterium *F. columnare* was isolated in every (dead) fish from the *F. columnare* exposed tanks after the experiment. Only one fish from one of the control tanks died but *F. columnare* could not be isolated from that individual. Thus, mortality of fish in this experiment can be connected specifically to *F. columnare*. This justifies the conclusion that higher survival of *M. margaritifera* infected fish—and the positive relationship between glochidia number and survival of fish—was attributed to higher resistance (lower exposure or susceptibility) of glochidia infected individuals against *F. columnare* bacteria.

All fish challenged with *F. columnare* fish died within 29 hours, and the difference in the mean survival time between *M. margaritifera* infected and control fish was about one hour in both experiments. The current challenge method is widely used when studying the infectivity and virulence of bacterial pathogens, including *F. columnare*, in fish, and it is not exceptional that a virulent *F. columnare* strain can cause 100 % mortality in juvenile salmonids within hours (Kunttu et al., 2009; Kunttu et al., 2012; Kinnula et al., 2017; Pulkkinen et al., 2018). Although the survival of glochidia-infected fish was on average only 1 h longer than that of control fish in this experiment with a highly virulent bacterial strain, this can provide a substantial survival benefit with a less virulent pathogen or in less stressful (natural) conditions. Thus, it is safe to conclude that *M. margaritifera* infection may decrease the virulence of *F. columnare* in natural and in aquaculture conditions—or at least that *M. margaritifera* infection does not increase the susceptibility of fish to *F. columnare*.

Co-infection of salmonid host with *M. margaritifera* glochidia and bacteria is probable during the captive breeding of *M. margaritifera* (e.g., Buddensiek, 1995; Thomas and Garcia de Leanis, 2010; Gum et al., 2011; Eybe et al., 2015; Moorkens, 2018). The protective effect of *M. margaritifera* against a bacterial pathogen should increase the willingness of commercial aquaculture units to be involved with captive breeding of *M. margaritifera*. As *F. columnare* is one of the worst fish pathogens harming freshwater fish farming e.g. in Finland, leading to high mortality of salmonids and intensive use of antibiotics, any new means to mitigate *F. columnare* problems are welcomed by the fish farming industry and the environment. Glochidia-infested fish stockings could be used to strengthen or restore *M. margaritifera* populations (see Wellmann, 1943). Use of glochidia-infested juvenile salmonid hosts of *M. margaritifera* in stocking programmes would be more attractive for the landowners, fishing managers, fishery collectives and salmonid conservation programmes if the glochidia of *M. margaritifera* provide protection against fish diseases—or at least would not increase disease risk.

The mechanism of protection against *F. columnare* is not known. It could be enhancement of unspecific immunity of fish due to *M. margaritifera* infection. In teleost fishes, unspecific immune defense (primary immune system, innate immunity) includes cellular components, i.e. phagocytotic cells (marcophages and granulocytes), natural killer cells, and humoral components, i.e. defense molecules, such as cytokines, interferons and the complement system (e.g. Jørgensen, 2014). *M. margaritifera* infection has been shown to induce transitory spleen enlargement (Thomas et al., 2014). The spleen is the major antibody producing organ in teleost fish (Manning, 1994), but spleen enlargement can be a signal

of infection—rather than a signal of enhanced immunocompetence—in fish (Seppänen et al., 2009). In addition, relative spleen size can decrease due to stress in fish (Kortet et al., 2003) Kunttu et al. (2009) failed to create protection against *F. columnare* in rainbow trout, even though the applied immunostimulant treatments raised values of several parameters of innate immunity in fish. However, immunostimulation as an explanation for the current result cannot be rejected and requires further investigation. If enhancement of unspecific immune defense is behind the result, it suggests that the immunostimulating effect of *M. margaritifera* glochidia is long-lasting, as the exposure to *Flavobacterium* in the long term (post-parasitic period) experiment took place 14 months after infection with glochidia and 3-4 months after detachment of glochidia from the host fish.

Alternatively, structure of the gills may change due to *M. margaritifera* infection, so that the entry of the bacterium through the gills, or the establishment of the bacterium on the gills is weakened. It is known that *M. margaritifera* glochidia cause hyperplasia and fusion of gill filaments (Treasurer and Turnbull, 2000; Thomas et al., 2014) and lessen mucous cells of gills (Thomas et al., 2014), but it is not known if these changes would increase resistance of trout against *F. columnare*. Furthermore, especially the protective effect of *M. margaritifera* infection after the glochidia drop-off is surprising. Metamorphosed glochidia rupture the gill epithelium when detaching (Waller & Mitchel, 1989), which should increase the vulnerability to secondary infections—especially to *F. columnare*, which enters the fish body mainly through the gills (Declercq et al. 2013, 2015).

In theory, it is possible that the observed protective effect of glochidia is an adaptive feature of *M. margaritifera* to increase its own survival and fitness (Poulin, 2010; Hughes et al., 2012; Gopko et al., 2015; Gopko et al., 2017a). It is not unprecedented that a parasite would enhance the immune defense of its host, or in some other way impair the ability of second parasite/microbe to enter the host (e.g. Ashby et al., 2017), but why would the effect remain several months after the glochidia are shed?

If there is an immunostimulation behind this finding, this could be produced by any parasite, not just *M. margaritifera*. However, this is not supported by results of Gopko et al. (2018) who showed that brown trout pre-infected with trematode parasites were either as vulnerable (2 weeks post-infection) or more vulnerable (20 h post-infection) to subsequent glochidial infection. Moreover, a number of studies have found a lowered resistance to bacterial infections in fish pre-infected with parasites (Kotob et al., 2016). In any case, if there is an immunostimulation, it could be produced not only by penetrating parasites, but by whatever foreign object, or damage, in addition to *M. margaritifera* glochidia. For instance, Henrich et al. (2014) found that tagging caused a significant, although ephemeral, increase in immune response of three-spined stickleback. It is, thus, important to verify that the present protective effect of glochidia infection is specific to *M. margaritifera.* Moreover, it is important to study if *M. margaritifera* glochidia provide protection also against other bacterial fish pathogens than *F. columnare*.

Increased susceptibility of fish to bacterial infection has been shown in co-infection by monogenean gill parasites (Busch et al. 2003), tissue-penetrating trematode metacercaria (Pylkkö et al. 2006), fish lice (Bandilla et al. 2008; Lhorente et al., 2014) and different ciliated ectoparasites (Evans et al., 2007; Xu et al., 2009; Shoemaker et al., 2012; Xu et al., 2012). A chronical myxosporean parasite infection decreased the resistance of rainbow trout to bacterial disease still 12 months after exposure to the parasite (Densmore et al., 2004). Thus, *M. margaritifera* glochidia, increasing resistance to *F. columnare* in trout, is a notable exception among co-infections between parasites and bacterial pathogens, urging further investigations on the relationship between fish and glochidium parasites.

The protective effect of *M. margaritifera* glochidia is also exceptional if compared to the results of Gopko et al. (2018) who showed that infection of brown trout with *M. margaritifera* glochidia increased susceptibility to subsequent infection—ten months after infected with glochidia—with the trematode parasite *Diplostomum pseudospathaceum* (Trematoda). The enhancing effect of *M. margaritifera* on subsequent infection with *Diplostomum* was explained by the increased ventilation of the glochidia-infected fish, which increases exposure to *Diplostomum* cercariae floating in the water and entering fish mainly through gills (Mikheev et al., 2014). The gills of the fish are the gateway — the first and main site of infection for both *F. columnare* (Declercq et al. 2013) and *Diplostomum* parasite (Mikheev et al., 2014). However, opposite to *Diplostomum* parasite, *M. margaritifera* glochidia did not enhance subsequent infection with *Flavobacterium* but suppressed it. Either the interaction between fish gills or immune system with a macroparasite larva is different than with a bacterium, or there is something special in *M. margaritifera* glochidia in this respect. To conclude, present results are in accordance with the Ziuganov (2005) proposal for some beneficial effects of *M. margaritifera* infection on fish health.

Freshwater mussels provide a variety of ecosystem services and perform many important functions in aquatic ecosystems, such as biofiltration, nutrient cycling and storage, food web dynamics and bottom quality modification, leading to improved water quality, habitat structure and biodiversity, in addition of direct provision of food, tools and jewelry (e.g. Vaughn & Hakenkamp, 2001; Strayer, 2014; Vaughn et al., 2008; Haag, 2012; Vaughn, 2018). For example, it was recently found that filtration by the unionid mussel *Anodonta anatina* reduces the density of cercarial larvae of the harmful fish parasite, *Diplostomum*, which causes problems in fish farming (Gopko et al., 2017b). In the light of the present results, protection of salmonid host against very harmful *Flavobacterium* fish pathogen by *M. margaritifera* can be added to the list of beneficial services provided by freshwater mussels. If so, this illustrates the risk of lost important (unknown) ecosystem services with decline or extinction of endangered species and declined biodiversity, in general. More specifically, this emphasizes the possible valuable functions and previously unknown interactions of freshwater mussels in aquatic communities and urges further studies on ecosystem services by the endangered freshwater pearl mussel, *M. margaritifera*. The result increases our knowledge and understanding of the interactions between mussels, their fish hosts and fish pathogens.

The freshwater pearl mussel *M. margaritifera* has become a flagship species, a symbol and leading element of conservation of river habitats and freshwater biodiversity (Geist, 2010). Our findings should increase the interest and willingness of commercial fish farms, fishing authorities, fishery collectives and landowners. These stakeholders is to participate in the conservation activities involving infection of salmonids with *M. margaritifera* since it may protect fish against severe bacterial disease and turn public opinion in favor of *M. margaritifera* conservation.

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