Master's thesis

Immunization of trout (S*almo trutta*) against freshwater pearl mussel (*Margaritifera margaritifera*) by duck mussel (*Anodonta anatina*) glochidia

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Chowdhury Motiur: Immunization of trout (*Salmo trutta*) against freshwater pearl mussel (*Margaritifera margaritifera*) by duck mussel (*Anodonta anatina*) glochidia
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ABSTRACT

Freshwater pearl mussel *Margaritifera margaritifera* is endangered throughout its range of distribution in Europe. In some rivers *M. margaritifera* may co-occur with the duck mussel *Anodonta anatina*. Brown trout *Salmo trutta* serves as suitable fish host for both mussel species. Thus it is possible that exposure of brown trout to *A. anatina* glochidia might increase resistance of fish against *M. margaritifera* glochidia. The aim was to test this 'cross immunity hypothesis'. Brown trout were immunized by duck mussel (*Anodonta anatina*) glochidia in late May. Then brown trout were challenged by pearl mussel (*Margaritifera margaritifera*) glochidia in late August after the *A. anatina* glochidia were developed and dropped off from the fish. The mean number and size of *M. margaritifera* glochidia were compared between immunized and control fish in 4 different time points over 10 months. Besides of these, effect of marking of fish (fin-clipping), fish length and body side (left vs. right) on glochidia number was also studied. The mean number of attached glochidia was significantly lower in immunized fish than in the unimmunized control fish in the first time point, in September, but not in December, May or June. No effect of immunization on growth (size) of glochidia was observed in any time point. Unexpectedly, right side gills contained more glochidia than left side gills of fish regardless immunization. Fish length had a significant positive effect on glochidial abundance. Fin-clipped fish had less glochidia than non-clipped fish. Result indicates that fin-clipping may activate non-specific immunity that may have given protection against *M. margaritifera* glochidiosis. Results of the present study suggest that cross immunization by exposer to *A. anatina* glochidia may not possess a significant threat for *M. margaritifera* conservation.

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TIIVISTELMÄ

Jokihelmisimpukka *Margaritifera margaritifera* on erittäin uhanalainen makean veden simpukkalaji. Jokihelmisimpukalla elämänkierrossaan on loisiva toukkavaihe, glokidium, joka elää lohen ja taimenen kiduksille tarttuneena. Joissakin tapauksissa pikkujärvisimpukka *Anodonta anatina* saattaa esiintyä samassa habitaatissa jokihelmisimpukan kanssa. Tästä syystä tässä työssä haluttiin tutkia aiheuttaako taimenen (*Salmo trutta*) altistaminen pikkujärvisimpukan (*Anodonta anatina*) glokidium-toukille immunisaation jokihelmisimpukan (*Margaritifera margaritifera*) glokidium-toukkia vastaan. Immunisointi: Glokidium-toukille altistumattomat, 1+ taimenet (300 kpl, Iijoen meritaimen, RKTL Taivalkoski) infektoitiin pikkujärvisimpukan (Koijärvi, Kerimäki) toukilla Konneveden tutkimusasemalla kesäkuun alussa 2012. Infektion onnistuminen (5 päivää infektoinnista), sekä toukkien kypsyminen ja poistuminen kaloista (2,5 kk infektoinnista) tarkastettiin mikroskooppisesti. Kalat merkittiin eväleikkauksin siten, että kahdessa koealtaassa immunisoidut kalat olivat eväleikattuja ja kahdessa altaassa puolestaan kontrollikalat olivat eväleikattuja (yhteensä 4 allasta). Kalat haasteinfektoitiin jokihelmisimpukan toukilla elokuun lopussa ja toukkien lukumäärä ja koko tutkittiin 2012 syyskuun lopussa ja joulukuussa sekä 2013 toukokuussa ja kesäkuussa, kun toukat alkoivat kypsyä. Immunisointi pikkujärvisimpukan toukilla antoi tilastollisesti merkitsevän suojan jokihelmisimpukan toukkia vastaan syyskuussa, mutta sen jälkeen vaikutus hävisi. Yllättäen tulokset viittasivat siihen, että eväleikkaus suojasi taimenia jokihelmisimpukkainfektiolta paremmin kuin immunisointi pikkujärvisimpukan toukilla. Eväleikkaus 2 viikkoa ennen haasteinfektiota saattoi tehostaa epäspesifin immuunijärjestelmän toimintaa kaloissa. Vielä yllättävämpi tulos oli se, että jokihelmisimpukka-glokidioitten määrä oli korkeampi kalojen oikean puolen kiduksilla verrattuna vasempaan puoliskoon. Tätä tulosta oli vaikea selittää, mutta se saattaa liittyä esimerkiksi kalojen kasvatusaikaisiin olosuhteisiin—ehkä oikean puolen kidukset olivat kookkaammat, jolloin niihin mahtui enemmän toukkia. Kaiken kaikkiaan tulokset viittavat siihen, että altistuminen pikkujärvisimpukan toukille ei saata muodostaa vakavaa uhkaa jokihelmisimpukan menestymiselle taimenisännässä.

Contents

1. INTRODUCTION

The freshwater pearl mussel (*Margaritifera margaritifera*) is an endangered bivalve mollusc that has obligate parasitic larval stage, known as glochidium. The species is being dramatically declined throughout its range of occurrence. The larvae of *M. margaritifera* are very host-specific and can only complete their development on Atlantic salmon (*Salmo salar*) or brown trout (*Salmo trutta*) (Hastie & Young 2001). On the other hand, the common duck mussel, *Anodonta anatina*, occurs abundantly throughout Finland and it has also a short parasitic larval stage being able to infect brown trout. Study shows that the acquired immunity of fish to one parasite species can result in immunity also to other species (cross resistance) (Buchmann 1998; Larsen et al. 2002; Dodd et al. 2005).

Life cycle of unionoida mussels requires a host fish. Based on structure the hooked glochidia (e.g., *Anodonta anatina*) are able to attach to the gills, fins or skin whereas hook free (e.g., *Margaritifera margaritifera*) attach only to gills of the host fish. It is possible that the fresh water pearl mussel *M. margaritifera* and duck mussel *A. anatina* species co-occur in most of the Europe, for example in southern Finnish rivers. Brown trout, which serves as the host for pearl mussel, is also a suitable host for the duck mussel. Therefore, exposure of brown trout to glochidium larvae of the duck mussel may induce acquired immunity that can impair development of pearl mussel glochidia in brown trout.

The generated knowledge from the experiment could have practical application in conservation efforts when searching for reasons for reduced reproductive success of the endangered freshwater pearl mussel. It would also increase our understanding of the mussel - fish host relationship. The main goals of this study were to determine whether host fish that have acquired resistance to one mussel species are cross-resistant to the freshwater pearl mussel species and effect on glochidial development.

2. BACKGROUND

Nearly all Unionoida have parasitic larvae (glochidia) that complete development to the juvenile stage while attached to fish. Based on the glochidium size (Bauer 1994) and water temperature the parasitic stage in unionids varies from 3 days to 10 months (Seshaiya 1969). According to Ziuganov et al. 1994 and Cosgove et al. 2000, the margaritiferid-salmonid relationship may be symbiotic. Glochidia obtain shelter and nutrition from the host fish (Fisher and Dimock 2002), to be able to complete their life cycle. Apart from the glochidial development, juvenile mussels gain most obvious advantage of upstream dispersal from their parasitic relationship with fish (Watters 2001).

On the other hand, filtration by adult mussels possibly clarifies river water to the benefit of other species, including juvenile Atlantic salmon (*Salmo salar*) and brown or sea trout (*Salmo trutta*) (Zuiganov et al. 1994). Providing substratum for algae and habitats for benthic invertebrates mussels also work as food source to Salmon parr. Moreover, high biomass of mussel plays important role in particle processing, nutrient release and sediment mixing (Vaughn and Hakenkamp 2001) According to Vaughn et al. 2008, by linking and influencing multiple trophic levels freshwater mussels are working as important components of food webs, as omnivores they feed on algae, bacteria, detritus, zooplankton and perhaps, dissolved organic matter. Mussels maintain water quality by storing toxicant in tissues and pseudofeaces (James 1987; Strayer et al. 1994). Mussels show characteristics of “sentinel organisms” (Philips and Rainbow 1992), and potentially are biomonitors of ecosystem health. (Jeffree et al. 1995).

Two stages, the early pre-parasitic (entering and attaching to the gills) and the post-parasitic (dropping off from gills to suitable environment of juvenile) stages, are the two critical phases during life cycles of *M. Margaritifera* and make the species vulnerable to changes in their habitats (Coker et al., 1919-20) Less than 10 out of 1 million glochidia can successfully be attached to the suitable host (Bauer 1989; Young and Williams 1984a). According to Young and Williams (1984b), found 95% juvenile mortality in Scotland causing from unfavourable habitats and predation. In addition to, eutrophication is also threat for juvenile mussels (Bauer et al., 1980; Buddensiek, in press).

The number of compatible host species varies among mussel species from 1 to 37 (Watters, 1994). Larvae of *Anodonta anatina* can complete their development on 15 fish species including brown trout (*Salmo trutta*) (Franke 1993). This species was also the most common host fish for pearl mussel revealed by Geist et al (2006).

Fish immune system provides fish individual with the ability to resist infectious agents, destroy neoplastic cells, and reject nonself component. The immune system is classified in two functional entities: the nonspecific (innate) and the specific (acquired). Natural resistance of fish is the result of a number of nonspecific humoral factors and body secretions (reviewed by Yano, 1996). Specific immune responses are directed against an agent to whom the organism has previously been sensitised. It is well known that fish possess lymphocyte populations analogous to T and B cells, and thus both cellular and humoral specific responses exist. Cytotoxic T-lymphocytes as a function of cell-mediated immunity (reviewed by Manning & Nakanishi, 1996; Nakanishi et al., 1999).

Cross immunization of trout by duck mussel may have an effect of glochidial attachment and development inside the trout is the main hypothesis of this study. More specifically, the hypothesis was that immunization will decrease number of *Maragritifera* glochidia in fish.

3. MATERIAL AND METHODS

3.1. Study area species

The study was conducted in Konnevesi research station of University of Jyvaskyla, Finland. The experiment took place from May 2012 to June 2013.

Brown trout (*Salmo trutta*) fry (>300 individuals), 1+ year old, Iijoki stock, were obtained from Finnish Game and Fisheries Research Institute Taivalkoski and transported to Konnevesi research station, University of Jyväskylä, on May 23, 2012. Fish were allocated into two tanks. Duck mussel (*Anodonta anatina*) (40 individuals) were obtained from Koijärvi, 350 km south from Iijoki, eastern Finland. *A. anatina* glochidia were obtained on May 24, 2012 by dissecting gills of mussels in the laboratory. By dissecting and checking the gills of a few trout, it was ensured that no previous glochidium infection was established among the experimental fish. Pearl mussel (*Margaritifera margaritifera*) glochidia were obtained mussels from Iijoki area and transporting the larvae shed by mussels to Konnevesi research station on 28 August 2012.

3.2. Infection by *Anodonta anatina* glochidia

Trouts were divided into four 163 litre flow-through tanks. Two immunizated tanks (163 L flow through) with 100 fish in one tank and 50 fish in another tank + two control tanks with 100 fish in one tank and 50 fish in another tank (Fig. 1). It was added 1, 8 L

(71000 larvae per L) glochidium suspension to tank holding 100 fish and 1,0 L to tank holding 50. Infection time was 2 hours; water had been rinsed for proportionate mixing of glochidial suspension throughout the tanks. Control fish were not given anything but water stopped equally. During immunization, water volume was 70 L in every tank and water temperature was 7.7 C.

3.3. *Anodonta anatina* infection success and glochidial development

It was examined 5 trout (from 100 fish tank) and 3 trout (from 50 fish tank) from both immunized and control fish 5 days post infection. Abundance of Anodonta glochidia in immunized varied between 90-232 glochidia, whereas no glochidium was found in control fish.

After 2.5 months (on 15 August 2012) of the first infection (immunization) one fish from each tank were examined and found no glochidia as *A. anatina* glochidia already developed and dropped off from the immunized/infected fish.

3.4. Infection by pearl mussel glochidia

Fish were marked on 15 August 2012, using "fin clipped and not-clipped" to mix primarily immunized and control fish together into 4 new tanks. Among the tanks, 2 tanks contained immunized and fin clipped with control and not-clipped fish whereas the other 2 tanks contained control and fin clipped with immunized and not clipped fish (Fig. 1). All the fish were anesthetized before marking and handled in similar ways for both clipped and not-clipped fish.

After 2 weeks of marking and mixing (on 28 August 2012), 0.5 L (584,600 glochidia per litre) pearl mussel glochidia suspension was added to all 4 tanks and mixed swiftly. Alike the first infection, the conditions in all 4 tanks was equal. The temperature varied between 1.1^0C to 16.8^0C throughout the experiment, being highest in September 2012 and June 2013.

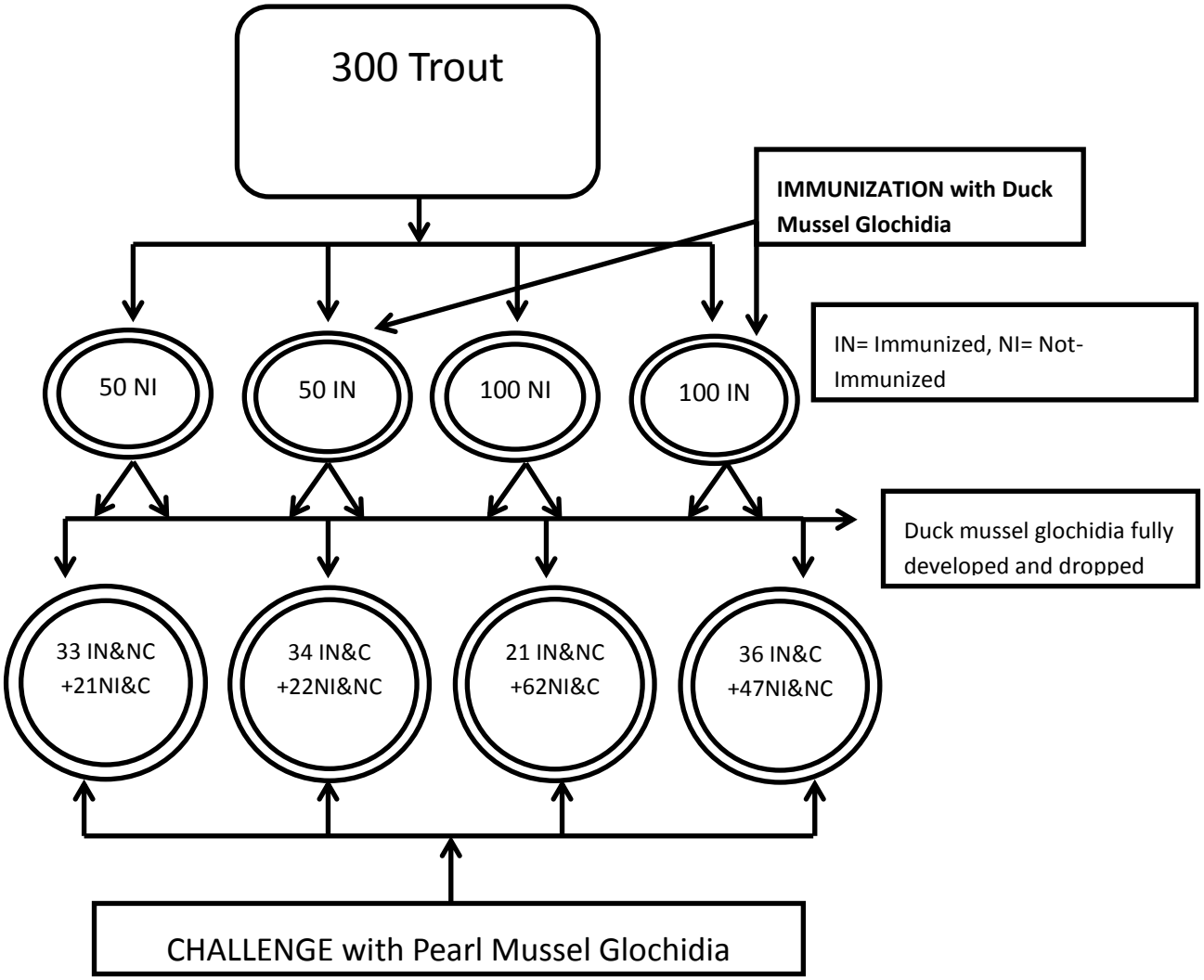

Figure 1. Experimental design; here IN, NI, C & NC were used for Infected, Not-infected, Fin cut/clipped & Not Fin-clipped accordingly.

3.6. Data collection and statistical analysis

Fish were killed and measured the length and weight of fish. Then gills were cut off for counting number of glochidia attached to the gills and measuring size of glochidia in micrometer by light microscope. In some cases we calculated only number from one side of gills, therefore the estimate of total number was this number multiplied by two. It is obvious that there are no systematic differences between left and right gills (Cunjak & McGladdery 1991). Data were collected in 4 different monitoring periods namely September 2012 (3 weeks post infection), December 2012 (3 months post infection):), May 2013(9 months post infection) and June 2013 (10 months post infection) to cover the whole 10 to 11 months period when glochidia are attached to fish.

Since fish grow and glochidia number is highly dependent on fish length, it was not possible to include all time points into same ANCOVA model while having fish length as a covariate. Thus, each time point was analysed separately by ANCOVA by having total glochidium number as the response variable, immunization (immunized, control) as a fixed factor, tank (4 tanks) as a random factor and fish length as a covariate. For each time point, first the interactions length x tank and length x immunization were analysed by conducting an ANCOVA with these interaction term included to verify that length can be used as the covariate; insignificant interactions indicate equal slopes with respect to fish length indicating that the use of covariate was allowed. In those cases, those interaction terms were excluded and the analysis was continued with a full factorial model.

When studying the effect of marking, fin-clipping, on glochidia numbers, ANCOVAs with marking (clipped vs. not clipped) as a fixed factor and fish length as a covariate were applied separately for control fish and immunized fish since fin clipping itself can interfere with host immune system. In addition, as in other analyses, time points were studied separately, as glochidium numbers may change over the ten months study period. Tank effect was not taken into account as the tank effect on glochidia numbers was found not significant.

In addition, paired t-test was studied for finding difference between right and left sides of the fish in glochidium abundance.

4. RESULTS

4.1 Glochidia numbers, effect of immunization and tank

In September the interactions length x tank and length x immunization were not significant. Results of the full factorial ANCOVA model indicated a significant, negative effect of immunization on glochidum abundance was significant in September ($F_{1,31} = 12.760$, $p = 0.046$) so that the mean length-adjusted abundance of glochidia was reduced by a factor of 0.82 in immunized fish as compared to unimmunized control fish (Fig 2). The effect of tank ($F_{3, 31} = 6.708$, $p = 0.070$) and the immunization x tank interaction ($F_{3, 31} = 0.228$, $p = 0.867$) were not significant indicating that the mean numbers of glochidia did not differ between tanks, and that the effect of immunization on glochidia numbers was consistent in all experimental tank. The effect of covariate fish length was highly significant ($F_{3, 31} = 18.858$, $p < 0.0001$). Numbers of glochidia increased by fish length (Fig. 3).

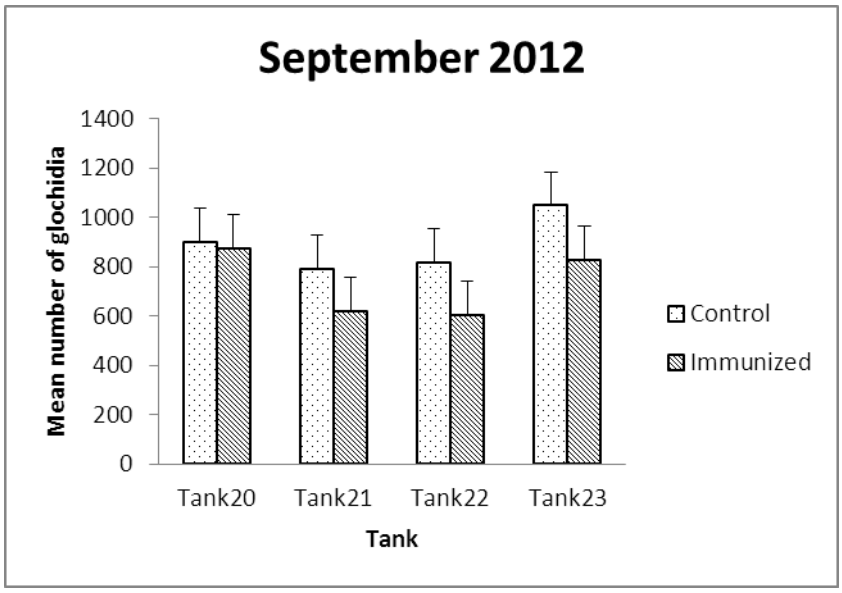

Figure 2. Length-adjusted mean (± s.e.) numbers of *Margaritifera margaritifera* glochidia among fish immunized with *Anodonta anatina* and among unimmunized control fish in four experimental tanks in September, three weeks after challenge infection.

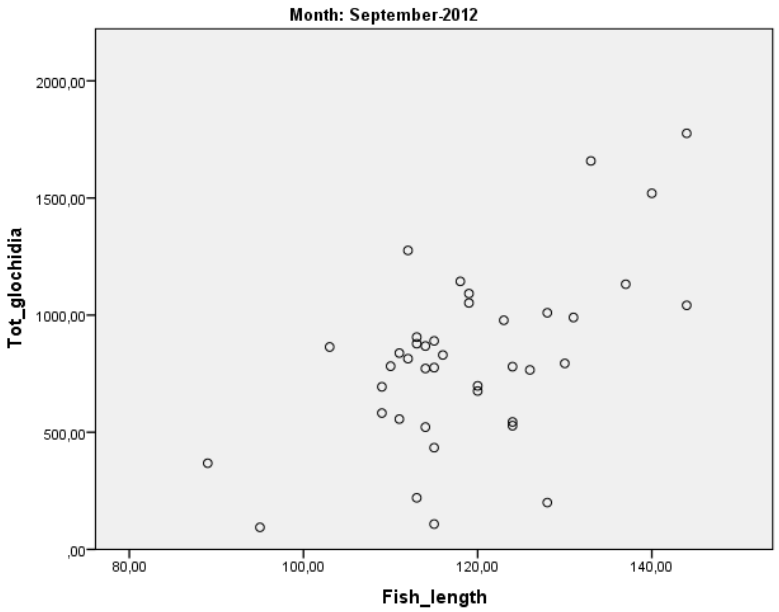

Figure 3. Total number of glochidia in relation to fish length in September

For December data, numbers of glochidia were logarithm-transformed (Log10(X+1)) to fulfil the assumptions of variance analysis. Also in December the interactions length x tank and length x immunization were not significant. Results of the full factorial ANCOVA model indicated no significant effect of immunization (Fig. 4) ($F_{1, 41} = 0.085$, $p = 0.786$) or tank ($F_{3, 41} = 0.198$, $p = 0.892$) or immunization x tank interaction ($F_{3, 41} = 1.369$, $p = 0.266$). The effect of covariate fish length was significant ($F_{3, 41} = 5.518$, $p = 0.024$) with increasing glochidia numbers by increased fish length (Fig. 5).

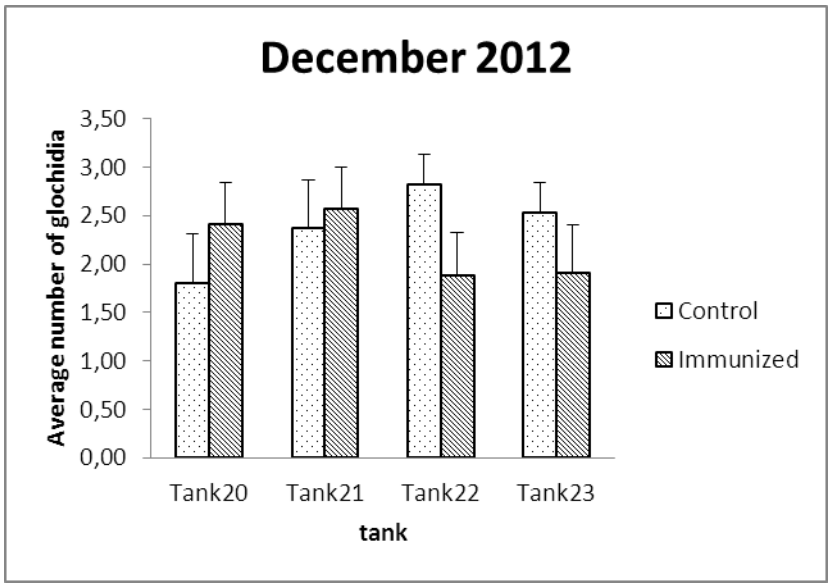

Figure 4. Length-adjusted mean (± s.e.) numbers of *Margaritifera margaritifera* glochidia among fish immunized with *Anodonta anatina* and among unimmunized control fish in four experimental tanks in December, three months after challenge infection.

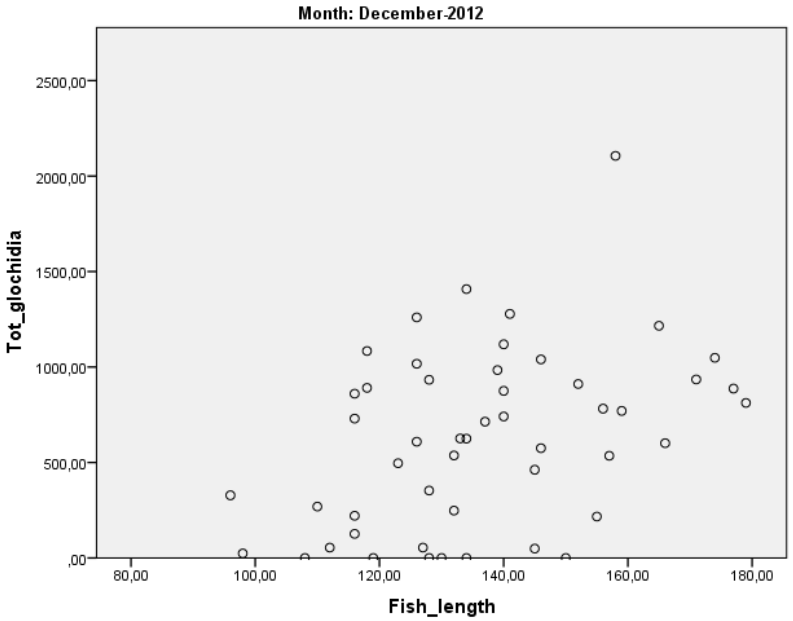

Figure 5. Total number of glochidia in relation to fish length in December

As in earlier time points, also in May the interactions length x tank and length x immunization were not significant. Like in December, results of the full factorial ANCOVA model indicated no significant effect of immunization (Fig. 6) ($F_{1, 65} = 0.693$, $p = 0.434$) or tank ($F_{3, 65} = 1.308$, $p = 0.418$) or immunization x tank interaction ($F_{3, 65} = 1.023$, $p = 0.388$). In May the effect of covariate fish length was not significant ($F_{1, 65} = 1.440$, $p = 0.235$) suggesting that the abundance of glochidia was not associated with fish length (Fig. 7).

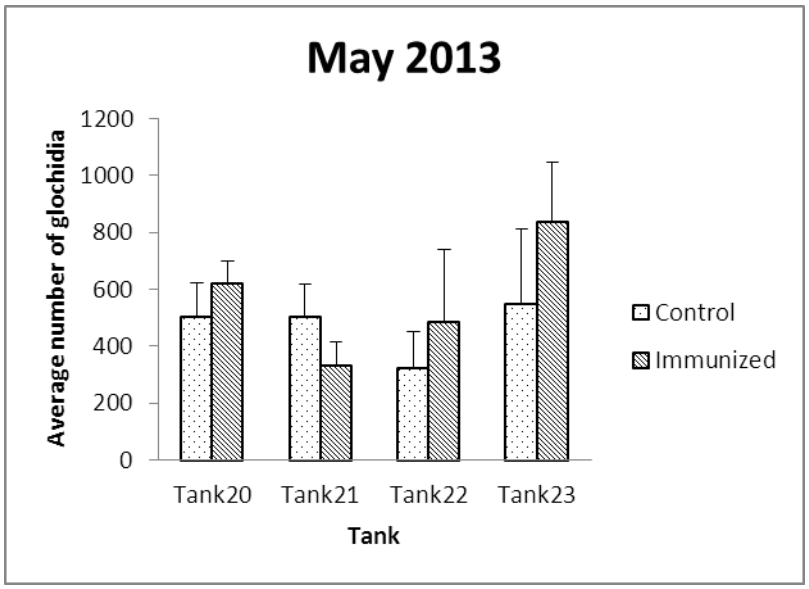

Figure 6. Length-adjusted mean (± s.e.) numbers of *Margaritifera margaritifera* glochidia among fish immunized with *Anodonta anatina* and among unimmunized control fish in four experimental tanks in May, nine months after challenge infection

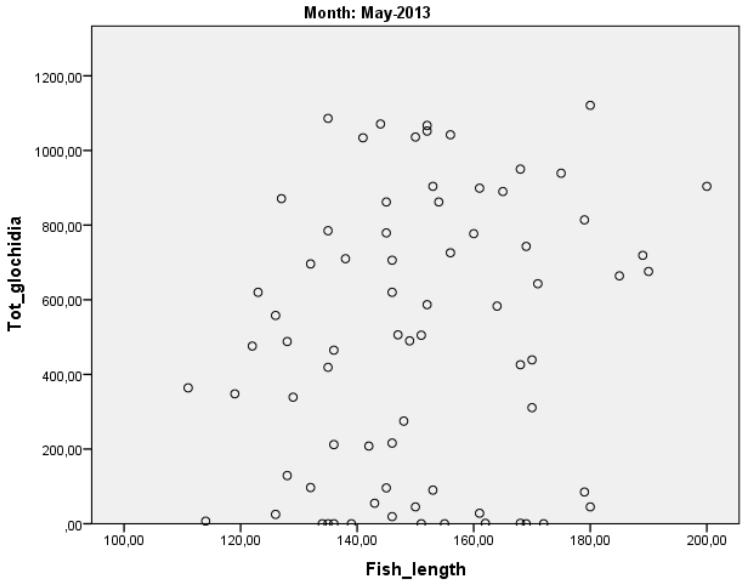

Figure 7. Total number of glochidia in relation to fish length in May

Also in June the interactions length x tank and length x immunization were not significant. Results of the full factorial ANCOVA model indicated no significant effect of immunization (Fig. 8) ($F_{1, 86} = 0.354$, $p = 0.672$) or tank ($F_{1, 86} = 3.086$, $p = 0.370$) or immunization x tank interaction ($F_{1, 86} = 1.178$, $p = 0.281$) In June the effect of covariate fish length was significant ($F_{1, 86} = 7.975$, $p = 0.006$) with a positive association between glochidium numbers and fish length (Fig. 9).

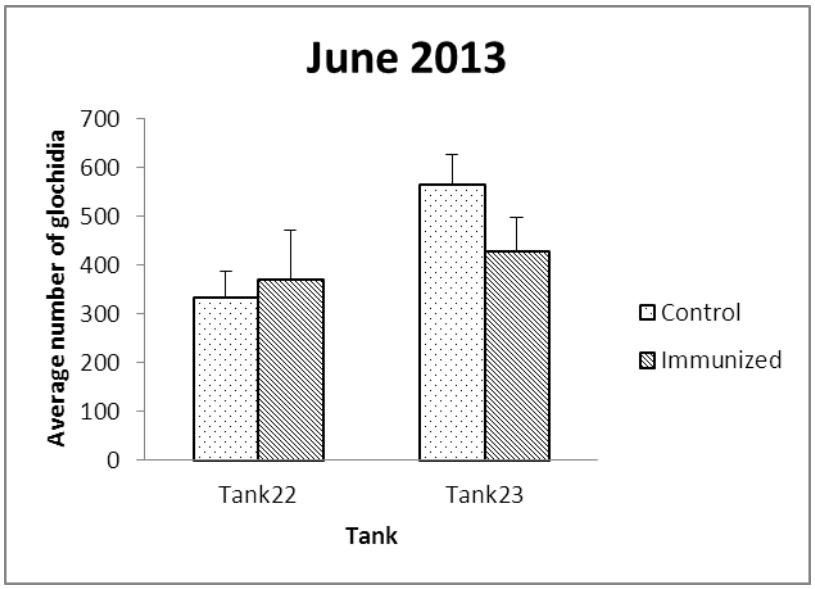

Figure 8. Length-adjusted mean (± s.e.) numbers of *Margaritifera margaritifera* glochidia among fish immunized with *Anodonta anatina* and among unimmunized control fish in four experimental tanks in June.

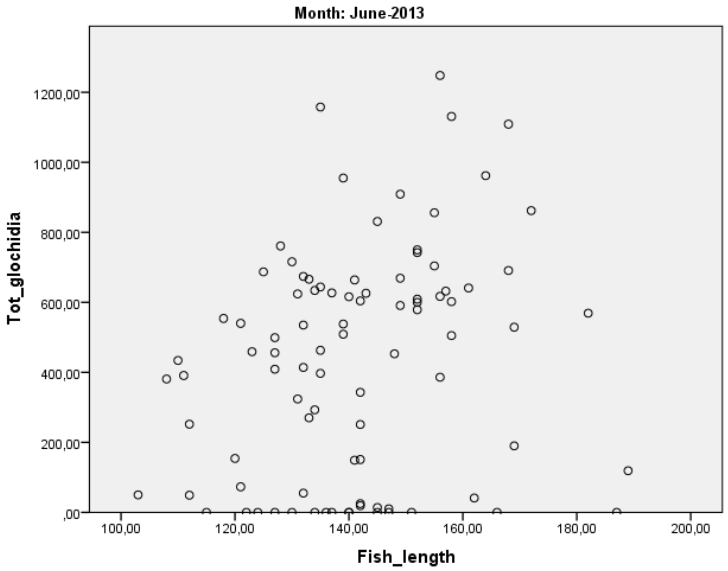

Figure 9. Total number of glochidia in relation to fish length in May

4.2 Glochidia size

In the present experiment which run over ten months, fish were growing and glochidia were growing by time. Thus, as the target was to analyse the effect of immunization of glochidium growth, the analyses were performed separately for each time points (September, December, May and June) using ANCOVA, with total glochidium number as the response variable, immunization (immunized, control) as a fixed factor, tank (4 tanks) as a random factor and fish length as a covariate. For each time point, first the interactions length x tank and length x immunization were analysed by conducting an ANCOVA with these interaction term included to verify that length can be used as the covariate; insignificant interactions indicate equal slopes with respect to fish length

indicating that the use of covariate was allowed. In those cases, those interaction term were excluded and the analysis was continued with a full factorial model

September data was squared to fulfil assumptions of variance analysis. In September the interactions length x tank and length x immunization were not significant. Results of the full factorial ANCOVA model indicated that neither the effect of immunization, tank or immunization x tank interaction was significant indicating that size of glochidia was not affected by these factors. Same pattern was observed in December and June. In May the effect of immunization was also not significant but there was a statistically significant tank effect ($F_{3, 57} = 14.237$, $p = 0.029$), although immunization effect was not. Pairwise comparisons revealed that glochidium size was lower in tank 21 than in tanks 20 and 22.

However, a clear relationship was observed between glochidium size and glochidium numbers (Fig. 10, 11, 12 &13). In all time points there was a statistically significant, positive correlation between glochidium size and number; September, Pearson $r = 0.428$, $n = 39$, $p = 0.007$; December, Pearson $r = 0.525$, $p < 0.001$, $n = 44$; May, Pearson $r = 0.566$, $p < 0.001$, $n = 66$; June, 0.492, $p < 0.001$, $n = 76$). Thus when glochidium number was high they also grew well.

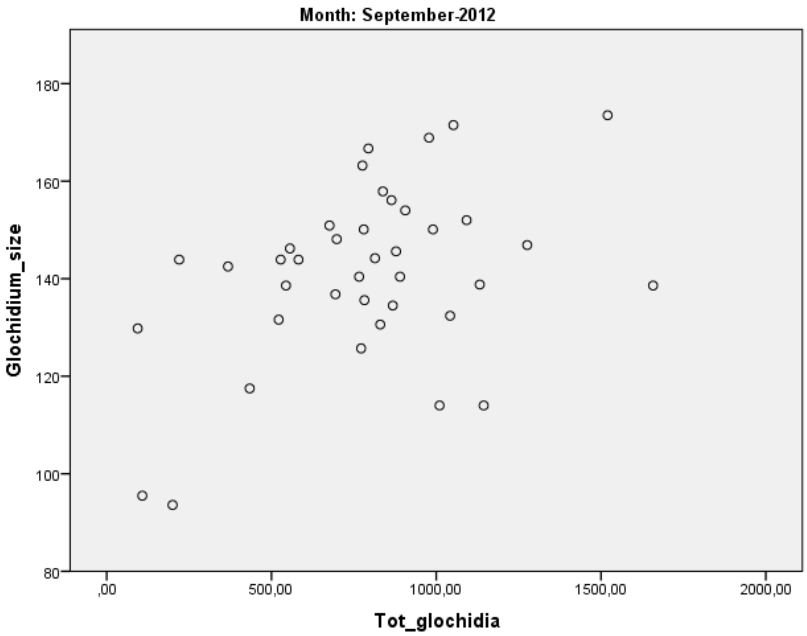

Figure 10. Glochidium size in relation to glochidium numbers in September

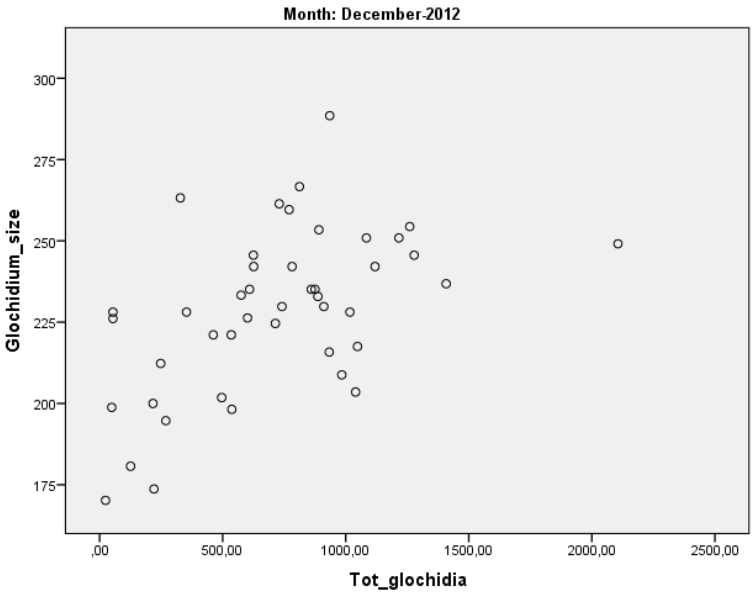

Figure 11. Glochidium size in relation to glochidium numbers in December

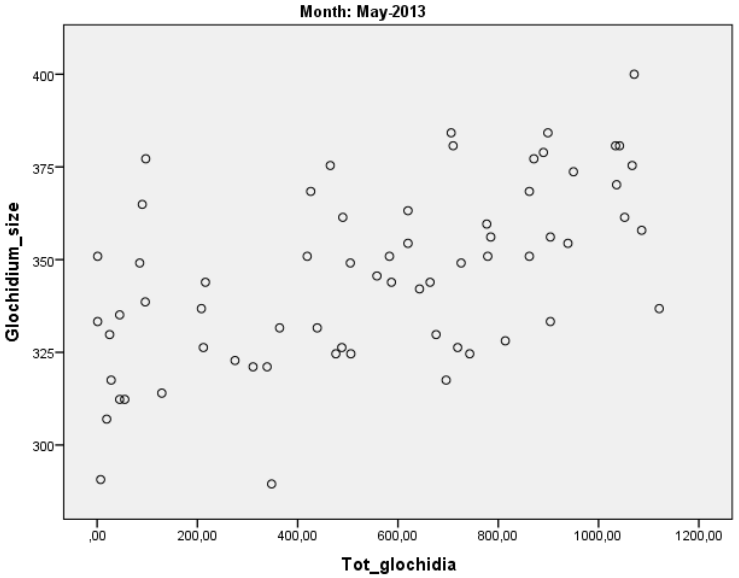

Figure 12. Glochidium size in relation to glochidium numbers in May

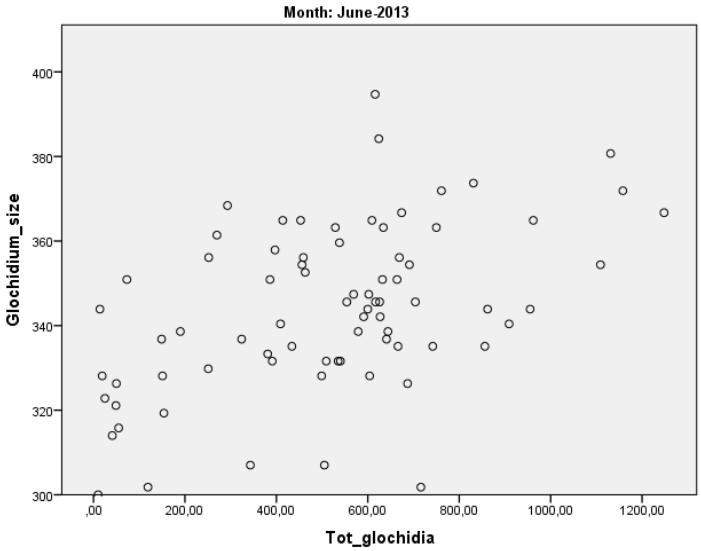

Figure 13. Glochidium size in relation to glochidium numbers in June

4.3 Left side versus right side

Difference between right and left sides of the fish in glochidium abundance was studied using paired t-test separately for each time point and tank since glochidia numbers probably change over ten months period of the present study. Mean number of glochidia was higher on the right side of fish than on the left side in all tanks in every time point (Fig. 14), the difference being statistically significant in December tanks 20 ($t = 3.591$, $df = 29$, $p = 0.001$) and 21 ($t = 2.092$, $df = 28$, $p = 0.046$), in May tanks 20 ($t = 3.591$, $df = 29$, $p = 0.001$) and 21 ($t = 2.092$, $df = 28$, $p = 0.046$)) and in June tank 23 ($t = 2.532$, $df = 49$, $p = 0.015$).

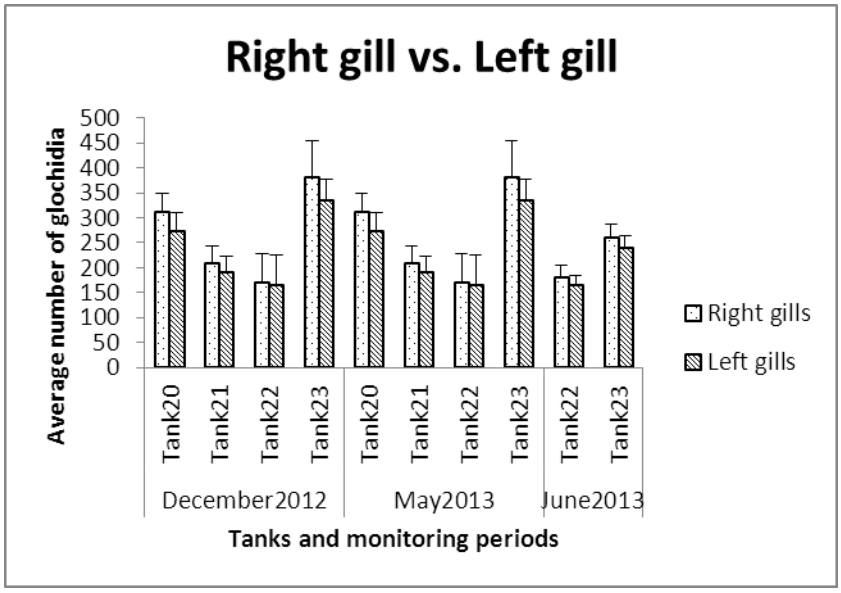

Figure 14. Mean number of glochidia in left vs. right gills in different tanks in different time points.

4.4 Fin-clipping

The mean abundance of glochidia was higher in fish not fin-clipped as compared fin-clipped individuals both in immunized fish and in control fish in every time point, except for immunized fish in June (Fig. 15). However, the difference between clipped and non-clipped fish was statistically significant only in control fish in June (Fig. 16) ($F_{1, 60} = 11.812$, $p = 0.001$) and among the immunized fish in May ($F_{1, 44} = 4.455$, $p = 0.041$), while the effect of fish length was always significant.

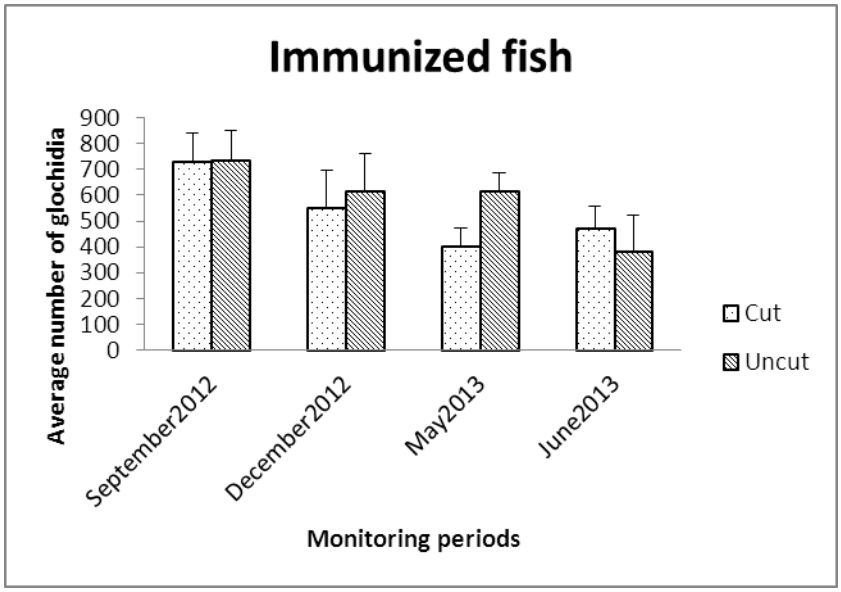

Figure 15. Length-adjusted monthly mean abundances of glochidia infection for fin-clipped and non-clipped fish in immunized fish group.

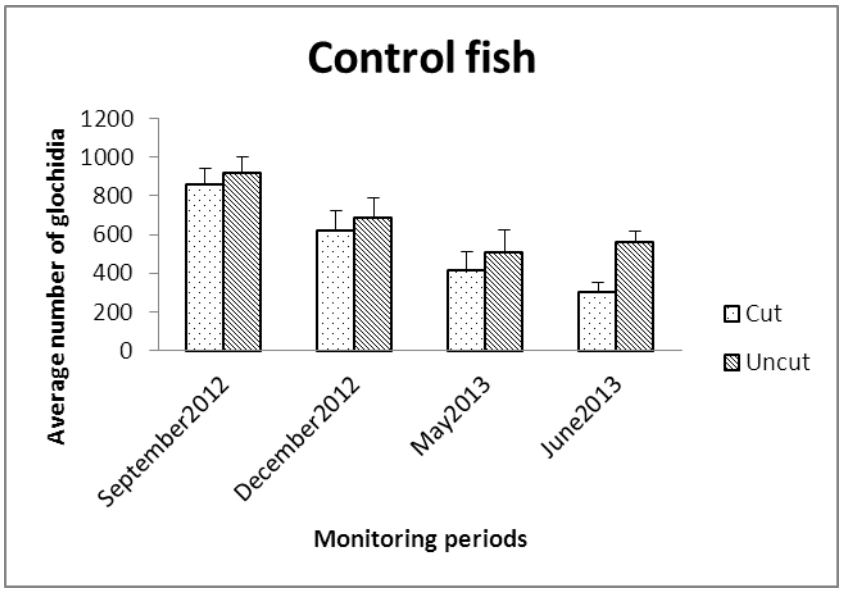

Figure 16. Length-adjusted monthly mean abundances of glochidia infection for fin-clipped and non-clipped fish in control fish group.

5. DISCUSSION

In this study, we examined our experimental fish in 4 different monitoring periods (September 2012, December 2012, May 2013 and June 2013) over 10 months. Number of glochidia was found significantly lower in immunized fish than in control fish only in first sampling point, September, 3 weeks after the infection. Several studies reported that attached glochidia to incompatible fish are sloughed from the host within hours or days after cyst formation (Scharsack 1994). The compatibility and incompatibility mechanisms are unknown, may be of innate resistance (Reuling, 1919; Arey, 1932; Meyers and Millemann, 1977; Young and Williams, 1984b; O'Connell and Neves, 1999). Besides of innate resistance, several studies revealed that suitable hosts acquire resistance to glochidia after repeated infections.

There is evidence that in nature fishes acquire resistance (Young and Williams, 1984a; Bauer, 1987; Watters and O'Dee, 1996; Hastie and Young, 2001). Acquired immunity to parasites involving antibodies is well documented in fishes. In case of different co-occurring mussel species, fish can develop cross-resistance to glochidia (Watters, 1994; Haag and Warren, 1997). In cases of cross-resistance in fish parasites, antibodies to shared antigens of parasites are involved in cross-resistance to these parasites (Ling et al., 1993; Sin et al., 1992; Goven et al., 1980; Wolf and Markiw, 1982; Dickerson et al., 1984). Nonspecific cytotoxic cells (NCC) in teleosts are capable of killing certain protists (Evans et al., 1998). Cell-mediated mechanisms are involved in acquired immunity of rainbow trout (*O. mykiss*) to hemoflagellates, Cryptobia salmositica (Mehta and Woo, 2002). Dodd et al (2005 & 2006) and Shiver (2002) also found higher percentages of transformation success (attached larval development to juvenile mussel) in control fish compared to immunologically primed fish after 4-5 successive infection and concluded it

as consequence of cross resistance. Again Dodd et al (2005) indicated cross immunization of host fish to different mussel species is possibly mediated by antibodies.

In contrast, in case of remaining 3 monitoring periods, number of glochidia did not differ significantly between immunized and control fish. Since a preceding infection increases mortality of the parasites, it can be assumed of an immunological memory by the host. Bauer & Vogel (1987) indicated mortality soon after infection is caused by a tissue response whereas after few weeks of infection is caused by specific parasite antibody. They also assumed immunological memory works for a much longer time. It may be even months to year — for example in mirror carp (*Cyprinus carpio*) it was found 8 months later (Hines & Spira 1974). In addition, live IP vaccination with *Cryptobia salmositica* provided rainbow trout (*Oncorhynchus mykiss*) protection for at least 24 months (Li & Woo 1995). Thus, in our study it was expected a specific response at last till to December 2012. Avtalion (1969) showed antibody is temperature dependent. He kept a group of immunized carp fish in two different temperatures (12^0C and 25^0C) and did not found any circulating antibody in lower temperature but in 25^0C it showed a rising titre of antibody. In our case, the highest temperature was 16.8^0C whereas the lowest temperature was 1.1^0C throughout the experiment. The temperature was above 15^0C only for 4 weeks (11 August 2012 – 6 September 2012). In the present study, lower water temperature, unable to activate the resistant antibody of host, may be a cause not to have significant cross immunization in last three time points.

Bauer & Vogel (1987) noted that in case of same glochidial density, *M. margaritifera* infection intensity in trout is size dependent. They assumed bigger fish pump more water through their gills and therefore will receive more glochidia. We have also similar results in case of fish length and number of attached glochidia. The effect of covariate fish length was found significant in 3 out of 4 monitoring periods except May 2013. This significance may be also for relatively large gill surface in large fish that facilitates more glochidial attachment besides the aforesaid water pumping capacity.

The effect of immunization on glochidial development (size of glochidia) was analysed separately for different monitoring periods since glochidia and fish were growing over time. According to our hypothesis, immunization may have effect on glochidial development regarding size. However, we did not find any significant effect on glochidial development by immunization throughout the monitoring periods. Barnhart et al (2008) hypothesized smaller juveniles might have difficulty settling in flowing water after dropping off from host. The relationships among juvenile size, current speed, and settlement deserve study, given evidence that flow strongly affects mussel recruitment (e.g., Howard and Cuffey 2006, Morales et al. 2006). Although it was not significant, in September mean glochidial size found bigger in control fish than that of immunized fish in all the 4 tanks. In case of glochidial abundance we found significant difference between control and immunized fish only in September in all the tanks. Glochidia obtain shelter and nutrition from the host fish (Fisher and Dimock 2002). Favourable temperature for antibody activation in September may explain lower glochidia numbers, and trend for reduced growth of glochidia in immunized fish in September.

Only in May 2013 tank effect was significant on glochidial size and found lower size glochidia in tank 21 than in tank 20 and 22. This suggests that randomization and marking of fish individuals in the experiment was successful.

Interestingly, statistically significant, positive correlation was found between glochidium size and glochidium numbers in all time points. This indicates that there is no trade-off between glochidium number and growth rate. In other words, high glochidium

number did not limit glochidia growth. In contrast, According to Bauer & Vogel (1987), the number of glochidia per fish and their development stage (size) was negatively correlated with fish size.

We also found in every tank and time point higher mean number of glochidia on right gills than that of left gills. In contrast, Cunjak & McGladdery 1991 noted that there are no systematic differences between left and right gills. However, study by Cunjak & McGladdery was performed using wild fish collected from a river while in the present study the fish were reared in hatchery tanks and the experiment was done in laboratory tanks. No more prior study found relevant to this present findings. However, it may be a cause of direction of water flow and swimming of fish in their past, which may have increased the size if right side gills—or unknown reason(s). More study is needed to reveal it.

The nonspecific (innate) pathway is a first defence mechanism following trauma or invasion by foreign pathogens, no prior contact with the pathogen is required (reviewed by Secombes, 1996). This mechanism is important in fish, as they have fewer specific (acquired) immune capabilities. Natural resistance of fish is the result of a number of nonspecific humoral factors and body secretions (reviewed by Yano, 1996). We found the mean number of glochidia was higher in not fin-clipped fish than that of fin-clipped fish in both immunized and control fish in every time point except in immunized fish from June, even though this difference was statistically significant only in control fish of June. However, this suggests that fin-clipping provided more effective protection against *M. margaritifera* infection than immunization with duck mussel glochidia. Our present study indicates fin-clipping may have altered the immune system of fish possibly by nonspecific pathway. This immunity can be considered in future study and avoided the marking system.

To conclude, cross-immunization of trout by duck mussel glochidia probably may not possess a real threat to success of *M. margaritifera*. However, the possibility that repeated exposures to duck mussel could elicit and boost such a cross-immunity remains open.

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