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

3

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10

11 **Abstract**

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

25

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

27

28 **Introduction**

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

37

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

46

47 The immune defense system in vertebrates includes innate and acquired (adaptive, specific) components so that
48 the acquired immunity is based on antibodies that bind to a specific antigen. In a repeated contact with the same
49 parasite or pathogen, the immune reaction is quicker and stronger (memory) due to a faster antibody production
50 and the antibody reserves left from the previous infection (Mutoloki et al. 2014). For example, fish hosts can
51 develop an acquired immunity against the glochidia of unionoid mussels (Bauer and Vogel 1987, Rogers and
52 Dimock 2003, Dodd et al. 2006, Treasurer et al. 2006). Sequential infection with parasites belonging to different
53 (often closely related) species can also provide protection, which is called cross-immunity or cross-resistance.
54 Thus, cross-immunity is a special type of acquired immunity. It has been shown, for instance, that infection with
55 glochidia of one unionid mussel can result in immunity against glochidia of another unionid species (Dodd et al.
56 2005). As the parasitic stage can largely contribute to the reproduction success of the endangered *M.*

57 *margaritifera*, the role of acquired immunity and cross immunity in conservation of *M. margaritifera* requires
58 attention.

59
60 Bauer & Vogel (1987) showed that brown trout can eliminate *M. margaritifera* glochidia by both tissue and
61 humoral reaction so that in repeated exposures to *M. margaritifera* glochidia the immunologic responses by
62 brown trout against glochidia are strengthened, indicating an acquired immunity against *M. margaritifera*. In the
63 case of *M. margaritifera*, lower infection rates have been found in older brown trout in the field or after second
64 infection in the laboratory, suggesting acquired immunity (e.g. Bauer 1987, Ziuganov et al. 1994, Hastie &
65 Young 2001). Nevertheless, even a higher infection success in the second infection has been recorded (see
66 Wächtler et al. 2001). In spite of these contradictory results, the acquired immunity in salmonid hosts against *M.*
67 *margaritifera* glochidia has not received much attention by experimental studies. For example, an acquired
68 immune response would mean that the success of *M. margaritifera* would largely depend on immunologically
69 naïve 0+ host fish in the environment – with important consequences for the conservation of the species.

70
71 The question remains whether the infection by *A. anatina* can cross-immunize brown trout against *M.*
72 *margaritifera* glochidia such a phenomenon could pose a threat to declined *M. margaritifera* populations in
73 rivers where these unionids live in sympatry. Nevertheless, this is still a widely under researched issue. Bauer et
74 al. (1991) conducted an experiment by infecting brown trout first with *A. piscinalis* (= *A. anatina*) glochidia, and
75 subsequently with *M. margaritifera* glochidia. No evidence for cross-immunity was achieved in this short-term
76 (35 days) experiment. However, as the length of parasitic period of *M. margaritifera* can be even more than 300
77 days (Young & Williams 1984), a thorough evaluation of the likelihood and strength of this phenomenon should
78 cover the whole parasitic period.

79
80 The aim of the present study is to investigate cross-immunity, i.e. whether the infection with glochidia of the
81 duck mussel, *A. anatina*, will induce immunity against *M. margaritifera* in an experiment covering the whole
82 parasitic period of *M. margaritifera*, as well as acquired immunity in salmonids hosts by *M. margaritifera*. The
83 aim of the acquired immunity experiment is to examine the magnitude of acquired immunity in brown trout and
84 the dose-dependent acquired immunity in Atlantic salmon against the glochidia of *M. margaritifera*, i.e. whether
85 the intensity of immunity depends on the number of glochidia to which fish are exposed. Our hypotheses are
86 that (i) *Anodonta* infection induces cross immunity against *M. margaritifera* glochidia, (ii) Atlantic salmon and

87 brown trout develop acquired immunity against *M. margaritifera* and that (iii) the acquired immunity is dose
88 dependent.

89

90 **Materials and Methods**

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

95

96 **Cross immunity experiment**

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

107

108 Five brown trout from the 100-fish-tanks and three from the 50-fish-tanks from both primed and control groups
109 were examined for glochidia five days post infection. All primed fish were infected and the number of *A.*
110 *anatina* in primed fish varied from 90 to 232 glochidia fish⁻¹, indicating a successful priming infection. No
111 glochidia were found from the control fish. On 15 August 2012, when the water temperature had increased to
112 15.7 °C (2.5 months post infection), one brown trout from each tank was examined and found uninfected,
113 indicating that *A. anatina* glochidia had already excysted (see also Douđa et al. 2013).

114

115 On 15 August 2012, fish were marked using fin clipping, and randomly re-allocated into four new 163 L flow-
116 through tanks so that all tanks received both primed and control fish. In every other tank the primed fish were

117 fin-clipped and control fish unclipped while in every other tank the primed fish were unclipped and control fish
118 fin-clipped. The number of primed fish per tank varied from 21 to 36 whereas the number of control fish per
119 tank varied from 21 to 62. Both the fin-clipped and unclipped fish were anesthetized using MS-222 before
120 marking and handled similarly, except for the clipping.

121

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

129

130 Data were collected at four time points; September 2012 (3 weeks post infection), December 2012 (3 months),
131 May 2013 (9 months) and June 2013 (10 months) (Table 1). Primed and control fish were randomly collected,
132 killed with a sharp blow on the head, and measured for the total length and fresh mass. The gills were cut off
133 and glochidia were examined microscopically for the number and size (length from a subsample of 10 random
134 larvae), except for September sampling when only the right side gills were examined. Therefore, only the data
135 for the right side gills were used in statistical analyses. Throughout the experiment, fish were daily fed with
136 commercial food pellets. During this phase of the experiment the minimum and maximum temperatures were
137 1.1 °C and 16.8 °C, being the highest in September 2012 and June 2013.

138

139 Acquired immunity experiment

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

144

145 In the brown trout experiment, fish were first randomly allocated to primed vs. control groups in two separate
146 163 L flow-through tanks with 50 fish per tank. Priming infection of trout was performed on August 28, 2012,

147 using similar methods and origin of *M. margaritifera* glochidia as in the cross immunity experiment, with $2.9 \times$
148 10^5 glochidia tank⁻¹. Control fish were not exposed to glochidia, but experienced otherwise the same treatment
149 as the primed fish. Next year, in August 26, 2013, when age of the fish was 1+, adipose fins of the primed fish
150 were cut, after which both the primed and control fish were put in one tank. On August 28, 2013, the fish were
151 infected with *M. margaritifera* glochidia (2.3×10^5 glochidia tank⁻¹) collected from the River Koivuojja (the
152 River Iijoki catchment), northern Finland. In November 25 (3 months post infection), all the fish were examined
153 with the methods described above (Table 1).

154
155 Atlantic salmon were primed at the same time (August 28, 2012), with same origin of glochidia and with the
156 same methods as brown trout mentioned above. Salmon were allocated to two tanks with three treatment groups
157 in each, (1) primed with a high dose (8.8×10^5 *M. margaritifera* glochidia, tip of the right pectoral fin clipped),
158 (2) primed with a low dose (1.7×10^5 *M. margaritifera* glochidia, tip of the left pectoral fin clipped), and (3)
159 control group (not prime infected, adipose fin clipped) with 17, 14 and 13 fish per tank, respectively. After one
160 year, in August 28, 2013, all salmon were infected with *M. margaritifera* glochidia collected from the River
161 Luttojoki (the River Tuuloma catchment), northern Finland. The challenge infection was performed with $6.0 \times$
162 10^5 glochidia tank⁻¹. As brown trout above, all salmon were examined on November 25, 2013 (3 months post
163 infection) (Table 1). Throughout the experiments, fish were daily fed with commercial food pellets. Due to lack
164 of logistic supports, the dose-dependence experiment was not performed for brown trout.

165
166 Statistical analyses

167 The effect of the previous infection with either *A. anatina* (cross immunity) or *M. margaritifera* (acquired
168 immunity) and other factors (month, dose, tank) on glochidia number in gills and the size of glochidia was
169 analysed by ANOVA. If the tank effect was not significant, the analysis was reduced to the effect of other
170 variables. If necessary, the response variables studied were transferred by Box–Cox-transformation $\{BCSN =$
171 $(N^{\lambda}-1)/\lambda\}$ to yield as normally distributed variable as possible within each treatment cell. In some cases the
172 distribution within a treatment cell still deviated significantly from normal, which induced a tendency for
173 incorrect rejection of H₀-hypothesis (bias for too low p-value). Therefore, if the H₀ was rejected ($p < 0.05$) the
174 hypothesis was also tested using more conservative non-parametric tests (e.g. Kruskal–Wallis). Fish were not
175 measured for length and weight at the time of exposure but when examined. Therefore, the number of *M.*

176 *margaritifera* in the gills was not standardised based on the size of the fish as the individual growth rate after
177 exposure, and consequently the size during exposure, was not known.

178

179 **Results**

180

181 Cross immunity experiment

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

187

188 Acquired immunity experiment

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

194

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

201

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

206 infection with *M. margaritifera* led to both lower intensity of infection and poorer growth of *M. margaritifera*
207 larvae when re-infected.

208

209 **Discussion**

210 In natural populations, individuals are usually infected not only by one but also with multiple parasitic species.
211 Interaction between the co-infecting parasitic species within a host individual can be negative (antagonistic),
212 leading – in extreme cases – to competitive exclusion (Holmes 1961). However, species can also be independent
213 of each other or the interaction can be even positive (co-operation, facilitation) (Poulin 2001, Lello et al. 2004).
214 Thus, the interaction between *A. anatina* and *M. margaritifera* could also lead to one of these three possible
215 outcomes. Because the earlier study by Dodd et al. (2005) showed that the previous infection of the host fish
216 with the glochidia of *Lampsilis reeveiana* lowered the infection success of other unionid mussels, *L. abrupta*,
217 *Villosa iris* and *Utterbackia imbecillis*, we hypothesised that the effect of *A. anatina* infection on *M.*
218 *margaritifera* would be negative, or at most insignificant. We also hypothesised that if the effect of *A. anatina*
219 infection on *M. margaritifera* would be negative, it would be due to cross-immunity – representing so-called
220 immune-mediated ‘apparent competition’ where one parasite species elicits an immune response which harms
221 its competitors (see Read & Taylor 2001). In natural conditions, the possible negative impact of *A. anatina* on
222 *M. margaritifera* could be also due to direct interference competition. *M. margaritifera* glochidia that occupy
223 brown trout gills in autumn could be interfered by the glochidia of *A. anatina* in the spring when the glochidia
224 shedding of the latter species takes place. However, in the present study, the exposure of brown trout was
225 sequential so that *A. anatina* infection occurred earlier (in spring/early summer) and that of *M. margaritifera*
226 started in autumn; glochidia of only one species was present in brown trout at a time.

227

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

236 activation of the adaptive immune system whereas the later arrival bears the full costs of the acquired immunity
237 (see Jackson et al. 2006, Hoverman et al. 2013, Klemme et al. 2016).

238

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

248

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

255

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

264

265 The acquired immunity could explain the previous contrasting findings of *M. margaritifera* infection rate with
266 respect to host fish age. In some studies the infection rate has been lower in older host fish (Bauer 1987, Hastie
267 & Young 2001) while in some studies the opposite was found (see Wächtler et al. 2001). For example, if
268 production of glochidia does not take place every year in a particular *M. margaritifera* population, there can be
269 years in which both the 0+ and 1+ age group fish are immunologically naïve with respect to *M. margaritifera*
270 glochidia. In such a condition, the larger sized 1+ fish, due to their large gill area, are probably more intensively
271 parasitized by *M. margaritifera* glochidia than the 0+ fish that is also supported by Geist et al. (2006). During
272 the year that follows production of *M. margaritifera* glochidia, the negative effect of acquired immunity may
273 override the positive effect of larger size among the 1+ age group fish, resulting in situation where the younger
274 and smaller but immunologically naïve 0+ individuals are more heavily infected by *M. margaritifera* than the
275 1+ fish. The dose dependence of acquired immunity can strengthen this process.

276

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

283

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

294 related to the dose of exposure. Thus, possibility of such a cross immunity cannot be ruled completely out, for
295 example, if brown trout is heavily exposed to *A. anatina* glochidia.

296

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

303

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309

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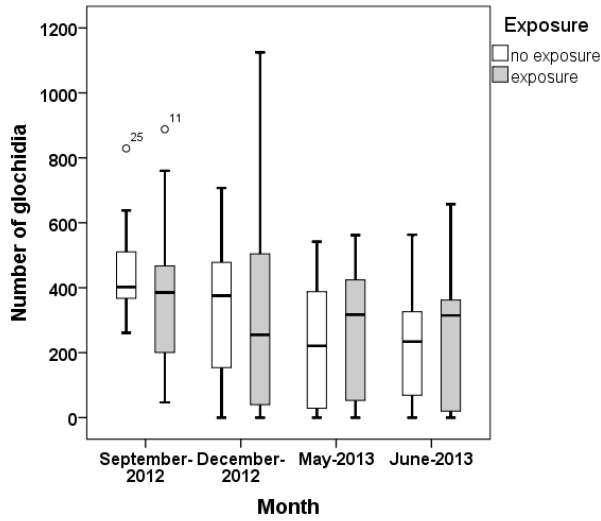
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401 **Figures**

402

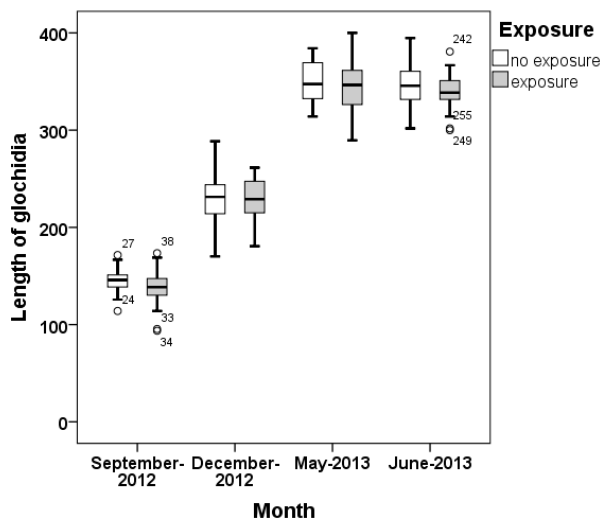


403

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

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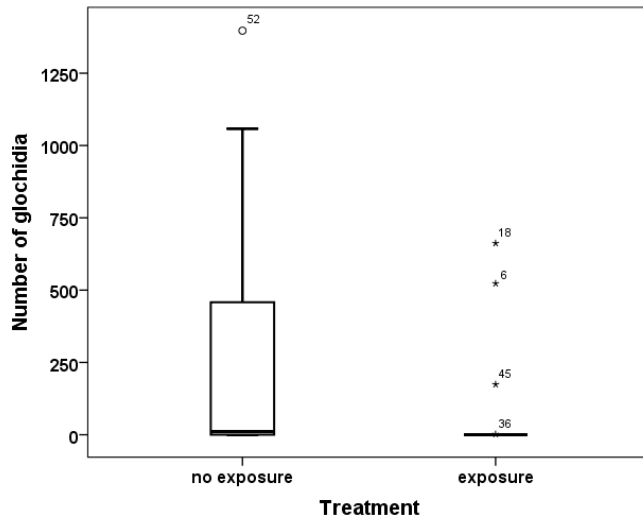


412

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

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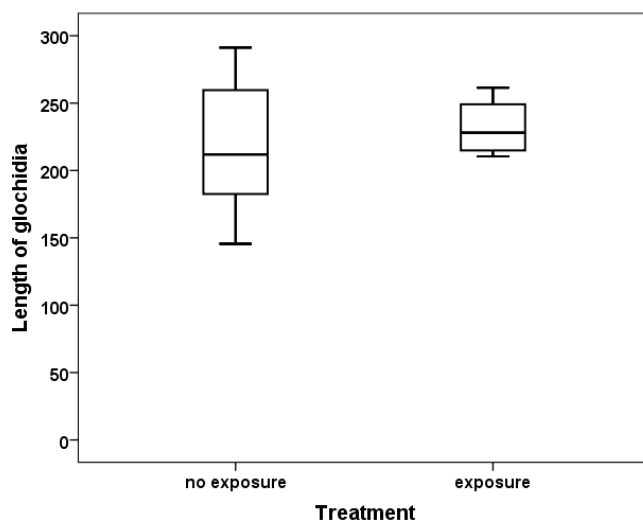


418

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

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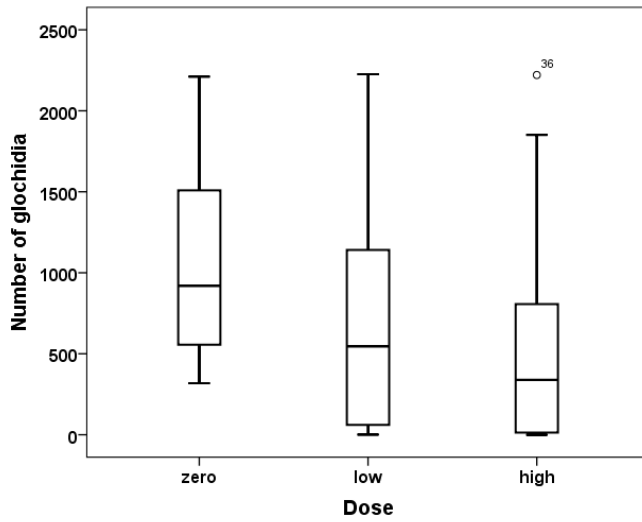


424

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

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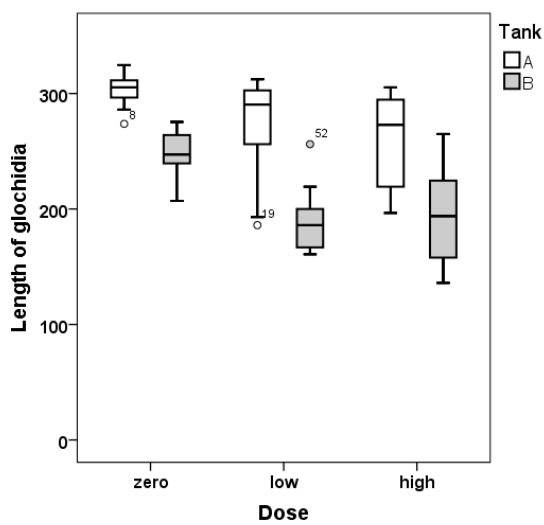


430

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

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436

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

440

441

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

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

444