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The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera* L.) I. Host response to Glochidiosis

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With 3 figures and 2 tables in the text

Abstract

Glochidia of the freshwater pearl mussel (*Margaritifera margaritifera* L.) are gill parasites on brown trout. A considerable loss of glochidia during the parasitic stage may occur, however, the underlying mechanisms are unknown. In this paper we therefore report the results of a study carried out to determine whether there is a humoral response by the host and whether repeated infection can lead to aquired immunity.

Introduction

Glochidia of the freshwater mussels Unionoidea are temporary parasites on fish hosts. The host range of a particular mussel species is more or less restricted, depending on the natural susceptibility of the fish (Arey 1923, Karna & Millemann 1978).

Though these large mussels with respect to biomass are important members of many aquatic communities (Bauer & Eicke 1986, Fish 1978, James 1985, Young & Williams 1984a), their host relationships are largely unknown. In many cases we even do not know the host species. The few studies on mechanisms governing survival of the glochidia during their parasitic stage indicate that a host response occurs, leading to glochidial mortality (Arey 1923, Meyers et al. 1980). One important component might be acquired immunity. An anamnestic response might be involved, however, an immunological memory to glochidiosis has been proved only for the hosts of some american *Lampsilis*-species (Arey 1923, Reuling 1919).

The freshwater pearl mussel (*Margaritifera margaritifera* L.) inhabits running waters poor in lime. Glochidia are very small (70 µm) and therefore when released are carried throughout the stream. They are gill parasites which reach the gills passively in the ventilating current of the fish. By pinching the tissue of a gill filament between the valves they externally become attached to the gills of a variety of fish species occurring in the rivers. However, they are shed within a few hours

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from unsuitable species (YOUNG & WILLIAMS 1984a, b). If the host is less resistant the parasites are surrounded by gill epithelium and come into close contact with the host tissue in this way.

The usual host in Central Europe is brown trout (BAUER 1979, UTERMARK 1973). But even on this host there is a considerable loss of glochidia during the parasitic stage (YOUNG & WILLIAMS 1984a, b). It is known from histological studies on encysted glochidia of *Margaritifera falcata* that this loss results from a destruction of the parasites (FUSTISH & MILLEMANN 1978). The underlying mechanisms, however, are largely unknown as the host – parasite relationship is only poorely understood. In this paper we report the results of a study carried out to determine some of these mechanisms. We were especially interested in whether there is a humoral response by the host to an infection and whether repeated infection can lead to acquired immunity.

Material and methods

All experiments were carried out with brown trout (*Salmo trutta* forma *fario*) of the same strain. The fish were obtained from a fish hatchery. The age classes are termed as 0+ (hatched in the same year) and 1+ (hatched one year before the experiment). Fish were infected by putting them into aquaria containing high numbers of glochidia (dose and exposure time are given below). All infections were conducted at a water temperature of 17°C. During infection the water was aerated to keep the glochidia in suspension.

Experiment I

For this experiment we distinguished three groups of fish (age class 1+) all kept under the same conditions during their whole life:

Reinfected fish (n=5): These fish had been infected one year before. Excystment of fully developed young mussels had occurred ca. 5 months before the experiment was started.

Infected fish (n=31): } Both groups had had no previous contact with glochidia.
Control fish (n=14): }

All fish except the controls were infected simultaneously by exposing them altogether for 5 minutes to a dose of 10×10^4 glochidia per litre. To determine the initial infection intensities, eight fish of the group "infected" were killed immediately after infection. The gills were dissected from the fish and the glochidia in each holobranch counted. The remaining fish were kept under identical conditions (17°C). Seven days post infection the reinfected, 14 infected and 7 control fish (for blood samples) were killed and the glochidia were counted. The remaining fish were killed 49 days p.i., the infection intensities were determined and the parasites were measured along their longest axis.

Experiment II

For this experiment we used two age classes (0+ and 1+, Tab. 1). The group "reinfected" consisted of 28 fish. They were slightly infected by exposing them for one minute to a dose of 10^3 glochidia per litre. After infection they were kept together with

Table 1. Design of experiment II.

Age of fish	Number in group "reinfected" receiving the first and second infection	Number in group "infected" receiving only the second infection
0+	10	14
1+	18	21

35 uninfected fish at high temperature (20°C) so that the parasites developed rapidly. Already three weeks p.i. inspection of anesthized fish showed that all parasites had disappeared from their hosts.

All fish (n = 63) were then simultaneously exposed to a dose of 40×10^4 glochidia per litre for five minutes. The initial glochidial load was determined by killing 20 fish of the group "infected" (7 class 0+, 13 class 1+) immediately after infection and counting the glochidia in their gills. A correlation between fish length and infection intensity was calculated for each age class. The remaining fish were kept at 18°C for four days, killed and the glochidia counted. Using the above correlations the initial number of glochidia was calculated and with these values the loss on infected and reinfected fish was estimated.

Serological procedures

Blood samples were taken from fish of experiment I. The blood which was obtained by caudal vein puncture was pooled for each group (infected, reinfected and control). It was allowed to clot and the clot was left to contract at 4°C overnight. The serum was then decanted. Sera were tested against glochidia, mashed in trisglycene buffer, pH = 8.2, using the double gel diffusion method of Ouchterlony. The gels were prepared using 0.75% (w/v) agarose in borate-buffered 1% (w/v) NaCl-solution (pH = 8.0). The preparations were incubated at room temperature in a moist chamber for two days. When this test is used in fish immunology, formation of a precipitate does not necessarily mean the presence of an antibody-antigen reaction as fish sera contain a variety of precipitating factors not identical with antibodies. After incubation the gels were therefore washed in sodium citrate (5%, w/v) or 0.1 M EDTA for four hours (ELLIS 1985, BALDO & FLETCHER 1973). In this way the lines not representing immune complexes were dissolved.

Results

Host response to the first infection (Exp. I, Fig. 1, infected fish):

When fish are exposed to the same parasite density, the infection intensities depend on the fish size, probably because bigger fish pump more water through their gills and therefore will receive more glochidia (Fig. 1, day 0 p.i.).

One week p.i. there was still a relationship between host size and infection intensity (Fig. 1). However, analysis of covariance shows that the intercepts of the regressions (day 0 and day 7 p.i.) differ significantly (P = 0.016), whereas the slopes cannot be distinguished (P = 0.97). Apparently in the first week p.i. the fish lost on the average 500 parasites each. The loss is independent of the fish size, i.e. of the initial parasite density.

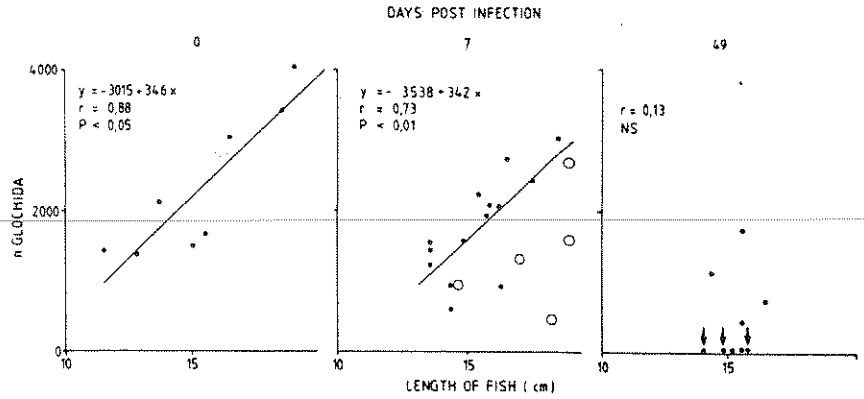

Fig. 1. Infection intensities of simultaneously infected brown trout. ● = infected (the fish had no previous contact with glochidia); ○ = reinfected (the fish had been infected one year before). The correlations are calculated only for infected fish. Arrows mark fish which had lost all glochidia.

Fourty-nine days p.i. the numbers had again decreased considerably showing high variability among the fish. Three had lost all glochidia whereas others still had retained fairly large numbers.

Glochidia of the freshwater pearl mussel grow from ca. 70 µm to ca. 400 µm during the parasitic stage. Fourty-nine days p.i. on some hosts the parasites were nearly fully developed whereas on others they were still very small. Correlation analysis shows that size depends on mortality but not on the initial infection intensity (Fig. 2), thus not on fish size. On fishes where mortality was low, the parasites had grown bigger, i.e., they had developed more quickly compared to glochidia on hosts where they suffered high mortalities.

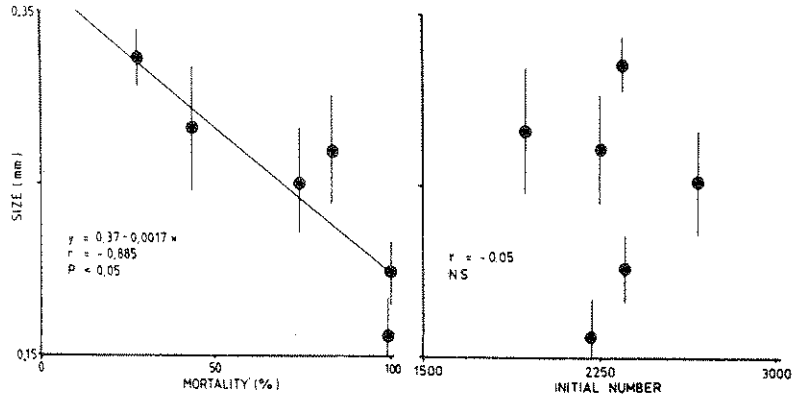

Fig. 2. The size of the parasites (mean ±95% c.i.) 49 days p.i. in relation to their mortality (left) and the initial infection intensity (right). (Each dot represents values from one host.)

Host response to a second infection:

According to the studies of MEYERS & MILLEMANN (1977) and FUSTISH & MILLEMANN (1978) on the host relationship of *Margaritifera falcata*, the initial infection intensities depend on the concentration of suspended parasites and on the fish size but not on the susceptibility of a particular fish species. REULING (1919) also states that attachment of *Lampsilis*-glochidia is not influenced by the degree of host immunity. Considering the process of attachment as described in the introduction, this seems quite logical. We therefore determined the initial infection intensities (= the number of glochidia externally attached to the gill filaments) only for infected fish. As the gill structure is not altered by a preceding infection (AREY 1913) and as both infected and reinfected fish were simultaneously exposed to the same dose, the relationship between size and glochidial load on day 0 p.i. will be identical in both groups. In the following section this relationship is used as basis to compare the fate of glochidial infection on infected and reinfected hosts.

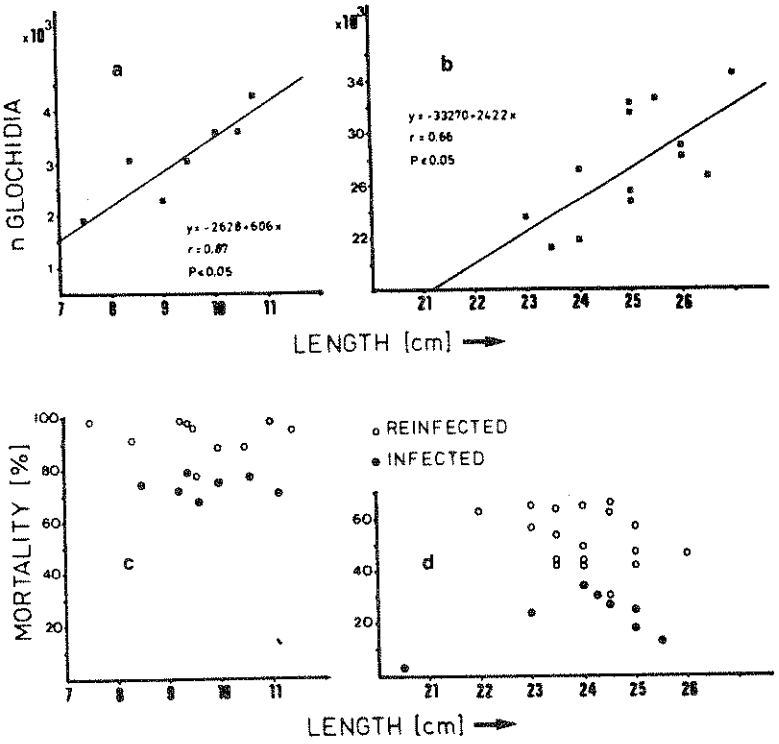

Fig. 3. The effect of a preceding infection (Experiment II).
a: relationship between host size and initial infection intensity for age class 0+.
b: the same for age class 1+.
c: mortality of glochidia 4 days p.i. (age class 0+).
d: mortality of glochidia 4 days p.i. (age class 1+).
(The reinfected hosts received their first infection 3 weeks before the second.)

Reinfected fish in experiment I (Fig. 1) had received their first infection one year ago and the fully developed mussels had been released five months before the experiment was started. In experiment II (Fig. 3) the first infection of reinfected fish dated back only three weeks. The experiments differ with regard to their conditions (host size, infection intensities, temperature, duration) and they were not simultaneously conducted, so the glochidia may have been differently viable. Mortality rates and their relation to glochidial density therefore are not comparable between the two experiments.

However, when infected and reinfected fish are compared, the results are the same in both cases: A preceding infection increases the mortality of the parasites. Both age classes in experiment II (Fig. 3) respond to a preceding infection. In Fig. 1 the reinfected fish do not show a relationship between size and infection intensity, whereas the infected fish do seven days p.i. By means of the sign test the number of fish above and below the regression line was compared within both groups. No difference can be proved for infected fish, however, the test is significant at the 5% level for the group "reinfected". Thus reinfected fish had lost more glochidia than infected ones.

Serology

In agarose gel plates no precipitates were observed between glochidia and a control serum (rabbit, Tab. 2). Non specific lines occurred as diffuse rings with all the fish plasma. When the gels were washed in sodium citrate or EDTA the precipitates with infected fish 7 days p.i. and with control fish dissolved completely within two hours. The lines with serum from reinfected fish 7 days p.i. and infected fish 49 days p.i., however, were stable for at least two days (Tab. 2).

Table 2. Results of Ouchterlony double diffusion tests in agarose with serum from brown trout (and rabbit) tested against glochidia.

	Trout					Rabbit
Days p.i.	7			49		
Group	infect.	reinfect.	contr.	infect.	contr.	
Number of fish	8	5	7	9	7	
washed in						
Sodium citrate	–	+	–	+	–	–
EDTA	–	+	–	+	–	–

infect. = fish which were infected for the first time
reinfect. = fish which were infected for the second time
contr. = control fish
\+ = stable precipitin line
– = no precipitation or diffuse line disappearing after washing

Discussion

At 17°C mortality of pearl mussel glochidia on brown trout starts very soon p.i. and proceeds until excystment of the young mussels. The parasites do not develop equally well on all hosts. Their growth is related to mortality: Development is delayed on those hosts which loose a great deal of their glochidia whereas parasites develop quickly when the loss is low (Fig. 2). Thus there are hosts offering worse conditions to the parasites than others.

These results obtained from the infected fish in experiment I indicate that mortality must be attributed to a host response. This response apparently is caused by two different mechanisms:

(a) Seven days p.i. the number of glochidia had already decreased significantly. Each fish had lost ca. 500 parasites, so that there was still a correlation between host size and infection intensity (Fig. 1). The Ouchterlony test at this time was negative (Tab. 2).

(b) Fourty-nine days p.i. the number of parasites had decreased further, but not to the same extent in all hosts: some had lost all parasites whereas others were still infected with quite high numbers (Fig. 1). At this time we could detect a serum factor showing a specific precipitin reaction with glochidia (Tab. 2).

We therefore suppose that mortality of parasites soon after infection is caused by a tissue response occurring in all fish. Some weeks p.i. the fish produce a serum factor which might be a specific parasite antibody. The pattern of glochidial load in Fig. 1, 49 days p.i. suggests that the latter response is not developed equally well in all hosts.

This hypothesis is supported by FUSTISH & MILLEMANN (1978) and MEYERS et al. (1980). The authors noted that on the natural host species *(Oncorhynchus tshawytscha)* of *Margaritifera falcata* the number of parasites decreased from an average of 938 to 726 per fish by 4.5 days p.i. They found hyperblastic nodules on gill filament tips, probably the tissue remnants after parasite sloughing.

10–12 weeks p.i. the precipitin tests of MEYERS et al. (1980) with glochidial extract and fish serum showed a weak reaction, suggesting that the host had developed specific parasite antibodies.

Investigations into the immunological memory of fish have produced rather contradictory results depending on the species and the experimental conditions (AMBROSIUS & FRENZEL 1972, AVTALION 1969, DUNIER 1985, TRUMP & HILDEMANN 1970). Our results indicate that there is an anamnestic response in brown trout which may lead to aquired immunity to glochidiosis. Mortality of parasites is higher on hosts which had been previously infected. This response is already recognizable three weeks after the first infection when fish are kept at high temperature. Considering the effect of temperature on antibody production in fish (CORBEL 1975), increased mortality on reinfected fish in this case might already be due to a humoral response. However, we cannot exclude the possibility that a

more intense tissue response, resulting from the first infection, is responsible for this short term effect.

But as experiment I shows the immunological memory works for a much longer time: even when the parasites excysted 5 months before the experiment, mortality on these hosts is increased. The pattern of mortality on reinfected and infected hosts differs considerably (Fig. 1), suggesting that the loss of glochidia in both groups is caused by different mechanisms. This hypothesis is confirmed by the Ouchterlony tests which are already positive for reinfected fish 7 days p.i., whereas they are not for infected fish at this time (Tab. 2). We therefore assume that an important component in the acquired immunity of brown trout to glochidiosis is due to a serum factor.

Summary

Development of pearl mussel glochidia on brown trout is related to their survival rates; on hosts where mortality is high, development is delayed (Fig. 2). Glochidial mortality must be attributed to a host response. Our results indicate that this response is caused by two different mechanisms.

One week post infection the number of parasites had decreased equally in all fish (Fig. 1), no serum factor could be detected at this time (Tab. 2). Fourty-nine days p.i. the numbers had decreased further (Fig. 1) and a serum factor, probably a specific parasite antibody, was found (Tab. 2). We therefore suppose that mortality soon after infection is caused by a tissue response, whereas some weeks p.i. it is also due to a humoral response.

Mortality of parasites is increased by a preceding infection. This effect is already recognizable three weeks after the first infection (Fig. 3). In reinfected hosts which had released the young mussels five months before the experiment, the serum factor could already be detected one week after the second infection (Fig. 1, Tab. 2). Therefore an immunological memory of brown trout to glochidiosis must be assumed.

Zusammenfassung

Die Entwicklung von Glochidien der Flußperlmuschel auf Bachforellen steht in Beziehung zu ihren Überlebensraten; wenn die Mortalität hoch ist, ist die Entwicklung verzögert (Fig. 2). Die Mortalität der Glochidien beruht auf einer Wirtsreaktion, der offensichtlich zwei unterschiedliche Mechanismen zugrunde liegen.

Eine Woche nach der Infektion hatte die Zahl der Glochidien auf allen Fischen gleichermaßen abgenommen (Fig. 1), es konnte kein serologischer Faktor nachgewiesen werden (Tab. 2). 49 Tage nach der Infektion waren die Infektionsraten abermals zurückgegangen (Fig. 1), und es wurde ein serologischer Faktor, vermutlich Antikörper, gefunden (Tab. 2). Wir vermuten daher, daß die Mortalität kurz nach der Infektion durch eine Gewebsreaktion verursacht wird, während sie einige Wochen nach der Infektion auf einer serologischen Komponente beruht.

Die Mortalität wird durch eine vorhergehende Infektion erhöht. Dieser Effekt ist bereits eine Woche nach der ersten Infektion erkennbar (Fig. 3). Bei reinfizierten Fischen, deren Jungmuscheln 5 Monate vor dem Experiment abgefallen waren, konnte der Serum-Faktor bereits eine Woche nach der zweiten Infektion nachgewiesen werden (Fig. 1, Tab. 2). Wir nehmen daher an, daß Bachforellen ein „immunologisches Gedächtnis" gegen Glochidiosis besitzen.

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