Evidence of Immunological Responses by a Host Fish *(Ambloplites rupestris)* and Two Non-Host Fishes *(Cyprinus carpio* and *Carassius auratus)* to Glochidia of a Freshwater Mussel *(Villosa iris)*

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ABSTRACT

Immunological responses of fishes to glochidia were evaluated using glochidia of the rainbow mussel (*Villosa iris*) to infest a host species, rock bass (*Ambloplites rupestris*), and two nonhost species, common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). Ouchterlony double-diffusion tests showed that host and nonhost species expressed a humoral defense factor specific to glochidial antigens after induced infestation with glochidia. Precipitin bands were observed in tests on infested fishes but not in tests on uninfested fishes. Microagglutination tests showed that host and nonhost species that were uninfested, infested, or reinfested with glochidia all expressed some agglutination response to glochidial antigens. Experimental fishes had specific humoral defense factors that reacted immunologically to glochidia tissue.

INTRODUCTION

The larval forms (glochidia) of most freshwater mussel species are obligate parasites on fish species and typically exhibit host specificity (Zale and Neves 1982). Glochidia are discharged from the female mussel into the water, where contact and encystment on appropriate host fishes are essential for transformation to juvenile mussels. If attachment to the gills or fins of an appropriate host fish occurs, glochidia obtain sustenance from the host's blood to metamorphose to the free-living juvenile stage (Isom and Hudson 1984). The criteria by which host species are susceptible to glochidiosis of different mussel species are not defined, but the immunological reactions of host fishes are the most likely factors (Reuling 1919, Arey 1932, Zale and Neves 1982, Bauer 1987, Kirk and Layzer 1997). Although these studies identified an immune response as the mechanism providing resistance to glochidia, suitable methods to determine the immunological mechanism involved in glochidiosis have not been described.

Exploitation, pollution, habitat destruction, competition with introduced exotic species and loss of host fishes are all factors in the decline of freshwater mussels in this century (Bates 1962, Neves et al. 1985, Bauer and Vogel 1987). Knowledge of the immunological mechanism of host fish recognition could be

beneficial to recovery of threatened or endangered species of freshwater mussels. Immunological methods to allow rapid identification of host fish species would facilitate efforts to obtain and culture juveniles in the laboratory to augment depleted stocks of mussels (Isom and Hudson 1982, Neves et al. 1985). To determine whether there was a basis for pursuing serological methods of glochidia-host identification, we tested whether host and nonhost fishes produced specific humoral defense factors (Yano, 1996) after infestation with glochidia of the rainbow mussel (*Villosa iris*). Humoral defense factors in fishes can include antibodies, lysozyme, complement, interferon, C-reactive protein, transferrin, lectin, or other substances (Yano, 1996). We tested for the presence of specific anti-glochidial defense factors in hosts and nonhosts experiencing different levels of glochidial infestation. Both precipitation and agglutination tests were employed to determine whether these simple methods could yield information toward serological discrimination of host and nonhost species.

METHODS AND MATERIALS

All experiments were carried out using glochidia of the rainbow mussel (*Villosa iris*) and rock bass (*Ambloplites rupestris*), 100 to 250 g, as the host species (Zale and Neves 1982). Common carp (*Cyprinus carpio*), 115 to 500 g, and goldfish (*Carassius auratus*), 70 to 400 g, were used as nonhost species. To avoid using fishes previously exposed to glochidia of *V. iris*., fishes were collected from the New River drainage, Montgomery County, Virginia, a region outside the geographic range of the mussel. Gravid mussels were collected from the Clinch River, Tazewell Co. and Copper Creek, Scott Co., Virginia. All fishes were collected by electro-fishing and were treated for parasites and diseases with furacin and salt before experiments. Prior to experiments, fishes were held in Living Streams (Frigid Units)[a] at 20°C under a 12-h light-dark cycle.

Before the induced infestation with glochidia, fishes were held in 38-liter aquaria for at least seven days or until they fed readily. At the time of infestation, individual fish were removed from aquaria and anesthetized with tricaine methanesulfonate (MS-222). Glochidia were obtained from gravid mussels according to Bruenderman and Neves (1993). We used a pipette to transfer 1200 to 1500 glochidia onto the right gills of each anesthetized fish. As controls, uninfested fishes had their gills treated with distilled water. Fishes were then returned to their respective aquaria. Uninfested and infested fishes were bled seven days after infestation. For those fishes reinfested, the above procedure was repeated at seven days post-infestation, and these fishes were then bled seven days after the second infestation.

Fish sera were collected from host and nonhost species that had been either infested or not infested (controls). Collections were made at three, seven, and 14 days post-infestation (and at 49 days for four *A. rupestris*), to determine when specific humoral defense factors (e.g. antibodies), if present, occurred in the blood after infestation. These sera were tested for the presence of precipitating anti-

[a] Reference to trade names does not imply government endorsement of commercial products.

glochidia factors using the double gel diffusion method (Bauer and Vogel 1987). Glochidia were homogenized for 15 min in phosphate-buffered saline (PBS), pH = 8.2, to produce the antigen material (concentration was one glochidium per microliter of PBS). Blood was obtained by dorsal gill incision (Watson et al. 1989). Sera were obtained by spinning down the blood at 3000 rpm for 15 min and were stored at -20°C until the experiments (Neumann and Tripp 1986). The gels were prepared using agarose in PBS. The preparations were incubated for three days at 20°C in moist chambers. After incubation, the gels were washed with 5% (w/v) sodium citrate to remove nonimmune complexes. The gels were then observed for precipitation bands using backlighting.

To determine if any specific humoral defense factors in fish sera agglutinated with glochidial antigens, sera from different treatments of fishes were combined with a glochidial antigen solution. Homogenized glochidia (as described) constituted the antigen solution. Different dilutions of fish sera were combined with the antigen solution in wells of a microtiter plate, and the microtiter plates were incubated at room temperature for 24 h. After incubation, presence of agglutination was confirmed visually or microscopically (20X). Agglutination was determined by the formation of "sheets" (Stavitsky 1954) or matrices representing a reacted complex of soluble glochidia particles and some specific humoral defense factor.

RESULTS

The Ouchterlony double-diffusion tests showed that the host (*A. rupestris*) and nonhost (*C. carpio* and *C. auratus*) species exhibited humoral defense responses to glochidia. Precipitation bands formed between wells containing glochidial antigen solution and wells containing sera from infested fishes (Table 1). These bands remained stable after repeated washings with 5% sodium citrate, which is used to dissolved unreacted proteins and other nonimmune precipitins remaining in the agar gel (Ellis 1978, Bauer 1987). No differences were observed in size, shape, or quantity of bands among species.

Sera collected three days post-infestation from infested host and nonhost fishes did not react with glochidial antigens to form precipitin bands; however, bands formed in tests using sera collected seven and 14 days after infestation of all three species. Bands also formed in tests using sera collected from four infested *A. rupestris* bled 49 days post-infestation. No stable precipitin bands formed with sera from uninfested, control fishes. Humoral defense factors were found in the sera of host and nonhost fishes seven days after infestation with glochidia at water temperatures of about 20°C. Because factors were present at seven days post-infestation, tests for the presence of agglutinating factors were done using sera collected seven days post-infestation. Use of the shortest interval of time minimized the possibility of fish mortality between the times of infestation and blood collection.

When combined and incubated with the glochidial antigen solution, fish sera from each treatment produced an agglutination response (Table 2). Only sera from one animal, an uninfested *C. carpio*, did not exhibit an agglutination response when combined with the glochidial antigen solution.

TABLE 1. Results of Ouchterlony double diffusion tests in agarose with sera from control (C) and experimental (E) groups of fishes. A stable precipitin line (+) indicates that anti-glochidial factors were present in the sera; a (-) indicates no precipitation, or a diffuse line that disappeared after washing.

Species	Days post-infestation	Group	No. fish	Precipitation
Rockbass	3	C	3	-
		E	3	-
	7	C	6	-
		E	6	+
	14	C	3	-
		E	3	+
	49	C	6	-
		E	4	+
Carp	3	C	3	-
		E	3	-
	7	C	6	-
		E	6	+
	14	C	3	-
		E	3	+
Goldfish	3	C	3	-
		E	3	-
	7	C	6	-
		E	6	+
	14	C	3	-
		E	3	+

DISCUSSION

Individuals of *A. rupestris*, *C. carpio*, and *C. auratus* infested with *V. iris* glochidia expressed humoral factors that were specific to the antigens of these glochidia. The simple precipitation and agglutination tests do not characterize the specific anti-glochidia factors. However, uninfested fishes expressed positive reactions in the agglutination tests but not in the precipitation tests, which suggests that more than one factor is reacting to glochidial antigens. For example, the

experimental fishes may have reacted to glochidial antigens with both agglutinating factors (e.g. C-reactive protein) and with precipitating factors (e.g. specific antibodies). Before effective host identification methods are developed, the humoral defense factor that most strongly determines host specificity needs to be identified.

We suggest that the production of specific anti-glochidia antibodies by fish infested with glochidia is related to host specificity and would be important for identifying host species. We suspect that specific antibodies are produced by fishes because we found precipitating factors specific to glochidial antigens in sera of infested and reinfested fishes at periods of seven, 14, and 49 days post infestation. Although we could not determine if antibodies were the precipitating factors, other studies support the conclusion that fishes produce specific antibodies towards glochidia. Kirk and Layzer (1997) induced the metamorphosis of glochidia on a non-host fish by using an immunosuppressant, cortisol. The hypothesized effect of the cortisol on the fish was to decrease the number of antibodies and reduce inflammation around glochidial cysts. The successful metamorphosis of glochidia they achieved through immunosuppression of the non-host fish is evidence that the production of anti-glochidial antibodies is an important factor in determining whether a fish will be a host or a non-host in the wild.

Meyers et al.(1980) observed that the host coho salmon (*Oncorhynchus kisutch*) and nonhost chinook salmon (*Oncorhynchus tshawytscha*) of the western pearlshell (*Margaritifera falcata*) contained antiglochidia antibodies in their blood plasma after infestation. Bauer and Vogel (1987), using the pearlshell (*M. margaritifera*), reported antiglochidia antibodies in the sera of reinfested brown

TABLE 2. Results of microagglutination tests with sera from uninfested (U), infested (I), and reinfested (R) groups of fishes. The formation of a matrix in the microtiter plate well (positive result) indicates that agglutination occurred between anti-glochidial factors in the sera and the glochidial antigens.

Species	Group	number of fish	% fish expressing agglutination
Rockbass	U	10	100
	I	9	100
	R	9	100
Carp	U	10	90
	I	8	100
	R	11	100
Goldfish	U	10	100
	I	8	100
	R	10	100

trout (*Salmo trutta*). In rainbow trout (*Oncorhynchus mykiss*), specific antibodies may not occur in the blood for up to ten days after immunization with a foreign antigen, although activation of the immune system in most fish occurs from one to three days post-infestation (Anderson 1990). If the precipitating humoral defense factors we observed in the sera of rock bass, carp, and goldfish are antiglochidia antibodies, then they appeared earlier than antiglochidia antibodies reported for the salmonids in previous studies. This result may have been caused by differences in experimental conditions or the fish species used. Antibody production in fishes tends to increase at higher temperatures (Manning and Mughal 1985, Plumb et al. 1986, Suzuki et al. 1996). The fishes we used were warm-water species kept at 20 to 21°C throughout the experiments. Logically, antibody production should occur sooner under these conditions than with coldwater, salmonid species. Also, we used wild-caught adult fishes, as opposed to the hatchery-reared juvenile fishes in earlier studies. Adult fishes have better-developed immune systems and express stronger immune responses than juvenile fishes (Manning and Mughal 1985). Finally, assumptions regarding the decreased immunocompetency of fishes relative to higher vertebrates (e.g. slower antibody production) may not be justified. There is evidence that fishes "exhibit numerous functional similarities to the adaptive immune systems of 'higher' animals" (Clem et al. 1996).

Host identification can not be achieved with the simple serological methods explored here. That fish humoral defense factors can react with glochidial antigens does merit further study to use immunological procedures to identify host fishes. To better understand the antigenic composition of glochidia and fishes, we suggest using more immunologically advanced vertebrates (e.g., rabbit, goat) as the reacting hosts. Reactions to glochidia and fish antigens would be easier to measure and identify with well-studied laboratory animals instead of fishes, which have highly variable immune systems (Zeeman 1986). Identifying the antigens to which fish react during a glochidial infestation is probably the next important step in seeking a serological method of host fish determination.

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