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Appendix 2- Draft manuscript

Cross-resistance of largemouth bass to glochidia of unionid mussels

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Abstract: We tested whether host fish that acquired resistance to glochidia of one mussel species were cross-resistant to glochidia of other species. Largemouth bass (*Micropterus salmoides*) were primed with 4-5 successive infections of glochidia of *Lampsilis reeveiana*. The percentage of attached glochidia that survived and transformed to the juvenile stage (transformation success) was compared between primed fish and naïve controls. Transformation success of *L. reeveiana, Lampsilis abrupta, Villosa iris*, and *Utterbackia imbecillis* was significantly lower on primed fish (37.8%, 43.5%, 67.0%, and 13.2% respectively) than on control fish (89.0%, 89.7%, 90.0%, and 22.2% respectively). Immunoblotting was used to analyze the binding of serum antibodies from primed fish with glochidia proteins. Antibodies bound to glochidia proteins of similar molecular weight from *L. reeveiana* and *L. abrupta*. Bound proteins of *V. iris* differed in molecular weight from those of the *Lampsilis* species. There was no binding to specific glochidia proteins of *U. imbecillis* or *Strophitus undulatus*. Our results indicate that host acquired resistance can extend across mussel genera and subfamilies, and might involve both specific and nonspecific mechanisms. Understanding the specificity of acquired resistance of hosts to glochidia could enhance understanding of the evolutionary and ecological relationships between mussels and their host fishes.

INTRODUCTION

Freshwater mussels of the family Unionidae have an obligate, parasitic larval stage, the glochidium, which typically attaches to the gills or fins of a host fish. Glochidia that attach to a compatible host species are encysted by migration of host cells. The larvae remain encysted for days to months depending on species and temperature, and transform to the juvenile stage. When development is complete the juveniles leave the host and become benthic suspension-feeders (Arey, 1921; 1932a; Fustish and Millemann, 1978; Waller and Mitchell, 1989).

Mussels are host-specific and are generally compatible with only a limited number of host species (Watters, 1994). Glochidia that attach to incompatible (non-host) species are lost from the host within a few days after attachment because they either fail to be encysted, or are subsequently sloughed from the host before transformation is complete. Incompatibility is thought to be innate, but the mechanisms involved are unknown (Reuling, 1919; Arey, 1932a; Meyers and Millemann, 1977; Meyers et al., 1980; Young and Williams, 1984b; O'Connell and Neves, 1999).

In addition to innate resistance, several studies have shown that compatible hosts acquire resistance to glochidia after one or more infections (Reuling, 1919; Arey, 1924; 1932a; Bauer and Vogel, 1987; Rogers and Dimock, 2003). Compared to naïve hosts, resistant host fish kill and slough a larger number of the attached glochidia, thus reducing the proportion that transform into juveniles (Bauer and Vogel, 1987; Rogers and Dimock, 2003). The underlying mechanisms of acquired resistance of host fish to glochidia are not fully understood. Fish infected with glochidia produce anti-glochidia factors in their serum, presumably antibodies (Meyers et al., 1980; Bauer and Vogel, 1987; O'Connell and Neves, 1999). However, the relationship between serum antibody levels and resistance has not been investigated.

Acquired resistance of fish to one species of parasite can result in resistance to other species (cross-resistance) (Buchmann et al., 1999, Larsen et al., 2002). Cross-resistance to glochidia of different mussel species has been documented but little information is available (Reuling, 1919). Further understanding of acquired resistance and cross-resistance could have practical application in efforts to understand mussel host relationships and to propagate endangered species. Captive propagation of mussels on host fish is increasingly used in efforts to conserve rare species of mussels and is an objective in many federal recovery plans (NNMCC, 1998). Propagating multiple species on the same host fish could be used to reduce labor and costs associated with collecting and maintaining hosts.

The main goals of this study were to determine 1) whether host fish that have acquired resistance to one mussel species are cross-resistant to other mussel species, and 2) whether serum antibodies from fish primed with glochidia from one species of mussel would cross-react with glochidia proteins of different species.

MATERIALS & METHODS

Fish and mussels

Six-month-old largemouth bass were obtained from Chesapeake State Fish Hatchery, Chesapeake, MO. Fish were held in a recirculating aquarium system at 23-24 C in moderately hard synthetic freshwater (SFW) (USEPA 2002). We fed fish 1-2% of their body weight daily (AquaMax pellet feed, Purina Mills), except during infections, when they were fed every other day to reduce feces production. The body mass (g) of each fish was measured following each infection.

Gravid mussels were collected from Missouri and North Carolina, USA during 2003 and 2004. We collected Ozark broken-rays mussels (*Lampsilis reeveiana brevicula,* hereafter referred to as *L. reeveiana*), rainbow mussels (*Villosa iris*), and creeper (*Strophitus undulatus*) from Beaver Creek, Taney County, Missouri (UTM 15, 503804E, 4066693N). Pink muckets (*Lampsilis abrupta*) were collected from the Meramec River, Jefferson County, Missouri (UTM 15, 699328E, 4260349N). Paper pondshell (*Utterbackia imbecillis*) were collected from Lake Rockingham, Rockingham County, North Carolina (UTM 17, 625142E, 4026086N). *Lampsilis reeveiana* and *V. iris* were maintained at 19-21°C. *Utterbackia imbecillis* were kept at 10°C and *S. undulatus* were kept at 6.5°C to slow the release of glochidia. *Lampsilis reeveiana*, *V. iris*, and *S. undulatus* were maintained unfed in SFW. *Lampsilis abrupta* were kept in a flow-through raceway that received water from a pond at Chesapeake State Fish Hatchery, Missouri. *Utterbackia imbecillis* were fed once or twice per week with a mixture of algae, and maintained in SFW. Mussels and fish were kept on a 12:12 hour light dark photoperiod, except for *L. abrupta* and *U. imbecillis*, which were subject to natural photoperiod.

Infection procedure

We used glochidia from one female mussel per infection, and obtained glochidia from a different female mussel for each infection. We used a needle and syringe to perforate the marsupial gill and flush the glochidia into a beaker. The glochidia of *S. undulatus* were freed from the conglutinates (Ortmann, 1911) by spraying them with water through 400 µm mesh nylon fabric. Glochidia were suspended in a known volume of water which was sub sampled for counting. The water was stirred with a large, rubber-bulb syringe while ten 200-µL samples were removed using a volumetric pipette. Each 200 µL sample was placed as a drop on a plastic Petri dish. The glochidia in each drop were counted and classified as open or closed before and after adding NaCl. Open glochidia that closed after NaCl were classified as "viable". The sample counts were averaged and used to estimate the concentration and the total number of viable glochidia.

Fish were infected with glochidia by placing them as a group in a bath containing 2,000 viable glochidia L^{-1} of SFW. The volume of the suspension was 0.5 L $fish^{-1}$. Aeration and stirring with a baster were used to keep the glochidia in suspension. After 15 minutes the fish were immediately transferred by dip net into individual 2.75 L tanks.

Transformation success

We monitored transformation success of mussel glochidia on individual fish in a recirculating system (AHAB® Aquatic Habitats, Inc. Apopka, FLA) modified for that purpose. Each 2.75 L tank received water continuously from a manifold, and the overflow entered a filter cup with a 125μm nylon screen (Nitex®). Flow rate through each tank was 0.5 L min^{-1}. Before each count (see below) the tanks were "flushed" at 2 L min^{-1} for approximately 10 min. Filter cups rested upon gutters that returned the water to a sump. The water was conditioned by mechanical, biological, and carbon filtration and received ultraviolet sterilization before returning to the tanks. Temperature was recorded hourly (Optic Stowaway, Onset Computer Corporation) and remained at 23-24°C during the test infections.

We counted the glochidia and juveniles present in the filter cups to monitor the timing of drop-off and the number recovered from each fish. We counted at one day after infection and every 2 days thereafter until no more glochidia or juveniles were recovered from any fish for at least 4 d. The contents of each filter cup were rinsed into a finger bowl and transferred to a Bogorov plankton counting tray with a pipette. We used a stereomicroscope at 10.5-40X to count the number of glochidia and juveniles. An individual was classified as a live juvenile if foot activity was observed.

Priming and test infections

We infected largemouth bass 4-5 times in succession with *L. reeveiana* glochidia to induce resistance ("priming"). Primed fish and naïve control fish (never exposed to glochidia) were then infected with each batch of test glochidia. The controls allowed us to distinguish differences due to priming from differences in the viability of glochidia from individual mussels. For each fish, we determined infection intensity (the total number of glochidia and juveniles recovered from the fish), transformation success (the percent of recovered individuals that were live juveniles), and mean duration of successful parasitism (i.e. days from infection to excystment of live juveniles). Two-tailed T-tests were used to compare fish body mass and intensity of infection between primed and control fish in each experiment. One-tailed t-tests were used to compare the number of recovered juveniles, transformation success, and the mean duration of successful parasitism between primed and control fish. The results are expressed as mean ± 1 SD unless otherwise noted, and differences are considered significant if $p < 0.05$.

Antibody tests

We then used immunoblotting procedures to test whether anti-glochidia factors (presumably antibodies) in fish blood serum would recognize glochidia proteins of *L. reeveiana* and the other test species. Serum was obtained from a separate group of largemouth bass from the same source and of similar size (~ 13.5 g) that were primed with 3 successive infections of *L. reeveiana* glochidia. Naïve bass that had never been exposed to mussel glochidia were also used for comparison.

Extraction and preparation of sera from fish: Fish were anesthetized with Finquel (MS-222). The caudal peduncle was severed with scissors and blood collected from the caudal vein with a pipette. Blood from different fish of the same treatment was pooled in a centrifuge tube and refrigerated (4°C) for 24 hours. Serum was separated from the blood by centrifugation (Labnet Spectrafuge 16M) at 3000 rpm, for 5 min. The serum was decanted from the blood cells and stored in aliquots at -80°C. The samples were later thawed for immunoblotting and 0.05% sodium azide was added to allow temporary storage at 2-4°C.

Detection of bass antibody production: Bass antibodies were isolated using Protein A affinity column chromatography. Briefly, an ImmunoPure ® Immobilized Protein A column (Pierce, Rockford, IL, USA) was equilibrated with binding buffer (10 mM Tris, pH 7.5). Pooled sera from 5 naïve largemouth bass from a different source (Foster's Lake and Pond Management, Garner, NC) was diluted in binding buffer and applied to the column for 3 h. The Protein A column was washed with binding buffer and the bound largemouth bass antibodies were eluted with elution buffer (0.1M glycine, pH 2.0). Eluted protein fractions were immediately neutralized with 1 M Tris, pH 7.5. The first two 1-mL fractions contained 90% of eluted antibodies and were pooled for subsequent use. Protein concentrations were determined using Bradford's assay (Bio-Rad).

SDS-PAGE was utilized to determine the purity of the eluted largemouth bass antibodies. Samples of the elutant, containing purified antibodies, and whole largemouth bass serum were mixed with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% B-mercaptoethanol; Bio-Rad) and boiled for 4 minutes. The samples (4 ug total protein for purified antibodies and 10ug total protein for whole serum) were applied to a 4% stacking gel over a 12% resolving gel. Broad range SDS-PAGE molecular weight standards (Bio-Rad) were included. After electrophoresis, the gels were fixed and stained using Coomassie Brilliant Blue R250.

Polyclonal mouse antibodies were then used to detect the production of antibodies in primed bass. The polyclonal antibodies were produced in BALB-c mice exposed to purified antibodies from bluegill sunfish (Rogers-Lowery et al., *unpublished*). To determine whether anti-bluegill antibodies would recognize largemouth bass antibodies, samples of purified largemouth bass antibodies and whole serum were first electrophoresed as described and then electrotransferred to 0.45 um nitrocellulose membrane using a Mini Trans-Blot ® Electrophoretic Transfer Cell (Bio-Rad). Prestained SDS-PAGE molecular weight standards (Bio-Rad) were included on the gels. After blotting, the gels were stained with Coomassie Brilliant Blue to confirm transfer of proteins to membrane. Membranes were blocked overnight with PBS containing 5% non-fat dry milk (PBS-NFDM) and then washed with PBS containing 0.5% Tween-20 (PBS-Tween). The membranes were initially probed with mouse anti-bluegill antibodies diluted 1:1000 in PBS containing 3% bovine serum albumin (PBS-BSA) for 1 h. After thoroughly rinsing in PBS-Tween, membranes were incubated in goat anti-mouse antibodies conjugated to horseradish peroxidase diluted 1:1000 in PBS-BSA. Antibody binding was visualized using 4-chloro-1-napthol and hydrogen peroxide to produce a colored precipitate.

Preparation, electrophoresis, and immunoblotting of glochidia extract: Glochidia were removed from gravid mussels of each species as described above and washed several times in SFW. The glochidia were frozen at -4°C until further use. Glochidia proteins were extracted by thawing and refreezing the samples several times and then homogenizing in 0.1 M Tris buffer containing a protease inhibitor cocktail (Sigma) using a Dounce homogenizer. Approximately 500 uL packed volume of glochidia was homogenized in 1500 uL total volume. Bradford's assay (Bio-Rad) was utilized to determine protein concentrations.

Samples of extracted proteins (each 10 ug total protein) were boiled in Laemmli sample buffer (Bio-Rad) for 4 minutes, and separated by SDS-PAGE on a 4% stacking gel over a 12% resolving gel with broad range molecular weight standards (Bio-Rad) included.. Gels were stained with Coomassie Brilliant Blue R250.

Immunoblotting techniques were used to determine which glochidia proteins were recognized by antibodies from primed largemouth bass. Glochidia proteins were separated by SDS-PAGE and electrotransferred to 0.45 um nitrocellulose membrane. Prestained SDS-PAGE molecular weight standards (Bio-Rad) were included on the gels. Membranes were blocked overnight with PBS-NFDM. After washing with PBS-Tween, the membranes were initially probed with pooled sera collected from naïve (n=9) or primed (n=14) largemouth bass diluted 1:50 in PBS-BSA for 1 h. After thoroughly rinsing in PBS-Tween, membranes were incubated in mouse anti-bluegill antibodies diluted 1:1000 in PBS-BSA and subsequently incubated in goat anti-mouse antibodies conjugated to horseradish peroxidase. Antibody binding was visualized using 4-chloro-1-napthol and hydrogen peroxide as the substrate.

RESULTS

Transformation success

During the course of the investigation, three different groups of host fish were primed with 4-5 infections of *L. reeveiana* (Figure 1). The mean intensity of infection (number of glochidia that attached) for each priming infections was 495 ± 149 glochidia per fish. All three groups exhibited similar resistance (1-way ANOVA p=0.5; mean transformation 32% ± 25) in the last priming infection. Primed fish were tested with glochidia of *L. reeveiana* and 4 other species. The mean body mass of the host fish was 34.6 ± 7.2 g. The mean intensity of the test infections was 655 ± 108 glochidia per fish and did not differ significantly between primed and control fish in any test (2-tailed T-tests).

The control transformation success of the lampsiline species (*L. reeveiana*, *L. abrupta*, and *V. iris*) was similar at about 90%, while control transformation of the anondontine species was much lower (*U. imbecillis* 22%, *S. undulatus* 1%) (Table 1, Figure 3). Transformation success of *S. undulatus* on primed fish was similarly low to that of controls (Table 1-Figure 3). Transformation success of all the other species was significantly reduced on primed hosts and averaged about 56 % of control values (Table

1-Figure 3). The transformation success of *L. reeveiana* in the last two priming infections and the test infection were statistically similar (i.e. the priming appeared to have reached a plateau).

The majority of glochidia sloughed from control fish were lost during the first day after attachment for all mussel species except *S. undulatus* (Figure 2). In contrast, primed fish continued to slough glochidia until juveniles were recovered (Figure 2). Both primed and control fish with *S. undulatus* continued to slough glochidia up until the appearance of transformed juveniles (Figure 2).

The mean duration of successful parasitism was significantly reduced for *L. reeveiana* on primed fish, relative to controls (Table 1-Figure 2). The mean duration of successful parasitism was similar on primed and control fish for the rest of the test species (Table 1-Figure 2).

Antibodies

SDS-PAGE of largemouth bass antibodies purified on a Protein A column revealed two heavy chain bands with molecular weights of 78-85 kDa and a single light chain band with molecular weight of ~29 kDa. No other bands were present in the gels of purified antibodies. Both heavy chains and light chain were recognized by mouse anti-bluegill IgM polyclonal antiserum. Immunoblot of whole serum from largemouth bass probed with anti-bluegill IgM antiserum revealed a heavy chain, light chain, and a third band with a molecular weight of ~110 kDa, which may represent associated heavy and light chains.

Antibodies produced in primed largemouth bass bound antigens in extracts of glochidia from the *L. reeveiana* and the other test species (Figure 4); however, antibodies from naïve largemouth bass did not (data not shown). Control blots probed with largemouth bass serum and goat anti-mouse antibodies (no mouse anti-bluegill antibodies), mouse anti-bluegill and goat anti-mouse antibodies (no largemouth bass serum), goat anti-mouse antibodies only, and substrate only all produced negative results (data not shown).

Antibodies bound several high molecular weight proteins for *L. reeveiana*, an intensely stained band with molecular weight of 132.5 kDa and several less intense bands (120.1, 85.0, and 78.5 kDa). Only the 132.5 kDa band was recognized for *L. abrupta*. Additionally, 3 low molecular weight bands with molecular weights of 44.5, 41.2 and 38.1 kDa were recognized for both *L. reeveiana* and *L. abrupta*.

The antibodies bound a 81.7 kDa protein band of *V. iris*, which is lighter than the major heavy molecular weight band (132.5 kDa) of the *Lampsilis* species. There was no evidence in *V. iris* of the 132.5 kDa protein of the *Lampsilis* species. However, very faint bands corresponding to the 81.7 kDa protein of *V. iris* were present for the *Lampsilis* species. Additionally, antibodies bound 5 low molecular weight bands ranging from 46.0 kDa to 22.0 kDa of *V. iris*.

No distinct bands were produced by serum from primed fish and extract of *S. undulatus* or *U. imbecillis* glochidia. However, diffuse staining was observed in the high molecular weight range (~183-109 kDa) for both species.

DISCUSSION

Glochidia initially attach to the host by clamping to host tissue, mainly the gills and fin margins. Attached glochidia are encysted within hours by migrating cells of the host epithelial and connective tissues. Glochidia on a compatible host species remain encysted for days or weeks and transform into juveniles before excystment occurs. On non-compatible hosts (nonhosts), or on hosts that have acquired immunity, cysts may fail to form, may regress, or the cyst may grow and detach from the underlying epithelium, so that glochidia are "sloughed" before transformation is complete. Glochidia may be sloughed live or may be killed within the cysts before sloughing occurs (Arey, 1921; 1932a; 1932b; Fustish and Millemann, 1978; Waller and Mitchell, 1989).

Several studies have reported unusual cyst formation by resistant host fish. Largemouth bass resistant to fat mucket (*Lampsilis siliquoidea*) produced bulky and irregular shaped cysts around glochidia attached to their gills (Reuling, 1919; Arey, 1932a). Bluegills resistant to *U. imbecillis* produced cysts on fins more slowly than naïve fish, and the cysts were often thinner or incomplete (Rogers and Dimock, 2003). In the present study, we observed intact cysts containing glochidia that had been shed from resistant fish, as well as unencysted glochidia. Sloughing of cysts appears to result from weakening of the attachment to the underlying tissue (Arey, 1932a).

Both live and dead glochidia were recovered from primed and control hosts in our study. We have also observed dead, open glochidia within cysts still attached to the host. Live and dead glochidia have both been recovered in other studies as well (Reuling, 1919; Arey, 1932a; Fustish and Millemann, 1978; Meyers et al., 1980; Bauer, 1987; Bauer and Vogel, 1987; Waller and Mitchell, 1989; Roberts and Barnhart, 1997; O'Connell and Neves, 1999; Rogers and Dimock, 2003). Presumably, elements of the immune system are responsible for death within the cysts (see below).

The normal process of excystment of transformed juveniles is not fully understood. The cyst wall can become thinner late in the parasitism (Arey, 1932a, Waller and Mitchell, 1989). However, it is not known whether movements of the juvenile rupture the cyst or whether the cyst tissue simply regresses or disintegrates. Sloughing might involve an acceleration of processes that cause normal excystment. In the present study, duration of successful parasitism of *L. reeveiana* juveniles was reduced on primed hosts. This change was not evident for the other test species (Table 1). Another study also found shorter duration of successful parasitism on primed host fish (Rogers and Dimock, 2003). In contrast, Bauer and Vogel (1987) reported prolonged encystment of *Margaritifera margaritifera* on re-infected brown trout (*Salmo trutta*) when compared to naïve fish. Shortened duration of encystment could limit nutritional exchange, which occurs between the host fish and glochidia (Arey, 1932c; Fisher and Dimock, 2002), and might therefore affect nutritional status and perhaps survivorship of juveniles.

Both non-specific and specific (antibody-mediated) mechanisms are involved in acquired resistance and cross-resistance of teleost fish to parasites. Priming with interleukin (IL-1), bacterial polysaccharide (LPS), concanavalin A (Con A), and mannan provide rainbow trout (*O. mykiss*) partial protection against the parasitic ciliate *Ichthyopthirius multifiliis* (Buchmann et al., 1999). Complement binds and kills the ectoparasitic platyhelminth, *Gyrodactylus derjavini* (Buchmann, 1998). Non-specific 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 (*Oncorhynchus mykiss*) to haemoflagellates, *Cryptobia salmositica* (Mehta and Woo, 2002).

Acquired immunity to parasites involving antibodies is well documented in fish (Hines and Spira, 1974; Clark et al., 1987; Cross and Matthews, 1992; Xu et al., 2002). Antibodies to shared antigens of several different protist parasites are involved in cross-resistance to these parasites (Ling et al., 1993; Sin et al., 1992; Goven et al., 1980; 1981; Wolf and Markiw, 1982; Dickerson et al., 1984).

Our results indicate that cross-resistance of host fish to different mussel species may be at least partly mediated by antibodies. Antibodies bound to glochidia proteins of 2 of the 3 test species that showed cross-resistance. These proteins were similar to those of the priming glochidia. Antibody-mediated cross-resistance is likely to be correlated with phylogenetic relatedness, because distantly related species may have proteins sufficiently different that they are not recognized by antibodies of primed fish. In this study, similar antigens were evidently present among the lampsiline species (members of the subfamily Lampsilinae; Ortmann, 1919; Parmalee and Bogan, 1998), but not in the less closely related anondontine species *U. imbecillis* or *S. undulatus* (members of subfamily Anodontinae) (Figure 4).

Control largemouth bass were poor hosts for *U. imbecillis* and essentially incompatible with *S. undulatus*, yet no antibody binding with specific proteins of either species was observed (Figure 4). This observation indicates that innate resistance did not involve these antigenic proteins. In spite of the fact that no antibody binding could be shown to *U. imbecillis*, significant cross-resistance was observed (Table 1). This result indicates that non-specific mechanisms as well as antibodies may be involved in cross-resistance of fish to glochidia. Eosinophilic granulocytes (nonspecific immune cells) may be involved in the cross-resistance to *U. imbecillis* because these cells congregate around glochidial cysts on immune hosts (Arey, 1932a).

There are few previous studies regarding cross-resistance of host fish to unionid mussel glochidia. Reuling (1919) found that largemouth bass that acquired resistance to *L. siliquoidea* glochidia were cross-resistant to glochidia of a congener, *L. cardium* and to glochidia of *A. ligamentina*, also a member of the Lampsilinae. Our results agree with Reuling's findings.

The possibility of cross-resistance of fish to glochidia and unrelated parasites has not been investigated since the early 1900's. Wilson (1916) found that black sandshell

(*Ligumia recta*) glochidia had a lower attachment success on white crappie (*Pomoxis annularis*) infected with parasitic copepods (*Ergasilus caeruleus*) than on uninfected fish. Conversely, copepodid larvae had lower attachment to gills of *P. annularis* that had *L. recta* glochidia attached to them. Similar results were found using short-nosed gar (*Lepisosteus platostomus*), *Lernaea* copepods, and unspecified mussel glochidia (Wilson, 1917). The mechanism of interference is not known and deserves further attention.

In eastern North America, mussel habitats generally support large numbers of species living in close proximity (Vaughn, 1997). In many cases different mussel species may utilize the same species of host fish (Watters, 1994; Haag and Warren, 1997). Given that fish can develop cross-resistance to glochidia, interspecific as well as intraspecific competition for naïve hosts might occur. There is evidence that fish acquire resistance to glochidia in nature (Young and Williams, 1984a; Bauer, 1987; Watters and O'Dee, 1996; Hastie and Young, 2001). Competition for hosts would be favored by prolonged retention by the host of acquired resistance. We have observed that largemouth bass retain measurable acquired resistance for at least 11 months (unpublished data).

Competition for immunologically naïve host fish could be a factor in niche partitioning and perhaps in the evolutionary diversification of Unionidae. Many mussels in the subfamily Lampsilinae display mantle lures that attract host fish. In the Mobile Basin, the Alabama rainbow (*Villosa nebulosa*) displays a white lure primarily at night, while the sympatric southern rainbow (*Villosa vibex*) has a black lure and displays mostly during the day. Such differences in lures and in luring behavior might permit coexistence of species because they minimize immunological competition for hosts (Haag and Warren, 2000).

Graf (1997) presented a model by which shifts in host utilization could promote sympatric speciation of unionids. In Graf's model, individuals compatible with a new host might be distributed into different habitat because of habitat preferences of the new host. Non-random mating resulting from host-linked habitat use might lead to sympatric speciation. If acquired immunity of a host population to mussels were extensive, mussel variants that were compatible with a different host species, one less likely to encounter glochidia and acquire immunity, might be favored by natural selection. A new host with different habitat preferences from the parental mussel species might also be less likely to have acquired immunity to that species.

Cross resistance of fish to mussel glochidia may have practical implications for efforts to propagate endangered mussel species. It appears that propagating either the same or different mussel species consecutively on the same host fish would reduce transformation success. Another question, which has apparently not been investigated, is whether the immune response of the host might affect the viability of those juveniles that do successfully transform. The shortened duration of successful parasitism observed in primed fish could affect the nutritional status of the juveniles. Study is also needed to establish whether infection intensity affects transformation success, duration of parasitism, or juvenile viability. Hypothetically, higher infection intensity could result in a stronger immune response, perhaps affecting the success of glochidia even during the first infection of a host. Establishing the optimum intensity of infection might improve the efficiency of captive propagation.

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LITERATURE CITED

Arey, L. B. 1921. An experimental study on glochidia and the factors underlying encystment. Journal of Experimental Zoology 33:463-499.

--------. 1924. Observations on an acquired immunity to a metazoan parasite. Journal of Experimental Zoology 38:377-381.

--------. 1932a. A microscopical study of glochidial immunity. Journal of Morphology 53:367-379.

--------. 1932b. The formation and structure of the glochidial cyst. Biological Bulletin 62:213-221.

--------. 1932c. The nutrition of glochidia during metamorphosis. Journal of Morphology 53:201-221.

Barnhart, M. C., and A. D. Roberts. 1997. Reproduction and fish hosts of unionids from the Ozark Uplifts. In: Conservation and Management of Freshwater Mussels II. Proc. UMRCC Symp. 16-18 October 1995, St. Louis, Missouri. Cummings, K. S., Buchanan, A. C., Mayer, C. A., and Naimo, T. J., eds., pg 16-20. Upper Mississippi River Conservation Committee, Rock Island, Illinois.

Bauer, G., and C. Vogel. 1987. The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera L.*) I. Host response to glochidiosis. Archiv fuer Hydrobiologie Supplement 76:393-402.

Buchmann, K. 1998. Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes, Monogenea). Diseases of Aquatic Organisms 32:195-200.

Buchmann, K., T. Lindenstrom, and J. Sigh. 1999. Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout. Journal of Helminthology 73:189-195.

Clark, T. G., H. W. Dickerson, J. G. Gratzek, and R. C. Findly. 1987. *In vitro* response of *Ichthyophthirius multifiliis* to sera from immune channel catfish. Journal of Fish Biology 31:203-208.

Cross, M. L. and R. A. Matthews. 1992. Ichthyophthiriasis in carp *Cyprinus carpio* L.: fate of parasites in immunized fish. Journal of Fish Diseases 15:497-505.

Dickerson, H. W., J. Brown, D. L. Dawe, and J. B. Gratzek. 1984. *Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: comparison between isolates and ciliary preparations. Journal of Fish Biology 24:523-528.

Evans, D. L., J. H. Leary, Jr., P. Nadella, and L. Jaso-Freidmann. 1998. Evidence for antigen recognition by nonspecific cytotoxic cells: initiation of ^{3}H-thymidine uptake following stimulation by a protozoan parasite and homologous cognate synthetic peptide. Developmental and Comparative Immunology 22:161-172.

Fisher, G. R., and R. V., Dimock, Jr. 2002. Ultrastructure of the mushroom body: digestion during metamorphosis of *Utterbackia imbecillis* (Bivalvia: Unionidae). Invertebrate Biology 121(2):126-135.

Fustish, C. A., and R. E. Millemann. 1978. Glochidiosis of salmonid fishes. II. Comparison of tissue response of coho and chinook salmon to experimental infection with *Margaritifera margaritifera* (L.) (Pelecypoda: Margaritanidae). Journal of Parasitology 64(1):155-157.

Goven B. A., D. L. Dawe, and J. B. Gratzek. 1980. Protection of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunization. Journal of Fish Biology 17:311-316.

Goven B. A., D. L. Dawe, and J. B. Gratzek. 1981. In vitro demonstration of serological cross-reactivity between *Ichthyophthirius multifiliis* Fouquet and *Tetrahymena pyriformis* (Lwoff). Developmental and Comparative Immunology 5:283-289.

Graf, D. L. 1997. Sympatric speciation of freshwater mussels (Bivalvia: Unionoidea): a model. American Malacological Bulletin 14(1):35-40.

Haag, W. R. and M. L. Warren, Jr. 1997. Host fishes and reproductive biology of 6 freshwater mussel species from the Mobile Basin, USA. Journal of the North American Benthological Society. 16:576-585.

Haag, W. R. and M. L. Warren, Jr. 2000. Effects of light and presence of fish on lure display and larval release behaviors in two species of freshwater mussels. Animal Behaviour 60:879-886.

Hastie, L. C., and M. R. Young. 2001. Freshwater pearl mussel (*Margaritifera margaritifera*) glochidiosis in wild and farmed salmonid stocks in Scotland. Hydrobiologia 445:109-119.

Hines, R. S. and D. T. Spira. 1974. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.) V. Acquired immunity. Journal of Fish Biology 6:373-378.

Larsen, A. H., J. Bresciani, and K. Buchmann. 2002. Interactions between ecto- and endo-parasites in trout *Salmo trutta*. Veterinary Parasitology 103:167-173.

Ling, K. H., Y. M. Sin, and T. J. Lam. 1993. Protection of goldfish against some common ectoparasite protozoans using *Ichthyophthirius multifiliis* and *Tetrahymena* for vaccination. Aquaculture 116:303-314.

Mehta, M., and P. T. K. Woo. 2002. Acquired cell-mediated protection in rainbow trout, *Oncorhynchus mykiss*, against the haemoflagellate, *Cryptobia salmositica*. Parasitology Research 88: 956-962.

Meyers, T. R., and R. E. Millemann. 1977. Glochidiosis of salmonid fishes. I. Comparative susceptibility to experimental infection with *Margaritifera margaritifera* (L.) (Pelecypoda: Margaritanidae). Journal of Parasitology 63(4): 728-733.

Meyers T. R., R. E. Millemann, and C. A. Fustish. 1980. Glochidiosis of salmonid fishes. IV. Humoral and tissue responses of coho and Chinook salmon to experimental infection with *Margaritifera margaritifera* (L.) (Pelecypoda: Margaritanidae). Journal of Parasitology 66(2):274-281.

NNMCC (National Native Mussel Conservation Committee). 1998. National strategy for the conservation of native freshwater mussels. Journal of Shellfish Research 17(5):1419-1428.

O'Connell, M. T., and R. J. Neves. 1999. Evidence of immunological responses by a host fish (*Ambioplites rupestris*) and two non-host fishes (*Cyprinus carpio* and (*Carassius auratus*) to glochidia of a freshwater mussel (*Villosa iris*). Journal of Freshwater Ecology 14(1):71-78.

Ortmann, A. E. 1911. A monograph of the naiades of Pennsylvania. Parts I and II. Memoirs of the Carnegie Museum 4(6):279-347 + 4 plates.

Ortmann, A. E. 1919. A mongraph of the naiades of Pennsylvania. Part III: Systematic account of the genera and species. Memoirs of the Carnegie Museum 8(1):xvi 384p + 21 plates.

Parmalee, P. W. and A. E. Bogan. 1998. The freshwater mussels of Tennessee. University of Tennessee Press. 328p.

Reuling, F. H. 1919. Acquired immunity to an animal parasite. Journal of Infectious Diseases 24:337-346.

Rogers, C. L., and R. V. Dimock, Jr. 2003. Acquired resistance of bluegill sunfish *Lepomis macrochirus* to glochidia larvae of the freshwater mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) following multiple infections. Journal of Parasitology 89(1):51-56.

Sin, Y. M., K. H. Ling, and T. J. Lam. 1992. Protection against velvet disease in goldfish recovered from ichthyophthiriasis. Aquaculture 102:187-191.

USEPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed.

van Snik Gray, E., W. A. Lellis, J. C. Cole, and C. S. Johnson. 2002. Host identification for *Strophitus undulatus* (Bivalvia: Unionidae), the Creeper, in the Upper Susquehanna River Basin, Pennsylvania. American Midland Naturalist 147:153-161.

Vaughn, C. C. 1997. Regional patterns of mussel species distributions in North American rivers. Ecography 20:107-115.

Waller, D. L., and L. G. Mitchell. 1989. Gill tissue reactions in walleye *Stizostedion vitreum vitreum* and common carp *Cyprinus carpio* to glochidia of the freshwater mussel *Lampsilis radiata siliquoidea*. Diseases of Aquatic Organisms 6:81-87.

Watters, G. T. 1994. An annotated bibliography of the reproduction and propagation of the Unionoidea (Primarily of North America). Ohio Biological Survey Miscellaneous Contributions No.1. 158 pp.

Watters, G. T. and S.H. O'Dee. 1996. Shedding of untransformed glochidia by fishes parasitized by *Lampsilis fasciola* Rafinesque, 1820 (Mollusca: Bivalvia: Unionidae): evidence of acquired immunity in the field? *Journal of Freshwater Ecology* 11:383-389.

Williams, J. D., M. L. Warren, K. S. Cummings, J. L. Harris, and R. J. Neves. 1993. Conservation status of freshwater mussels of the United States and Canada. Fisheries 18(9):6-22.

Wilson, C. B. 1917. The economic relations, anatomy, and life history of the genus *Lernaea*. Bulletin of the United State Bureau of Fisheries 35:165-198 + 10 plates.

Wilson, C. B. 1916. Copepod parasites of fresh-water fishes and their economic relations to mussel glochidia. Bulletin of the United States Bureau of Fisheries (1914) 34:331-374 + 15 plates.

Wolf, K., and M. E. Markiw. 1982. Ichthyophthiriasis: Immersion immunization of rainbow trout (*Salmo gairdneri*) using *Tetrahymena thermophila* as a protective immunogen. Canadian Journal of Fisheries and Aquatic Science 39:1722-1725.

Xu, D.H., P.H. Klesius, and R.A. Shelby. 2002. Cutaneous antibodies in the excised skin from channel catfish, *Ictalurus punctatus* Rafinesque, immune to *Ichthyophthirius multifiliis*. Journal of Fish Diseases 25:45-52.

Young, M., and J. Williams. 1984a. The reproductive biology of the freshwater pearl mussel *Margaritifera margaritifera* (LINN.) in Scotland I. Field Studies. Archiv fuer Hydrobiologie 99(4):405-422.

Young, M., and J. Williams. 1984b. The reproductive biology of the freshwater pearl mussel *Margaritifera margaritifera* (LINN.) in Scotland II. Laboratory Studies. Archiv fuer Hydrobiologie 100(1):29-43.

Table 1. Cross-resistance test results. Control fish had never been previously exposed to glochidia, and primed host fish received 4-5 previous infections with *L. reeveiana* (Figure 1). The duration of successful parasitism indicates days from attachment to excystment of live juveniles. Transformation success indicates percent of attached glochidia that were recovered as live juveniles. Numbers are means ± SD. An asterisk indicates that the mean for primed fish was significantly lower (one-tailed t-test, $p < 0.05$) than the corresponding control fish.

Mussel species	Host group (n)	Number of juveniles recovered	Transformation success (%)	Duration of successful parasitism (days)
L. reeveiana	Control (4)	723 ± 194	89.0 ± 2.5	20.3 ± 0.5
L. reeveiana	Primed (3)	321 ± 198*	36.8 ± 17.5*	14.8 ± 0.8*
L. abrupta	Control (4)	618 ± 32	89.7 ± 1.4	16.4 ± 1.3
L. abrupta	Primed (4)	270 ± 131*	43.5 ± 21.8*	17.2 ± 0.4
V. iris	Control (4)	616 ± 85	90.0 ± 6.0	19.6 ± 1.3
V. iris	Primed (4)	469 ± 238	67.0 ± 18.5*	19.4 ± 2.2
U. imbecillis	Control (7)	137 ± 25	22.2 ± 7.5	9.4 ± 0.4
U. imbecillis	Primed (7)	61 ± 30*	13.2 ± 8.6*	9.1 ± 0.5
S. undulatus	Control (3)	8 ± 4	1.3 ± 0.6	9.3 ± 0.3
S. undulatus	Primed (3)	9 ± 3	1.9 ± 0.4	9.5 ± 0.7

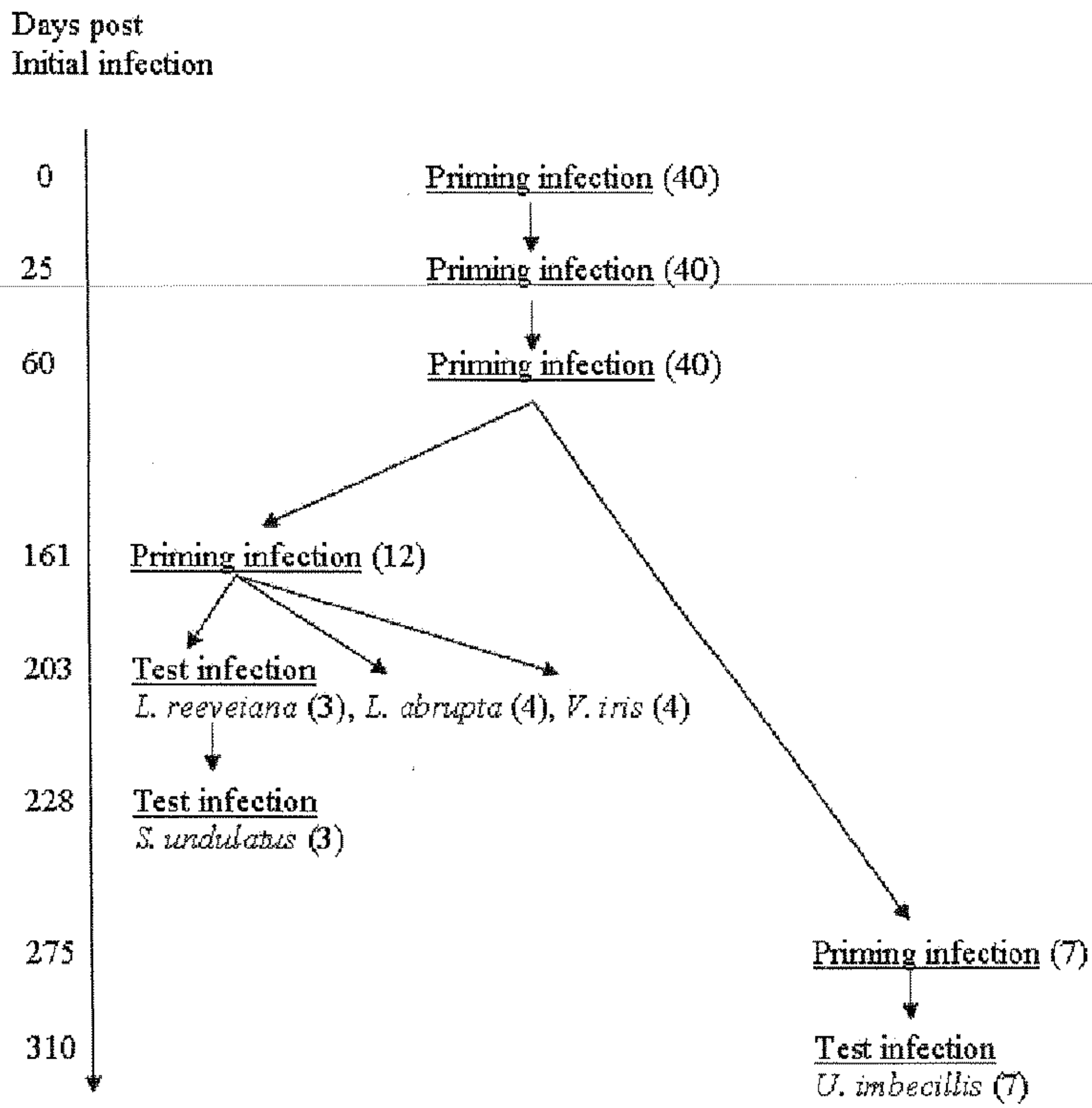

Figure 1. Experiment infection schedule. *L. reeveiana* glochidia were used for the priming infections. The timing of each infection is indicated. The numbers of host fish infected are shown in parentheses.

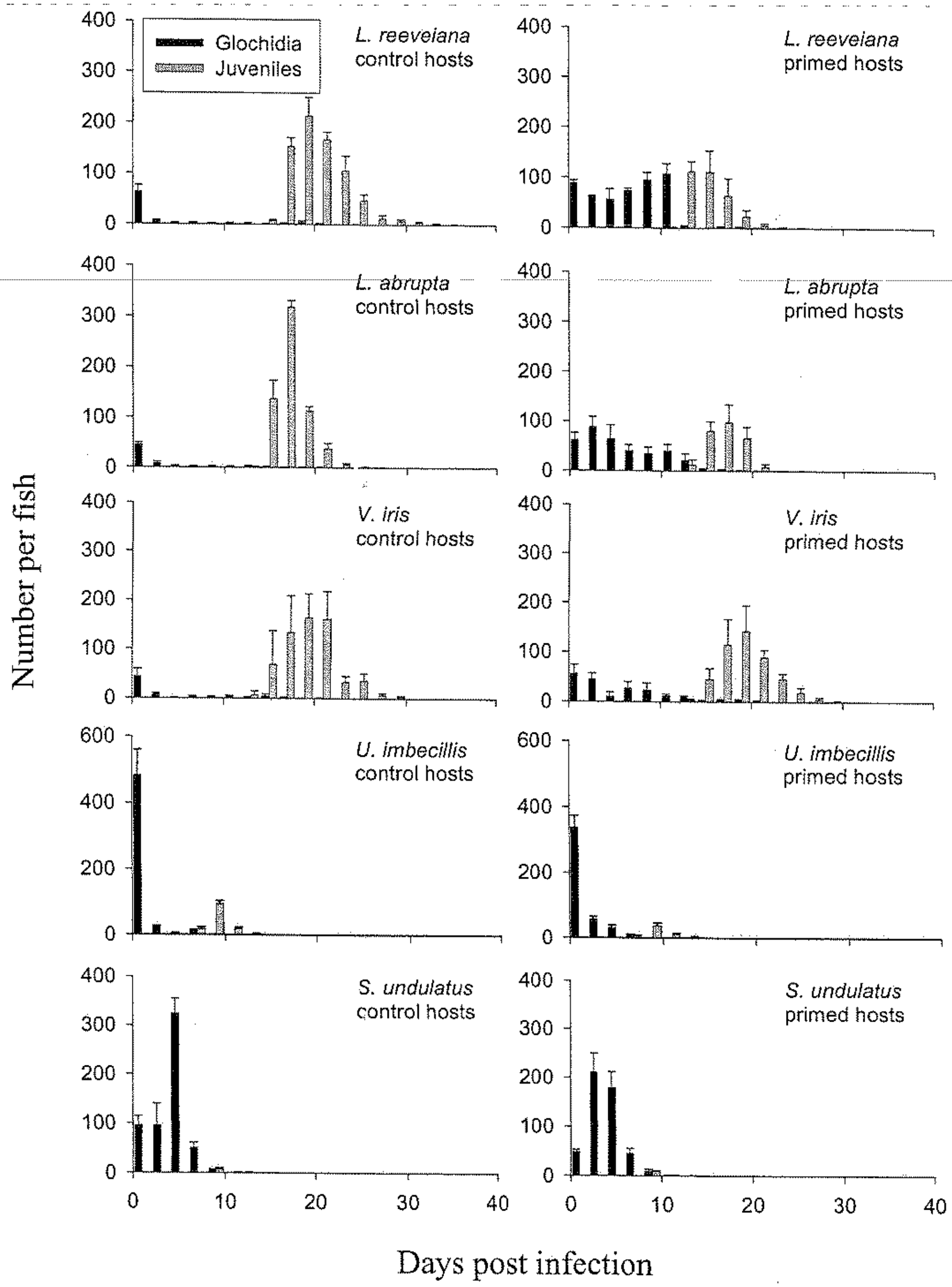

Figure 2. Time course of recovery of untransformed glochidia and of transformed juveniles from primed and control bass. Bars indicate the mean and standard error of the number of glochidia (black bars) or juveniles (grey bars) recovered per host fish.

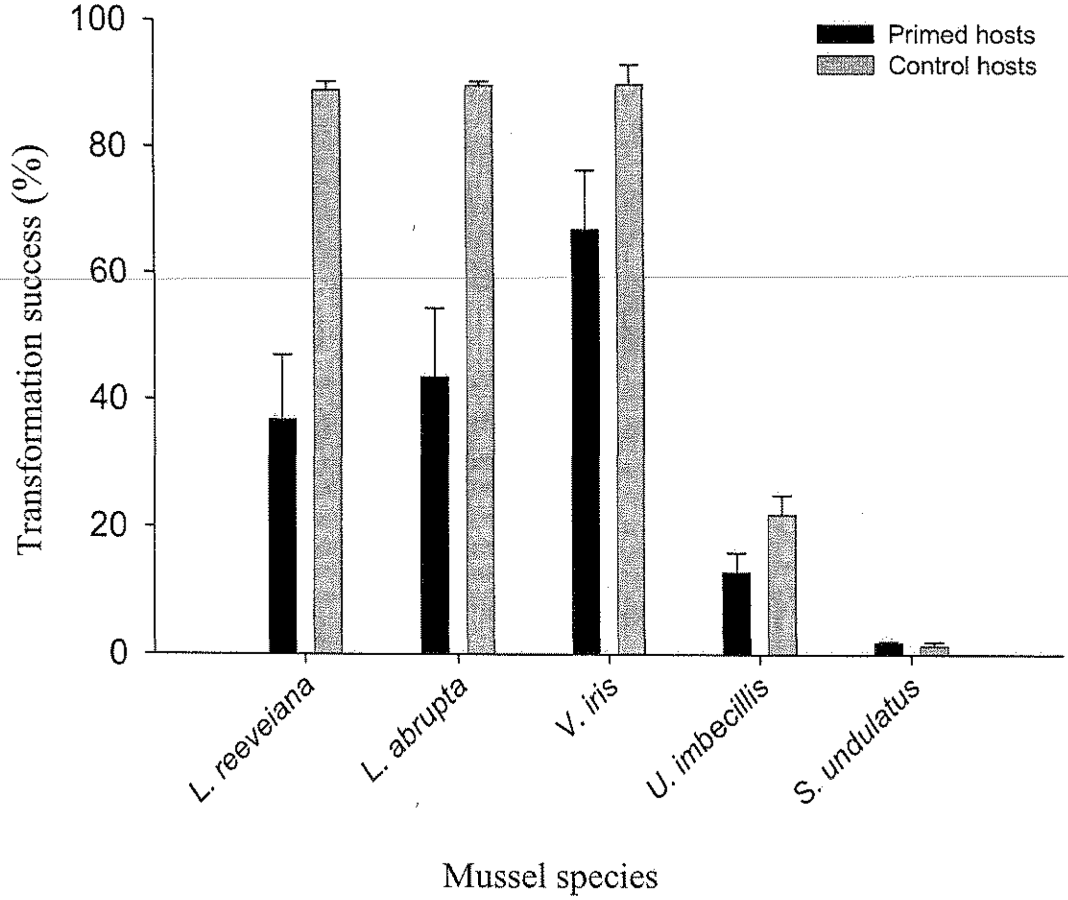

Figure 3. Effect of priming with *L. reeveiana* on the subsequent transformation success of *L. reeveiana* and other test species on largemouth bass. Bars indicate mean ± standard error. Black bars represent transformation success on primed hosts that previously received 4-5 *L. reeveiana* infections. Gray bars represent success on control (naïve) hosts.

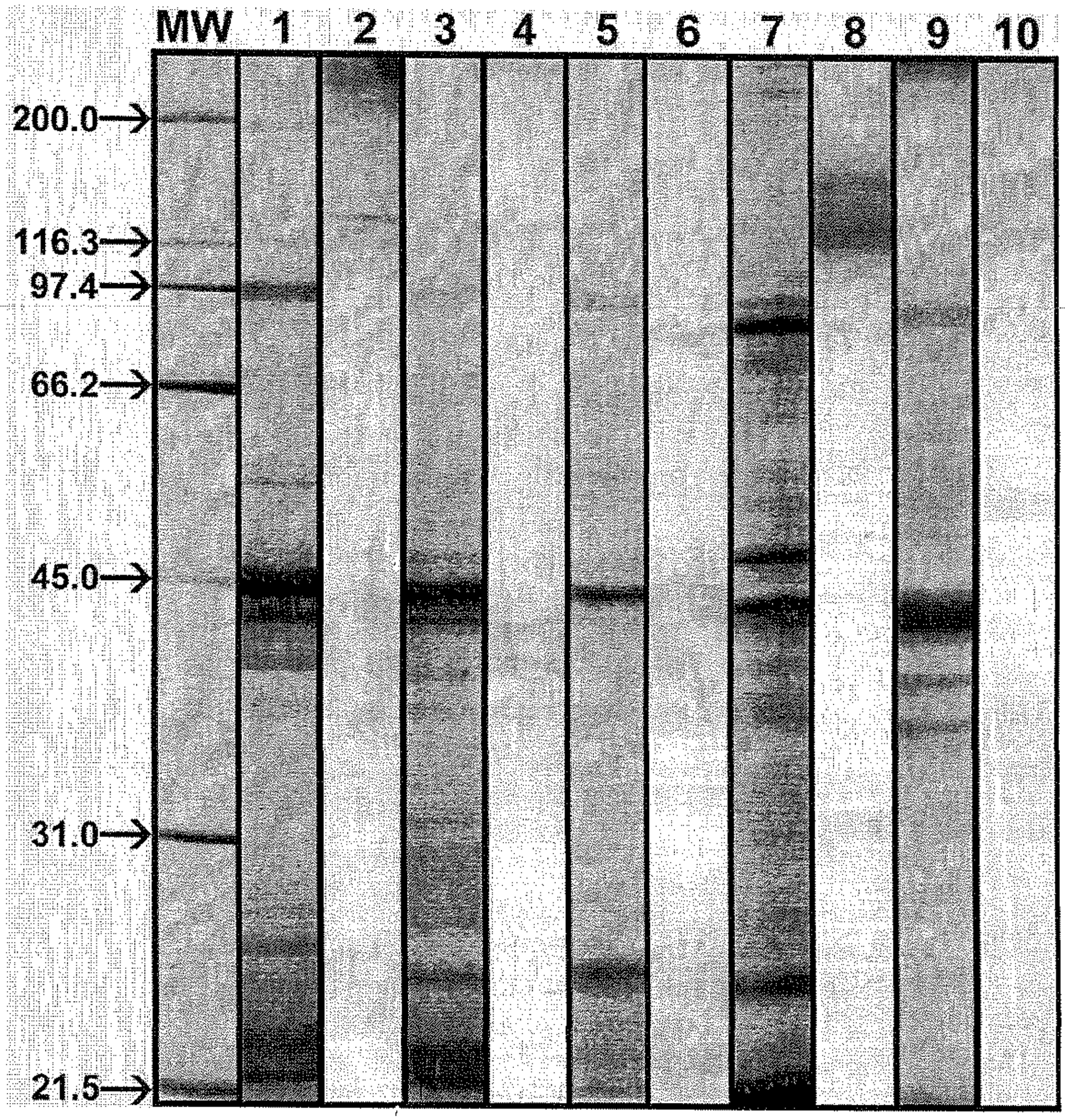

Figure 4. Western Blot of glochidia antigens recognized by serum antibodies of largemouth bass primed with *L. reeveiana* glochidia. The lanes are Molecular Weight standards (MW), *L. reeveiana* proteins (1), recognized *L. reeveiana* proteins (2), *L. abrupta* proteins (3), recognized *L. abrupta* proteins (4), *V. iris* proteins (5), recognized *V. iris* proteins (6), *S. undulatus* proteins (7), recognized *S. undulatus* proteins (8), *U. imbecillis* proteins (9), and recognized *U. imbecillis* proteins (10).