Induced Metamorphosis of Freshwater Mussel Glochidia on Nonhost Fish

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ABSTRACT

Intraperitoneal implants of cortisol, an immunosuppressant, suspended in liquid cocoa butter were administered to nonhost fish species. Fish were then infested with glochidia of freshwater mussels to determine if transformation would occur after immune system manipulation. Glochidia of *Venustaconcha sima* (Lea, 1838) (*Villosa iris* complex) transformed on orangethroat darters, *Etheostoma spectabile* (Agassiz, 1854), after injection of cortisol at concentrations ranging from 0.005 to 0.040 mg per g of fish weight. Banded sculpins, *Cottus carolinae* (Gill, 1861), transformed glochidia of *Villosa taeniata* (Conrad, 1834) following cortisol injections ranging from 0.005 to 0.020 mg/g. Creek chubs, *Semotilus atromaculatus* (Mitchill, 1818), failed to transform glochidia of either mussel species after cortisol administration. No juvenile mussels were collected from noninjected or sham injected fish during any experiment. Cortisol-induced immunosuppression facilitates metamorphosis of glochidia on some nonhost fish species. Refinement of this technique could provide an alternative means for propagating freshwater mussels, especially for those endangered species that utilize unknown hosts.

Key Words: cortisol, immunosuppression, transformation, glochidia, implants, Unionidae.

INTRODUCTION

The obligate parasitic stage in the life cycle of freshwater mussels on specific host fish may limit reproduction of many populations. Because adequate biological inventories were not conducted before impoundment of most river systems, many fish species were extirpated before identification of glochidial hosts could occur (Schindler, 1989). Hosts are known for only one-quarter of the North American species of mussels (Hoggarth, 1992; Watters, 1994). Although glochidia of a few mussel species have been transformed in a sterile culture medium (Isom & Hudson, 1982), metamorphosis of other species has been less successful. Consequently, artificial propagation of many freshwater mussel species that utilize unknown hosts has not been possible.

Other than the use of known hosts and artificial media, few advancements have occurred in methods of artificial propagation of freshwater mussels. With the relatively recent threat of the zebra mussel, *Dreissena polymorpha* Pallas, 1771, concern for the fate of our native fauna has increased efforts to sustain diversity and protect existing populations, especially endangered or threatened species without identified hosts. Efforts include assessment of relocation into areas with little risk of zebra mussel invasion and the use of mussel refugia such as hatcheries (Shannon *et al.*, 1993).

Host specificity of freshwater mussels is believed to have an immunological basis (Reuling, 1919; Arey, 1932; Meyers *et al.*, 1980). Isom and Hudson (1982; 1984) demonstrated that components in fish blood necessary for initiation of glochidial transformation are not species specific and can be found in the blood of all fish. Moreover, horse and neonatal calf serum are also suitable media for glochidial transformation (Keller & Zam, 1990). The implications of these studies suggest that the immune response of the host is the determining factor in mussel-host specificity.

Immune responses of both host and nonhost fish to glochidia of freshwater mussels have been documented (Reuling, 1919; Arey, 1932; Meyers *et al.*, 1980; Bauer & Vogel, 1987). Responses of host fish consist of host tissue proliferation which encysts the glochidium within a few hours after attachment (d'Eliscu, 1972; Waller & Mitchell, 1989). In host and nonhost species, humoral and cell mediated responses of the immune system occur. These immune responses include the presence of specific antibodies to the parasites (Reuling, 1919; Meyers *et al.*, 1980; Bauer & Vogel, 1987), an increase in eosinophils and other leukocytes around glochidial cysts (Arey, 1932), hyperplastic sloughing of host epithelial tissue (Meyers *et al.*, 1980), and in some cases, cytolitic destruction of the glochidium (Reuling, 1919; Arey, 1932).

Cortisol, a major corticosteroid in teleosts (Idler & Truscott, 1972) is released during stress (Bennett & Wolke, 1987; Schreck, 1990) and produces immunosuppressive

effects. Cortisol can be administered to produce a dose-dependent elevation of plasma cortisol and immunosuppression (Pickering & Duston, 1983). Cortisol reduces lymphocyte concentrations circulating in the blood, reduces lymphocyte participation in inflammation and reduces other inflammatory cells (Pearson *et al.*, 1978). We hypothesized that the effects of cortisol might inhibit the ability of nonhost fish to respond to glochidia by decreasing the number of antibodies and reducing inflammation around glochidial cysts, thus allowing glochidia to attach, encyst, and metamorphose into juveniles. In this paper, we evaluate the effects of cortisol, administered in a vehicle of liquefied cocoa butter, on glochidial metamorphosis.

MATERIALS & METHODS

Collection of fish and mussels: For our experiments, we selected two bradyticite species of mussels based on their narrow host specificity and availability. Gravid *Venustaconcha sima* (Lea, 1838)[1], were collected from the Collins River in Grundy County, Tennessee. Gravid *Villosa taeniata* (Conrad, 1834) were collected from the Roaring River in Overton County, Tennessee. Rockbass, *Ambloplites rupestris* (Rafinesque, 1817), is the only known host of *V. taeniata* (Gordon *et al.*, 1994), and banded sculpin, *Cottus carolinae* (Gill, 1861), is the only known host of *V. sima* (unpublished data). To avoid premature expulsion of glochidia, mussels were kept at approximately 4°C in pans of shallow water until needed. Laboratory established nonhost fish species were used as experimental fish, with the exception of creek chub, *Semotilus atromaculatus* (Mitchill, 1818). The suitability of creek chub as hosts for *V. sima* has not been previously tested in the laboratory; however, examination of wild fish did not reveal any infestations (unpublished data).

A backpack electrofishing unit was used to collect fish from the East Blackburn Fork, Putnam County, Tennessee. Populations of banded sculpins, creek chubs, rockbass, and orangethroat darters, *Etheostoma spectabile* (Agassiz, 1854), are abundant in this stream. No mussels occur in the East Blackburn Fork, and an impassable waterfall downstream prevents any possible upstream movement of fish that may have been exposed to glochidia. Fish were transported back to the laboratory, acclimated to laboratory conditions, and held in 38 liter aquaria.

Implantation and infestation: Pure cocoa butter was melted at a temperature of 40°C. A predetermined amount of cortisol was dissolved in ethanol. Ethanol was used at a rate of 10% the volume of cocoa butter. The dissolved cortisol was suspended in the liquid cocoa butter and allowed to mix in a water bath. Average fish weight was used to calculate the amount of cortisol per gram of fish weight needed to obtain the desired concentration within the fish. Orangethroat darters received 50 µl implants and creek chub and banded sculpins received 100 µl implants.

Infective glochidia were obtained from gravid females and exposed to salt to determine maturity (Zale & Neves, 1982). After fish were anesthetized with tricaine methanesulfonate (MS 222), they were placed on a wet paper towel to reduce the removal of mucus during the injection process. The liquid cocoa butter implant was injected into the peritoneal cavity of the fish with a 1 ml tuberculin hypodermic syringe. The liquid cocoa butter solidified within the cavity of the fish and acted as a solid implant during the experiment. Presumably, cortisol leaked slowly from the implant, but the kinetics of this transfer were not addressed. Pickering and Pottinger (1985) observed maximum mean plasma cortisol levels of 9 and 15 ng/ml for brown trout, *Salmo trutta* (Linnaeus, 1758), that received 10 and 20 mg cortisol respectively. Immediately after cortisol injection, glochidia were pipetted onto the left gills of each fish. Fish were then transferred to fresh water and revived. Each experimental group was kept in a separate aquarium.

Experimental design Treatments varied among experiments, but generally included a known host control group to measure glochidial viability. The no-injection (NI) group verified that the test species did not normally serve as a host. Sham injected fish received injections of pure cocoa butter only, and were used to determine if the injection process or cocoa butter had an effect on glochidial transformation. Cortisol injected fish received concentrations that ranged from 0.005 to 0.040 mg/g. Preliminary experiments determined the feasibility of working with these concentrations, which we initially based on those used by Pickering and Duston (1983) and Pickering and Pottinger (1985).

Aquaria were siphoned daily and the siphonate was examined under a dissecting microscope at 10X to 20X magnification. The number of glochidia and juveniles, water temperature and fish mortality were recorded. Criteria for recognizing juvenile mussels included the presence of two adductor muscle scars, closed valves, and movement within 24 hours of collection. If these criteria were not met, organisms were considered untransformed or partially transformed glochidia. Experiments were terminated when no juveniles were collected from host fish for one week after the last juvenile was collected, or examination of the gills revealed no encysted glochidia. All fish were used for only a single infection experiment.

RESULTS

Metamorphosis of *Venustaconcha sima* on nonhost fish: A total of 58 juvenile *Venustachona sima* were transformed on cortisol injected orangethroat darters. Transformation occurred only on orangethroat darters injected

[1]This species is generally considered a member of the *Villosa iris* complex but was elevated by Gordon (1995). In this paper, we use *Venustaconcha sima* to clearly identify which of the two members of the *Villosa iris* complex occurring sympatarically in the Collins River were used in our experiments.

Table 1. Numbers of juveniles collected from cortisol injected orangethroat darters infested with glochidia of *Venustaconcha sima* in each experiment. Dates indicate when fish were injected and infested. Numbers in parentheses are the average number of juveniles transformed per fish (NI = No-injection).

	Date				
Cortisol (mg/g)	June 6	August 5	March 31	May 17	Total
NI	0	0	0	0	0
0.000	—	0	0	0	0
0.005	17 (1.89)	0	5 (0.36)	3 (0.25)	25
0.010	—	0	4 (0.29)	—	4
0.020	—	—	7 (1.00)	5 (0.50)	12
0.030	—	—	0*	—	0
0.040	—	—	17 (1.70)	0*	17

* Fish mortality was ≥88%.

Table 2. Numbers of juveniles collected from cortisol injected banded sculpins (October and May) and orangethroat darters (December) infested with *Villosa taeniata* in each experiment. Dates indicate when fish were injected and infested. Numbers in parentheses are the average number of juveniles transformed per fish (NI = No-injection).

	Date			
Cortisol (mg/g)	October 27	December 28	May 27	Total
NI	0	0	0	0
0.00	0	0	0	0
0.005	1 (0.13)	—	0	1
0.010	0	0	1 (0.10)	1
0.020	—	0	4 (1.00)	4
0.030	—	0	—	0

in March, May, and June (Table 1). Glochidia from *V. sima* transformed on fish injected with 0.005 to 0.040 mg/g cortisol. Metamorphosis never occurred in sham injected and no-injection (NI) treatment groups during any experiment. Glochidia did not transform on either orangethroat darters or creek chubs injected in August.

Juvenile transformation varied among experiments. For instance, the average number of juveniles transformed per orangethroat darter injected with 0.005 mg/g cortisol ranged from 0.00 to 1.89 among experiments (Table 1). There was a positive correlation between cortisol concentration and metamorphosis within experiments; however, this relationship was confounded by high mortality in some treatment groups. Mortality varied within and among experiments; most deaths occurred soon after injection, and were likely the result of physical injury.

Metamorphosis of *Villosa taeniata* on nonhost fish: A total of six juvenile *Villosa taeniata* were collected from banded sculpins injected with cortisol (Table 2). Transformation of *V. taeniata* glochidia did not occur on orangethroat darters or creek chubs injected with cortisol. In one experiment, nonhost species sloughed glochidia quickly after the infestation. After examination of their gills revealed no remaining glochidia, the fish were reinfested 11 days after the initial infestation. Although creek chubs and banded sculpins retained glochidia longer after the second infestation, metamorphosis did not occur.

Metamorphosis of glochidia on host fish: Transformation of glochidia of *Venustachoncha sima* on the host, banded sculpins, varied between experiments. Banded sculpins transformed 35 juvenile mussels in March and six juvenile mussels transformed on banded sculpins in May. When used as a host control, all banded sculpins were held in one aquarium during each experiment, and individual variation in juvenile transformation could not be assessed.

Because of the aggressive nature of rockbass, the host of *Villosa taeniata*, individuals were kept in separate aquaria. Transformation of glochidia of *V. taeniata* on rockbass varied greatly among individuals and among experiments (Table 3). Juvenile transformation on individual fish ranged from 0 to 13 juveniles in one experiment, to a range of 7 to 262 juveniles in another experiment. There was no clear relationship between the number of glochidia used to infest host fish or water temperature and the number of juveniles recovered from individual rockbass.

DISCUSSION

Cortisol affects leukocyte circulation, influences immune effector mechanisms in lymphocytes, modulates activities of inflammatory mediators, and modifies protein, carbohydrate, and fat metabolism (Tizard, 1988). In fish, this results in immunosuppression and an increased susceptibility to infectious diseases such as furunculosis and bacterial fin-rot (Pickering & Duston, 1983; Pickering & Pottinger, 1985).

The present study has shown that cortisol-induced immunosuppression can facilitate glochidial metamorphosis on nonhost fish species. Presumably, cortisol suppressed the humoral and cell-mediated responses observed in glochidial infestations by Arey (1932) and Meyers *et al.* (1980). Although the strength of fish immune responses after cortisol administration was not measured, transformation occurred on experimental nonhost species only when the immune system was compromised. These results are consistent with the hypothesis that host specificity of freshwater mussels is immunologically controlled.

Glochidia of *Venustachoncha sima* transformed on orangethroat darters in three of four experiments at the lowest concentration (0.005 mg/g) used. Transformation of glochidia of *Villosa taeniata* occurred on banded sculpins during two experiments. For this species, fewer juveniles were collected from fish injected with the same concentration used for orangethroat darters. Creek chubs failed to transform glochidia from either mussel species

Table 3. Total length, number of glochidia recovered, number of juveniles recovered, metamorphosis period, and water temperature (±S.D.) for individual rockbass infested with glochidia of *Villosa taeniata*. Dates indicate when fish were infested.

Date	Total Length (mm)	Temperature (±S.D.)	Number of Glochidia Recovered	Number of Juveniles Recovered	Metamorphosis Period (Days)
Oct. 27, 1993	165	23.75 ± 1.54	986	0	—
	165		798	0	—
	155		693	13	29–31
	169		659	3	29–32
	115		741	1	32
Dec. 28, 1993	51	22.04 ± 1.80	230	7	43–52
	77		124	12	39–52
	63		121	2	34–52
May 27, 1994	160	22.32 ± 1.33	453	249	21–31
	118		421	14	22–28
	98		441	7	23–26
	177		1,221	262	21–39

when injected with cortisol concentrations of 0.005 and 0.010 mg/g cortisol, suggesting that responses to (or uptake of) cortisol varies with species. Threshold levels of cortisol in the blood may exist that allow glochidial transformation, and these levels may not have been reached in creek chubs.

Fish experience periods of natural elevation of plasma cortisol (Idler & Truscott, 1972). Spring and summer elevations of plasma cortisol and other circulating corticosteroids coincide with migration, smoltification of juvenile anadromous salmonids, sexual maturation and spawning of salmonids (Thorpe *et al.*, 1987; Pickering & Pottinger, 1983; Pickering & Christie, 1981; Anderson, 1990). Cortisol concentrations used in this study should have elevated cortisol levels to the physiological range of fish undergoing stress or periods of natural cortisol elevation (Pickering & Duston, 1983; Pickering & Pottinger, 1985; Thorpe *et al.*, 1987). Periods during the life cycle of fish where cortisol elevation has been observed may correspond with the presence of some species of host fish over mussel beds during the discharge of glochidia (Farzaad, 1991). Perhaps mussels evolved to take advantage of weakened immune responses of host species during spawning or other periods of natural immunosuppression.

Host and nonhost fishes exhibit humoral responses of similar strengths, but host and nonhost species reject glochidia at different rates (O'Connell, 1991). Although cortisol injected fish transformed relatively few juveniles during these experiments, transformation per fish was comparable to hosts in some cases. For instance, 0.020 mg/g cortisol injected orangethroat darters transformed an average of 0.50 juvenile *V. sima* per fish in May. Host fish (banded sculpins) only transformed 1.00 juvenile per fish in the same experiment. Individual variations within and among experiments in glochidial transformation of *V. taeniata* on host fish may also reflect a seasonal aspect in the susceptibility of the host (e.g., host spawning season) or the ability of glochidia to be infective at times of the year other than the normal discharge period. We have observed similar variation among trials and individuals in the numbers of juveniles transformed per host for other mussel species. Variation of an order of magnitude or more in the mean number of juveniles transformed per fish has been reported in other studies as well (e.g., Zale & Neves, 1982). Although the number of glochidia attaching to fish is difficult to control and may contribute to some of the variation in the numbers of juveniles transformed, there was no relationship in the apparent numbers (glochidia + juveniles recovered) of *Villosa taeniata* glochidia attaching to individual rock bass and the numbers of juveniles produced.

Additional development of the cortisol-induced immunosuppression of nonhost fish is needed before the technique can be applied on a large scale as a culture method for freshwater mussels. Nonetheless, refinement of this technique could have widespread management implications, especially as a means for propagation of endangered species. This method could also be used to reestablish populations of nonendangered mussel species into new and reclaimed habitat. Application of cortisol immunosuppression techniques to host species could perhaps increase juvenile transformation for reintroduction studies. Also, application of this technique to non-host fish would provide an alternative means of propagating mussel species that have rare or endangered hosts.

The need for further research on the ecological requirements, including identification of fish hosts, habitat, and physicochemical information of mussel species cannot be over emphasized. These ecological requirements hold the answers to developing self-sustaining populations of freshwater mussels. Because survival and fitness of juvenile mussels produced by means of artificial propagation has not been determined (O'Connell, 1991), artificial propagation methods should be used with caution. Any long-term artificial propagation could alter the genetic integrity of mussel species, and possibly prove more harmful than beneficial (Kennedy, 1975; O'Connell,

1991). As a short-term solution to the immediate problem of endangered species, the technique developed in this study offers hope for propagating many species for which hosts have not been identified.

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