Behavioral responses of glochidia of freshwater mussels (Bivalvia: Unionidae) to chemical cues of fish

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Abstract: Glochidia of wavyrayed lampmussels, *Lampsilis fasciola* Rafinesque, 1820 and rainbow mussels, *Villosa iris* (Lea, 1829) were exposed to mucus, blood, blood by-products, and amino acids of host smallmouth bass, *Micropterus dolomieu* Lacepède, 1802 and non-host carp, *Cyprinus carpio* Linnaeus, 1758 to measure their chemosensory responses. A valve closure index (VCI), the product of percentage of glochidia closed 1 min after exposure times the reciprocal of the time (sec) to closure of the first glochidium upon exposure, was developed and used as the response variable. The median VCI of carp mucus exposures was significantly greater than that of bass mucus for *V. iris* ($p < 0.04$) and for L. fasciola ($p < 0.04$). We believe that this result was due to blood in the carp mucus samples. Glochidia of *V. iris* also were exposed to carp blood, carp serum, carp plasma, amino acids, and fibrinogen. The VCI values for carp serum treatments were significantly less than those of carp blood ($p < 0.04$). The VCI values for carp serum treatments were significantly less than those for carp plasma ($p < 0.04$). These results indicate that fibrinogen may be a principle response cue for glochidia. From lowest to highest VCI values, statistically significant ($p = 0.05$) treatment groupings included: water and heparin; L-threonine/L-serine and L-alanine/L-proline; carp serum, saline, thrombin, and bass mucus; carp mucus, blood, and plasma; and a fibrinogen/thrombin mixture, and fibrinogen alone. VCI values were highest in fibrinogen treatment exposures, indicating that glochidia may respond initially to chemicals involved in the encystment process, rather than to host fish status or nutrients required for metamorphosis.

Key Words: Freshwater mussels, Unionidae, chemosensory, host fish, valve closure index.

The ability to detect and respond to chemical stimuli emanating from fish hosts has been demonstrated in glochidia. Heard and Hendrix (1964) reported that glochidial valve closure in three species of Lampsilinae was affected by the blood of many fish species and various amino acids. They hypothesized that amino acids in fish blood stimulated valve closure of glochidia. Lukacosovics and Labos (1965) determined that fish serum and mucus increased the likelihood of valve closure in the swan mussel, *Anodonta cygnea* (Linnaeus, 1758). Young and Williams (1984) observed that the opening and closing of valves greatly increased when glochidia of the eastern pearlshell, *Margaritifera margaritifera* (Linnaeus, 1758), were exposed to host mucus, blood, and gill and fin tissue of brown trout, *Salmo trutta* Linnaeus, 1758.

Wood (1974) investigated glochidial response to fish hosts and determined that increased valve-snapping behavior in glochidia of *A. cygnea* is related to the detection of "substances found in the mucus and epidermal layers of fish." She also reported that sustained valve closure in glochidia was dependent on chemical cues. Mantle cell sensory hairs were determined to contain the responsive chemoreceptors. Wood (1974) isolated amino acids from fish mucus, exposed glochidia to them, and found that these substances were not important for behavioral response. She described the closure responses of glochidia as occurring in two phases, initial and delayed. Two small molecules, identified in fish mucus, were responsible for these valve closure phases. The first was an uncharged molecule, and was termed an "initial response substance." The second was a positively charged "delayed response substance." These unidentified molecules were responsible for initial and delayed valve closure, respectively.

The purpose of this study was to investigate valve closure behavior of glochidia of wavyrayed lampmussels, *Lampsilis fasciola* Rafinesque, 1820 and rainbow mussels, *Villosa iris* (Lea, 1829) in response to mucus and amino acids of smallmouth bass, *Micropterus dolomieu* Lacepède, 1802, which are hosts, and carp, *Cyprinus carpio* Linnaeus, 1758, which are not hosts (Henley, 1996). Because a higher

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percentage of glochidia elicited valve closing behavior when exposed to non-host mucus, additional experiments were conducted using *C. carpio* blood and blood components.

MATERIALS AND METHODS

The abilities of glochidia of *Villosa iris* and *Lampsilis fasciola* to respond to host (*Micropterus dolomieu*) and non-host (*Cyprinus carpio*) mucus, blood, blood components, and amino acids were tested. Blood was drawn from the fish using an 18-gauge needle and syringe. In an exploratory test to determine whether blood of anesthetized fish stimulated different behavioral responses in glochidia, a subsample of the total number of carp sampled was anesthetized using tricaine methanesulfonate (Finquel[2], Argent Chemical Laboratories, Redmond, Washington). Mucus was scraped from fish, frozen at -60 °C, and thawed for experimental use. Unless otherwise noted, all commercially purchased chemical compounds and test kits were obtained from Sigma Chemical Co.[2], St. Louis, Missouri. Mucus was analyzed for total protein, as well as total and free amino acid content. Total protein content (mg/l) was measured using a Bicinchoninic Acid Protein Assay Kit[2]. Chromatographic analysis of total and free amino acids was conducted using a Pico-Tag Amino Acid Analysis System[2] (Millipore Corp., Bedford, Massachusetts). Glochidia were extracted from *V. iris* and *L. fasciola* by gill irrigation using a 26-gauge needle and syringe, and tested for viability using a weak saline solution (Bruenderman and Neves, 1993). Components of blood from carp were separated using 10 ml Vacutainer Evacuated Blood Collection Tubes[2] (Becton Dickinson, Rutherford, New Jersey) containing 143 USP Units of sodium heparin.

Glochidia from 3 females with 5 replicates per female were placed in separate petri dishes. Thus, the sample size was 3 per treatment, and a replicate consisted of 2 drops of distilled water containing 30 to 168 glochidia placed in a petri dish. These replicates of glochidia were exposed to 2 drops of test solution (described below) from a 26-gauge needle and syringe. Time (sec) was recorded to first valve closure after solution introduction, as was the percentage of glochidial valves closed after 1 min. A multiplicative valve closure index (VCI) for each replicate was developed and calculated by multiplying the reciprocal of time to first valve closure by the percentage (decimal) of glochidia closed after 1 min. The rationale for this method was that a higher percentage of valve closure and greater speed to first closure should be related to the sensitivity of glochidia to test substances. The reciprocal of time to first closure was calculated and used in the VCI formula, so that greater weighting could be applied to VCI values in experiments with faster initial response times.

Glochidia of *Villosa iris* and *Lampsilis fasciola* were first exposed to host and non-host mucus. Because VCI values were higher for glochidia subjected to carp mucus (non-host) than to bass mucus (host), glochidia of *V. iris* were tested against carp blood, blood components, amino acids, and sheep fibrinogen (Table 1). These tests were conducted because of suspected blood in carp mucus samples; *i.e.*, mucus scraping often caused surface epidermal bleeding in the carp. Sparse data were available on the amino acid composition of fish mucus, and none was found for the fish species used in this experiment. The mixtures of amino acids used in experiments were L-alanine/L-proline and L-threonine/L-serine based on an analysis of gill mucin of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) by Lumsden and Ferguson (1994). Since fish fibrinogen was not available commercially, sheep fibrinogen was used. This substitute was selected since fibrinogen is similar among vertebrate taxa (Doolittle, 1990). Fibrinogen is important in the blood clotting process through the thrombin-catalyzed conversion of fibrinogen to fibrin (Doolittle, 1990). A fibrinogen/thrombin mixture also was tested to determine whether VCI responses to fibrin differed significantly from those of fibrinogen. The thrombin used in this mixture also was from sheep, as previously described. With these substances, an attempt was made to determine whether a primary chemical cue was responsible for differences in VCI ratings.

Results of test groups were compared nonparametrically using the Kruskal-Wallis test for differences among group medians, and the Multiple Comparisons Test for differences among level medians in the MOOD command in Minitab 10.5[2] (Minitab Inc., State College, Pennsylvania). Individual nonparametric comparisons were conducted using the Mann-Whitney test for differences among group medians.

RESULTS AND DISCUSSION

The Kruskal-Wallis test detected differences among treatment medians ($p < 0.0001$); therefore, nonparametric multiple comparisons were protected at this alpha level. The range of VCI medians for all treatments was from 0.0 for water and heparin to 209.0 for fibrinogen (Table 1). The components of the VCI; namely, the reciprocal of the time (sec) to first valve closure in treatment replicates, and the percentage of glochidial valves closed in replicates after 1 min, were not related to the number of glochidia used in treatment replicates ($r^2 = 0.02$, $p = 0.06$ and $r^2 = 0.00$, $p = 0.73$, respectively). The reciprocal of the time to first valve

[2]Use does not imply endorsement by the U. S. government.

Table 1. Median Valve Closure Index (VCI) values for *Lampilis fasciola* and *Villosa iris* and concentrations of treatment substances used. Multiple comparisons are for exposures with *V. iris*; family error rate = 0.05. Treatments with the same letter are not significantly different. ND = not determined.

Substance	Concentration Analysis	Species		Multiple Comparison Analysis
		L. fasciola	*V. iris*	
distilled water	ND	0.0	0.0	a
sodium heparin	143 USP units		0.0	a
L-threonine / L-serine	threonine=10.0% and L-serine=8.0%		1.0	b
L-alanine / L-proline	alanine=17.6% and proline=9.9%		5.7	b
carp serum	ND		10.1	c
thrombin	144 NIH units		13.6	c
saline	0.85%		20.4	c
bass mucus	1.24 mg protein/ml	23.8	43.7	c
carp mucus	0.907 mg protein/ml	50.1	74.6	d
carp blood without Finquel[3]	ND		75.1	d
carp plasma	ND		89.3	d
fibrinogen / thrombin	thrombin=144 NIH units and fibrinogen = 1.0%		174.9	e
fibrinogen	1.0%		209.0	e

closure component of the VCI was most predictive of calculated VCI values ($r^2 = 0.88$, $p < 0.0001$).

Surprisingly, VCI values for both *Villosa iris* and *Lampsilis fasciola* glochidia were higher with exposure to non-host (carp) mucus than to host (bass) mucus. For *V. iris*, the VCI of carp mucus exposures was significantly greater than that of bass mucus ($p < 0.04$), as was the similar comparison for *L. fasciola* ($p < 0.04$). We believe that this result was due to blood in the carp mucus samples. The carp mucus was pink, but we saw no evidence of blood in the bass mucus. Also, there were differences in VCI values between the glochidia of both mussel species within similar mucus types. The VCI values of *V. iris* were significantly higher with exposure to bass mucus than VCI values of *L. fasciola* ($p < 0.04$), and VCI values of *V. iris* were significantly greater than those of *L. fasciola* in carp mucus exposures ($p < 0.04$) (Table 1). These findings reaffirm that glochidia possess chemosensory abilities. Despite this result, it was surprising that the VCI values of these glochidia were higher with exposure to carp (non-host) mucus than to bass (host) mucus. These results infer that the responses of glochidia may not relate to host/non-host status of the fish from which mucus-based cues originate.

The total protein content in bass mucus and carp mucus was 1.24 mg/l and 0.91 mg/l, respectively. Total (hydrolyzed) amino acid composition of the two types of mucus was very similar (Table 2). The only substantive differences between total (hydrolyzed) amino acids found in bass mucus and carp mucus were that bass mucus included B-aminoisobutyric acid, whereas carp mucus did not; and carp mucus included hydroxyproline, whereas bass mucus did not. A comparison of free amino acids (mg/l) contained in bass mucus and carp mucus samples shows differences in occurrence and concentrations (Table 2). There were five free amino acids in carp mucus that were missing in bass mucus; hydroxyproline, anserine nitrate, histidine, methionine sulfone, and arginine. Most free amino acid concentrations were much greater in carp mucus than in bass mucus (Table 2). Identification of amino acid(s) possibly responsible for an increase or decrease in VCI values is difficult because the concentration of an amino acid may not be reflective of its effectiveness as a chemical cue. A free amino acid may be in low concentration but readily soluble; therefore, it may be a more potent chemical cue than an amino acid in greater concentration but relatively insoluble.

Because VCI values were significantly greater in carp mucus than in bass mucus exposures, and because we suspected that blood was present in the carp mucus samples, glochidia of *Villosa iris* were tested with carp blood and blood components. For comparison, we also tested glochidia of this mussel species for responses to various amino acids (Table 1). When testing the constituent components of carp blood, we found that the VCI values associated with blood-based tests were ordered and significantly different. The VCI values for tests using carp serum (lacking clotting factors such as fibrinogen) were significantly less than those for carp blood ($p < 0.04$), but VCI values for carp blood tests were not significantly less than those for carp plasma (both of which contain clotting factors) ($p < 0.19$). The VCI values for carp serum tests were significantly less than those for carp plasma ($p < 0.04$). Noteworthy is that the VCI values for fibrinogen exposures were the highest of all tested substances (Table 1). The VCI values for thrombin/fibrinogen exposures were not significantly different from those for fibrinogen ($p = 1.0$), substantiating the hypothesis that the higher VCI medians associated with fibrinogen were due to chemical stimulation, rather than tactile stimulation from fibrin. VCI values associated with blood exposures from anesthetized versus unanesthetized carp did not differ significantly ($p = 1.0$).

Table 2. Mean concentrations (mg/l) of free and hydrolyzed amino acids in carp (*Cyprinus carpio*) and bass (*Micropterus dolomieu*) mucins.

	Free Amino Acids (mg/l)		Hydrolyzed Amino Acids (mg/l)	
Amino Acid	Bass	Carp	Bass	Carp
phosphoserine	0.00	0.00	0.00	0.00
aspartic acid	3.34	5.32	78.52	83.06
glutamic acid	7.69	25.35	146.52	143.53
α-aminoadipic acid	0.00	0.00	0.00	0.00
hydroxyproline	0.00	1.56	0.00	1.07
phosphoethanolamine	3.14	2.12	1.78	1.03
serine	5.39	13.06	74.99	79.92
asparagine	0.00	6.61	0.00	0.00
glycine	3.90	7.44	62.85	67.47
glutamine	1.98	7.17	0.00	0.00
B-alanine	0.00	0.00	0.00	0.00
taurine	38.72	51.74	33.56	46.76
histidine	0.00	3.52	23.65	24.43
γ-aminobutyric acid	0.00	0.00	0.00	0.00
citrulline	0.00	0.00	0.00	0.00
threonine	5.12	10.15	55.58	51.48
alanine	5.91	14.32	62.16	65.99
B-aminoisobutyric acid	1.27	0.49	1.57	0.00
carnosine	0.00	0.00	0.00	0.00
arginine	0.00	23.42	54.87	56.31
proline	1.74	6.73	44.83	54.46
1-methylhistidine	0.00	0.00	0.00	0.00
anserine nitrate	0.00	0.00	0.00	0.00
3-methylhistidine	0.00	0.00	0.00	0.00
ethanolamine	1.66	2.49	3.55	3.34
α-amino-n-butyric acid	0.00	0.00	0.00	0.00
tyrosine	3.01	14.08	38.97	34.19
valine	2.33	12.46	45.60	47.44
methionine	0.00	5.70	27.96	29.83
cystathionine	0.00	0.00	0.00	0.00
cystine	0.00	0.00	0.00	0.00
cysteine	0.00	0.00	8.80	7.31
isoleucine	1.41	9.87	33.63	34.96
leucine	3.03	20.57	87.13	88.31
hydroxylysine 1	0.00	0.00	0.00	0.00
hydroxylysine 2	0.00	0.00	0.00	0.00
phenylalanine	2.40	14.59	46.64	50.09
tryptophan	0.28	3.35	0.00	0.00
ornithine	1.37	0.89	1.99	1.24
lysine	2.93	28.45	90.78	96.24

Further analysis of test results, using multiple comparisons of treatment VCI values, revealed that there were 5 distinct and significantly different substance groupings (Table 1). From lowest to highest response, treatment groupings of VCI medians included: water and heparin; L-threonine/L-serine and L-alanine/L-proline; carp serum, saline, thrombin, and bass mucus; carp mucus, carp blood, and carp plasma; and fibrinogen/thrombin and fibrinogen (Table 1). From this analysis, it was apparent that VCI values increased with an increase in the concentration of fibrinogen. Test results showed that the VCI values for the amino acid group were among the lowest measured, with VCI scores slightly greater than water and heparin (Table 1). This is a clarification of the work of Heard and Hendrix (1964) and Wood (1974); *i.e.* although amino acids can induce valve closure response in glochidia, they are seemingly marginal cues. In fact, glochidia were observed in pre-testing experiments to respond to substances unrelated to the encystment process, including heparin and human saliva (VCI ranges were 0.0 to 0.99 and 33.4 to 110.5, respectively). In this context, the VCI provides a refinement on methods previously used in chemosensory experiments with glochidia because it uses group, as well as individual, responses. Thus, the VCI allows measurement of the strength of responses to various treatments, rather than simply answering a yes or no question concerning the chemosensory responses of glochidia.

It may be appropriate to partition chemical cues into strength-of-response categories. Minor chemical cues may be those that induce marginal valve closing responses from glochidia, whereas principle cues may be those that most readily initiate responses. This is to say that a minor cue may one to which a smaller percentage of glochidia elicit valve closing behavior with a slower initial closing time, and vice-versa for principal cues. Substances such as heparin, thrombin, fish mucus, aqueous forms of amino acids, and blood plasma are seemingly minor chemical cues, while fibrinogen seems to be a principle cue. This is not to say that amino acids are not included in the chemical structure of fibrinogen; indeed, it may be that binding sites, such as the Gly-Pro-Arg-ending peptides on the fibrinogen molecule are principle chemical cues for glochidia (Doolittle, 1990). The level of tissue damage that occurs when glochidia attach to fish is undescribed, but some must occur, especially with the attachment of hooked glochidia. Because fibrinogen is vital in the process of fluid retention after injury (Van Vliet *et al.*, 1985; Doolittle, 1990) and, therefore, in the encystment process, the glochidial response to fibrinogen is seemingly appropriate because glochidia may have an inherent biochemical recognition system that is based on fibrinogen or similar substances. Fibrinogen also plays a vital role in masking bacteria from host immune assault (Whitnack and Beachey, 1982; Whitnack *et al.*, 1984; Horstmann *et al.*, 1992). In several types of streptococci, fibrinogen binds to M protein in surface fabrillae and prevents the binding of complement (C3) to the streptococcal cell surface. Thus, fibrinogen impedes access of complement proteins to cell wall receptors (Whitnack and Beachey, 1982). In this way, streptococci are masked from phagocytosis. Because clotting factors may be involved in the encystment process, the strong behavioral response of glochidia to fibrin and fibrinogen could be related to these important biochemical processes.

The process of the thrombin-catalyzed conversion

of fibrinogen to fibrin has apparently been in place for 450 million years in primitive fishes, and has been an evolutionary constant for all vertebrates (Doolittle and Surgenor, 1962; Doolittle, 1990). All vertebrate fibrinogens are large proteins with molecular weights between 320,000 and 400,000. They are composed of 3 pairs of polypeptide chains (α_2 β_2 γ_2) that vary in amino acid composition (Doolittle, 1990). The amino acids of the β and α chains are highly variable among species, but those of the γ chain are highly conservative among species (Doolittle, 1990). Since relatively high VCI values were associated with glochidia exposed to carp (non-host) plasma and fibrinogen (sheep) exposures, it is probable that the primary cue that stimulated these responses is found on the more conservative chain. The constituent components of fibrinogen could be tested in future chemosensory work using fragmentation procedures outlined by Spraggon *et al.* (1997).

Preliminary results with artificial media infer that plasma components may be important for increasing glochidial transformation success. Keller and Zam (1990) reported the transformation of glochidia to juveniles in an *in vitro* culture medium that included only serum. However, Hudson and Shelbourne (1990) reported that transformation rate for glochidia was increased with the inclusion of fish plasma. Isom and Hudson (1984) reported the importance of adding fish plasma to artificial media; glochidial transformation could occur in artificial media with plasma from confirmed non-host fish species. Their conclusion was that the key component in fish blood, necessary to initiate glochidial transformation, was contained in the blood of all fish tested. The important substance in plasma may be fibrinogen or related compounds. Fibrinogen may not be essential to transform glochidia, but substances in plasma that are not in serum seem to have a positive effect on transformation success, as well as a stimulatory effect on the behavior of glochidia. Our findings, that glochidia are strongly predisposed to respond to fibrinogen, suggest that further research on the hematological and immunological aspects of the parasite-host relationship between glochidia and fish is needed to isolate those blood components essential to both closure and transformation of glochidia on host fishes.

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