Transactions of the American Fisheries Society 110:514–518, 1981

Agglutinating Antibody to *Aeromonas hydrophila* in Wild Largemouth Bass

TERRY C. HAZEN

Department of Biology, Faculty of Natural Sciences, University of Puerto Rico Río Piedras, Puerto Rico 00931

GERALD W. ESCH AND MARK L. RAKER

Department of Biology, Wake Forest University Winston-Salem, North Carolina 27109

Abstract

Among largemouth bass *Micropterus salmoides* in Par Pond, South Carolina, a significantly larger percentage of those with red-sore disease were positive for anti-*Aeromonas hydrophila* agglutinin than of uninfected fish. Highest titers occurred during summer and fall, when the prevalence of the disease was declining. Most agglutinin activity was associated with a single serum fraction; the agglutinin has an apparent molecular weight of >340,000 daltons, suggesting it may be a macroglobulin-like antibody. Homologous agglutinin reacted better with *A. hydrophila* than heterologous agglutinin. Differences in severity and duration of red-sore epizootics in the southeastern United States may be due to differing virulence among strains of *A. hydrophila*.

Aeromonas hydrophila is the primary etiological agent for red-sore disease (Hazen$_3$ et al. 1978), a serious problem for game and commercial fish species in the southeastern United States (Miller and Chapman 1976). In the epizootiology of red-sore disease among largemouth bass *Micropterus salmoides*, there is a significant positive correlation between densities of *A. hydrophila* free in the water column and the prevalence of the disease (Hazen 1979). There also is a significant negative correlation between prevalence of red-sore disease and host body condition (Esch and Hazen 1978, 1980), suggesting an important relationship between host susceptibility and stress. Largemouth bass with low condition factors have reduced hematocrit, hemoglobin, and total red and white blood cell counts, and elevated serum cortisol and thyroxin concentrations (Hazen$_1$ et al. 1978; Esch and Hazen 1980). These observations imply that immune mechanisms and susceptibility to infection by *A. hydrophila* could be altered by stress. In the present report, we characterize anti-*A. hydrophila* agglutinin in largemouth bass and assess seasonal changes in humoral immunity.

Methods

The primary study site was Par Pond, a 1,012-hectare reservoir that receives heated effluent from a nuclear power plant; it is on the Savannah River Plant near Aiken, South Carolina. Water quality and biotic characteristics of Par Pond are given by Hazen (1978, 1979). Largemouth bass also were taken from Lake Norman, an impoundment on the Catawba River in North Carolina, from Badin Lake located on the Yadkin River, and from the Chowan River, a tributary of Albemarle Sound in northeastern North Carolina.

Twenty-five largemouth bass were angled or electrofished quarterly from heated and unheated parts of Par Pond; temperatures at the heated site generally were >5 C above those in unheated areas. Fish from the Chowan River, Badin Lake, and Lake Norman were taken by angling during spring and summer months.

All fish were bled within 1 hour of capture by cardiac puncture; blood was drawn into heparinized 10-ml vacutainers. Serum was obtained by centrifugation after addition of 1% $CaCl_2$ to whole blood.

The agglutination assay was modified from Kuhn and Vaughn (1976). Pure cultures of *A. hydrophila* were harvested by centrifugation after being grown 24 hours in TSB (Difco, Detroit, Michigan) at 35 C. Cells were suspended in Locke's solution and the density was adjusted to 1×10^9 cells·ml^{-1} with the aid of a Coulter counter, model ZB (Coulter Electronics, Hialeah, Florida). The cell suspension then was

incubated in 5 μcuries·ml^{-1} ^{51}Cr-sodium (Amersham, Chicago, Illinois) with constant shaking for 2 hours at 35 C. Labeled cells were centrifuged and washed three times with cold (4 C) Locke's solution (Kuhn and Vaughn 1976). Final suspensions were prepared with 0.85% saline and the cell densities adjusted to 1×10^9 cells·ml^{-1}. Labeled cells were then incubated 1 hour with two-fold serial dilutions of fish sera. The supernatant of each serum reaction was then counted with a Beckman automated gamma counter, model 4000 (Beckman Company, Irvine, California). The amount of antibody present was assumed to be inversely proportional to the count obtained. Titers were calculated as the point of 50% reduction in count from control values.

Sera were characterized by column chromatography with Sephadex G200 and DEAE-cellulose (Pharmacia Fine Chemicals, Piscataway, New Jersey). One milliliter of largemouth bass serum having a high titer was applied to a Sephadex G200 column (3 × 50 cm). The serum was fractionated in a descending direction with 0.1 M Tris-HCl buffer (pH 8.0) at a flow rate of 10 ml·hour^{-1} at room temperature. One milliliter of largemouth bass serum was also applied to a DEAE-cellulose (coarse mesh—0.9 mequivalent·g^{-1}) column (3 × 50 cm). The serum was fractionated in a descending direction in 0.01 M to 0.3 M phosphate buffer (pH 8.4). The eluted serum fractions from both procedures were pooled according to absorbance at 280 nm, measured on a Perkin-Elmer double beam spectrophotometer. The pooled fractions were returned to original whole-serum volumes by ultrafiltration with a 1,000 nominal molecular weight Pellicon filter (Millipore Corporation, Bedford, Massachusetts). After concentration, each fraction was tested for anti-*A. hydrophila* agglutinin by the previously described technique. To aid in molecular weight determinations, the gel filtration column was precalibrated with blue dextran (2×10^6 daltons), human fibrinogen (340,000 daltons), human gamma globulin (146,000 daltons), and cytochrome C (12,500 daltons). Serum with high titer also was diluted with 0.1 M 2-mercaptoethanol and anti-*A. hydrophila* agglutinin was measured as before.

Parametric data were subjected to analysis of variance. Any probability less than 0.05 was considered significant.

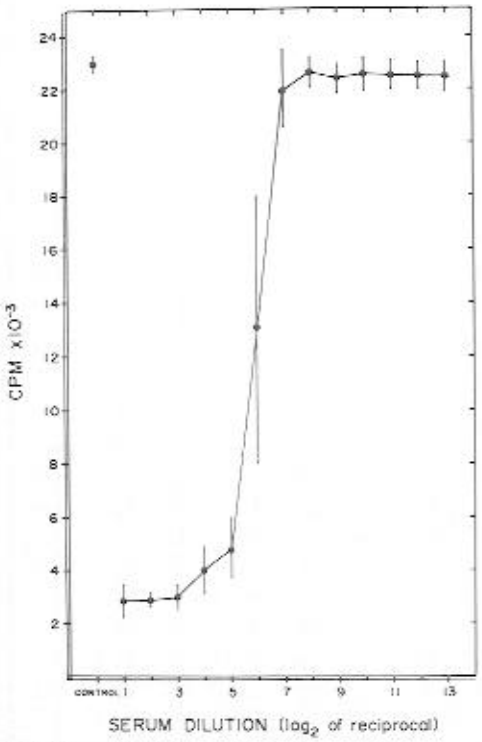

FIGURE 1.—*A typical profile for largemouth bass serum with high titers of* ^{51}*Cr-labeled anti-*Aeromonas hydrophila *agglutinin. Each point represents a mean of four determinations (CPM = counts per minute); vertical bars represent one standard deviation. The control consisted of labeled cells mixed with 0.85% saline.*

Results

Dilutions of largemouth bass sera with saline indicate that the technique used to estimate agglutinin concentration was highly sensitive (Fig. 1). Ten replicates at each dilution had less than a 10% coefficient of variation (100·SD/mean), so titers could be estimated to 0.1 unit. Titers ranged from 0 to 16 ($\log_2$ of the reciprocal of the last dilution). Because of the large variation observed in the fish titers, data were analyzed nonparametrically; any titer greater than 20 was considered a positive agglutinin response.

Eluate profiles of serum from 20 largemouth bass on Sephadex (G200) columns all showed three distinct optical peaks (Fig. 2). Fraction 1 was the only region in which anti-*A. hydrophila* agglutinin could be found. Comparison with compounds of known molecular weight indicates the agglutinin is slightly more than 340,000 daltons (Fig. 3). The DEAE-cellulose

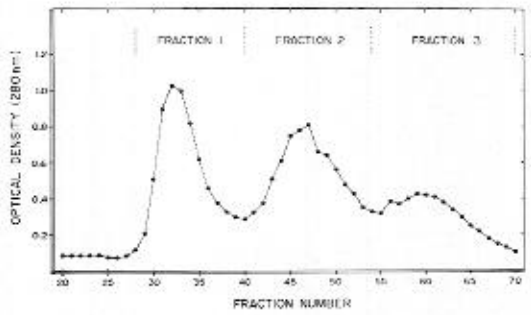

FIGURE 2.—*A typical Sephadex G200 fractionation profile of largemouth bass serum.*

eluate showed four less distinct peaks; only the second peak had significant amounts of anti-*A. hydrophila* agglutinin. When sera from 10 largemouth bass having high concentration of anti-*A. hydrophila* agglutinin were diluted with 0.1 M 2-mercaptoethanol, agglutinin activity was significantly reduced compared with activity of sera diluted with 0.85% saline (Fig. 4).

Largemouth bass in Par Pond had highest incidences of red-sore disease in spring and summer. Overall, the disease was significantly more prevalent in heated parts of the reservoir (20% of fish) than in unheated areas (12%; chi-square test; $P < 0.02$), but its seasonal trends were similar in both thermal and ambient environments.

The development of anti-*A. hydrophila* agglutinins in largemouth bass sera also varied seasonally (Fig. 5). The correlation between numbers of fish with positive titers and the seasonal

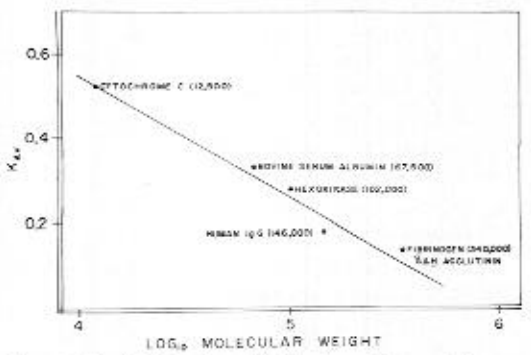

FIGURE 3.—*Calibration of Sephadex column with compounds of known molecular weight. Anti-*Aeromonas hydrophila *agglutinin in largemouth bass serum is indicated by open circles (IgG = gamma globulin; K_{av} = diffusion constant).*

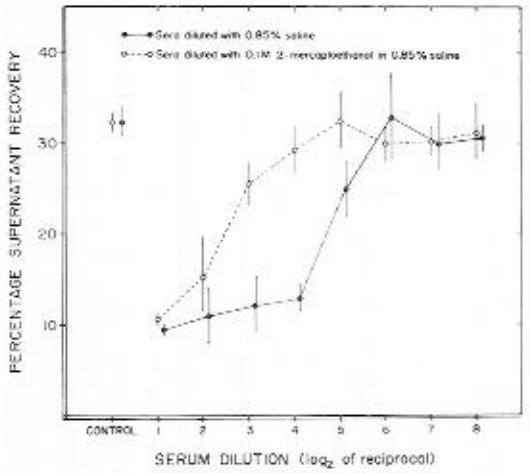

FIGURE 4.—*Effect of 0.1 M 2-mercaptoethanol on activity of anti-*Aeromonas hydrophila *agglutinin. Each point is the mean of four determinations; vertical bars indicate standard deviation.*

prevalence of red-sore disease was significant ($r = 0.65$; $P = 0.03$); when the prevalence of red-sore disease was lagged by one season, the correlation was not significant. Positive agglutinin responses occurred in 42% of fish infected with red-sore disease ($N = 81$) versus 19% in noninfected fish, a significant difference (chi-square test; $P < 0.01$). Slightly more fish from heated areas showed the response than from unheated areas of Par Pond (27% versus 23%), though the difference was not significant.

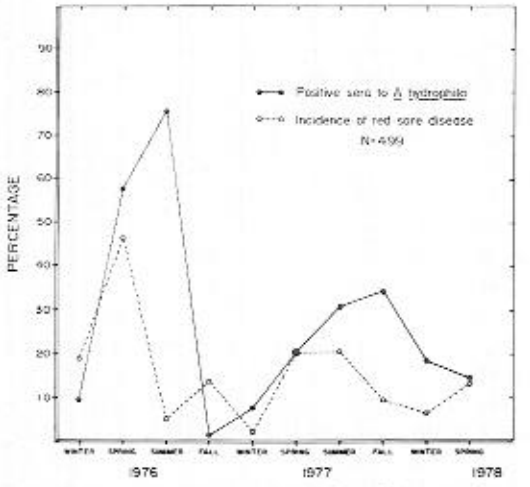

FIGURE 5.—*Overall prevalence of red-sore disease and positive anti-*Aeromonas hydrophila *sera in largemouth bass from Par Pond, by season.*

TABLE 1.—*Reactivity of largemouth bass sera with* Aeromonas hydrophila *isolates from four sources in North and South Carolina. Values are percent of sera with positive reactions; sample sizes are in parentheses. All chi-square values are significant ($P < 0.01$) except that for Lake Norman isolates. Homologous reactions (isolate and serum from the same source) are underlined.*

Serum source	Isolate of *Aeromonas hydrophila*				
	Badin Reservoir	Par Pond	Lake Norman	Chowan River	χ^2
Badin	63% (46)	17% (24)	24% (34)	23% (13)	12.7
Par	9% (58)	57% (47)	15% (47)	2% (49)	45.8
Norman	0% (20)	0% (20)	35% (20)	0% (20)	21.0
Chowan	0% (12)	0% (49)	37% (30)	40% (50)	23.7
χ^2	40.8	42.1	4.3	23.8	

When *A. hydrophila* isolates from Chowan River and three reservoirs were compared with sera from largemouth bass taken in the four locations, homologous agglutinin (from the same source as the serum) reacted significantly better than heterologous agglutinin in nearly all cases (Table 1). In the lone exception, the Lake Norman isolate reacted the same with Chowan River and Lake Norman sera.

Discussion

The agglutinin in serum from largemouth bass that reacted with *A. hydrophila* occurred in fraction 1 after separation with Sephadex G200 and in fraction 2 after separation with DEAE-cellulose. This corresponds with fractions from mammals known to have IgM immunoglobulins. Based on calibration of the Sephadex column, with solutions of known molecular weight, the anti-*A. hydrophila* agglutinin appears to have a molecular weight greater than 340,000 daltons. These observations conform to those of Litman (1976) who reported that many other teleost species produce an IgM-like antibody having a molecular weight of 400,000, and consisting of four 100,000-dalton subunits. In addition, a significant reduction in activity occurred when sera were diluted with 2-mercaptoethanol, suggesting that only a polymeric form is reactive. Corbel (1975), in an extensive literature review, reported nearly all studies have shown that fish immunoglobulins are sensitive to 2-mercaptoethanol.

Our data show, for the first time, that largemouth bass can produce specific humoral antibodies in response to infection by *A. hydrophila*. Rainbow trout *Salmo gairdneri* forms specific antibodies to *A. hydrophila* (Post 1963), and common carp *Cyprinus carpio* will do so to *A. punctata* (Szakilczai 1969).

The seasonal changes of red-sore disease and antibody titers in largemouth bass suggest that epizootics of red-sore disease may be followed by a decrease in susceptibility to infection resulting from an increase in the number of individuals with some degree of immunity. Such a notion is supported by long-term observations that showed a strong positive correlation between densities of *A. hydrophila* in the water column and prevalence of red-sore disease (Hazen 1979). However, during the summer, densities of *A. hydrophila* are always slightly higher than would be expected from the prevalence of red-sore disease that occurs. Concurrent with the decline in prevalence of infected largemouth bass was the increase in circulating agglutinin levels, especially obvious during the epizootic of spring 1976. As densities of *A. hydrophila* decline in the fall, and antigenic stimulation decreases, circulating agglutinins remained high and then fell to low levels in winter. Thus, as for IgM immunoglobulins in mammals, the IgM-like agglutinin is useful to the fish during recovery from red-sore disease and prevents reinfection, but only for two seasons.

The number of largemouth bass positive for anti-*A. hydrophila* agglutinin was greater in heated parts of the reservoir but not significantly so, even though the prevalence of red-sore disease was significantly higher. There are strong indications that fish from these areas of the reservoir are under substantial stress due to elevated temperatures (Hazen, et al. 1978). It is believed that stress in largemouth bass within thermally altered parts of the reservoir causes the specific antibody response to be a less sufficient deterrent to *A. hydrophila* infection.

Anti-*A. hydrophila* agglutinins in largemouth bass from the Chowan River and the three reservoirs are specific to the *A. hydrophila* strain causing red-sore disease in each of the four locations. Little, if any, cross reactivity was observed between sera and isolates of *A. hydrophila* from the four systems. Thus, it is apparent that largemouth bass are able to recognize strain differences of *A. hydrophila* and respond accordingly by production of strain-specific anti-*A. hydrophila* agglutinin. Fliermans and

Hazen (1980), using specific fluorescent antibodies, have shown that the dominant isolate of *A. hydrophila* in the water is not the dominant isolate obtained from infected largemouth bass or infected American alligators *Alligator mississippiensis*. These investigations suggested that several "strains" of *A. hydrophila* may thus be present simultaneously in the same habitat. Several variables, including temperature, dissolved oxygen, phosphates, nitrogen, ammonia, and chlorophyll *a*, are correlated with *A. hydrophila* density (Hazen$_2$ et al. 1978; Hazen 1979; Hazen and Fliermans 1979). If changes in these variables increase the density of certain virulent "strains," they could influence the occurrence of red-sore disease. It is possible that the variability in severity and duration of epizootics observed throughout the southeastern United States could be due to differences in virulence among *A. hydrophila* strains.

Acknowledgments

Raymond E. Kuhn provided the technique and equipment for this study. James R. Matthews, William F. Crawford, Melanie Trogdon, Ann F. Mansfield, and Gerald Smith gave technical assistance. J. W. Gibbons and M. H. Smith, of the Savannah River Ecology Laboratory provided equipment and space. Members of the Twin Cities Bass Masters club of Winston-Salem, North Carolina and the Franklin, Virginia B.A.S.S. club helped catch largemouth bass for this study. This work was supported by contract EY-76-S-09-0900 between the United States Department of Energy and Wake Forest University, by the Office of Coordination of Graduate Studies and Investigation, University of Puerto Rico, by grant RR-8102 from the National Institute of Health, and by contract EY-76-C-09-0819 between the United States Department of Energy and the University of Georgia.

References

Corbel, M. J. 1975. The immune response of fish: a review. Journal of Fish Biology 7:539–563.

Esch, G. W., and T. C. Hazen. 1978. Thermal ecology and stress: a case history for red-sore disease in largemouth bass (*Micropterus salmoides*). Pages 361–363 *in* J. H. Thorpe and J. W. Gibbons, editors. Energy and environmental stress in aquatic systems. Department of Energy Symposium Series, CONF-71114, Washington, District of Columbia, USA.

Esch, G. W., and T. C. Hazen. 1980. Stress and body condition in a population of largemouth bass: implications for red-sore disease. Transactions of the American Fisheries Society 109:532–536.

Fliermans, C. B., and T. C. Hazen. 1980. Immunolluorescence of *Aeromonas hydrophila* as measured by fluorescence photometric microscopy. Canadian Journal of Microbiology 26:161–168.

Gibbons, J. W., G. W. Esch, D. H. Bennett, and T. C. Hazen. 1978. A longterm study of body condition of largemouth bass in a thermally altered reservoir. Nature (London) 274:470–471.

Hazen, T. C. 1978. The ecology of *Aeromonas hydrophila* in a South Carolina cooling reservoir. Doctoral dissertation. Wake Forest University, Winston-Salem, North Carolina, USA.

Hazen, T. C. 1979. The ecology of *Aeromonas hydrophila* in a South Carolina cooling reservoir. Microbial Ecology 5:179–195.

Hazen$_1$, T. C., G. W. Esch, A. B. Glassman, and J. W. Gibbons. 1978. Relationship of season, thermal loading and red-sore disease with various hematological parameters in *Micropterus salmoides*. Journal of Fish Biology 12:491–498.

Hazen, T. C., and C. B. Fliermans. 1979. Distribution of *Aeromonas hydrophila* in natural and man-made thermal effluents. Applied and Environmental Microbiology 38:166–168.

Hazen$_2$, T. C., C. B. Fliermans, R. P. Hirsch, and G. W. Esch. 1978. The prevalence and distribution of *Aeromonas hydrophila* in the United States. Applied and Environmental Microbiology 36:731–738.

Hazen$_3$, T. C., M. L. Raker, G. W. Esch, and C. B. Fliermans. 1978. Ultrastructure of red-sore lesions on largemouth bass (*Micropterus salmoides*): the association of the peritrich *Epistylis* sp. and the bacterium *Aeromonas hydrophila*. Journal of Protozoology 25:351–355.

Kuhn, R. E., and R. T. Vaughn. 1976. The use of ^{51}Cr-labelled *Trypanosoma cruzi* in agglutination titrations. International Journal for Parasitology 6:223–225.

Litman, G. W. 1976. Physical properties of immunoglobulins of mammals. Pages 239–275 *in* J. J. Marchalonis, editor. Comparative immunology. John Wiley and Sons, New York, New York, USA.

Miller, R. M., and W. R. Chapman. 1976. *Epistylis* and *Aeromonas hydrophila* infections in fishes from North Carolina reservoirs. Progressive Fish-Culturist 38:165–168.

Post, G. 1968. The immune response of rainbow trout (*Salmo gairdneri*) to *Aeromonas hydrophila*. State of Utah Department of Fish and Game, Departmental Information Bulletin, Salt Lake City, Utah, USA.

Szakilczai, J. 1969. Untersuchungen zum Nachweis von sessilen Antikörpern bei Karpten (*Cyprinus carpio*) II. Nachweis sessiler Antikörper gegenuber *Aeromonas punctata* bei unter naturlichen Umstandeu umgestimmten Karpfen. Zeitschrift fuer Immunitaetsforschung und Experimentelle Therapie 137:377–381.