Journal of Aquatic Animal Health 17:304–314, 2005

DOI: 10.1577/H04-028.1

[Article]

Effects of Factors Related to Water Quality and Population Density on the Sensitivity of Juvenile Largemouth Bass to Mortality Induced by Viral Infection

KATE R. INENDINO AND EMILY C. GRANT

Department of Natural Resources and Environmental Sciences, University of Illinois, Champaign, Illinois 61820, USA

DAVID P. PHILIPP

Center for Aquatic Ecology, Illinois Natural History Survey, and Program in Ecology and Evolutionary Biology, University of Illinois, Champaign, Illinois 61820, USA

TONY L. GOLDBERG*

Department of Veterinary Pathobiology, College of Veterinary Medicine, and Program in Ecology and Evolutionary Biology, University of Illinois, Champaign, Illinois 61820, USA

Abstract.—Environmental stressors can predispose fish to mortality from infectious disease. This study examined the effects of two factors, water quality and physical crowding, on the responses of fish to viral infection. Juvenile largemouth bass *Micropterus salmoides* were experimentally inoculated with largemouth bass virus (LMBV), an emerging pathogen in the family Iridoviridae. In separate experiments, fish were exposed to various concentrations of nitrate (0, 40, 200, and 400 mg/L) and were housed at either high or low population densities. Survival time, viral load (quantity of virus in tissues), and body condition were measured as outcomes. Nitrate, as well as other water quality parameters such as ammonia, nitrite, and pH, affected mortality rates and viral loads in complex ways. Paradoxically, increased nitrate concentrations were associated with reduced mortality rates in juveniles exposed to LMBV. In general, rapid fluctuations in the concentrations of dissolved toxins had greater impact on sensitivity to viral infection than did persistently high levels of these toxins. Fish housed at high density experienced increased mortality rates, elevated viral loads, and reduced body condition compared with fish held at low density. These results demonstrate that both physiochemical and social environmental stressors can affect the survival and condition of largemouth bass infected with LMBV.

Adverse environmental conditions can affect the health and growth of fish both in captivity and in the wild. Suboptimal water quality and high population densities, in particular, are known to affect fish health negatively (Nicholson et al. 1990; Carballo and Munoz 1991; Wedemeyer 1996). Reduced water quality exacts a direct physiological toll on fish. Both concentrations of noxious chemicals and how long the fish are exposed to them determine the extent of physiological damage (Carballo and Munoz 1991). Fish living in crowded conditions can become stressed from either altered social interactions or restrictions of their ability to move freely or to otherwise behave normally (Wedemeyer 1996).

* Corresponding author: tlgoldbe@uiuc.edu

Received May 13, 2004; accepted March 4, 2005
Published online August 24, 2005

Water quality reductions and demographic factors may also affect the ability of fish to resist infectious disease. Sublethal concentrations of toxins or pollutants are associated with immunosuppression and susceptibility to infectious disease in fish (e.g., Hetrick et al. 1979; Knittel 1981; Carballo et al. 1995; Arkoosh et al. 1998). Nitrate, in particular, may suppress fish immunity either directly, through negative effects on functional immunity, or indirectly, through general physiological stress (Hrubec et al. 1996). Fish held in high densities are known to suffer immunosuppression (Meade 1986; Pickering and Pottinger 1987). High fish density has been associated with disease epidemics in coho salmon *Oncorhynchus kisutch* (Fagerlund et al. 1986), Chinook salmon *O. tshawytscha* (Mazur et al. 1993; Banks 1994), white sturgeon *Acipenser transmontanus* (LaPatra et al. 1996), and rainbow trout *O. mykiss* (Bebak-Williams et al. 2002). Nevertheless, the mecha-

nisms by which water-quality reductions and demographic factors reduce resistance to infectious disease are poorly understood.

Viruses of the family Iridoviridae are of increasing concern for both wild and farmed fish (Williams 1996; Georgiadis 2001; Chinchar 2002). Iridoviruses are large, complex DNA viruses that cause systemic infections and mortality in fish (Wolf 1988), amphibians (Daszak et al. 1999; Docherty et al. 2003), reptiles, and insects (Williams 1996; Chinchar 2002). Large-scale mortality events attributable to iridoviral disease have occurred in wild populations of amphibians and fish (Daszak et al. 1999). Iridovirus epidemics have also caused economic losses in commercial aquaculture systems (Langdon and Humphrey 1987; Williams 1996; Essbauer and Ahne 2001; Georgiadis 2001). Iridoviral infection causes extremely varied clinical responses in fish, ranging from lethal to subclinical. The causes of this variability are poorly understood, although environmental factors are suspected to be critical determinants of clinical disease in fish infected with iridoviruses (LaPatra et al. 1996; Chinchar 2002; Goldberg 2002).

This study addresses the hypotheses that reduced water quality and crowded conditions can alter the sensitivity of fish to mortality from iridoviral infection and that such effects operate through immunosuppressive mechanisms to facilitate viral replication within fish. We performed two separate viral challenge experiments to test this hypothesis. The first experiment investigated the effects of selected water quality parameters on the severity of clinical disease and viral loads in experimentally infected fish. The second experiment investigated the effects of high population density on the severity of clinical disease and viral loads in similarly infected fish. We used as a study pathogen largemouth bass virus (LMBV), an emerging iridovirus that has caused mass mortality events in largemouth bass *Micropterus salmoides* and Florida bass *M. floridanus* throughout the southern and eastern United States (Goldberg 2002; Grizzle and Brunner 2003). Like other iridoviruses, LMBV varies widely in the nature and severity of clinical disease it causes (Hanson et al. 2001; Goldberg 2002). We predicted that reduced water quality and increased population density would both adversely affect the ability of infected fish to control viral replication and to resist clinical disease.

Methods

Study organisms.—The juvenile largemouth bass used in all experiments came from experimental rearing ponds at the Illinois Natural History Survey's Aquatic Research Field Laboratory, which is located on the University of Illinois Urbana-Champaign campus. All fish were between 4 and 6 months of age with a mean total length of 59.0 ± 11.7 mm (± SE) and a mean weight of 2.55 ± 1.35 g. Fish were originally collected from the Kaskaskia River basin in Illinois. This stock has been reared for several generations at the pond site and has not been exposed to LMBV. Before the study, we tested a representative sample of fish from this population for LMBV, using virus isolation. All fish tested were negative. Experimental fish were held in either 10-gal (37.8-L; water quality experiment) or 20-gal (75.6-L; density experiment) aquaria housed inside a controlled environment chamber kept at constant temperature (25°C) and photoperiod (12 h light : 12 h dark). Fish were fed bloodworms (approximately 0.8 g/fish) daily at 0900 hours, except on the day of exposure to LMBV.

Virus.—The viral isolate used for inoculation of largemouth bass was a sixth cellculture passage of the original type isolate recovered from the 1995 LMBV Santee Cooper Reservoir fish kill in South Carolina (Plumb et al. 1996). Virus was grown and harvested according to previously published methods (Goldberg et al. 2003).

Experimental inoculation.—Experimental fish received 0.1 mL of Hank's Balanced Salt Solution (HBSS) containing live virus at a concentration of $10^{5.16}$ genomes of LMBV per dose, measured by using real-time quantitative polymerase chain reaction (PCR). This is the dose calculated to kill half the experimental subjects (LD50) within 2 weeks postexposure (Goldberg et al. 2003).

Sham-injected control fish received the same volume (0.1 mL) of undiluted virus-free cell culture supernatant. Fish received injections in the peritoneal cavity, along the ventral midline halfway between the pelvic and anal fins.

Monitoring and processing fish.—We monitored survival of the fish for 2 weeks after injections. At 24-h intervals, we recorded the number of dead fish in each tank and recorded qualitative data on the behavior and appearance of surviving fish. We fed fish daily with approximately 0.2 g of pure frozen bloodworms (red mosquito larvae; San Francisco Bay Brand, Newark, California) per fish.

We removed dead and moribund fish daily from

the tanks. We killed moribund fish (fish that were listless or swimming with marked loss of equilibrium) in a solution of clove oil emulsified in ethanol (1:9 by volume; Taylor and Roberts 1999). We recorded the total weight (g) and total length (mm) of each fish, as well as the presence of any external or internal gross pathological lesions. We quantified the body condition of each fish at the time of death according to Fulton's index'' [K = weight in g · (total length in mm)$^{-3}$ ·100,000 $^{-1}$], a common measure for comparing the condition of fish within populations (Bolger and Connolly 1989).

We then removed the viscera (liver, spleen, stomach, intestines, kidney, swim bladder, heart, and reproductive organs) and diluted them 1:50 in HBSS containing antibiotics (penicillin, 100 units/mL; gentamycin sulfate, 50 g/mL; streptomycin, 100 μg/L; and amphotericin B, 0.25 μg/L). We homogenized the viscera with a ''stomacher'' (Stomacher 80 Biomaster automatic stomacher; Steward, Ltd., Worthington, UK) at normal speed for 1 min and froze the tissue homogenates at −80°C. We quantified viral load by using real-time quantitative PCR, expressing the results as the number of viral genomes per gram of fish tissue, according to published protocols (Goldberg et al. 2003).

Water quality experiment.—We performed a preliminary experiment to determine what concentration of nitrate was toxic to the fish used in this study. We placed 10 juvenile largemouth bass each into four 10-gal (37.8-L) aquaria equipped with recirculating water supplies and air stones and held them at 25°C. We then added sodium nitrate solution (1 M) to each tank, at different rates (0, 10, 40, and 100 mg/L per day). The final concentrations of sodium nitrate added to these tanks ranged from 0 to 1,600 mg/L. We observed fish for toxic effects and terminated the experiment when fish displayed overt signs of nitrate toxicosis (disequilibrium, spiral swimming). Fish used in this experiment were not inoculated with LMBV.

We then conducted an experiment to determine the effects of selected water quality parameters (elevated levels of dissolved nitrate in particular) on the sensitivity of bass to mortality from LMBV infection. For this experiment, we used twelve 20-gal (75.6-L) aquaria, each holding 10 fish. We kept the aquaria, which were bare except for an aerated filter sponge, inside a controlled environment chamber at 25°C and on a timed 12-h-light : 12-h-dark photic period. We allowed fish to acclimate to these conditions for 48 h before any manipulation.

We randomly selected 8 of the 12 tanks to serve as experimental tanks (LMBV-injected fish). The remaining four tanks served as controls (sham-injected fish). We added nitrate to tanks to the following four final concentrations of NO_3-N: 0, 40, 200, and 400 mg/L. We chose this range of concentrations based on our preliminary toxicity trial, selecting a maximum concentration well below that which caused direct toxic effects to bass but well above that typically seen in fish farms or in the natural environment. We gradually introduced a solution of 1 M sodium nitrate into the aquaria at various rates so that the tanks attained their particular target concentrations after 3 d. For each target nitrate concentration, we used two of the eight experimental tanks and one of the four control tanks, such that 20 experimental fish and 10 control fish were exposed to each nitrate concentration. We allowed fish to acclimate to their target nitrate concentration for four additional days before injecting them with LMBV.

Water chemistry measurements.—We took daily samples (50 mL) of water from each aquarium and froze them at −20°C in nonsterile plastic conical centrifuge tubes with screw caps (Fisherbrand; Fisher Scientific, Pittsburgh, Pennsylvania). We stored samples for no more than 2 months before analysis. For analysis, we thawed samples in a 25°C water bath and filtered them to remove debris and bacteria through a 0.45-μm pore-size syringe filter unit (millex-hv; Millipore, Molsheim, France), using disposable sterile plastic syringes. We determined the pH of each water sample with a pH meter ion electrode at 25°C (the water temperature at which the samples were collected). We determined nitrate and nitrite concentrations by ion chromatography (Dionex DX-120 Chromatograph, IonPac AS4A-SC Anion Exchange Column, Dionex Corporation, Sunnyvale, California). When necessary, we diluted samples with deionized water to adjust concentrations to be within the limits of the machine standards (NO_3-N, NO_2-N <4.0 mg/L). We measured total ammonia nitrogen concentrations (TAN; mg/L) by using methodology based on the Berthelot Reaction, in which addition of sodium nitroprusside forms a blue color that can be measured with a colorimeter at 660 nm (Bran+Luebbe TRAACS 2000 Analytical Console, Bran+Luebbe Industrial Method No. 780–86T; G. Bran+Luebbe Inc., Delavan, Wisconsin). For all measurements (NO_2-N, NO_3-N,

TAN), the minimum detectable level was 0.01 mg/L.

Effect of nitrate on viral growth in vitro.—To measure the direct effects of nitrate on viral growth, we grew LMBV in cell cultures at a series of nitrate concentrations identical to those used for the in vivo study. We inoculated the Santee–Cooper LMBV isolate at a multiplicity of infection of 1.0 onto confluent monolayers of fathead minnow cells in 25 cm^2 flasks and added sodium nitrate to the cell culture supernatant. We used three virus-inoculated replicate flasks and one virus-free control flask per nitrate treatment level (NO_3-N at 0, 40, 200, and 400 mg/L) and incubated them at 25°C. We removed 250-μL aliquots of cell culture supernatant from the flasks at 24-h intervals for 5 d. We measured viral concentrations in these supernatants by real-time quantitative PCR (Goldberg et al. 2003).

Population density experiment.—To determine the effects of physical crowding on the sensitivity of bass to mortality from viral infection, we used eight 20-gal (75.6-L) aquaria, each containing 12 fish. Fish were housed at either high or low density (four tanks for each treatment). The low-density treatment involved placing 12 fish in each aquarium such that they had unencumbered access to the entire water volume. The high-density treatment involved placing the same number of fish in aquaria to which a plastic fenestrated barrier had been installed at one average fish length (4.5 cm) from the front glass. The barriers were made of medium-strength shatter-resistant acrylic sheets (Lucite-ES) and were anchored in place with 100% silicon sealant (GE, Huntersville, North Carolina). Fish were placed in the space between the barrier and the front aquarium wall. This design crowded fish in the high-density treatment while keeping water volume equal between the two treatments. To control for the presence of the acrylic sheet, we installed identical barriers in each of the low-density tanks but placed them flush with the rear wall of the aquarium such that they did not restrict the movement of fish. Three aquaria (one at each density) served as experimental tanks (housing virus-injected fish), and one aquarium at each treatment level served as a paired control (housing sham-injected fish).

We placed all four aquaria in a controlled environment chamber and held them at a constant temperature of 25°C. Aquaria were bare except for an aerated filter sponge and the plastic barriers. Fish were kept on a timed 12-h-light : 12-h-dark photic period. Fish were allowed to acclimate to density treatments for 24 h before injection with LMBV or sham-injection.

Statistical analyses.—The outcome variables included survival times of individual fish, viral loads in individual fish, body condition scores of individual fish, gross pathology in individual fish, and mortality rates in aquaria both over the entire experiment and for specific time intervals. For the water quality experiment, predictor variables included nitrate, nitrite, ammonia, and pH concentrations for each aquarium on each experimental day. We also created predictor variables to represent daily changes in each water quality parameter, as well as changes over the 3 and 5 d preceding a mortality event. For the density experiment, the single predictor variable was treatment (low or high density).

We performed all statistical analyses with the computer program SAS (SAS Institute 2004). We used survival analysis (Cox proportional hazard regression) to investigate associations between mortality rates and either water quality parameters or density treatment. We also used logistic regression, where appropriate, to investigate associations of death events with various water quality parameter predictors. We used linear regression to investigate relationships between tank-specific mortality rates (proportion of remaining fish dying on any given day) and water quality parameters, as well as for analyses of viral loads and body condition. We adopted repeated-measures models throughout our analyses to account for multiple observations from the same tanks over time. We evaluated assumptions and goodness-of-fit for all statistical models and performed appropriate transformations of the data as necessary to ensure that model assumptions were satisfied.

Results

Preliminary Nitrate Toxicity Trial

Fish in the aquarium to which nitrate was added at the fastest rate (100 mg/L per day) displayed obvious behavioral abnormalities when nitrate concentration reached 1,600 mg/L. The fish lost equilibrium, had increased respiratory effort, and swam without directional control. These effects may have been a direct response to nitrate toxicity, or they may have resulted from other toxins that covary with nitrate (e.g., ammonia, nitrite). No adverse effects were observed in the three aquaria containing lower final concentrations of added nitrate (0, 160, and 640 mg/L).

TABLE 1.—Means (ranges) of water quality parameters in experimental tanks subjected to different nitrate treatments. All values are milligrams per liter except for pH.

Nitrate treatment (mg/L)	pH	Total ammonia nitrogen	NO_2-N	NO_3-N
0	7.81 (7.71–7.91)	0.53 (0.01–1.98)	5.77 (1.65–9.00)	0.20 (0.10–0.27)
40	8.00 (7.88–8.20)	0.20 (0.01–1.94)	6.29 (3.60–7.56)	43.3 (36.3–52.0)
200	7.89 (7.73–8.12)	2.37 (0.01–3.99)	3.70 (0.35–10.5)	212 (172–267)
400	8.11 (7.94–8.25)	2.84 (0.54–4.01)	3.01 (0.42–9.82)	385 (271–452)

Water Quality Experiment

Water quality parameters in the aquaria before the addition of nitrate were similar to the values in the rearing ponds from which the fish were collected (see Table 1, zero nitrate treatment). Water quality parameters varied within and among aquaria over the 2-week observation period. Means and ranges of water quality parameters across all aquaria during the experiment are summarized in Table 1.

Fish injected with LMBV began dying on day 3 postinoculation (Figure 1). Cumulative mortality of LMBV-injected fish at 14 d postinoculation was 90% for all treatments except the 200 mg/L treatment, where cumulative mortality was 70%. Median survival times for LMBV-injected fish in the 0, 40, 200, and 400 mg/L nitrate treatments were 6, 6, 11, and 10 d, respectively. No sham-injected control fish died in any of the treatments except in the 400 mg/L treatment, where three fish were killed before the end of the experiment because of their clinical signs suggestive of nitrate toxicity (spiral swimming). Virus-injected fish died at a significantly faster rate than sham-injected fish for

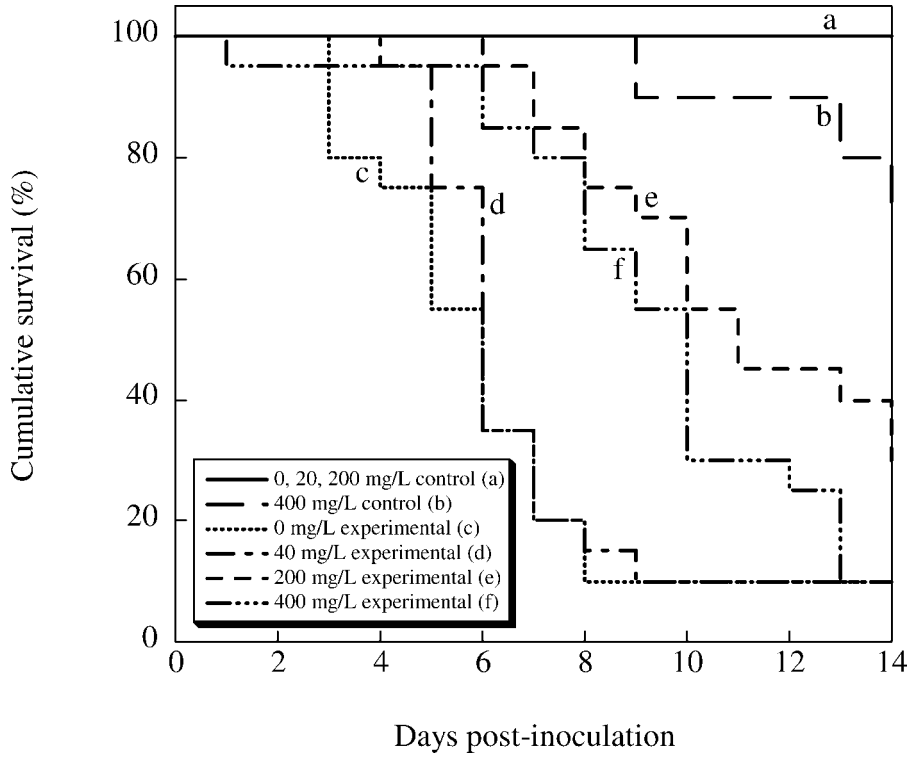

FIGURE 1.—Survival of juvenile largemouth bass injected with largemouth bass virus and held in tanks with different concentrations of sodium nitrate added (0, 40, 200, or 400 mg/L). Sham-injected fish were exposed to virus-free cell culture supernatant and served as controls. No mortality occurred in control fish held in the 0, 20, or 200 mg/L tanks, so their results were collapsed into a single (solid) line.

TABLE 2.—Statistically significant relationships between water quality parameters and mortality events within tanks. Results are from logistic regression analyses in which the outcome variable was whether or not any mortality was observed in a given tank on a given day. Only statistically significant results are shown.

					Odds ratio		
Parameter	Estimate[a]	SE[a]	Chi-square	*P*-value	Mean	Lower limit	Upper limit
NO_3[b]	−0.0208	0.00834	6.2204	0.0126	0.979	0.964	0.996
pH[c]	−4.1522	1.8589	4.9891	0.0255	0.016	<0.001	0.601
TAN[d]	−0.8550	0.4040	4.4793	0.0343	0.425	0.193	0.939
NO_2[e]	−0.5140	0.2600	3.9077	0.0241	1.672	1.004	2.784

[a] Estimates and SEs refer to regression parameter estimates from the final multiple logistic regression model.
[b] Value of NO_3-N on the day on which mortality was measured.
[c] Value of pH 24 h before the day on which mortality was measured.
[d] Value of the 24-h change in total ammonia nitrogen.
[e] Value of the change in NO_2-N over the 5 d before the day on which mortality was measured.

each corresponding nitrate treatment (Figure 1). Gross lesions seen included white exudate in the coelomic cavity, whitening and thickening of the swim bladder, and discoloration of the liver, but these lesions were not consistently present in experimental or control fish.

Nitrate concentration was negatively associated with mortality for LMBV-injected fish (hazard ratio = 0.998, $\chi^2 = 7.6259$, $P = 0.006$). LMBV-injected fish in the two lower (0 and 40 mg/L) nitrate treatment groups died at a rate 2.7 times faster did fish in the higher (200 and 400 mg/L) nitrate treatment groups ($\chi^2 = 15.58$, $P < 0.0001$). Mortality rates for fish in the 0 and 40 mg/L nitrate groups were statistically indistinguishable, as were those for fish at the 200 and 400 mg/L levels. These effects could have resulted from nitrite being present in higher concentrations in the tanks with low added nitrate than in the high-added nitrate tanks (Table 1).

The mean values (over 2 weeks) of water quality parameters within tanks had no statistically significant association with mortality rate. However, several statistically significant relationships emerged between time-specific mortality events (whether or not a tank experienced any mortality on a given day) and water quality parameters. Table 2 summarizes these results. Finally, the magnitude of change in nitrate concentration over a 5-d period (positive or negative) was positively associated with the proportion of fish dying within those 5 d ($t = 0.057$; $P = 0.03$). No other water quality parameters were significantly associated with proportions of fish dying within tanks.

TABLE 3.—Mean ± SE viral loads ($\log_{10}$ viral genomes per gram of fish tissue) of LMBV-injected largemouth bass by nitrate treatment and outcome. Values with asterisks are significantly greater than the other value in that row.

Nitrate treatment (mg/L)	Found dead or moribund		Survived until end of experiment	
	n	Viral load	*n*	Viral load
0	18	9.72 ± 0.36*	2	5.32 ± 0.47
40	18	9.62 ± 0.29*	2	5.54 ± 1.85
200	14	9.03 ± 0.78*	6	5.16 ± 0.78
400	18	8.30 ± 1.35	2	8.33 ± 0.99

Across all treatments, viral loads of LMBV-injected fish that were found dead (9.0 ± 1.18 log viral genomes per gram of fish tissue) did not differ significantly from viral loads of LMBV-injected fish that were found moribund and subsequently killed (9.4 ± 0.59 log viral genomes per gram). Viral loads of dead and moribund fish together (9.2 ± 0.99 log viral genomes per gram), however, were significantly higher than those of nonmoribund fish that were killed at the end of the experiment (5.8 ± 1.46 log viral genomes per gram). Viral load data are summarized in Table 3.

Viral loads were greater in fish that presented with external lesions than in fish that presented without external lesions ($t = 2.79$; $P = 0.007$). Fish in the two lowest nitrate treatment groups (0 and 40 mg/L) had higher viral loads than fish in the two highest nitrate treatment groups (200 and 400 mg/L; $t = 2.42$; $P = 0.018$). No other associations between viral load and nitrate treatment groups were significant. Table 4 describes statistically significant associations between water quality parameters and viral loads.

All fish, regardless of injection type or nitrate treatment, had statistically indistinguishable body condition scores. The mean Fulton scores for the 0, 40, 200, and 400 mg/L treatments were 1.11, 1.12, 1.13, and 1.06, respectively. Body condition

TABLE 4.—Statistically significant associations between water quality parameters and viral loads of fish. Results are from linear regression analyses in which the outcome variable was the final viral load of individual fish. Only statistically significant results are shown.

Predictor	Parameter estimate[a]	SE[a]	*t*-value	*P*-value	Correlation[b]
pH					
24-h change	−0.43310	0.15480	−2.80	0.0067	0.068
5-d change	−3.08499	1.30432	−2.37	0.0212	0.064
TAN (3-d change)	0.59634	0.27820	2.14	0.0361	0.054
NO_2					
3 d before	−0.60141	0.15620	−3.85	0.0003	0.128
3-d change	0.46118	0.13847	3.33	0.0015	0.129
5-d change	0.40758	0.15307	2.66	0.0099	0.081
NO_3					
24-h change	0.00703	0.00302	2.32	0.0231	0.047
5-d change	−0.01000	0.00352	−2.84	0.0061	0.092
Change up to death	0.00744	0.00260	2.86	0.0055	0.095
Total[c]	0.00726	0.00357	2.03	0.0456	0.049

[a] Estimates and SEs refer to regression parameter estimates from the final multiple linear regression model.
[b] Squared semipartial correlation coefficient, type II, which indicates the proportion of variation in the final regression model uniquely accounted for by each individual predictor variable.
[c] Range of nitrate concentrations over the entire 2-week experiment.

score did not vary in association with any measured water-quality parameter.

Effect of Nitrate on Viral Growth in Vitro

Flasks in each nitrate treatment had statistically indistinguishable final viral concentrations (*P*-values ranging from 0.055 to 0.482 for comparisons with results for control flasks). Rates of viral replication were statistically unaffected by nitrate concentrations. Table 5 summarizes the effects of nitrate concentration on viral growth in vitro.

Population Density Experiment

Fish injected with LMBV and held at low density began to die on day 1 postinoculation. The median survival time of fish held at low density was 5 d. At the end of the 2-week observation period, 32 of 36 fish (89%) held at low density had died or were found moribund. Fish exhibited behavior typical of LMBV infection, including disequilibrium and lethargy (Plumb and Zilberg 1999). No sham-injected controls in the low-density treatment died over the 14-d observation period.

Fish injected with LMBV and held at high density began dying on day 3 postinoculation. Again, the median survival time of fish held at high density was 5 d. At the end of the 2-week observation period, 35 of 36 fish (97%) held at high population density had died or were found moribund. One sham-injected fish held at high density died during the 2-week observation period. LMBV-injected fish held at high density were at a significantly higher risk of dying than LMBV-injected fish held at low density ($\chi^2 = 3.57$; hazard ratio = 1.6; $P = 0.029$; Figure 2). Gross lesions were similar to those described for the water quality experiment.

Viral loads of LMBV-injected fish found dead (mean of 9.4 log viral genomes per gram) did not differ significantly from viral loads of moribund virus-injected fish that were subsequently killed (mean, 9.4 log viral genomes per gram; $F = 0.01$; $P = 0.996$). However, fish that died during the course of the experiment (high and low density combined) had significantly higher viral loads (mean, 9.4 log viral genomes per gram) than did fish that survived to the end of the experiment (mean, 7.5 log viral genomes per gram; $F = 62.41$;

TABLE 5.—Viral concentrations (mean ± SE) in cell culture flasks inoculated with LMBV at different nitrate concentrations. Values are expressed as $\log_{10}$ viral genomes per milliliter of cell culture supernatant at the end of the 5-d growth period. No statistically significant differences existed either in final viral concentrations or in the rate of viral replication among nitrate levels.

Nitrate level (mg/L)	*n*[a]	Viral load
0	3	8.37 ± 0.06
40	3	8.38 ± 0.04
200	3	8.33 ± 0.08
400	3	8.54 ± 0.09

[a] Number of replicate flasks per nitrate level.

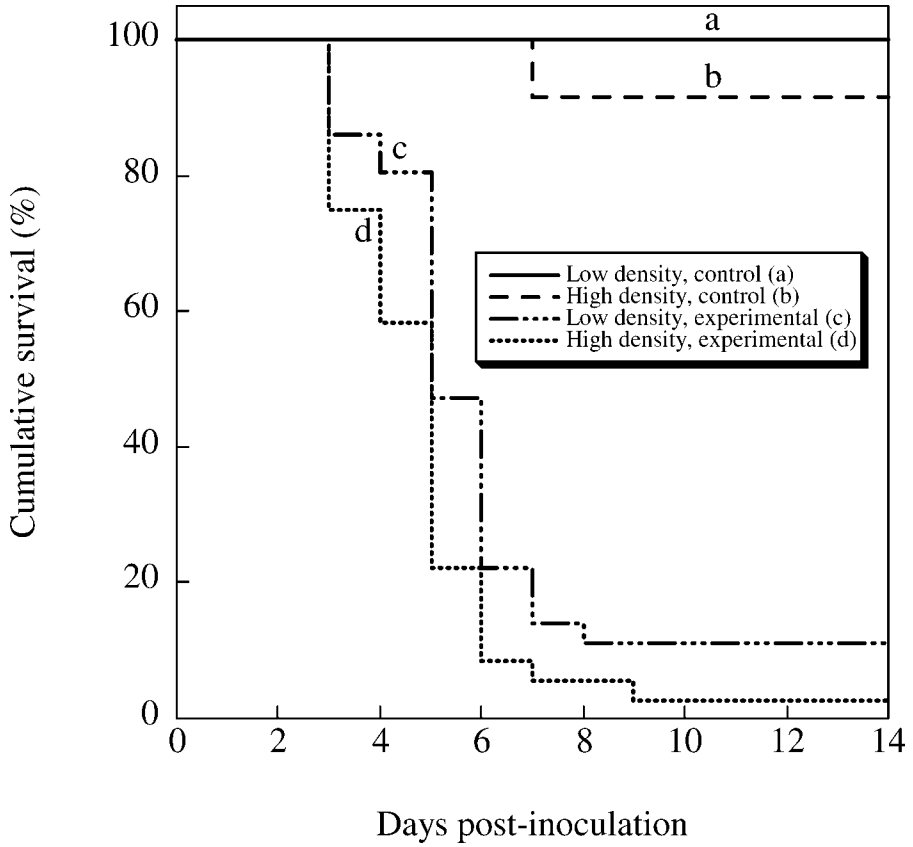

FIGURE 2.—Survival of juvenile largemouth bass injected with largemouth bass virus and held at high or low population density. Sham-injected fish were exposed to virus-free cell culture supernatant and served as controls.

$P < 0.0001$). Fish injected with LMBV and held at high density had significantly higher viral loads than LMBV-injected fish held at low density ($t = 2.19$; $P = 0.016$). Mean viral loads are summarized by density treatment in Table 6.

External and internal lesions showed no significant association with viral load. However, body condition scores of fish held at high density were significantly lower for virus-injected fish (1.12 ± 0.1) than for sham-injected controls (1.26 ± 0.1; $t = -3.82$; $P = 0.0002$; Table 7). This difference did not occur for fish held at low density (Table 7).

TABLE 6.—Mean ± SE viral loads ($\log_{10}$ viral genomes per gram of fish tissue) of juvenile largemouth bass injected with LMBV and held at low or high population density (see text) by outcome. The asterisk indicates that the value was significantly greater than the other value in that column.

	Found dead or moribund		Survived until end of experiment	
Density	*n*	Viral load	*n*	Viral load
Low	32	9.30 ± 0.36	4	7.28 ± 1.75
High	35	9.52 ± 0.39*	1	8.19 ± 0.00

TABLE 7.—Mean ± SE Fulton body condition scores of juvenile largemouth bass injected with LMBV and held at low or high population density (see text). The asterisk indicates that the value was greater than the other value in that row. There were no significant differences within columns.

	Sham injected		Virus injected	
Density	*n*	Fulton score	*n*	Fulton score
Low	12	1.19 ± 0.15	36	1.12 ± 0.13
High	12	1.26 ± 0.12*	36	1.12 ± 0.11

Discussion

Although we expected that increased concentrations of dissolved nitrate would have adverse effects on the survival of LMBV-inoculated largemouth bass, nitrate was paradoxically protective

in this study. Higher nitrate levels were associated with lower mortality and lower viral loads in fish exposed to LMBV. Examination of the effects of nitrate on viral growth in vitro demonstrated that nitrate concentration does not affect viral replication directly. The protective effect of nitrate in vivo must have occurred, therefore, from the action of nitrate on the fish themselves. We speculate that nitrate may alter physiological processes within the fish, causing an inhibition of viral replication within host cells (perhaps by generally retarding intracellular metabolism, on which viral replication depends).

In general, relationships between water quality parameters, survival, and viral loads were varied and inconsistent. Associations between viral load and pH, nitrite, and nitrate were most robust. The direction of these relationships varied, however, such that generalization across parameters was difficult (Tables 2 and 4). Furthermore, the number of statistical analyses conducted makes it likely that some marginally significant results appeared to be significant because of type I error. Nevertheless, the most robust associations that emerged involved changes in water quality parameters over time, rather than absolute water quality values (Tables 2 and 4). This observation suggests that rapid fluctuations in water quality may have had greater impact on sensitivity to viral infection than persistently high levels of dissolved toxins.

The effect of water quality on an individual fish is influenced by its species, age, size, and previous exposure to the water parameter in question (Wedemeyer 1996). Water chemistry parameters within this experimental system were dramatically higher than typical levels found in unpolluted natural waters or levels recommended by aquaculture standards. Most recommended maximum or minimum water-quality standards have been based on the physiological needs of salmonids, which are a highly sensitive group of fish (Wedemeyer 1996). Largemouth bass, however, like other centrarchids or members of the cyprinid family, have a greater tolerance to nitrate than salmonids do and have been able to adapt to adverse water quality situations, including eutrophic waters (Smith and Williams 1974; Lewis and Morris 1986; Hendricks et al. 1995). That the bass stock used in this experiment exhibited high tolerance to nitrate (as illustrated by the preliminary nitrate toxicity experiment) is therefore not surprising.

The results of the population density experiment indicate that increased fish population density can affect the survival, viral load, and body condition of fish infected with an iridovirus. Fish injected with LMBV and held at high density were at a 1.6-fold greater risk of dying than LMBV-injected fish held at low density. At the end of 2 weeks, total mortality in LMBV-injected fish held at high density was 8% higher than total mortality in LMBV-injected fish held at low density. Although not dramatic (Figure 2), these trends were statistically significant. The range of densities examined in this study was limited, however, and the dose of virus was chosen to cause relatively rapid mortality. That an effect of crowding on mortality was nevertheless observed suggests that population density could be a biologically significant factor for determining the survival of largemouth bass infected with a potentially lethal virus.

Previous studies have documented negative effects of high fish density on disease-related outcomes (Fagerlund et al. 1986; Mazur et al. 1993; Banks 1994; LaPatra et al. 1996; Bebak-Williams et al. 2002), but the mechanisms accounting for these associations have remained unclear. Specifically, past studies have not controlled for water volume, such that it is unknown whether increased pathogen transmission rates, sociobehavioral stress, or combinations of the two account for the trends observed. Our experimental design forced fish into high density without altering water volume. The critical parameter that varied, therefore, was the physical space in which fish were housed. The social stress that accompanies crowded conditions can cause subordinate fish to have impaired leukocyte function and suppressed immunity (Cooper et al. 1989). Suppressed immunity could, in turn, lead to an inability to inhibit viral replication. The observation that fish held at high density not only died more quickly but also had significantly higher viral loads than fish held at low density supports such a mechanism. We did not examine the effects of physical contact on the transmission of the virus between individuals in this study. Increased fish density could lead to increased physical contact and therefore increased transmission. In a separate study (Grant et al. 2005, this issue), we have documented that direct contact does indeed facilitate viral transmission in this system.

The body condition scores of virus-injected fish held at high density were lower than those of sham-injected fish held at high density. This difference did not exist for fish held at low density. The combination of high population density and the presence of an infectious agent resulted in reduced weights and poor body condition, while high pop-

ulation density alone did not. This observation supports the idea that elimination of infectious agents from intensive rearing systems could increase the overall health and productivity of fish populations.

The combined results of our water quality and population density studies suggest that both physiological and sociobehavioral stressors can modify the clinical effects of viral infection in fish. The mechanisms by which these effects operate are still unknown, but the results of this study implicate immunosuppression, modulated either through direct physiological pathways (as in the case of water quality effects) or through indirect neuroendocrine pathways (as in the case of population-density effects). The fact that both physical and social factors can alter the clinical manifestations of viral infection underscores the need for ''holistic'' approaches to the management of fish populations (both captive and wild) that harbor infectious agents. Strategies that maximize the quality of the physical environment in which fish live while simultaneously minimizing sociobehavioral stress should most effectively increase health and productivity.

Acknowledgments

We thank Todd Royer for assistance with equipment and Jens Sandberger for technical support with water-quality laboratory analyses. The Illinois Council on Food and Agriculture Research and the Conservation Medicine Center of Chicago provided funding for this research.

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