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[Article]

Effects of Practices Related to Catch-and-Release Angling on Mortality and Viral Transmission in Juvenile Largemouth Bass Infected with Largemouth Bass Virus

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Abstract.—Largemouth bass virus (LMBV; family Iridoviridae) has recently emerged as a causative agent in fish kills of largemouth bass *Micropterus salmoides*. Little is known about how the virus is transmitted or what factors predispose fish to mortality subsequent to infection. Concern has nevertheless arisen that activities related to recreational angling may affect transmission dynamics and may alter the susceptibility of infected fish to clinical disease. This study examined the separate effects of two angling-related factors on the susceptibility of juvenile largemouth bass to mortality from LMBV infection and on the transmission of LMBV from infected to uninfected fish. The first factor was hook-and-line angling. Infected fish that underwent a simulated angling treatment did not experience higher mortality or have higher viral loads in their tissues than those that were not angled. The second factor was direct contact between infected and uninfected fish, as would occur in live wells and holding tanks. The LMBV was transmitted from infected to uninfected fish through water, even when direct contact was prevented. Transmission of LMBV between infected and uninfected fish separated by a fenestrated barrier was nearly as efficient as LMBV transmission between infected and uninfected fish that were allowed direct contact. These results imply that angling itself may have only minimal effects on the survival of largemouth bass infected with LMBV but that angling-related practices that place infected and uninfected fish together in a limited water volume may facilitate viral transmission. Partitioning or cooling of live wells and holding tanks, as well as limiting their use, could reduce LMBV-associated mortality and viral transmission.

Largemouth bass virus (LMBV; family Iridoviridae; Plumb et al. 1996) is the only virus known to cause epidemic mortality in wild largemouth bass *Micropterus salmoides* (Grizzle and Brunner 2003). The LMBV has been associated with large-scale fish kills in largemouth bass, particularly during warm seasons (Plumb et al. 1996; Hanson et al. 2001). While certain populations of infected largemouth bass have suffered fish kills, other infected populations suffer no discernible clinical signs, suggesting that key environmental factors may play a role in the pathogenesis of LMBV. Such factors may also play a role in the transmission of LMBV between fish and between bodies of water (Goldberg 2002).

Concern has arisen among fisheries biologists and anglers alike that hook-and-line angling and related activities may enhance LMBV-associated mortality or transmission (Goldberg 2002). Such effects could operate by reducing the immune ca-

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pacity of fish or by increasing contact rates between infected and uninfected individuals. For example, catch-and-release angling has been associated with physiological changes that affect homeostasis (Gustaveson et al. 1991; Suski et al. 2003, 2004; Cooke et al. 2004), and these changes could result in immunosuppression (Anderson et al. 1982; Anderson 1990). Regardless of the mechanisms involved, it would be important to know whether and how angling and related activities alter the susceptibility of LMBV-infected fish to clinical disease and how such activities might affect viral transmission. Such information would be critical for making recommendations aimed at limiting the impact of LMBV and similar pathogens.

The goal of this study was to examine the effects of two factors, both associated with hook-and-line angling, on the survival of largemouth bass infected with LMBV and on the transmission of the virus from infected to uninfected individuals. Both factors have been widely implicated in LMBV-related mortality and in the transmission of the virus. The first factor examined was angling itself. We inoculated juvenile largemouth bass with LMBV and subjected them to an angling simulation conducted at two different temperatures. We then measured mortality rates and viral loads in fish. The second factor was direct contact between infected and uninfected individuals as would occur in live wells or other settings where fish are housed together at high density. We housed uninfected juvenile fish in aquaria with infected fish but separated them by a fenestrated barrier that prevented direct contact. We measured the efficiency of transmission from infected to uninfected fish by quantifying mortality rates and viral loads that were then compared to those of uninfected fish that were allowed direct contact with infected fish. We hypothesized that angling would increase mortality and viral loads in infected fish and that transmission would be more efficient between fish that were allowed direct contact than between fish that were kept physically separate.

Methods

Study organisms.—The largemouth bass used for experimentation were harvested from experimental ponds owned and operated by the Illinois Natural History Survey's Center for Aquatic Ecology. This population of largemouth bass has been maintained in an isolated experimental pond complex for over 7 years and has not been exposed to LMBV. A sample of the population ($N = 15$ adults) was initially tested for LMBV by use of virus isolation and was found to be LMBV-negative. The fish used for experimentation were 4–9 months of age; the mean ± SE length of experimental fish was 68 ± 1.7 mm, and the mean weight was 3.8 ± 0.2 g. Although these fish were smaller and younger than fish typically angled in the wild, they were amenable to experimental manipulation at sufficient numbers for meaningful statistical analysis.

Experimental setup.—After their removal from rearing ponds, fish were housed in one of twelve 75.6-L aquaria (76.2 × 30.48 × 30.48 cm) in a controlled environment chamber. Each aquarium was equipped with a simple air-driven sponge filter and was devoid of substrate or plant life. Aquaria were filled with tap water (pH = 8.0) that was allowed to age for 3 d prior to introduction of fish. A thermometer was placed in each aquarium, and individual glass tops covered all aquaria. Conditions inside the chamber were strictly controlled, including temperature (ambient at 25°C), constant humidity (60%), and constant photoperiod (12 h light: 12 h dark, provided by overhead fluorescent bulbs). Lights were on from 0700 to 1900 hours each day.

Virus.—The viral isolate used for each experiment was the Santee Cooper Reservoir isolate recovered from South Carolina in 1995 (Plumb et al. 1996). The virus used for inoculation was a sixth cell culture passage isolate. The isolate was derived from a fifth passage virus inoculated at a multiplicity of infection of 1.0 onto a confluent monolayer of fathead minnow *Pimephales promelas* cells and was grown, harvested, and titered according to previously published methods (Goldberg et al. 2003).

Exposure to LMBV.—Each experimental fish was injected intraperitoneally with LMBV at the ventral midline between the anal opening and the pelvic fins. Injections were made with a sterile, 1-cm^3 tuberculin syringe and 1/2-in (1.27-cm) needle containing cultured LMBV diluted with Hank's balanced salt solution (HBSS) (injection = 0.1 mL/fish). The infectious dose was 1×10^5 tissue culture infectious doses with 50% cytopathic endpoint of LMBV. Control fish were injected intraperitoneally with 0.1 mL of virus-free cell culture supernatant.

Simulated angling experiment.—Twelve fish per tank were acclimated to 25°C for 7 d in 10 aquaria inside the controlled environment chamber. Submersible 500-W heaters were used to heat five of the aquaria to 30°C. The fish were then given seven more days to acclimate to the elevated test tem-

peratures before exposure to LMBV. Fish were fed bloodworms (approximately 0.8 g/fish) at 0900 hours every morning except on the day of exposure to LMBV. Fish were monitored for morbidity and mortality for 14 d subsequent to exposure. Fish at each temperature were experimentally angled 2 d after LMBV injection.

The angling simulation consisted of netting out all the fish in a tank and transferring them to a temporary holding tank. Fifty percent of fish from each tank were hooked through the lower jaw on a single hook attached to a commercial monofilament line and pulled in the opposite direction of natural motion. The line was held consistently taut for 1 min. Fish were then held out of the water and suspended from the mouth by the hook for 45 s before the hook was removed. After hook removal, each fish was given a partial fin clip for treatment identification and was returned to its original tank. The remaining 50% of fish in each experimental tank were not angled. Twelve fish in each of two tanks (one 25°C tank and one 30°C tank) were subjected to the identical angling simulation but were sham-injected with virus-free cell culture supernatant. These fish served as controls.

This simulation was designed to be representative of a catch-and-release angling event in which the fish is reeled into a boat after a sufficient fighting sequence and held out of the water prior to release. Application times were based on physiological exhaustion and recovery studies of angled largemouth bass (Cooke et al. 2004). The application times of 1 min of angling and 45 s of air exposure also caused obvious disequilibrium and were adopted based on preliminary trials indicating that longer application times caused immediate death.

Transmission experiment.—Eight aquaria, all maintained at 25°C, were used in the transmission experiment. In four aquaria, a double fenestrated barrier (two perforated Plexiglas sheets with a 1-in separation between them) was placed in the center of the tank to divide the tank in half. The other four aquaria had identical Plexiglas sheets placed 0.5 in from each end of the tank, creating an undivided water space equivalent to that in the divided tanks. Ten largemouth bass were placed in each of the eight tanks. In three of the divided tanks, five infected fish were placed on one side of the barrier and five uninfected fish were placed on the other side of the barrier. In three of the undivided tanks, five infected and five uninfected fish were placed together in the central space, thereby allowing direct contact between infected and uninfected individuals. The remaining two tanks (one divided and one undivided) housed control fish; half of the fish in each tank were inoculated with virus-free cell culture supernatant, while the remaining fish were not inoculated.

Monitoring and processing of fish.—Fish were monitored for 14 d postinjection. Once each day before feeding, all dead or moribund fish were removed from each aquarium and immediately processed in the laboratory. Moribund fish were euthanatized by submersion in a solution of clove oil and ethanol (1:9; 0.25 L) added to 1.5 L of water (Taylor and Roberts 1999). The date of death, state at death (dead or moribund), temperature or angling treatment and tank, total length (mm), total weight (g), and the presence of gross external or internal lesions were recorded for each fish during processing. Body condition was calculated as Fulton's index ($10^5 \times \text{weight/length}^3$), a common metric for comparing body condition of fish within a population (Bolger and Connolly 1989).

Processing consisted of removing visceral tissues (liver, spleen, kidneys, stomach, intestines, and swim bladder) and diluting these tissues (1 g: 50 mL) in HBSS containing antibiotics (100 units of penicillin/mL, 100 μg of streptomycin/mL, 0.25 μg of amphotericin B/mL, and 50 μg of gentamycin sulfate/mL). The tissue solution was then homogenized with a Stomacher 80 Biomaster automatic stomacher (Steward, Ltd.) for 1 min at normal speed, and the homogenate was frozen at −80°C for future analysis.

Quantification of viral load.—The DNA was extracted from fish tissue homogenates using a QIAGEN QIAamp DNA blood minikit (250) and a QIAvac 6S vacuum manifold (QIAGEN, Inc.) according to the protocol provided by the manufacturer. Real-time quantitative polymerase chain reaction (RT-qPCR; Higuchi et al. 1992, 1993) was used to measure the concentration of viral genomes in each sample of tissue homogenate (viral genomes per gram of fish tissue). The RT-qPCR was performed according to previously published protocols (Goldberg et al. 2003).

Statistical analyses.—Statistical analyses were performed by use of version 8.2 of the Statistical Analysis System (SAS Institute, Inc., Cary, North Carolina). Survival data were analyzed with Cox proportional hazard regression. Student's *t*-tests, analyses of variance, and multiple linear regression analyses were performed on viral load data. Statistical models included contrasts between injected and uninjected fish exposed to different angling treatments, those with different Fulton's in-

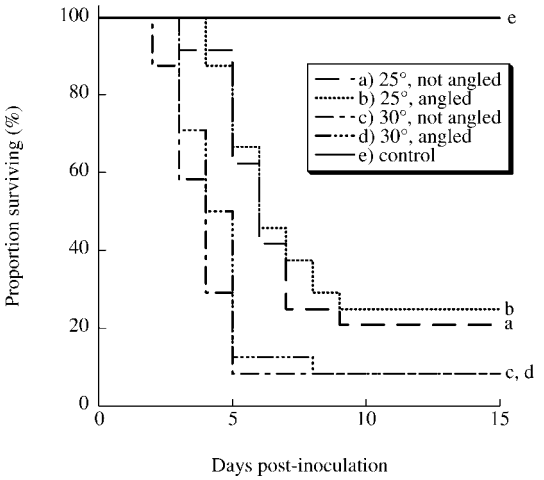

FIGURE 1.—Cumulative survival of juvenile largemouth bass that were injected with largemouth bass virus, either angled or not angled, and held at either 25°C or 30°C. No mortality was observed in control (sham-injected) fish from any treatment; therefore, control data from all treatments were pooled.

dex values, and those that differed in status at the time of sampling (i.e., dead, moribund, or survived to the end of the experiment). Post hoc Tukey's and least-significant-difference tests were performed to identify significant differences between specific treatments in analyses of variance. Associations were considered significant at an α level of 0.05.

Results

Effects of Simulated Angling

Median survival times for both angled and nonangled fish were 4 d postinoculation for fish held at 30°C and 6 d postinoculation for fish held at 25°C. Largemouth bass that were held at 25°C and that underwent angling simulation experienced 75.0% cumulative mortality at 14 d, while nonangled fish held at 25°C experienced 79.2% cumulative mortality at 14 d (Figure 1). Angled fish held at 30°C experienced a cumulative mortality of 91.7% at 14 d, and nonangled fish that were held at 30°C also experienced 91.7% cumulative mortality at 14 d (Figure 1). At both 25°C and 30°C, LMBV-injected fish died significantly more quickly than did sham-injected controls (25°C: $\chi^2 = 18.92$, $P < 0.0001$; 30°C: $\chi^2 = 27.70$, $P < 0.0001$). Angling did not have a significant effect on survival at either temperature (25°C: $\chi^2 = 0.32$, $P = 0.57$; 30°C: $\chi^2 = 1.44$, $P = 0.23$; Figure 1). Temperature, however, did have a significant effect on survival. The LMBV-injected fish held at 30°C died more quickly than did LMBV-injected fish held at 25°C ($\chi^2 = 17.93$, $P < 0.0001$; hazard ratio = 4.41). No sham-injected fish died during the observation period.

Fish that died developed clinical signs of viral infection consistent with those described by Plumb and Zilberg (1999), such as spiral swimming, abdominal distention, and lethargy. External gross lesions included visibly distended abdomens, inflammation at the injection site, and localized or generalized hyperemia. External lesions were present in 33% of fish that died during the observation period but 0% of fish that survived to the end of the experiment ($\chi^2 = 13.97$, $P \leq 0.0001$). Internal gross lesions included a thick, white exudate in the coelomic cavity, a whitening and thickening of the swim bladder wall (suggestive of pneumocystitis), and color changes to various visceral organs, especially the liver. Internal lesions were present in 55% of fish that died during the observation period but only 2% of fish that survived to the end of the experiment ($\chi^2 = 11.01$, $P \leq 0.0001$). Neither external nor internal lesions were observed in any sham-injected control fish.

Fulton's index for both experimental and control fish was 1.2. Similarly, fish that were found dead or moribund and fish that survived to the end of the trial also exhibited Fulton's indices equal to 1.2. Values of Fulton's index were statistically indistinguishable across treatments and outcomes.

There was no significant difference in viral load between dead and moribund fish ($t = 0.12$, $P = 0.90$; Table 1). However, when pooled, dead and moribund fish had significantly higher viral loads than did fish that survived to the end of the experiment ($t = 11.98$, $P < 0.0001$). Fulton's index ($t = -0.23$, $P = 0.82$) and the presence of internal gross lesions ($\chi^2 = 0.61$, $P = 0.44$) or external gross lesions ($\chi^2 = 0.13$, $P = 0.72$) were not significantly associated with viral load in fish that died or were euthanatized during the observation period.

Viral loads of experimental fish held at 25°C did not differ significantly from viral loads of experimental fish held at 30°C ($t = 1.20$, $P = 0.23$). Viral loads of angled fish held at 25°C or 30°C did not differ significantly from viral loads of nonangled fish held at 25°C or 30°C ($t = 0.30$, $P = 0.76$). There was no significant first-order interaction between temperature and angling ($t = -0.72$, $P = 0.48$). Mean viral loads were not statistically different between fish held at 30°C and those held at 25°C (Table 1). The RT-qPCR was

TABLE 1.—Mean ± SE viral loads of juvenile largemouth bass exposed to equal doses of largemouth bass virus at 25°C and 30°C and subjected to experimental hook-and-line angling. Viral loads are expressed as $\log_{10}$ transformed viral genomes per gram of tissue. Similar letters signify no statistically significant difference at the 0.05 level (letters in the final column refer only to comparisons among values within that column).

	State of fish					
	Found dead or moribund		Survived to end of experiment		All fish combined	
Treatment	*n*	Viral load	*n*	Viral load	*n*	Viral load
Angled at 25°C	18	9.19 ± 0.08 z	6	5.95 ± 0.58 x	24	8.38 ± 0.33 z
Not angled at 25°C	20	9.12 ± 0.10 z	4	5.74 ± 0.70 x	24	8.55 ± 0.29 z
Angled at 30°C	22	9.13 ± 0.13 z	2	6.50 ± 2.43 x	24	8.91 ± 0.24 z
Not angled at 30°C	22	9.19 ± 0.11 z	2	7.68 ± 0.15 y	24	9.06 ± 0.13 z

also run on the control fish from each treatment. Viral loads were found to be below minimum detection limits and were statistically indistinguishable from negative control RT-qPCRs.

Effects of Direct Contact on Transmission

The median survival time of all fish injected with LMBV was 6 d (Figure 2). Only one control fish and one uninjected experimental fish died during the course of the experiment (Figure 2). The difference in mortality rates between injected and uninjected fish (both experimental and controls) was statistically significant ($\chi^2 = 21.226$, P 0.0001). Mortality rates did not, however, differ significantly between uninjected experimental fish and sham-infected controls. Mortality rates did not differ significantly between LMBV-injected fish in divided tanks and those in undivided tanks.

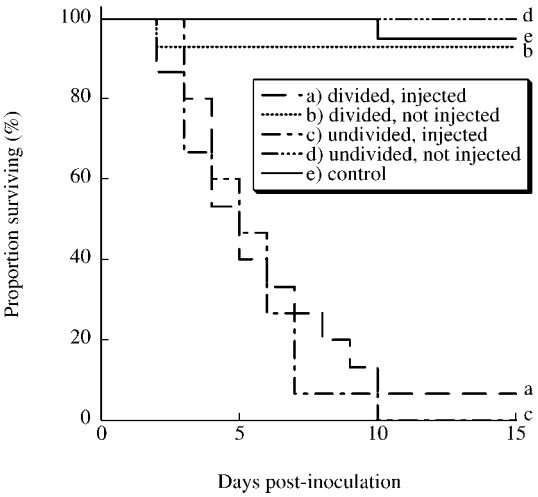

FIGURE 2.—Cumulative survival of juvenile largemouth bass that were injected or not injected with largemouth bass virus and held either in tanks divided by a fenestrated barrier (preventing direct contact between infected and uninfected fish) or in undivided tanks. Only one control fish (sham-injected) died; therefore, control data from all treatments were pooled.

Fish that died during the observation period displayed clinical signs and gross lesions consistent with LMBV infection, as described above. External lesions were present in 48% of fish that died during the observation period but 0% of fish that survived to the end of the experiment ($\chi^2 = 10.99$, $P \leq 0.0001$). Internal lesions were present in 28% of fish that died during the observation period but only 3% of fish that survived to the end of the experiment ($\chi^2 = 4.77$, $P = 0.03$). Neither external nor internal lesions were observed in any sham-injected control fish.

The mean ± SE Fulton's index of LMBV-injected fish (1.16 ± 0.18) was significantly lower than that of the uninjected fish housed with them (1.29 ± 0.10; $t = -3.393$, $P = 0.002$). However, the mean Fulton's index of LMBV-injected fish did not differ significantly from that of sham-injected control fish (1.15 ± 0.17; $t = -0.142$, $P = 0.889$).

Mean ± SE viral loads differed significantly between fish injected with LMBV (divided tanks: 8.96 ± 0.35 $\log_{10}$ viral genomes [LVGs] per gram of tissue; undivided tanks: 9.42 ± 0.12 LVGs/g) and the uninjected fish housed with them (divided tanks: 4.72 ± 0.08 LVGs/g; undivided tanks: 4.95 ± 0.07LVGs/g; $t = 23.216$, $P \leq 0.001$; Table 2). Among fish that survived to the end of the experiment, uninjected fish in divided tanks had marginally significantly lower viral loads (4.73 ± 0.08 LVGs/g) than did uninjected fish in undivided tanks, where direct contact with infected individuals was possible (4.95 ± 0.07 LVGs/g; $t = 2.211$, $P = 0.036$; Table 2). Although statistically significant, the absolute value of this difference (0.22 LVGs/g) was small. Viral loads of control fish were below minimum detection limits and were statistically indistinguishable from negative control RT-qPCRs.

TABLE 2.—Mean ± SE viral loads of juvenile largemouth bass either uninjected or injected with largemouth bass virus and held in equal numbers in divided tanks (preventing contact between injected and uninjected fish) or undivided tanks. See Table 1 for additional details.

	State of fish					
	Found dead or moribund		Survived to end of experiment		All fish combined	
Treatment	*n*	Viral load	*n*	Viral load	*n*	Viral load
Divided tank, injected	14	8.92 ± 0.35 z	1	9.54 ± 0.00 z	15	8.96 ± 0.35 z
Divided tank, not injected	1	4.53 ± 0.00 y	14	4.73 ± 0.08 x	15	4.72 ± 0.08 y
Undivided tank, injected	15	9.42 ± 0.12 z			15	9.42 ± 0.12 z
Undivided tank, not injected			15	4.95 ± 0.07 y	15	4.95 ± 0.07 y

Discussion

Effects of Simulated Angling

The LMBV-injected largemouth bass that were experimentally angled experienced no higher mortality than did LMBV-injected fish that were not angled. This result was consistent both at 25°C and 30°C. Largemouth bass injected with LMBV and held at 30°C, whether angled or not, experienced higher mortality than did bass held at 25°C (Figure 1), confirming that temperature elevations approaching 30°C increase the susceptibility of fish to the virus under controlled experimental conditions (Grant et al. 2003).

Given that the survival of angled and nonangled fish did not differ significantly, it is not surprising that the viral loads of angled fish did not differ from those of nonangled fish. The viral loads of fish that died during the observation period did not differ significantly between fish held at 25°C and those held at 30°C, even though survival rates at these two temperatures were significantly different. This was surprising considering previous results that did, in fact, demonstrate such a trend (Grant et al. 2003). Experimental exposure of fish to LMBV is known to cause inconsistent clinical outcomes (Grizzle and Brunner 2003). We cannot explain these inconsistencies, but they could result from differences among fish and fish populations in levels of innate immunity, which in turn may be influenced by temporally variable environmental factors (Goldberg 2002).

Within each treatment, fish that survived to the end of the experiment had lower viral loads than those found dead or moribund. This observation suggests that viral replication may have been inhibited in some fish. It is not known why certain fish within treatments were able to withstand infection while others were not. In both experiments, fish that died during the observation period were significantly more likely to have internal and external gross lesions than fish that survived to the end of the experiment. However, the presence of gross lesions was not correlated with viral loads in fish that died or were found moribund during the observation period. These results suggest that gross pathology may indicate LMBV infection but is a poor indicator of infection intensity.

Concern about the potential effects of catch-and-release angling on the survival of LMBV-infected fish has been based on the assumption that angling is a stressful event that can increase the sensitivity of fish to infection through immunosuppression or similar effects. Although we did not measure stress directly in this study, evidence indicates that catch-and-release angling does indeed increase stress-related physiological characteristics in largemouth bass (Suski et al. 2003, 2004; Cooke et al. 2004). The fact that angling did not affect the subsequent survival of LMBV-infected fish in our study suggests that not all stressful events are sufficient to increase mortality in fish infected with a potentially lethal pathogen. We used only a single, relatively short (approximately 2 min) angling protocol, however; perhaps repeated angling episodes or more protracted stressors would have produced the predicted trend.

The principal limitation to the generalization of our results is the size and age of the fish used in the experiment. Juvenile largemouth bass may respond differently to angling than would larger fish, which are also more frequently angled. Similarly, the effects of angling may differ between the controlled experimental setting of this study and the complex ecosystems in which largemouth bass are typically angled. For example, increased postrelease recovery times in LMBV-infected fish would be of little consequence for mortality in an environment devoid of predators. Clearly, future studies of the interactions between angling and LMBV in larger fish and in naturalistic settings are warranted.

Effects of Direct Contact on Transmission

The LMBV was transmitted nearly as efficiently through water alone as between infected and uninfected fish that were allowed direct contact with each other. Uninfected fish in direct contact with infected fish had higher viral loads at the end of the experiment than did uninfected fish separated from infected fish by a fenestrated barrier. Although this difference was statistically significant, it was nevertheless small. Its biological significance is therefore uncertain, especially since mortality rates of uninfected fish in direct contact with infected fish were very low and did not differ significantly from mortality rates of uninfected fish that were prevented from direct contact with infected fish.

Values of Fulton's index were lower in fish injected with either LMBV or virus-free cell culture supernatant than in fish that were uninjected. It would appear that the intraperitoneal injection of fluid, whether it contained LMBV or not, was responsible for this reduction in Fulton's index.

The known modes of transmission of LMBV include through water (Plumb and Zilberg 1999) and by ingestion of experimentally infected prey (Woodland et al. 2002). The presence of LMBV in the mucus of infected fish (Woodland et al. 2002) provides a mechanism whereby direct contact might be expected to facilitate transmission. Our study confirms this hypothesis and implies that keeping infected fish physically separate from uninfected fish could reduce transmission of the virus. Live wells and holding tanks, which are common features in recreational angling settings, may enhance viral transmission. Compartmentalization of such containers or decreasing their usage could reduce transmission rates. We also suggest that reducing temperatures in live wells and holding tanks might have beneficial effects. Our current data demonstrating a strong effect of temperature on the survival of LMBV-infected fish and our previous data demonstrating that increased temperatures may facilitate viral replication (Grant et al. 2003) support this management recommendation.

We documented a small effect of direct contact on viral transmission that was evident in the analysis of viral loads but not in the mortality rates of fish to which the virus was transmitted. As in the experimental angling trial, these effects could differ in magnitude for larger fish in actual live wells and holding tanks. For example, the ratio of fish volume to water volume in a typical live well would be considerably higher than that simulated in this study, such that the magnitude of the effect of direct contact on viral transmission might be correspondingly higher. Again, experiments on larger fish in naturalistic settings are warranted.

Conclusions

The results of our experimental angling study suggest that a single angling event may not be sufficient to increase mortality rates in LMBV-infected largemouth bass. However, the results of our transmission study suggest that certain angling-related practices that place infected and uninfected fish together in close proximity can facilitate transmission of the virus. Altering such practices (e.g., partitioning or cooling live wells and holding tanks or discouraging their use) might decrease transmission of the virus and thereby reduce its prevalence in infected populations. Verification of our findings will have to await future studies of angling and LMBV conducted on larger fish in naturalistic settings.

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