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Effects of Temperature on the Susceptibility of Largemouth Bass to Largemouth Bass Virus

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, University of Illinois, Champaign, Illinois 61820, USA

KATE R. INENDINO

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

TONY L. GOLDBERG

College of Veterinary Medicine, Department of Pathobiology, University of Illinois, Urbana, Illinois 61802, USA

Abstract.—Temperature is an environmental variable thought to influence the susceptibility of fish to infectious diseases. This study demonstrated that juvenile largemouth bass *Micropterus salmoides* that were experimentally infected with largemouth bass virus (LMBV; family Iridoviridae) experienced greater mortality at 30°C than at 25°C. Juvenile largemouth bass were exposed to equal doses of LMBV and held at three temperatures: 25, 30, and 35°C. Fish held at 30°C suffered mortality at a higher rate than fish held at 25°C and had higher viral loads (viral genomes per gram of tissue) at the time of death. The LMBV-injected fish held at 35°C suffered mortality at rates equal to those of sham-injected controls, suggesting that stressful manipulation at temperatures approaching the upper lethal limit can induce mortality in fish independently of viral infection. These results suggest that temperature is an important determinant of host survival and viral replication in the LMBV system.

Largemouth bass virus (LMBV) is a recently discovered pathogen that has become widely distributed throughout the southeastern and midwestern United States (Plumb et al. 1996; Goldberg 2002). This virus infects several warm water fish species, including bluegill *Lepomis macrochirus*, striped bass *Morone saxatilis*, spotted bass *Micropterus punctulatus*, smallmouth bass *M. dolomieu*, and most notably, the northern and Florida strains of largemouth bass *M. salmoides* (Goldberg 2002). Although LMBV has been associated with large-scale fish kills in bass, it has caused no known episodes of epidemic mortality in other species (Plumb and Zilberg 1999a; Goldberg 2002).

The fact that some infected populations of largemouth bass do not suffer mortality suggests that environmental factors play a role in the pathogenesis of LMBV. It is becoming apparent that LMBV tends to strike during the warmest summer months (Goldberg 2002). Physiological stressors that come into play during the summer months include elevated water temperature, low dissolved oxygen, and increased angling pressure. The interaction of such environmental stressors with viral infection may precipitate fish kills. Environmental stressors may reduce the immune capacity of fish and increase the potential for the virus to replicate. Identifying the specific host- and pathogen-related factors that precipitate LMBV fish kills is critical to understanding and managing the disease.

Temperatures outside of normal thermal regimes have been documented to have immunosuppressive effects in fish (Bly et al. 1997; LaPatra 1998). The average habitat temperature range for largemouth bass is from 10°C to approximately 34°C (Davis and Lock 1997). The critical thermal maxima (the temperatures at which death occurs when the environmental temperature is raised 0.2°C/min) for northern and Florida largemouth bass acclimated to 24°C are 36.5 ± 0.5°C (mean ± SD) and 37.5 ± 0.6°C, respectively (Fields et al. 1987).

* Corresponding author: ecgrant@uiuc.edu

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According to Eaton and Scheller (1996), the lethal habitat temperature limit for largemouth bass is 35.5°C.

To assess the effect of temperature on the susceptibility of largemouth bass to LMBV, we performed a challenge experiment, experimentally inoculating the bass with LMBV and holding them at varying temperatures. We hypothesized that temperature differences among treatment groups would affect the mortality caused by LMBV infection as well as the viral loads in infected fish. We also examined the viral replication of LMBV at different temperatures in vitro to determine whether the observed differences in viral loads were due to direct effects of temperature on viral replication or to indirect immunosuppressive effects on fish.

Methods

Study organisms.—The largemouth bass used in the experiment were offspring of broodstock collected from the Kaskaskia River in Illinois. Fish were harvested from experimental ponds owned and operated by the Illinois Natural History Survey, Center for Aquatic Ecology. This population has been maintained in an isolated experimental pond complex for over 7 years and has not been exposed to LMBV. Representative samples of these bass were tested for LMBV by means of standard tissue culture methods and were confirmed to be negative. The fish used in the experiment were less than 1 year old and ranged from approximately 70 to 105 mm in length and from approximately 2 to 13.5 g total weight.

Experimental environment.—Fish were housed in a controlled environmental chamber in 12 identical 75.6-L aquaria (76.2 × 30.48 × 30.48 cm), each equipped with a simple, air- driven sponge filter. No substrate or plant life was included in any of the aquaria. Aquaria were filled with tap water (pH 8.0) that was allowed to age for 3 d before introduction of the fish. The temperature in the chamber was strictly controlled (ambient at 25°C), and the environment included constant humidity (60%) and photo periods (12 h light: 12 h dark).

Four aquaria were randomly assigned to each test temperature. Three were designated as experimental aquaria, and one was a temperature-matched control. Ten fish were placed in each of the aquaria and acclimated for 7 d at 25°C. Submersible, 500-W heaters were placed in 8 of the 12 tanks and the temperature increased (over a 48-h period) to 30°C and 35°C in those tanks. The remaining four aquaria were kept at 25°C. The fish were then acclimated to the elevated test temperatures before being exposed to LMBV. Fish were fed bloodworms (~0.76 g/fish) every morning except for the morning of injection.

Exposure to LMBV.—Largemouth bass virus was cultured in vitro by infecting fathead minnow cells and harvesting virus after the maximum cytopathic effect was observed (Piaskoski et al. 1999). Each experimental fish was injected intracoelomically (along the ventral midline) with 1×10^5 tissue culture infectious doses with 50% endpoint of cultured LMBV diluted in Hank's balanced salt solution (HBSS; injection volume = 0.1 mL/fish). Control fish were sham-injected intracoelomically with 0.1 mL of virus-free cell culture supernatant per fish. Injections were performed using 1-mL tuberculin syringes and 27-gauge, 1.27-cm (1/2-in) needles.

Necropsy.—Fish were monitored closely for 14 d following injection. Once a day, before feeding, all dead and moribund fish were removed from each aquarium and immediately processed in the laboratory. Moribund fish were euthanatized by submersion in a solution of approximately 0.45 L of clove oil and ethanol (1:9) added to approximately 1.5 L of water. The date of death, condition upon collection (dead or moribund), temperature treatment and tank, total length (mm), total weight (g), and presence of gross external or internal lesions were recorded for each fish during processing. Processing consisted of extracting the visceral tissues (liver, spleen, kidneys, stomach, intestines, and swim bladder), diluting these tissues (1 g per 50 mL) in HBSS containing antibiotics (100 units of penicillin, 100 μg of streptomycin, 0.25 μg of amphotericin B, and 50 μg of gentamycin sulfate, all per milliliter), homogenizing this tissue solution with a Stomacher 80 Biomaster automatic stomacher (Steward, Ltd.) for 1 min at normal speed, and finally freezing the homogenate at −80°C for future analysis.

Quantifying viral load.—Viral DNA was extracted from fish tissue homogenates using the QIAamp DNA blood mini-kit (Qiagen, Inc.) according to the recommendations of the manufacturer. A real-time quantitative polymerase chain reaction (qPCR) was used to measure viral load as the number of viral genomes per gram of visceral tissue. The details of the real-time qPCR are given elsewhere (Goldberg et al. 2003).

Measuring viral growth rates in vitro.—Fathead minnow cells were grown to the monolayer stage in fifteen 25-cm^2 cell culture flasks according to

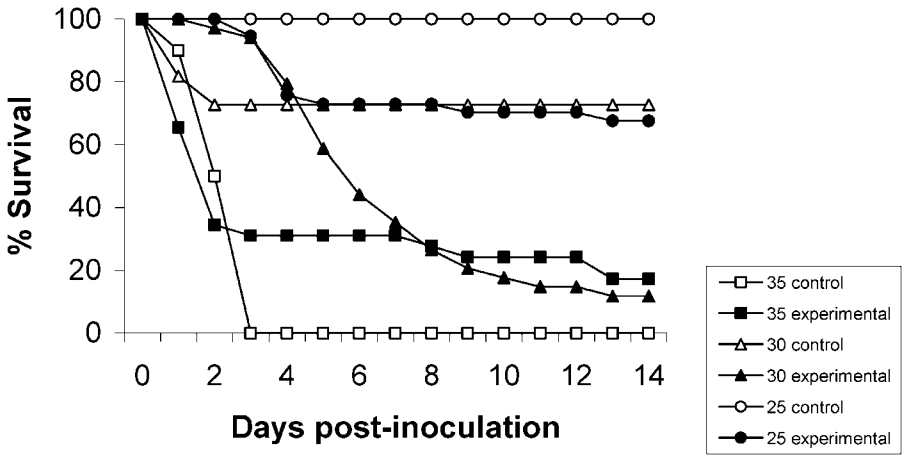

FIGURE 1.—Survival curves for control juvenile largemouth bass and those injected with largemouth bass virus (LMBV) that were held at 25°C (N = 12 and 37, respectively), 30°C (N = 11 and 34), and 35°C (N = 10 and 29).

the methods of Goldberg et al. (2003). Five flasks were randomly assigned to each of the three temperature treatments (25, 30, and 35° C). Four flasks from each temperature were used as experimental replicates, and one was used as a control. Largemouth bass virus was inoculated simultaneously into each flask at a multiplicity of infection of 1.0 on day 0. After inoculation with the virus, each flask (including the controls) was incubated at the appropriate temperature (25, 30, or 35° C). At 24-h intervals beginning on day 0, 250- mL aliquots were removed from each flask. DNA was extracted and quantitative PCR was performed on all aliquots as described above.

Statistical analysis.—Statistical analyses were performed using SAS (SAS Institute 1999). Survival data were analyzed by means of Cox proportional- hazard regression. Student's *t*-tests and multiple linear regression analyses were performed on viral load data. Statistical models included contrasts between LMBV-injected and sham-injected fish exposed to different temperatures; body condition scores (calculated as Fulton's index $\{[\text{weight}/\text{length}^3] \times 10^5\}$; Bolger and Connolly 1989); and the states of the fish at the time of death (i.e., found dead, found moribund, or survived to the end of the experiment). Associations were considered significant at the 0.05 level.

Results

Virus-injected fish held at 30°C began dying on day 2 postexposure. Virus-injected fish held at 25°C began dying on day 3 postexposure. The fish developed clinical signs of viral infection consistent with those described by Plumb and Zilberg (1999b), such as spiral swimming, abdominal distention, and lethargy, before death. Largemouth bass held at 25°C and 30°C experienced cumulative mortalities of 32.4% and 88.2%, respectively, at 14 d. At both temperatures, LMBV-injected fish died more quickly than sham-injected controls (25°C: $\chi^2 = 5.23$, $P = 0.01$ (one-tailed test), df = 1; 30°C: $\chi^2 = 8.50$, $P = 0.002$ (one-tailed test), df = 1). The LMBV-injected fish held at 30°C died 1.3 times faster than virus-injected fish held at 25°C (hazard ratio = 1.30, $P < 0.0001$ (one-tailed test), df = 1; Figure 1).

Virus-injected fish held at 35°C began dying on day 1 postexposure (Figure 1). By day 14, 83% of LMBV-injected fish held at 35°C were found dead or moribund, as were all of the sham-injected control fish held at 35°C. There was no significant difference in the rate at which experimental and control fish held at 35°C suffered mortality ($\chi^2 = 0.18$, $P = 0.68$, df = 1).

External gross lesions observed in the LMBV-injected fish and (to a lesser degree) in the sham-injected control fish included visibly distended abdomens, inflammation at the injection site, and localized or generalized hyperemia. At the time of processing, 26% of the LMBV-injected fish had external gross lesions, compared with only 9% of the sham-injected fish. This difference was statistically significant (Fisher's exact test; $P = 0.03$). The internal gross lesions observed included exudative polyserositis, pneumocystitis, and color changes to various visceral organs, especially the liver. At the time of processing, 49% of the LMBV-injected fish had internal gross lesions, compared

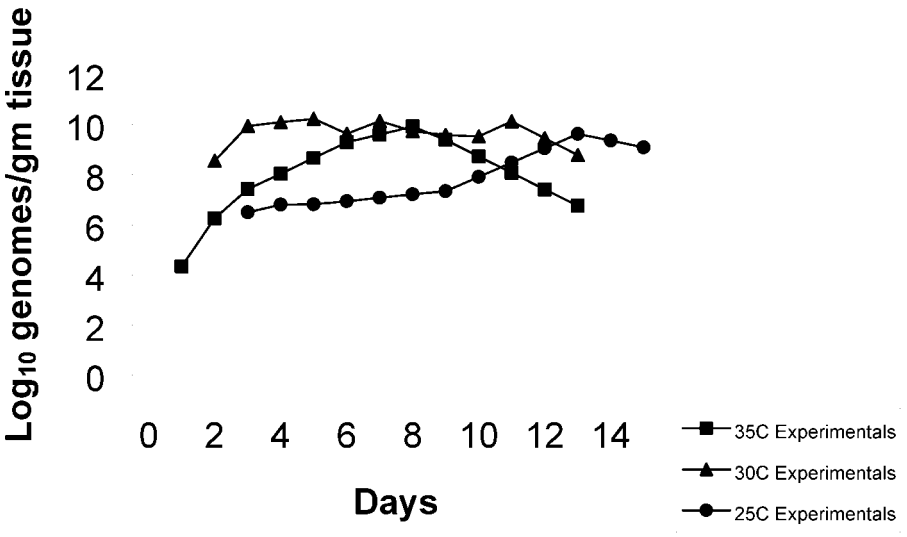

FIGURE 2.—Relationship between viral load and length of survival. The points represent the average viral loads of all fish found dead or moribund on that day.

with only 12% of control fish. This difference was also statistically significant (Fisher's exact test; $P < 0.001$). The mean body condition scores of the experimental and control fish were both 0.90 and not statistically different (t-value = 0.19; $P = 0.85$). While no control fish held at 25°C suffered mortality before day 14, three control fish held at 30°C died by day 3. We attribute the mortality of control fish at 30°C to temperature-related stress, since no evidence of viral infection was found in these fish. Gross necropsy was consistent with this interpretation, since physical lesions were minimal but consistent with starvation in one case.

Multiple linear regression analyses of viral load data indicated that the viral loads of the fish that died or were found moribund during the 14 d of experimentation increased with the number of days that a fish survived postinfection (t-value = 2.56, $P < 0.01$ (one-tailed test), squared semipartial correlation coefficient type II < 0.01; Figure 2). There was no significant difference in viral load between dead and moribund fish (t-value = −0.03, $P = 0.97$, squared semipartial correlation coefficient type II < 0.01). However, fish that were found dead or moribund within 14 d of injection had significantly higher viral loads than fish that survived to the end of the experiment (t-value = 7.06, $P < 0.01$, squared semipartial correlation coefficient type II = 0.06). In both the 25°C and 30°C experimental fish, the presence of internal gross lesions was positively associated with viral load (t-value = 1.69, $P = 0.04$) and Fulton's body condition index was negatively associated with viral load (t-value = −2.08, $P = 0.02$). However, the presence of external gross lesions (t-value = −0.43, $P = 0.67$) was not significantly associated with viral load. Experimental fish held at 30°C had significantly higher viral loads than those held at 25°C (t-value = 9.59, $P < 0.0001$, squared semipartial correlation coefficient type II = 0.11; Table 1).

TABLE 1.—Viral loads ($\log_{10}$ transformed number of viral genomes per gram of tissue [mean ± SE]) of juvenile largemouth bass exposed to equal doses of largemouth bass virus at three different temperatures, by state of fish.

Temperature (°C)	State of fish: Found dead or moribund		State of fish: Survived to end of experiment	
	N	Viral load	*N*	Viral load
25	13	7.19 ± 0.29	24	4.33 ± 0.19
30	30	9.87 ± 0.08	4	6.17 ± 0.38
35	24	5.83 ± 0.36	5	4.39 ± 0.29

Quantitative PCR was also run on the control fish from each treatment. Viral loads were found to be below minimum detection limits and statistically indistinguishable from negative control qPCRs (Goldberg et al. 2003).

Figure 3 shows the rate of replication of LMBV at three different temperatures in vitro. Largemouth bass virus incubated at 25°C did not replicate at a significantly different rate from LMBV incubated at 30°C (t-value = 1.55, $P = 0.13$). Virus

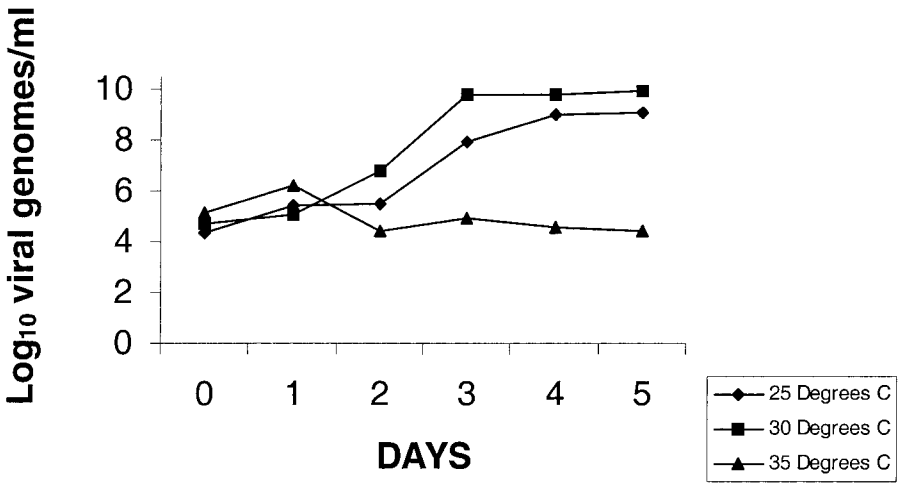

FIGURE 3.—Replication rate of LMBV incubated in monolayers of fathead minnow cells at three different temperatures. The points represent the mean values for four replicate tissue culture flasks per temperature.

incubated at 25°C and 30°C, however, replicated at a significantly higher rate than LMBV incubated at 35°C (t-value = 12.86, $P < 0.0001$), which did not appear to replicate beyond day 1 of incubation. Largemouth bass virus was not detected in control flasks.

Discussion

Largemouth bass injected with LMBV at 30°C experienced higher mortality rates than those injected at 25°C, indicating that elevated ambient temperature increases the susceptibility of fish to the virus under controlled experimental conditions. The mechanism for this increased susceptibility could operate at the level of the host, the pathogen, or both. Elevated temperature could cause immunosuppression in bass or facilitate replication of the virus directly.

Our data also indicate that LMBV replicates more quickly at 30°C than at either 25°C or 35°C, confirming previously published findings (Piaskoski et al. 1999). This pattern indicates that the differences in mortality observed between bass held at 25°C and and those held at 30°C probably resulted from differences in the efficiency of viral replication at these two temperatures. The lack of a statistically significant difference in mortality rate between experimental and control fish held at 35°C is consistent with the observation that 35°C is above the maximum temperature at which LMBV can replicate.

The results of our study, in combination with those of Piaskoski et al. (1999), indicate that 30°C is the optimal temperature for the replication of LMBV both in vivo and in vitro. This result is intriguing because 30°C is at the high end of the range of temperatures under which largemouth bass occur naturally (Eaton and Scheller 1996). It was evident in this study that 30°C was a more stressful temperature not only for the virus- infected fish but also for the control fish.

It has been argued that LMBV was recently introduced as an exotic pathogen of Southeast Asian tropical fish (Mao et al. 1999). A temperature optimum of 30°C for viral replication would be consistent with the hypothesis of a naturally warm climatic origin for the virus. However, channel catfish virus also replicates optimally at 30°C and is not generally considered a ''tropical'' pathogen (Bowser and Plumb 1980). Further data on the biology and epidemiology of this virus are needed to determine its origin.

Fish held at 35°C died at equal rates regardless of exposure to LMBV. Because the lethal temperature limit for largemouth bass is very close to 35°C, the mortality experienced by the fish in this treatment may have been a response to the stress of handling and had little to do with viral infection. In support of this conclusion, and according to the results of quantitative PCR, the viral loads of LMBV-injected fish held at 35°C were lower than those of fish held at the two cooler temperatures.

As the survival time (postinoculation) of LMBV-injected fish increased, the viral load also increased. This observation implies that viral replication is ongoing in infected fish until the time of death. However, the fish from each treatment

that survived to the end of the experiment had lower average viral loads than those found dead or moribund. This observation suggests that viral replication was inhibited in some fish. It is currently unclear why certain fish within treatments were able to withstand viral inoculation while others were not.

There were significant associations between viral load and the presence of internal lesions and body condition but not between viral load and the presence of external lesions. A significantly higher proportion of experimental fish than control fish did, however, display external gross lesions at the time of processing. One explanation for this finding is that the external gross lesions observed in experimental fish were not caused by viral replication but by local inflammatory responses directed against viral proteins or the injection wound. Further investigation into the gross lesions observed in LMBV-injected largemouth bass would be needed to explain the significance of this association.

Fifteen percent of the variance in viral load observed among the fish in this study was accounted for by temperature (25°C versus 30°C). The effect of temperature on survival and viral load is therefore both statistically and biologically significant. These results imply that elevated temperature may be an important factor in precipitating LMBV-associated fish kills in the field. The observation that LMBV-associated fish kills typically occur during hot summer months lends credence to this conclusion.

Temperature, however, is only one factor that might contribute to an increased susceptibility to LMBV during summer months. In North America, warm weather is also associated with low dissolved oxygen levels and high angling pressure. These factors may interact in the field to precipitate fish kills, either by causing immunosuppression in fish or by facilitating viral replication. Further research is needed to confirm the importance of such factors and elucidate the mechanisms by which they increase the susceptibility of largemouth bass to LMBV.

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