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Influence of temperature on Ranavirus infection in larval salamanders *Ambystoma tigrinum*

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ABSTRACT: Temperature strongly influenced percent mortality and time to death of salamanders exposed to the *Ambystoma tigrinum* virus (iridovirus) (ATV). Most salamanders survived when exposed at 26°C, whereas all died at 18°C and nearly all died at 10°C. Some asymptomatic salamanders that survived 60 d at 10 or 26°C were found to be carrying virus. Polymerase chain reaction (PCR) confirmed the presence of virus in ATV-exposed salamanders but was found to be less sensitive than cell culture in detecting ATV at low concentrations. PCR products were 100% identical to ATV in the major capsid protein sequence. Virus titer was higher in salamanders held at 10°C than at 18°C but little virus, if any, was present in the small number of salamanders that died at 26°C. These results may help explain periodic viral epizootics in field populations of *A. tigrinum* where water temperatures fluctuate widely.

KEY WORDS: Epizootic · Cell culture · Iridovirus · Mortality

INTRODUCTION

Massive periodic die-offs of the endangered Sonoran tiger salamanders *Ambystoma tigrinum stebbinsi* in the San Rafael Valley in southern Arizona, USA (Collins et al. 1988), were found to be caused by *Ambystoma tigrinum* virus (ATV) (family Iridoviridae, genus Ranavirus; Jancovich et al. 1997). In the laboratory at 20 to 22°C, salamanders generally die about 10 to 14 d following exposure to ATV. Symptoms include white skin polyps, lethargy, bloody gills, mucus from the cloaca and internal bleeding. Similar viruses have been found in Saskatchewan (Canada), North Dakota, Colorado and several other sites in Arizona (Bollinger et al. 1999, Carey et al. 2003, Docherty et al. 2003, Jancovich et al. 2005).

Ambystoma tigrinum experience daily and seasonal water temperature fluctuations ranging from 5 to 30°C in their natural habitats in Southern Arizona (Snyder 1998). In Northern Arizonan *A. tigrinum* habitats, summer temperatures range from ca. 14°C at 2 m depth to as high as 23.5°C in the littoral zone, with temperatures varying 3 to 5°C during each day (Holomuzki 1986). *A. tigrinum* is reported to prefer 25°C and its activity level increases above 15°C. This salamander has been observed to move to shallow water during the day and deep water at night during the winter, but movement was random during the spring and summer (Lucas & Reynolds 1967, Whitford & Massey 1970, Stauffer et al. 1983).

Environmental temperature influences physiological mechanisms, including metabolism, behavior and potentially immunity of poikilotherms. For example, allograft rejection in newts is significantly delayed at temperatures below 23°C (Cohen 1966). In cell culture, ATV can multiply between 15 and 31°C (Jancovich 1999), which is very close to the reported preferred temperature range of *Ambystoma tigrinum* (16 to 31°C) (Lucas & Reynolds 1967). Our objective in this study was to determine whether infectivity of ATV and survival of infected salamanders differ at 3 temperatures within the range normally found in Arizona salamander habitats in an effort to better understand periodicity of epizootic events in the field.

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MATERIALS AND METHODS

General methods. ATV was cultured using *epithelioma papilloma cyprini* (EPC) cells (Fijan et al. 1983). Plaque and $TCID_{50}$ (tissue culture infectious $dose_{50}$; Reed & Muench 1938) assays employing EPC cells were used to estimate the concentration of virus used in each experiment. Three to 4 mo old *Ambystoma tigrinum nebulosum* larvae originated from stock held in the laboratory for at least 2 generations. No mortality due to possible virus infection was observed in the rearing facility during this period. During the experiments, each salamander was held individually in 300 ml of water in a Zip Lock® (S. C. Johnson) container and water was changed weekly. Salamanders were fed brine shrimp *Artemia* sp. 3 times a week and observed daily. Salamanders that died were stored at –70°C. At the end of each experiment, body wall samples were taken from all dead larvae and tail clips from surviving salamanders for virus detection. Each sample was placed in a Stomacher® (Seward) bag containing 2 ml of Eagle's Minimum Essential Medium (MEM) + 2% Fetal Bovine Serum (FBS) + penicillin-streptomycin-neomycin (PSN; Sigma) + diatomaceous earth powder, and homogenized in a Stomacher device. The homogenate was centrifuged at 9000 × *g* for 10 min and the supernatant was stored at –80°C. Supernatant (100 µl) was inoculated onto EPC cells in each well of 12- or 24-well plates, the preparation was rocked for 1 h and then 1 ml of MEM + 10% FBS + PSN was added. Cells were incubated at 22°C under 5% CO_2 and observed for 2 wk for cytopathic effect (CPE). Samples showing no CPE were passed through 2 further sets of EPC cells to confirm the presence or absence of virus.

To determine the multiplication of virus at the 3 experimental temperatures, replicate EPC cultures in 24-well plates were inoculated with dilutions of ATV and held at 10, 18 or 26°C for 10 to 16 d. Development of CPE was observed and $TCID_{50}$ was calculated during incubation.

In order to confirm the results of cell culture assays, homogenized body wall and tail clip tissue from infected and control larvae were subjected to polymerase chain reaction (PCR). PCR was performed on samples of body wall or tail clip tissue from selected salamanders from Expts 1 and 2 (below) to verify virus identity. In total, 75 specimens were subjected to PCR analysis, including 3 untreated controls. Samples included homogenized tissue used previously for virus culture or tissue samples from salamanders frozen at –70°C. Two 3 mm^2 tissue samples were extracted using 10 µl STE (0.1 M NaCl, 0.05 M Tris-HCl pH 7.5, 0.001 M EDTA disodium) and 1 µl Proteinase K (Promega) and incubated at 58°C for 3 h, then boiled for 5 min to inactivate Proteinase K. DNA was extracted using the GeneReleaser (BioVentures) protocol: 20 µl GeneReleaser, 65°C for 30 s, 8°C for 30 s, 65°C for 1.5 min, 97°C for 3 min, 8°C for 1 min, 65°C for 3 min, 97°C for 1 min, 65°C for 1 min and held at 80°C. Samples were spun for 3 min at 16 000 × *g* and supernatant containing DNA was subjected to PCR. Major capsid protein (MCP) primers 4 and 5 (Mao et al. 1997) were used to amplify approximately 500 bp of the 5′ end of the MCP gene in a MJ Research Peltier Thermal Cycler. The following steps were used: denaturing at 94°C for 5 min followed by 29 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Upon completion, the amplified DNA fragments were visualized by 1.5% agarose gel electrophoresis. Ten PCR products (5 from each experiment), were sequenced using automated equipment (ABI 377) at the Arizona State University DNA laboratory.

Confirmation of the presence of ATV was based upon identical sequence within the 5′ region of the MCP, which differs from that of other published iridovirus sequences (Jancovich et al. 2003). This region exhibits low divergence among 19 isolates from North America but has 21 bp and 9 amino acid differences from the Ranavirus type species, FV3, and greater differences from other iridoviruses (Jancovich et al. 2003, Jancovich et al. 2005).

Expt 1. Thirty 3 mo old salamander larvae were acclimated in refrigerated chambers at 10 or 18°C or in an incubator at 26°C for 2 wk before half of each group was exposed by water bath immersion to 10^3 plaque-forming units (pfu) of virus ml^{-1}. Half of each group was held as untreated controls. Water was changed with water held at the same temperature as the larvae except that water at 18°C was changed with water at room temperature (21°C) due to lack of holding space. This water dropped to 18°C within 10 min. After 60 d of exposure to ATV, half of the surviving salamanders at all the temperatures were euthanized with tricaine methanesulfonate and tested for virus. The other half of surviving salamanders was euthanized after 90 d and tested for virus.

Expt 2. In order to confirm the results of Expt 1 and to determine whether rapid mortality observed at 18°C was due to thermal shock, we performed a second experiment 1 yr later. In Expt 2, the water at 18°C was changed with 18°C water, salamanders were held at 10, 18 and 26°C, and each experimental group consisted of 16 control and 16 treated 4 mo old salamanders. Sixty days following exposure to 10^4 pfu ml^{-1} of ATV, tissue samples from all surviving larvae and all frozen, dead salamanders were tested for the presence of ATV.

Statistical analyses. Systat 10® statistical package was used to analyze data. Days to death were compared within Expts 1 and 2 using 1-way ANOVA; multiple comparisons were made with the Tukey-Kramer procedure. For each tempe-rature, a *t*-test was used to compare percent mortality across experiments assuming unequal variances.

RESULTS

Virus multiplication in cell culture at 3 temperatures

Viral replication, detected as CPE, occurred very rapidly at 26°C, reaching maximum $TCID_{50}$ of 10^7 ml^{-1} within 8 d incubation. Control cells did not survive beyond 10 d at 26°C. Virus titer developed more gradually at 18°C, reaching a maximum titer of $10^{6.5}$ ml^{-1} by 12 d incubation. At 10°C, however, CPE developed very slowly, appearing to plateau at a $TCID_{50}$ of ca. $10^{4.75}$ ml^{-1} by 16 d (Fig. 1).

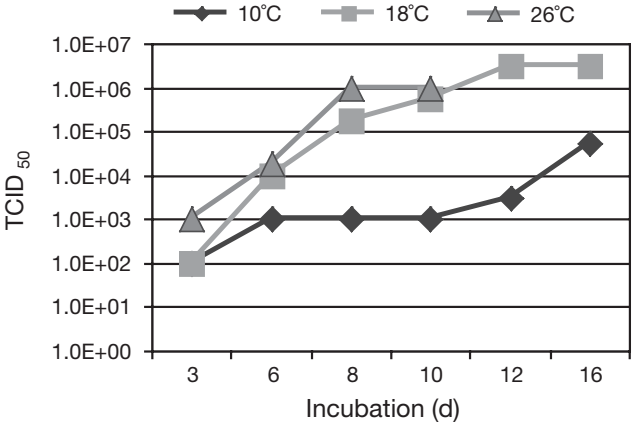

Fig 1. $TCID_{50}$ of *Ambystoma tigrinum* virus (ATV) infected *epithelioma papilloma cyprini* (EPC) cells incubated at 3 temperatures

Expt 1

All control salamanders survived at 10 and 18°C, whereas 20% of the controls died at 26°C. No virus was detected in any control salamander by either tissue culture or PCR. By 60 d after exposure to ATV, 87% of the salamanders had died at 10°C, 100% at 18°C and 33% at 26°C (Table 1, Fig. 2).

At 26°C, the first ATV-exposed salamander died after only 7 d but no virus was found in this animal. Tissue samples from the remaining 4 ATV-exposed salamanders that died at 26°C during the 60 d experimental period demonstrated CPE only after at least 1 wk of incubation, suggesting low virus titer in these animals. Virus was detected in 2 of the salamanders euthanized after 60 d at 26°C only on the second cell culture passage, also indicative of low virus concentration. The remainder of samples from salamanders held at 26°C produced no virus after 3 cell culture passages.

All salamanders exposed to ATV at 18°C died between 15 and 38 d post-exposure (Fig. 2). Of the salamanders held at 10°C, 80% died between 49 and 63 d post-exposure. All salamanders that died during 60 d, and one of the exposed survivors held at 10°C, were positive for virus by both tissue culture and PCR (Table 1). Cell cultures exposed to tissues from salamanders held at 10 or 18°C developed CPE within 2 to 4 d.

Expt 2

As in Expt 1, no control salamanders died at 10 or 18°C but 25% of controls died at 26°C during the 60 d experimental period. All salamanders exposed to ATV at 18°C died between 16 and 28 d post-exposure, and 94% of those exposed at 10°C died between 35 and 56 d post-exposure (Fig. 2). All salamanders that died during the experiment at 10 or 18°C were positive for virus by cell culture assay, but the single surviving individual exposed at 10°C was negative. Of 16 salamanders exposed to ATV at 26°C, 6 died during the 60 d exposure period but only 1 that died and 2 that survived were positive for virus on first passage through cell culture. Virus was detected in one of the 26°C-exposed salamanders after second passage and one at third passage through cell culture (Table 1).

Mean days to death in Expt 1 were not statistically different from results of Expt 2 at $\alpha = 0.05$ (10°C, $p = 0.97$; 18°C, $p = 0.96$; 26°C, $p = 0.17$). In both experiments, mean days to death at 10°C were significantly greater than at 18 or 26°C (Table 1).

In Expt 2, we observed that CPE occurred more rapidly in cell culture

Table 1. Mortality and infection status of *Ambystoma tigrinum* larvae at 3 temperatures

Treatment	Expt 1			Expt 2		
	10°C	18°C	26°C	10°C	18°C	26°C
Control mortality (%)	0	0	20	0	0	25
Exposed mortality (%)	80	100	33	93.8	100	38
Virus-positive (%)	86.7	100	40	93.8	100	31
Mean days (%) to death (SD)	45.8 (4.0)	20.8 (3.5)	15.0 (12.7)	46 (6.9)	20.9 (3.9)	24.6 (4.5)

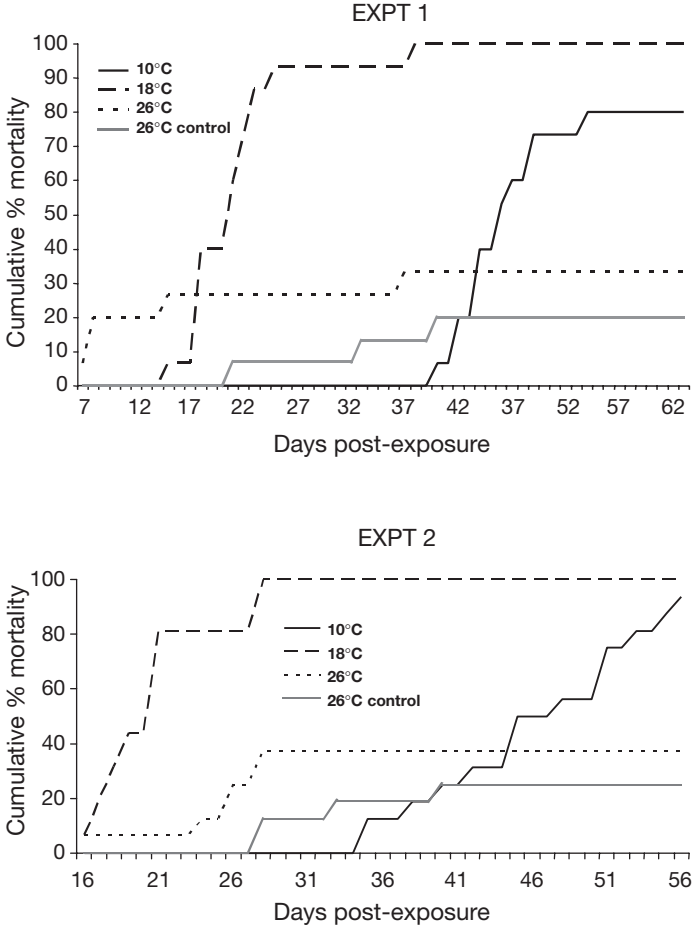

Fig. 2. Cumulative mortality in Expts 1 and 2 at 3 temperatures

assays of specimens that died at 10°C than in ones that died at 18°C, leading to the hypothesis that the salamanders held at 10°C may have developed a higher virus titer than those at 18°C. Samples were taken from the ventral body wall from these salamanders and weighed before homogenization. Samples from 18°C salamanders (X = 0.196 g; SD = 0.046 g) produced $TCID_{50}$ values ranging from $10^{4.5}$ to $10^{6.25}$, with the majority of samples at or below $10^{6.0}$. Body wall samples from salamanders exposed at 10°C (X = 0.276 g; SD = 0.139 g) produced $TCID_{50}$ values ranging from $10^{5.5}$ to $10^{6.75}$, with the majority of samples above $10^{6.0}$. The highest virus titers were found in samples from salamanders that died more than 30 d after exposure at 10°C (Fig. 3).

Salamanders exposed to ATV that eventually died exhibited symptoms of infection similar to those described earlier (Jancovich et al. 1997), including reddening and hemorrhaging on feet and legs, sloughing of skin, heavy mucus excretion from the cloaca, floating and refusal to feed.

Polymerase chain reaction

Salamanders total of 75 specimens were subjected to PCR analysis. All PCR products from salamanders exposed to ATV ran parallel to ATV positive control products on 1.5% agarose gels. A small proportion of these bands ran slightly lower than ATV; however, sequencing revealed 100% identity to ATV in MCP sequence. No control tissue from untreated salamanders produced a PCR product. Tissues from salamanders exposed at 26°C that gave positive results after 3 passages in cell culture did not produce detectable PCR products and none of the samples that were negative in cell culture were positive by PCR. All 10 sequenced products were identical to ATV MCP sequence (Jancovich et al. 2003).

DISCUSSION

We explored the effects of 3 constant temperatures similar to those found in Arizona field sites on survival of *Ambystoma tigrinum* infected with low dosages of ATV. Our results demonstrate that temperature strongly influences the infectivity, percent mortality and time to death of ATV-infected salamanders (Table 1, Fig. 2). Although salamanders may be killed by ATV at high virus concentrations at 25°C (Jancovich 1999), lower dosage exposure more closely mimics the situation in the field, where salamander larvae are probably exposed to virus carried by conspecifics, since no other host or reservoir for ATV has yet been found (Jancovich et al. 2001). Water collected near heavily infected, symptomatic salamanders exposed to ATV in laboratory experiments did not exceed 10^3 to 10^4 pfu ml^{-1} (E. W. Davidson unpubl. data). In cell culture, ATV caused CPE more rapidly at 26°C than at

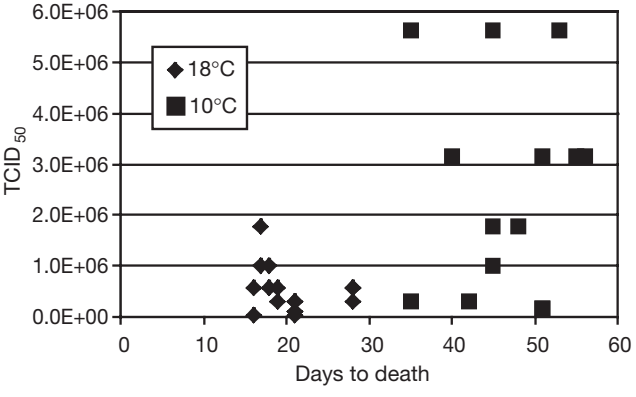

Fig. 3. $TCID_{50}$ of ATV in body wall samples from *Ambystoma tigrinum* infected at 18 or 10°C, versus days to death. Sample weights: 18°C, X = 0.196 g (SD = 0.046 g); 10°C, X = 0.276 g (SD = 0.139 g)

lower temperatures (Fig. 1) and has been shown to multiply in cell culture up to 31°C (Jancovich 1999). Failure of salamanders to develop lethal viral infections at 26°C, therefore, was not due to the inability of the virus to multiply at higher temperatures, but probably to enhanced immune function of the animal itself. ATV was not detected in control larvae by either cell culture or PCR. Samples from infected larvae held at 26°C that required 2 or 3 passages in cell culture for detection of virus were negative by PCR. These results suggest that cell culture is a more sensitive method than PCR for detection of low levels of virus in salamander tissue. PCR confirmed that control mortality at 26°C was not due to inapparent virus infection triggered by higher holding temperatures and that experimentally challenged salamanders indeed died of ATV infection. MCP primers used in this study are capable of detecting and differentiating between many species of Ranaviruses and should have detected such viruses if they were present (Mao et al. 1997, Marsh et al. 2002).

In Expt 1, weekly water changes of 18°C larvae using 21°C water could have led to thermal shock, although 10 min or less were required for the temperature to drop to 18°C. However, mortality at 18°C was quite similar in Expt 2, in which water temperature remained constant, suggesting that thermal shock was not a factor leading to the rapid mortality at 18°C. At lower temperatures, the salamander immune system is apparently less able to inhibit multiplication of the virus. Humoral-/cell-mediated immune defense mechanisms in frogs and some fish have been shown to be reduced at lower temperatures (Green & Cohen 1977, Hardie et al. 1994, Maniero & Carey 1997, Jozkowicz & Plytycz 1998, Watson et al. 1998, Carey et al. 1999). Synthesis of antimicrobial skin peptides by *Rana sylvatica* is inhibited by cold (Matutte et al. 2000), although frog skin peptides can inactivate Ranaviruses at temperatures as low as 0°C (Chinchar et al. 2001). Adult newts *Diemictylus viridescens* required 1.7 times longer to reject allografts at 20°C than at 23°C (Cohen 1966). The change in allograft rejection time mirrors the dramatic difference found in our study between virus-induced mortality and infectivity at 26 and 18°C. Taken together, prolonged allograft rejection and enhanced ATV mortality at temperatures below 20°C suggest that the function of one or more components of the urodele immune system is sharply inhibited below ca. 20°C.

Temperatures at or near 18°C appear to be optimum for ATV to infect and rapidly kill *Ambystoma tigrinum*. The mortality of salamanders at 18°C appears to be a result of the interaction of rapid viral multiplication with reduced host immune response at this temperature. At colder temperatures (10°C), time to death is greater but virus titer per animal is increased. Apoptosis of infected cells may be reduced at lower temperatures (Barker et al. 1997), leading to enhanced virus production per cell. A related ranavirus, Frog Virus 3, induces apoptosis in cultured cells within 6 to 7 h post-infection at 26°C; however, apoptosis at lower temperatures was not tested (Chinchar et al. 2003). Massive die-offs related to ATV observed in the field in the spring and fall may be related to these temperature-influenced interactions between the salamander immune system and viral multiplication.

The 20 to 25% control mortality at 26°C may be due to lowered oxygen concentrations in the small holding containers, increased bacterial content or general stress, as 26°C is near the upper limit of temperatures experienced by *Ambystoma tigrinum* in its natural habitat (Snyder 1998). Although higher temperature may result in recovery from infection, explaining the low viral titer found in 26°C-infected salamanders, it may also provide a greater chance for secondary microbial infections (Watson et al. 1998). No evidence of viral infection was found in the 26°C control larvae by either cell culture or PCR and these salamanders did not display the characteristic symptoms of ATV infection (Jancovich et al. 1997). Our results suggest that if infected salamanders moved to warmer regions of the habitat, such 'behavioral fever' could inhibit viral mortality due to ATV. *A. tigrinum* may select specific temperature regions within a pond, but no evidence of basking was reported (Brattstrom 1963). In response to environmental hypoxia, *A. tigrinum* may seek lower temperatures (Dupré & Wood 1988). Hypoxia may occur in eutrophic ecosystems such as the small ponds that provide habitat for Arizona salamanders, suggesting a possible link between hypoxia, salamanders seeking lower water temperature and viral epizootics.

Four ATV-exposed salamanders in Expt 1, and 2 in Expt 2 survived at least 60 d and were asymptomatic but were found to have low levels of virus as detected by cell culture. These results, combined with other recent results from our laboratory (Brunner et al. 2004) demonstrate that sublethal infections can result in viral carrier status in both the laboratory and the field. Such carrier animals can be important reservoirs for virus infection in the field.

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