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Susceptibility of the Endangered California Tiger Salamander, *Ambystoma californiense*, to Ranavirus Infection

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ABSTRACT: Emerging infectious diseases are implicated in the declines and extinctions of amphibians worldwide. Ranaviruses in the family *Iridoviridae* are a global concern and have caused amphibian die-offs in wild populations in North America, Europe, South America, and in commercial populations in Asia and South America. The movement of amphibians for bait, food, pets, and research provides a route for the introduction of ranaviruses into naive and potentially endangered species. In this report, we demonstrate that the California tiger salamander, *Ambystoma californiense*, is susceptible to *Ambystoma tigrinum* virus (ATV). This virus has not been previously reported in California tiger salamander, but observed mortality in experimentally infected animals suggests that California tiger salamander populations could be adversely affected by an ATV introduction.

Key words: *Ambystoma californiense*, *Ambystoma tigrinum* virus, ranaviruses, tiger salamander.

Amphibians are declining and becoming extinct on a global scale, at a rate exceeding that of birds and mammals (Stuart et al., 2004). Three relatively well understood causes of declines include the introduction of exotic species, commercial overexploitation, and land use change. However, amphibians also have declined in protected parks and reserves where these factors should not be operating, and in such areas, three additional factors are suspected: global climate change, contaminants, and emerging infectious disease (EID) (Collins and Storfer, 2003; Stuart et al., 2004). Emerging infectious diseases have been implicated in amphibian declines in Central America and Australia (Lips et al., 2006; Pounds et al., 2006), and they are increasingly reported in amphibian populations around the world (Retallick et al., 2004; Ron, 2005).

Amphibians are moved globally for bait, food, pets, and research (Jancovich et al., 2005; Schlaepfer et al., 2005), which can potentially move amphibian pathogens from their native range into naïve populations (Cunningham et al., 2003). These pathogens include ranaviruses, such as *Ambystoma tigrinum* virus (ATV; *Iridoviridae*), that have caused amphibian die-offs (Chinchar, 2002; Jancovich et al., 2005). The potential for moving these viruses through the commercial trade of amphibians, such as fishing bait, has been demonstrated by the detection of ranavirus-infected tiger salamander larvae in 85% of surveyed bait shops in Arizona during the 2005 fishing season (Picco, unpubl. data). Jancovich et al. (2005) reported that an ATV isolate from tiger salamanders purchased from a bait shop in Phoenix, Arizona, was most closely related to isolates from Colorado. There is clear evidence that ATV is present in the tiger salamander bait trade, but the impact of potential introduction to naïve hosts is less clear. Thus, we tested whether the endangered California tiger salamander, *Ambystoma californiense*, from which ranaviruses have not been isolated, is vulnerable to ATV.

Adults of the endangered California tiger salamander and three subspecies of *Ambystoma tigrinum* (*A. t. nebulosum*, Arizona tiger salamander; *A. t. mavortium*, barred tiger salamander; and the endangered *A. t. stebbinsi*, Sonora tiger salamander) were experimentally infected with ATV. Three subspecies of tiger salamanders were included in this study to compare mortality and time course of infection between known susceptible species/subspecies and the endangered California tiger salamander. The *A. t. stebbinsi*

and *A. t. nebulosum* were obtained from our breeding colony, and the *A. californiense* and *A. t. mavortium* were provided by H. B. Shaffer (University of California, Davis, California) (12 salamanders of each species/subspecies); all animals were surplus animals that could not be reintroduced into natural populations. The *A. t. mavortium* were originally collected from Clearlake Oaks, California, and the *A. californiense* were from White Slough and Great Valley Grasslands in California. The *A. t. stebbinsi* were laboratory-bred animals from stock originally collected from the San Rafael Valley, Arizona. The *A. t. nebulosum* were offspring of stock originally collected from the White Mountains, Arizona. Sample sizes were limited due to the endangered status of *A. californiense*. Of the 12 individuals from each species/subspecies, six individuals (three males and three females) were assigned to the treatment group, and the other six individuals (three males and three females) were assigned to the control group. Animals from similar localities and clutches were evenly distributed between treatment and control groups to avoid confounding factors associated with locality and clutch.

All animals were individually housed at 20 C in 5.7-l plastic boxes (35 × 21 × 11 cm) placed at an incline filled with 700 ml water on one side, and fed five crickets once per week. Animals were acclimated to laboratory conditions for 2 mo before the experiment. Thirty days before the challenge experiment, tail and toe tissue samples were taken from all animals for ATV screening to verify that animals were not infected before the experiment.

Treatment animals were intraperitoneally injected with 200 µl of inoculum containing 1,000 virions (determined by plaque assay) of ATV suspended in amphibian phosphate-buffered saline (APBS) solution (6.6 g of NaCl, 1.15 g of anhydrous Na_2HPO_4, 0.2 g of KH_2PO_4, and sterile water to 1 l). Control animals were injected with 200 µl of APBS solution. The ATV isolate used was collected from an *A. t. nebulosum* on 23 July 2002 near Prescott, Arizona, and it was grown on epithelioma papilloma cyprini cells (Fijan et al., 1983) for three passes before this experiment (Jancovich et al., 2005). Prescott, Arizona, is an area with salamanders known to be involved in tiger salamander translocations for bait purposes (Collins, 1981).

All animals were observed daily for signs of infection for 60 days and then twice a week for an additional 90 days. Signs of ranavirus infection were recorded, including lesions, papules, and exudates from cloaca (Jancovich et al., 1997). To monitor infections, tail clips were taken from all surviving control and treatment animals each month and screened by polymerase chain reaction (PCR). The experiment was terminated after 150 days, at which point all surviving animals were euthanized with an overdose of MS-222 (Poole, 1987). Upon death, samples were immediately collected from liver, stomach, spleen, intestine, and body wall using sterile equipment for each individual. All samples were homogenized in 2 ml of APBS in individual sterile bags with a Stomacher 80® (Seward Ltd., London, England) and frozen at −80 C for later screening.

DNA was extracted from 150 µl of each of the homogenized samples by using a salt extraction protocol (Sambrook and Russell, 2001). Extracted DNA was tested for the presence or absence of ranaviruses using PCR primers amplifying an ~500 base pair (bp) region of the major capsid protein (MCP) of ranaviruses using primers 4 and 5 described in Mao et al. (1997). Samples were visualized by electrophoresis on 1.5% agarose gels. Quantitative PCR also was used to test animals for ATV infection. Animals were considered infected if either diagnostic or quantitative PCR was positive. The quantitative PCR protocol amplified a 70-bp region of the ranavirus MCP (Brunner,

TABLE 1. Mortality rates and detection of *Ambystoma tigrinum* virus (ATV) in experimentally exposed and control animals of each of four species/subspecies of tiger salamander.

Species	Mortality[a]		PCR results[b]	
	Control	ATV-exposed	Control	ATV-exposed
Ambystoma californiense	2/6	5/6	0/6	6/6
A. t. stebbinsi	0/6	5/6	0/6	6/6
A. t. mavortium	0/6	4/6	0/6	5/6
A. t. nebulosum	1/6	6/6	0/6	6/6

[a] Number dead/number injected.

[b] Number positive/number injected.

2004). Each sample was analyzed in triplicate in 20-μl reactions containing 10 μl of 2× TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, California, USA), 2 μl (100 ng) of template DNA, 300 nmol rtMCP-for (5′-ACACCACCGCCCAAAAGTAC-3′) and 900 nmol rtMCP-rev (5′- CCGTTCATGATGCGGATAATG-3′), and 250 nmol fluorescent probe rtMCP-probe (5′-FAM-CCTCATCGTTCTGGCCATCAACCAC-TAMRA-3′). Quantitative PCR analyses were conducted in 384-well optical PCR plates on an ABI Prism 7900 Sequence Detection system (Applied Biosciences). Negative and positive controls were included in each run for both diagnostic PCR and quantitative PCR. No attempt was made to quantify the amount of viral DNA in the samples; quantitative PCR was only used in this study to detect the presence or absence of ranaviruses.

Differences in mortality rates among species/subspecies were analyzed with likelihood ratio chi-square tests, Kaplan-Meier estimates of mean survival times (number of days to death) were compared with a Wilcoxon sign-rank test, and differences in the number of signs of infection presented by each species/subspecies were analyzed using a Kruskal-Wallis test. All analyses were performed using JMP 5.1 (SAS Institute, Cary, North Carolina, USA).

All six ATV-injected California tiger salamanders displayed signs consistent with ranavirus infection, including papules, hemorrhaging from lesions in the skin, and exudate from the cloaca. Five of the six salamanders died, three of them on day 14, and one each on day 16 and day 18. ATV was detected by PCR in all six of these animals, confirming their susceptibility to ATV infection (Table 1). Two of six control *A. californiense* died on days 2 and 68, but ATV was not detected by PCR in these or any other control animals (Table 1).

Mortality differed between treatment and control animals across all species/subspecies ($\chi^2=25.696$, df=1, $P<0.0001$), but it did not differ among species/subspecies (Table 1; $\chi^2=3.175$, df=3, $P=0.36$). The mean time to death did not differ among species/subspecies ($\chi^2=3.8063$, df=3, $P=0.28$).

California tiger salamanders were infected with ATV; all six ATV-exposed animals became infected and most (five of six) died. The infection and mortality rates of the three *A. tigrinum* subspecies were similar to those of *A. californiense*. Given that we found no difference in mortality rates or time course of infection among the species/subspecies, we would expect mortality in populations of California tiger salamanders to be similar to mortality associated with ranavirus infections in other states if ATV were introduced.

Given the limited number of experimental California tiger salamanders available, we chose to use intraperitoneal injection as our experimental route of

infection. Intraperitoneal injection is not a normal route of infection in wild populations of tiger salamanders, but it was used to demonstrate susceptibility. Further experiments should be conducted to test the susceptibility of California tiger salamanders to ATV infection through more normal routes, such as water bath exposure.

Ranaviruses have been isolated from tiger salamanders throughout the Rocky Mountains and Intermountain West of the United States and Canada as well as from several midwestern states and provinces (Green et al., 2002; Jancovich et al., 2005). Current regulations in many states allow for the unlimited and unregulated collection and movement of tiger salamander larvae as fishing bait. No current estimate exists of the number of tiger salamander larvae sold annually, but in 1968, 2.5 million tiger salamander larvae were sold as bait in the lower Colorado River area alone (Collins et al., 1988). Although California currently prohibits the use of tiger salamanders for fishing bait, it is difficult to enforce such regulations, and there is a risk that tiger salamanders from other locations could be introduced into native populations of *A. californiense*, and with them ATV or other pathogens. In North American locations where ATV has been documented, high mortality rates illustrate the possibility that recurrent epidemics might increase local extinctions and hamper the ability of the larger metapopulation to recover.

Concern about pathogen-related amphibian declines also has prompted a search for surrogate species that might be used as models for threatened or endangered species (Collins et al., 2004). The barred tiger salamander is often used as a laboratory surrogate for studying the endangered Sonora tiger salamander and the endangered California tiger salamander. Our results suggests that the nonthreatened tiger salamander subspecies, *A. t. mavortium* and *A. t. nebulosum*, are suitable models for studying ATV in threatened and endangered tiger salamanders.

Considering the vulnerability of *A. californiense* and other tiger salamanders to ranaviruses, a clear understanding of the risk of ATV introduction is needed. We are currently testing animals in the bait trade for ranavirus infection and describing the patterns of movement of salamanders and their viruses. With this understanding it may be possible to control the spread of this and other diseases that may result from the trade of amphibians.

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