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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Pathogenicity of Frog Virus 3-like Virus in Red-eared Slider Turtles (*Trachemys scripta elegans*) at Two Environmental Temperatures

M. C. Allender*, M. A. Mitchell†, T. Torres*, J. Sekowska* and E. A. Driskell‡

** Department of Comparative Biosciences, † Department of Veterinary Clinical Medicine and ‡ Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802, USA*

Summary

Ranaviral disease has affected several species of reptiles, but disease progression and mortality in relation to environmental temperature has yet to be determined. In this study, two separate trials challenged adult female red-eared slider turtles (*Trachemys scripta elegans*) with a ranavirus (frog virus 3-like virus; FV3) isolate at environmental temperatures of 22°C ($n = 4$) and 28°C ($n = 4$). The mortality rates in the turtles in the 22°C and 28°C trials were 100% and 50%, respectively. Median survival time for turtles exposed to FV3 at 22°C was 24 days, while it was 30 days in the group kept at 28°C. Consistent microscopical lesions were observed only in the group inoculated at 22°C and included fibrinoid necrosis of vessels in the spleen, vascular and sinusoidal thrombi in the liver, necrotizing myositis and a mild heterophilic interstitial pneumonia. Quantitative polymerase chain reaction, targeting a conserved portion of the major capsid protein, was able to detect virus copies in whole blood, oral and cloacal swabs, tongue, skeletal muscle, lung, heart, liver, spleen, ovary and kidney. Viral copy number in ante-mortem clinical samples was non-significantly highest in whole blood, while kidney had the highest viral copy number in post-mortem samples. All samples had higher virus copy number in turtles exposed to FV3 at 22°C compared with 28°C. This study determined that environmental temperature affects the survival and disease progression in ranavirus-infected red-eared slider turtles, which will aid in managing animals in a clinical or free-ranging setting.

Keywords: chelonian; iridovirus; pathogenesis; turtle

Introduction

Wildlife diseases are observed increasingly across the world, with some resulting in a dramatic impact on local populations (Daszak *et al.*, 1999). Ranaviruses are the cause of a disease that threatens biodiversity and which has resulted in over 80 documented epizootics involving reptiles and amphibians since the early 1990s (Green *et al.*, 2002; Miller *et al.*, 2011). These outbreaks have occurred throughout the world on five continents (Miller *et al.*, 2011). The majority of the epizootics have involved amphibians, but there have also been increases in cases in chelonians, and specifically box turtles, in the USA (De Voe *et al.*, 2004; Allender *et al.*, 2006; Johnson *et al.*, 2008).

Ranaviruses, a member of the family Iridoviridae, are large, icosohedral, DNA viruses that can be found in an enveloped or non-enveloped form (Chinchar *et al.*, 2009). Frog virus 3 or frog virus 3-like virus (FV3) is the type species in the genus and has accounted for most reports of the known cases of disease in chelonians based on the conservative detection of a major capsid protein (MCP) gene segment. Disease events have been scattered between captive and free-ranging animals with no apparent pattern. Amphibian outbreaks of FV3 infection have been the focus of most studies and much of the disease ecology in chelonians is unknown.

Correspondence to: M. C. Allender (e-mail: mcallend@illinois.edu).

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A previous report describing an experimental challenge in red-eared slider turtles (*Trachemys scripta elegans*) fulfilled Koch's postulates, establishing Burmese star tortoise virus BSRTV (genus *Ranavirus*) as the causative agent of disease (Johnson *et al.*, 2007). However, this study did not evaluate the effect of temperature on the development or outcome of disease. In fish and amphibians, temperature has been shown to have a profound impact on survival and pathology following iridoviral infections (Rojas *et al.*, 2005; Jun *et al.*, 2009).

The aim of this study was to evaluate the role of temperature in the pathogenesis of FV3-like infection in red-eared slider turtles in order to address key gaps in knowledge of the epidemiology of this virus in chelonians.

Materials and Methods

Animals and Husbandry

Animals in this study were sampled concurrently as part of a separate study (Allender, 2012). Sixteen adult female red-eared slider turtles were acquired in groups of eight. Using computer-generated randomization, four animals were assigned to receive virus inoculation and four animals were assigned to remain as uninfected controls in each of the two environmental temperature trials. The first trial was carried out in a 4.8 × 3.65 m environmental chamber (Rheem Puffer Hubbard, Atlanta, Georgia, USA) held at 22°C. This trial was carried out with both the inoculated and control animals in the same room, but separated by a double plastic barrier and 0.6 m wide hallway created between the plastic barriers. The second trial was carried out with the inoculated and control animals housed in separate, but identical, environmental chambers at 28°C. Each temperature trial included a 7-day acclimation period and was terminated at 31 days postinoculation (dpi), at which point surviving turtles were killed. The first trial began in July 2011 and the second began in November 2011. Animals were housed singly in 170 or 190 l plastic enclosures with approximately 80 l of water and a dry docking spot (cinder block). No additional thermal support was provided. There was a 12 h light cycle set to a timer. Animals were fed a commercial aquatic turtle diet (Fluker Farms, Port Allen, Louisiana, USA) every other day. Complete (100%) water changes were performed twice weekly. All activities were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol: 11050).

Clinical signs were evaluated daily and recorded. The presence or absence of clinical signs compatible with those previously described for *Ranavirus* infection or other non-specific systemic disease were documented. Specific clinical signs evaluated daily included: cutaneous abscessation, nasal discharge, ocular discharge, oral plaque and lethargy. Each sign was scored as absent (0) or present (1).

Virus Preparation

A *Ranavirus* isolate originally from an infected free-ranging box turtle in Tennessee was grown to confluence in *Terrapene* heart cells (TH-1) as described by Allender *et al.* (2006). The isolate was characterized using electron microscopy and conventional polymerase chain reaction (PCR) targeting a 531 base pair (bp) conserved segment of the FV3 MCP and was therefore called an FV3-like virus isolate. When cells exhibited 100% cytopathic effects the flasks were frozen and thawed three times, being thoroughly vortexed before and after each freeze cycle. The contents were then transferred to 50 ml conical tubes and centrifuged at 4,000 g for 20 min. The cell pellet was discarded and quantitative PCR (qPCR) was then performed on the supernatant to confirm presence and quantity of viral DNA. Viral titres ($TCID_{50}$) were determined from serial dilutions of virus in TH-1 cell culture in four technical repeats and calculations were performed as previously described (Reed and Muench, 1938). The virus was frozen at −80°C in 5×10^5 $TCID_{50}$ aliquots until the day of inoculation, when they were thawed to room temperature.

Animal Inoculation and Sampling

At 7 days (−7 d) and 4 days (−4 d) before virus inoculation, the turtles were examined, weighed and confirmed to be negative for FV3-like virus through qPCR in whole blood, oral swabs (OSs) and cloacal swabs (CSs). On each day, blood was collected from the subcarapacial sinus, immediately placed into a lithium heparin microtainer and stored at −20°C until analyzed. Additionally, OSs and CSs (plastic handled cotton-tipped applicator, Fisher Scientific, Hanover Park, Illinois, USA) were collected, placed in separate 2.0 ml polypropylene Eppendorf tubes and stored dry at −20°C until analyzed. Quantitative PCR was performed on the whole blood and both swabs from both days to confirm negative status. On day 0, each treatment animal was given 5×10^5 $TCID_{50}$ FV3-like virus (0.6 ml) intramuscularly in the right forelimb. Each control animal was administered an equal volume (0.6 ml) of uninfected TH-1 cell lysate on the same day. Animals were weighed and clinical samples (blood, OS and CS) were

collected from each animal at 3, 7, 10, 14, 17, 21, 24, 28 and 31 dpi unless they were killed prior to the sampling period.

Pathology

Each animal was subjected to necropsy examination separately in a biological safety cabinet. Sterile procedures (ethanol rinse and flame sterilization) were used to collect samples of tongue, right forelimb skeletal muscle, liver, heart, lung, spleen, kidney and ovary for qPCR. Duplicate samples of tissues were collected and placed in neutral buffered formalin. Samples were processed routinely and sections (3 μm) were stained with haematoxylin and eosin (HE). The pathologist was blinded to the treatment and control status of the animals.

Quantitative Polymerase Chain Reaction

Quantitative PCR was performed as described by Allender *et al.* (2012). Briefly, DNA was extracted from whole blood, OS and CS (QIAmp Blood Mini Kit, Qiagen, Valencia, California, USA) and tissues (DNeasy kit, Qiagen) using the manufacturer's instructions. TaqMan-MGB assay (FAM dye label) was performed using forward (5′AACGCCGACCG AAAACTG3') and reverse (5′GCTGCCAAGATG TCGGGTAA3') primers and a probe (CCGGCTTT CGGGC) targeting a 54 bp segment of the MCP of FV3. Assays were performed on dilutions of turtle-derived positive control plasmid of FV3 MCP DNA (5.29×10^9–5.29×10^1 copies/reaction) and a non-template control within each run. All samples were assayed in three repeats using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, California, USA), analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems) and results averaged.

Statistical Analysis

Animals were classified as either inoculated (administered FV3-like virus) or control (administered uninfected cell lysate), then further categorized as either infected (developed disease and qPCR positive) or uninfected (did not develop disease and qPCR negative) for each temperature trial. Sample size was determined using the following a priori information: alpha = 0.05, power = 0.8 and an expected difference in disease prevalence of 50% between the inoculated and control groups (≥75% of the inoculated animals would be infected and ≤25% of the control animals would be infected within the same trial). Descriptive statistics were produced for each continuous variable (i.e. weight, virus copy number) at each time point, including the mean/median, 95% confidence interval (CI)/10–90% percentiles and minimum/maximum. Normality of data was determined using the Shapiro–Wilk test. Associations between disease status and categorical variables (i.e. clinical signs, sample type) were assessed using Fisher's Exact/Chi-squared test. In the experimental design, to account for missing values from individuals that died, only four time points were included in the final analysis: the 'presample' (two pre-inoculation samples were averaged), the immediate 'post-inoculation sample' and the 'terminal sample' (the last sample taken prior to death). The timing of the presample and the post-inoculation samples occurred on the same days for every turtle; however, the final sample was collected at the last time point prior to death (7–31 dpi). Repeated measures analysis (Friedman's for non-normally distributed data or repeated measures analysis of variance [ANOVA] for normally distributed continuous variables described above) was performed over time, independent of treatment and temperature exposure in the turtles. Then, separately, the same analysis was performed for turtles in each temperature and turtle treatment trial, respectively. The sensitivity and specificity of detecting ranaviral infection in clinical samples was determined based on virus detection in the post-mortem tissues. Repeated measures analysis of categorical variables (i.e. clinical signs) were evaluated using a Cochran's Q test and, if significance was found, specific time point differences were further evaluated using the McNemar test. Differences between temperatures (22°C and 28°C) for continuous variables (listed above) at the same time point were evaluated using an independent samples t-test. Life tables were constructed for days to death (median survival time [MST]) in inoculated turtles and those individuals that survived to the completion of the study were censored. Kaplan–Meier estimates were used to determine any difference in survival based on environmental temperature. Statistical significance was considered at $P \leq 0.05$. All analysis was performed using commercial software (SPSS 20, IBM statistics, Chicago, Illinois, USA).

Results

Clinical Features

All turtles were free of disease at the start of the study. Duplicate OS and CS and whole blood samples collected 7 and 4 days prior to inoculation were negative for FV3-like DNA.

Survival. MST for turtles inoculated experimentally with FV3-like virus and maintained at 22°C was 24

days (range 14–30 days) and for turtles maintained at 28°C the median survival was 30 days (range 17–31 days) (Fig. 1). All inoculated turtles in the 22°C trial were killed due to the severity of clinical signs (between 14 and 30 dpi), while only two (50%) animals were killed in the 28°C trial due to severity of clinical signs (on days 17 and 18). The remaining two inoculated animals in the 28°C trial survived with no apparent clinical signs and were killed at the termination of the study (31 dpi). One control animal (turtle number 6) died in the 28°C study due to bacterial septicaemia and was negative for ranavirus. There was no significant difference in MST between environmental temperatures ($P = 0.35$). For turtles in the 22°C trial, there was a significant difference in infection between inoculated and control animals ($P = 0.01$). There was no significant difference in mortality between inoculated and control turtles in the 28°C trial ($P = 0.21$).

Natural History. There were no significant differences in body weights between animals kept at either temperature at the same time points (pre-inoculation, $P = 0.12$; initial post-inoculation, $P = 0.13$; terminal, $P = 0.15$). There also was no significant difference in body weight at any one point between inoculated and control turtles during the 22°C study (pre-inoculation, $P = 0.50$; post-inoculation, $P = 0.43$; terminal, $P = 0.31$) or 28°C study (pre-inoculation, $P = 0.66$; post-inoculation, $P = 0.94$; terminal, $P = 0.90$), although there was a significant trend for increasing body weight in the infected animals at both temperatures (22°C, $F = 11.1$, $P = 0.04$; 28°C, $F = 7.13$, $P = 0.03$) over time and no similar significant trend in the control turtles (Table 1).

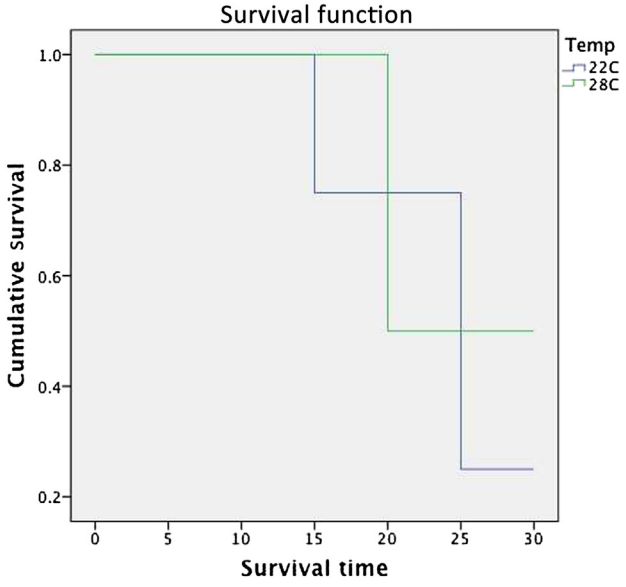

Fig. 1. Survival analysis of red-eared slider turtles infected experimentally with frog virus 3-like virus at 22°C (1, blue) and 28°C (2, green).

Clinical Signs. Descriptive statistics for clinical signs observed in exposed turtles are presented in Table 2. There were no clinical signs recorded that were significantly different between infected and uninfected turtles using repeated measures analysis evaluating each day in the study. However, when evaluating clinical signs between infected and uninfected turtles (both temperatures combined) at the pre-inoculation, initial post-inoculation and terminal time points, there were significant changes over time for nasal discharge ($P = 0.02$) (Table 2), ocular discharge ($P = 0.02$), lethargy ($P = 0.002$) and inoculation site swelling ($P = 0.007$). There were no significant differences observed over time in inoculated animals for the presence of oral plaques ($P = 0.22$) (Fig. 2A) or skin abscesses ($P = 0.13$). The two 28°C inoculated animals that failed to develop disease at the pre-inoculation, post-inoculation or terminal time points did exhibit lethargy (one animal) and leg swelling (both animals). The only clinical signs that were present in any control animal (either temperature) were leg swelling and lethargy. There was no significant change over time in the control animals for lethargy ($P = 0.37$) or leg swelling ($P = 0.13$).

Virology

There were no significant differences in DNA concentration between temperature trials for samples of whole blood (pre-inoculation, $P = 0.82$; post-inoculation, $P = 0.50$; terminal, $P = 0.34$), OSs (post-inoculation, $P = 0.71$; terminal, $P = 0.71$) or CSs (pre-inoculation, $P = 0.54$; post-inoculation, $P = 0.67$;

Table 1
Distribution of body weights of virus-infected turtles

		22°C		*28°C*	
Pre-inoculation (day −7)	Mean (g)	1693.56*		2063.12†	
	95% CI	1244.13	2142.99	1582.69	2543.55
	Min/max	1397.45	2077.50	1654.50	2300.00
Initial post-inoculation (day 0)	Mean (g)	1692.50*		2082.50†	
	95% CI	1252.99	2132.01	1519.77	2645.23
	Min/max	1390.00	2060.00	1606.00	2404.00
Terminal (10–30 dpi)	Mean (g)	1802.50*		2159.50†	
	95% CI	1310.57	2294.43	1686.49	2632.51
	Min/max	1535.00	2245.00	1772.00	2423.00

g, body weight in grams.
*Significant increase over time ($F = 11.1$, $P = 0.045$).
†Significant increase over time ($F = 7.13$, $P = 0.026$).

Table 2
Proportion of infected turtles showing specific clinical signs

	22°C	*28°C*
Nasal discharge	4/4	0/2
Ocular discharge	4/4	0/2
Oral plaque	4/4	0/2
Skin abscess	3/4	1/2
Lethargy	4/4	2/2
Leg Swelling	4/4	2/2

The proportions represent the presence or absence of clinical signs at any time during the course of the study.

terminal, $P = 0.33$) (Table 3). There were significantly higher DNA concentrations in OSs of inoculated turtles compared with control turtles for the pre-inoculation samples ($P = 0.01$). There were no significant differences in DNA concentration over time within whole blood ($P = 0.06$, power = 0.562), OSs ($P = 0.28$, power = 0.180) or CSs ($P = 0.33$, power = 0.155) (Table 3).

All turtles inoculated in the 22°C trial were qPCR positive for FV3-like DNA in whole blood, OS samples, CS samples and each of the eight tissues collected (Table 4). The proportion of positive turtles varied with sample type and date (Fig. 3). Only the two ranavirus-inoculated turtles that died due to severity of clinical signs in the 28°C trial had detectable FV3-like DNA. These 28°C infected turtles were positive in whole blood, OSs and all eight post-mortem tissues, but only one turtle had a positive CS. All control turtles in both temperature trials and the two inoculated turtles in the 28°C trial that failed to develop clinical signs had no detectable FV3-like DNA in any clinical or post-mortem sample at any time point.

There was no significant change over time in the DNA concentration or FV3-like DNA copy number in whole blood, OSs or CSs for either temperature trial ($P > 0.05$).

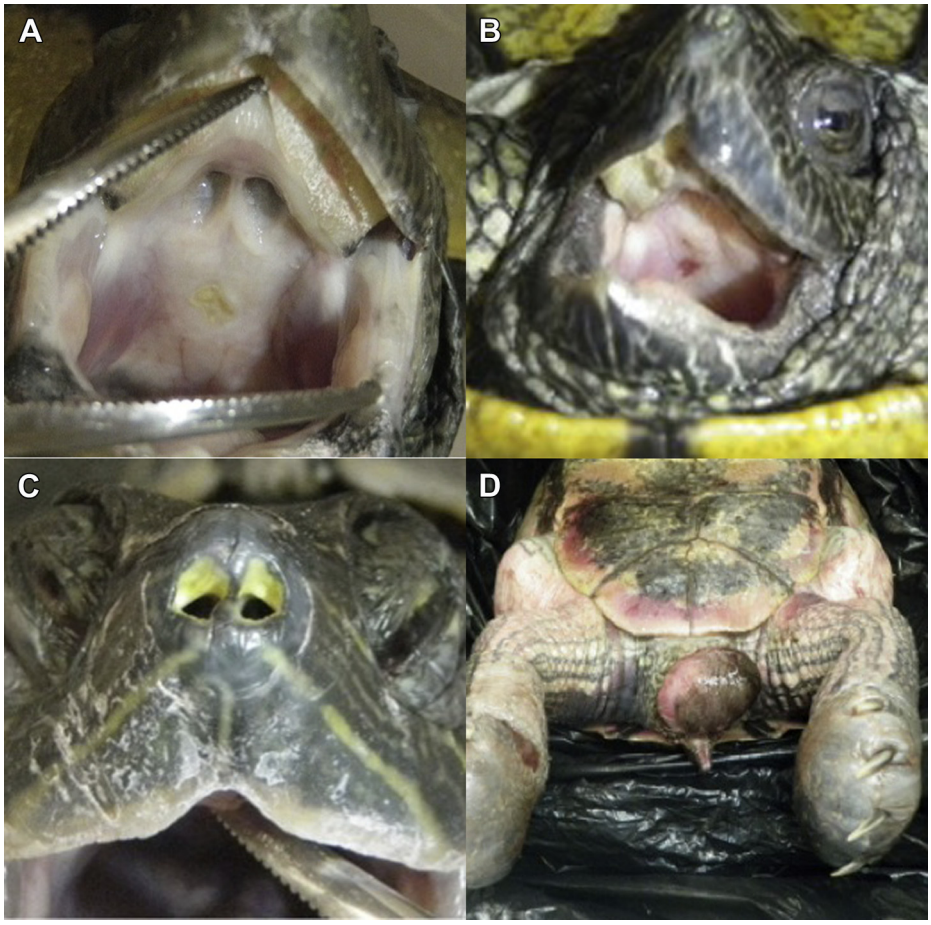

Fig. 2. (A) Oral plaque on the dorsal palate of the oral cavity of a red-eared slider turtle inoculated experimentally with ranavirus in the 22°C challenge trial. (B) Oral ulceration in a red-eared slider turtle inoculated experimentally with ranavirus in the 22°C challenge trial. (C) Widening of the nares observed in a red-eared slider turtle inoculated experimentally with ranavirus in the 22°C challenge trial. (D) Ranavirus-infected red-eared slider turtle demonstrating severe oedema that occurred in less than a 24 h period at 28°C. The animal was also confirmed to be septic by histopathology.

Table 3
DNA concentration and DNA purity from swabs collected from infected turtles

		22°C		28°C	
Concentration (μg/ml) of DNA					
Whole blood	Mean	97.35[*,§]		25.31[*,§]	
	95% CI	27.84	166.86	15.48	35.14
	Min/max	40.1	139.26	17.07	31.98
OS	Mean	26.66[†,§,¶]		12.56[†,§,¶]	
	95% CI	17.9	35.41	7.3	17.83
	Min/max	21.06	33.41	9.71	16.24
CS	Mean	11.39[‡,§,¶]		5.81[‡,§,¶]	
	95% CI	9.11	13.67	3.23	8.38
	Min/max	9.87	12.92	4.44	7.97
DNA purity (ratio of absorbance at 260:280 nm)					
Whole blood	Mean	1.82		1.81	
	95% CI	1.79	1.85	1.75	1.86
	Min/max	1.81	1.85	1.77	1.84
OS	Mean	1.91		1.83	
	95% CI	1.75	2.08	1.77	1.89
	Min/max	1.77	2.02	1.79	1.86
CS	Mean	1.86		1.96	
	95% CI	1.65	2.06	1.71	2.22
	Min/max	1.68	1.99	1.79	2.17

[*]Statistically significant $P = 0.044$.
[†]Statistically significant $P = 0.005$.
[‡]Statistically significant $P = 0.002$.
[§]Statistically higher DNA concentration in whole blood than OSs ($P = 0.026$) and CSs ($P = 0.014$).
[¶]Statistically higher DNA concentration in OSs than CSs ($P = 0.001$).

The sensitivity and specificity of whole blood was 100% when comparing the terminal sample collected with the presence of ranavirus in post-mortem tissues within the same animal. The sensitivity and specificity for OSs was 67% and 100%, respectively, for the same comparison, but 100% and 100% when evaluating the FV3 DNA presence at any time point. CS samples were 83% sensitive and 100% specific when comparing the terminal sample with post-mortem tissues. All infected turtles in the 22°C trial were positive for FV3-like DNA in the terminal sample of whole blood, OS and CS (Fig. 3). In the 28°C trial, both infected animals were positive in whole blood with the terminal sample and both were positive in the penultimate samples with OS samples, but only one had a positive CS sample (terminal sample). Over the course of the last six sampling points, the infected animal in the 22°C trial that survived the longest (30 days) was positive for detectable FV3-like DNA in OS and CS twice, then negative twice and then positive for the last two samples. The PCR was positive in whole blood samples over the entire sampling period.

Table 4
Median virus copy numbers recovered from infected turtles

		22°C	28°C
Pre-inoculation	Whole blood	0	0
	OS	0	0
	CS	0	0
Initial post-inoculation	Whole blood	0	0
	OS	0	0
	CS	0	0
Terminal	Whole blood	1.23×10^7	6.74×10^3
	OS	7.23×10^6	0*
	CS	1.37×10^6	$3.35 \times 10^{3\dagger}$

[*]Both turtles had detectable DNA on the penultimate day before death.
[†]Only one animal had detectable levels.

Pathology

Gross necropsy examination revealed few changes, apart from oral plaques and ulceration of the hard palate in two (25%) animals (Figs. 2A and B), widening of the nares in four (50%) animals (Fig. 2C), intracoelomic fluid in three (37.5%) animals and severe disseminated subcutaneous swelling in three (37.5%) animals (Fig. 2D). Oral plaques and ulcers were observed clinically over the course of the study; however, these lesions had resolved by the time of necropsy examination.

The most consistent and significant microscopical lesions were observed in the spleens of the turtles in the 22°C inoculated trial and were present in all four animals, while the spleens of the control animals were unaffected (Figs. 4A–C). No significant microscopical lesions were observed in any tissue of any control animal in the 22°C group. Microscopical lesions included total effacement of the splenic architecture with haemorrhage, fibrin and heterophils in two of the four turtles (Fig. 4B). Remaining vessels contained abundant fibrin and heterophils in the vessel wall and there was lymphoid depletion (Figs. 4B and C). Liver lesions included thrombi in small vessels and in sinusoids, which was observed in all four turtles of the 22°C group (Figs. 4E and F). All turtles had hepatocellular intracytoplasmic vacuolar change (accumulation of glycogen and lipid) and collections of hepatic melanomacrophages; however, there were no differences in the magnitude of vacuolar change or melanomacrophage aggregates between the animals in the two temperature trials. Capillary thrombosis was present in the lung of one animal in the 22°C trial. Additionally, the lung of two turtles in this trial had a mild to moderate heterophilic

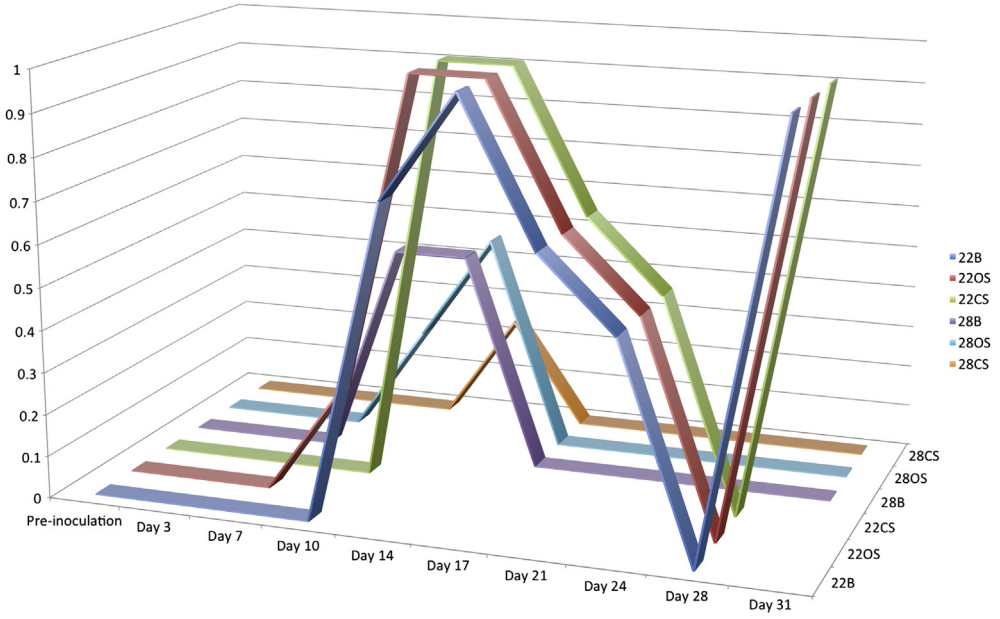

Fig. 3. Proportion (out of the total animals alive at the measured time point) of frog virus 3-like DNA detected in whole blood (B), OSs and CSs at 10 time points before and after experimental inoculation in the six red-eared slider turtles that became infected after FV3 inoculation at 22°C and 28°C. Animals are removed from the graph after they died.

interstitial pneumonia, consisting of heterophils in the faveolar interstitium and increased heterophils in the faveolar capillaries (Fig. 4D). Two of the turtles in the 22°C trial also had necrotizing myositis, characterized by lytic to degenerating skeletal myocytes that were effaced with fibrin and heterophils, while control animals were unaffected (Figs. 4G and H). Rare scattered heterophils were present in the liver and ovary of some turtles.

Microscopical lesions in the turtles kept at 28°C were less striking. The two inoculated turtles in this group that were PCR negative for FV3-like virus did not have any significant microscopical lesions. One of the two turtles that were PCR positive for FV3-like virus had intravascular bacilli in multiple vessels that were associated with thrombi, indicating bacteraemia and sepsis. This turtle also had fibrinoid necrosis of the splenic vessels. The other FV3-like PCR-positive turtle in this group had heterophilic interstitial pneumonia and necrotizing myositis, but no lesions were observed in the spleen. Viral inclusions were not observed in any of the turtles from either temperature group. Turtles from both groups had a few granulomas in various organs, which were centred on spirorchid-type trematode eggs. Additionally, some turtles had rare protozoal cysts within skeletal myocytes, but no evidence of associated necrosis or inflammation. The trematode eggs and protozoal cysts were interpreted as incidental findings.

Eight tissues were collected from each animal at necropsy examination for FV3-like virus qPCR analysis (Table 5). There was a significantly higher DNA concentration in heart tissue in turtles exposed at 22°C compared with those kept at 28°C ($P = 0.01$) and while there was no significant difference in virus copy number found for the other tissues (all $P > 0.05$ for all 63 comparisons), there was a reduction in the number of virus copies found when comparing tissues from animals maintained at 28°C compared with 22°C (Table 5). DNA concentration and purity (absorbance at 260:280 nm) were not significantly different for samples from animals kept at either environmental temperature and therefore the data were combined ($P > 0.05$ for all eight tissue types). There were significantly lower DNA concentrations in heart tissues (38.24 μg/ml) compared with the liver (151.78 μg/ml; $P = 0.02$), spleen (97.18 μg/ml; $P = 0.03$), ovary (71.06 μg/ml; $P = 0.05$) and kidney tissues (109.99 μg/ml; $P = 0.05$). There were significantly higher DNA concentrations in the lung tissues (107.5 μg ml) compared with skeletal muscle tissues (23.85 μg ml; $P = 0.05$). The purity of DNA for lung (1.84), heart (1.72), liver (1.7), spleen (1.86), skeletal muscle (1.72) and ovary (1.79) fell within the range recommended by the manufacturer, but tongue (2.61) and kidney (3.8) were outside the ideal range.

Discussion

This study determined that environmental temperature affects the survival and disease progression in

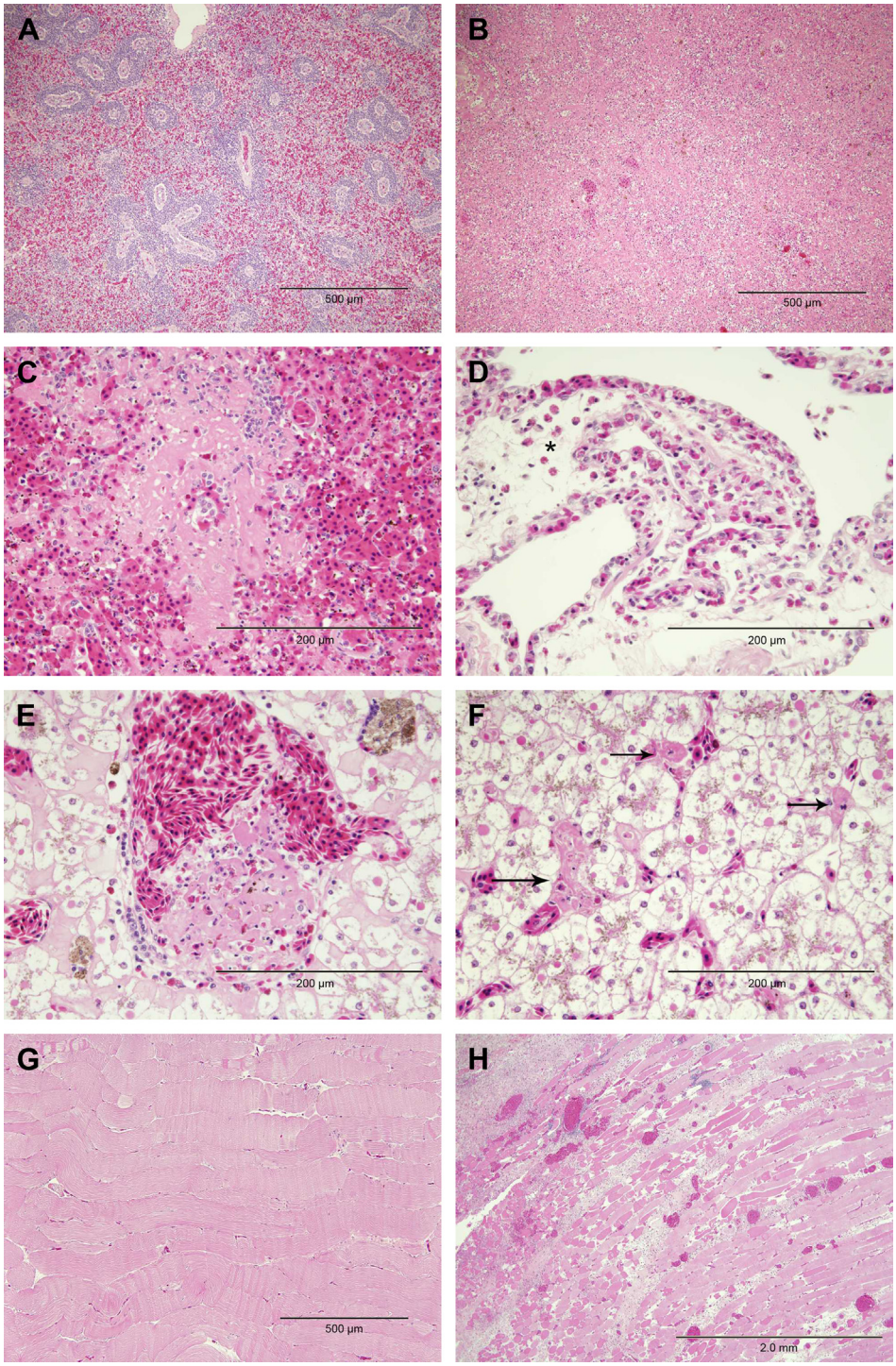

Fig. 4. (A) Red-eared slider turtle that was not inoculated with ranavirus in the 22°C challenge trial with no lesions in the spleen. HE. (B) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with splenic necrosis that effaces the splenic architecture. HE. (C) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with fibrinoid necrosis of a splenic vessel. HE. (D) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with heterophilic interstitial pneumonia. Heterophils fill faveolar capillaries and are present in the interstitium (asterisk). HE. (E) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with a thrombus filling a hepatic vessel. HE. (F) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with thrombi in hepatic sinusoids (arrows). HE. (G) Red-eared slider turtle that was not inoculated with ranavirus in the 22°C challenge trial with no lesions in the skeletal muscle. HE. (H) Red-eared slider turtle that was inoculated with ranavirus in the 22°C challenge trial with necrotizing myositis. Fibrin and heterophils efface the skeletal myocytes and myocytes exhibit necrosis and degeneration. HE.

Table 5
Copies of virus detected per gram of tissue in samples collected at necropsy examination of infected turtles

Tissue	*Value*	*22°C*		*28°C*	
Tongue	Mean/median‡	1.25×10^{9}‡		5.94×10^{6}‡	
	95% CI/10–90% percentiles‡	3.58×10^{5}	2.56×10^{10}‡	3.58×10^{5}	5.94×10^{6}‡
	Min/max	6.19×10^{5}	3.37×10^{10}	3.58×10^{5}	1.15×10^{7}
Skeletal muscle	Mean/median‡	3.7×10^{10}‡		3.64×10^{8}‡	
	95%CI/10–90% percentiles‡	1.76×10^{10}	1.9×10^{11}‡	7.40×10^{7}	3.64×10^{8}‡
	Min/max	1.76×10^{10}	2.39×10^{11}	7.40×10^{7}	6.53×10^{8}
Lung	Mean/median‡	6.29×10^{9}‡		5.01×10^{9}‡	
	95%CI/10–90% percentiles‡	3.27×10^{9}	1.94×10^{10}‡	2.61×10^{8}	5.01×10^{9}‡
	Min/max	3.27×10^{9}	2.32×10^{10}	2.61×10^{8}	9.76×10^{9}
Heart*	Mean/median‡	2.92×10^{10}		1.27×10^{9}‡	
	95%CI/10–90% percentiles‡	1.58×10^{10}	4.36×10^{10}	7.05×10^{6}	1.27×10^{9}‡
	Min/max	1.89×10^{10}	3.72×10^{10}	7.05×10^{6}	2.54×10^{9}
Liver†	Mean/median‡	2.15×10^{9}		1.70×10^{7}‡	
	95%CI/10–90% percentiles‡	1.13×10^{9}	3.16×10^{9}	1.87×10^{6}	1.70×10^{7}‡
	Min/max	1.32×10^{9}	2.85×10^{9}	1.87×10^{6}	3.21×10^{7}
Spleen	Mean/median‡	2.23×10^{10}‡		5.44×10^{7}‡	
	95%CI/10–90% percentiles‡	6.43×10^{9}	1.4×10^{11}	1.52×10^{8}	5.44×10^{8}‡
	Min/max	6.43×10^{9}	1.77×10^{11}	1.52×10^{8}	9.36×10^{8}
Ovary	Mean/median‡	8.93×10^{9}‡		9.06×10^{6}‡	
	95%CI/10–90% percentiles‡	4.53×10^{8}	1.28×10^{10}‡	0	9.06×10^{6}‡
	Min/max	4.52×10^{8}	1.31×10^{10}	0	1.81×10^{7}
Kidney	Mean/median‡	3.46×10^{10}‡		2.54×10^{8}‡	
	95%CI/10–90% percentiles‡	1.77×10^{9}	5.16×10^{10}‡	2.43×10^{7}	2.54×10^{8}‡
	Min/max	1.77×10^{9}	5.69×10^{10}	2.43×10^{7}	4.84×10^{8}

*Significant difference between environmental temperatures, $P = 0.012$.
†Significant difference between environmental temperatures, $P = 0.011$.
‡Value in the row is a median and not the mean; data are not normally distributed.

ranavirus-infected red-eared slider turtles. These turtles are known to be susceptible to experimental infection with ranavirus, but in the reported study temperature was not controlled (Johnson *et al.*, 2007). In the current study, an increase in mortality of the turtles exposed to a ranavirus isolate was seen at the lower temperature compared with uninfected controls held at the same temperature, while a similar significant increase in mortality was not observed at the higher environmental temperature, supporting the hypothesis that temperature has an effect on progression of ranaviral infection in this species. Mortality (50%) still occurred in the inoculated turtles at the higher environmental temperature, but it also occurred in one of the control turtles held at the same temperature, so no significant difference in survival was found. It is possible that with higher sample sizes, a trend towards increased mortality at higher temperatures may still be observed. Increased mortality at low temperature was similarly reported in salamanders (Rojas *et al.*, 2005).

The affected turtles developed a range of clinical signs. The body weight of turtles exposed to ranavirus increased throughout the course of the study at either temperature, but there was no difference in body weight of the control turtles over time. Disease processes in most species lead to anorexia and subsequent weight loss. However, in this study, it is unlikely that increased appetite led to the weight gain because lethargy was a significant finding. It is possible that the vasculitis seen microscopically may have led to leakage of fluid, but this was not observed microscopically and was only noted in three animals grossly. None of those three animals had a significant fluid volume that could be measured. Nasal discharge, ocular discharge, lethargy and inoculation site swelling were also observed in the ranavirus-infected turtles. These signs are consistent with other reports in turtles, but are also consistent with general signs of respiratory disease seen with other pathogens such as *Mycoplasma* spp. and chelonian herpesvirus infection in tortoises (Brown *et al.*,

1994; Christopher *et al.*, 2003; Origgi *et al.*, 2004; Johnson *et al.*, 2005). Another clinical sign reported in box turtles and red-eared slider turtles with ranavirus infection is the development of oral plaques (De Voe *et al.*, 2004; Johnson *et al.*, 2007). While oral plaques were observed in all of the turtles infected at 22°C, they were not seen consistently over time. The turtles would commonly present with an oral plaque that might be present for several days, but then resolve or result in pinpoint oral ulcers on the hard palate. Nevertheless, the severity of the oral plaques was not consistent with other reported cases in box turtles and red-eared slider turtles (Allender *et al.*, 2006; Johnson *et al.*, 2007) or with tortoises with herpesvirus infection (Origgi *et al.*, 2004). This difference may be related to the virulence of the virus, sampling time or technique. While it may not be possible to rely on the presence of oral ulcers as a pathognomonic sign for ranavirus infection in turtles, their presence might be used by a clinician to pursue possible diagnostics (e.g. qPCR) for the disease.

The microscopical lesions of fibrinoid necrosis and vasculitis with thrombosis in multiple organs of inoculated turtles are consistent with previous studies of ranavirus in turtles (Johnson *et al.*, 2007). This study, like others, demonstrated that the spleen was the organ that was most frequently and most severely affected microscopically. Consistent with the clinical and ranavirus qPCR findings, turtles inoculated at 22°C all had severe microscopical changes within the spleen and in contrast, the only turtle inoculated at 28°C had splenic lesions, but that animal was also bacteraemic. Microscopical lesions in other organs for both temperature groups were inconsistent between turtles. Viral inclusions within tissues were not observed in any of the turtles from either temperature group. Although ranaviral inclusions are a prominent feature of infections in some fish, amphibians and turtles, other studies have also found that they are not prominent in ranaviral infections in red-eared slider turtles (Johnson *et al.*, 2007).

Turtles exposed to a temperature of 22°C survived for a median of 24 days in this study and all developed signs of disease, while only two animals in the 28°C trial were killed due to disease. If the study had been maintained past 30 days, the MST in the 28°C trial would likely have been longer and may have resulted in a significant difference between temperatures. Furthermore, there was no DNA evidence of ranavirus in any sample (ante-mortem or post-mortem) from exposed, but uninfected 28°C trial turtles, suggesting that the elevated temperature may have been protective against development of disease in this group of inoculated turtles. In cell culture, ranaviruses do not survive above 32°C and it is likely that as temperatures approach this, the in-vivo mortality will also decrease. This may explain the lack of detectable virus DNA in the two turtles in the 28°C trial that failed to develop disease. These two turtles may have had clearance of the virus through either cell-mediated or humoural immune responses that were protective. Previous studies have demonstrated a very low prevalence of anti-viral antibodies in free-ranging chelonians (Johnson *et al.*, 2010) and this suggests that humoural immunity may not be the predominant mechanism for initial viral clearance. Future studies are needed that address these gaps in knowledge of reptile immunity and response to infection.

Quantitative PCR was able to detect ranaviral DNA in every sample type tested in this study. While detection in post-mortem tissues is invaluable, the use of PCR in determining infection in animals prior to death is equally important. The evaluation of ante-mortem sampling of turtles with ranavirus had not been determined previously. Common clinical samples collected from turtles are whole blood and OS and CS (Allender *et al.*, 2006; Johnson *et al.*, 2007). In the current study, it was determined that whole blood and OSs have 100% sensitivity and specificity and would be appropriate clinical samples for red-eared slider turtles, while CSs had only 83% sensitivity and might therefore lead to false-negative results for these turtles. However, OSs were not positive in the terminal sample despite positive PCR from post-mortem tissues and so OSs might also lead to false-negative results, similar to CSs. Conversely, a cross-sectional survey of box turtles demonstrated that 50% of the positive box turtles were OS positive and whole blood negative (Allender, 2012). Therefore, there may be species differences that lead to differences in virus shedding, differences between experimentally exposed or naturally exposed turtles or differences in assay specificity in other species due to non-specific inhibitors present in box turtles and not in red-eared slider turtles.

The virus copies in the clinical samples (i.e. whole blood, OS and CS) went from undetectable immediately post inoculation to billions of copies within 7–14 dpi. All infected turtles had between two and four positive samples prior to death that corresponded to 1–2 weeks duration of infection. This indicates that ranaviral disease in red-eared slider turtles is an acute process that offers little chance to intervene in a clinical setting and it would be even less likely to be manageable in a wild population. Furthermore, this acute disease duration may lead to disease events occurring in free-ranging reptiles without being detected. Conversely, in solitary animals like the box

turtle, the acute disease process may also lead to minimal interactions with other individuals that could spread the disease.

All eight tissue types tested had detectable ranaviral DNA in all infected turtles, indicating systemic viral distribution. The virus copies in tissues were non-significantly different from the copy numbers in whole blood samples. The highest ranaviral DNA copy numbers were found in skeletal muscle, which is not surprising as this was the site of inoculation. The kidney had the next highest virus copy numbers. The kidney has been proposed as the site of viral persistence in *Xenopus* (Robert *et al.*, 2007). The site of viral persistence has not been established for any other species and further work is required to determine the site or persistence in reptiles. Virus loads were higher in all tissues from turtles in the 22°C study compared with tissues from turtles inoculated at 28°C, but were only significantly different for the heart and liver. There was no correlation between virus copy number and severity of pathology for the examined organs.

Ranavirus has been suggested to be a threat to biodiversity of amphibian populations (Pearman and Garner, 2005; Gray *et al.*, 2009; Teacher *et al.*, 2010; Miller *et al.*, 2011). The effect on reptile populations is less well understood, largely due to a lack of epidemiological studies of ranavirus infection. The red-eared slider turtle has been shown to be a competent model for ranaviral disease; however, whether findings in this species will translate adequately to other species is unknown. The red-eared slider turtle has an expanding range and likely shares habitat with other animals experiencing ranavirus outbreaks, yet there have been few cases of ranaviral infections documented in free-ranging red-eared slider turtles. With the wide species susceptibility to FV3, red-eared slider turtles may actually facilitate the spread of FV3 across landscapes if they are a competent carrier of the virus. Red-eared slider turtles have been shown to spread other pathogens (Verneau *et al.*, 2011) and investigations into this species, as well as others, are needed for the characterization of ranaviral disease ecology.

In summary, red-eared slider turtles infected with a ranavirus isolate exhibit higher mortality and decreased survival time at lower environmental temperatures. However if not initially cleared, through mechanisms still unknown, the clinical course of disease is similar but less severe. Pathological findings of vasculitis are consistent with other reports in turtles. The clinical sample with the highest sensitivity and specificity for diagnosis of the infection in red-eared slider turtles was whole blood and this sample should be collected from suspected cases in the future. This study helps to elucidate the epidemiology of ranavirus in chelonians by demonstrating the importance of temperature differences in the development of FV3 disease in red-eared slider turtles.

Conflict of Interest Statement

The authors declare no conflicting interests.

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