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Vol. 126: 173–183, 2017
https://doi.org/10.3354/dao03172

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published November 21

Serological survey of Australian native reptiles for exposure to ranavirus

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ABSTRACT: Ranaviruses have been isolated from many ectothermic vertebrates, and serological surveys of both amphibians and reptiles have shown the presence of ranaviral antibodies in a proportion of these populations. An enzyme-linked immunosorbent assay (ELISA) was developed to measure serum antibodies against ranavirus in Australian reptiles. The ELISA was validated with serum from challenge trials with Bohle iridovirus (BIV) in 6 reptilian species. A preliminary serosurvey of northern Queensland riparian reptile fauna (saw-shelled turtles *Myuchelys latisternum*, Krefft's river turtles *Emydura macquarii krefftii*, freshwater crocodiles *Crocodylus johnstoni*, as well as the snakes *Boiga irregularis*, *Dendrelaphis punctulatus*, *Tropidonophis mairii*, *Morelia spilota*, *Liasis childreni* and *L. fuscus*) revealed evidence of past exposure to Bohle iridoviral antigens in part of the population at several locations sampled. Furthermore, in Krefft's river turtles and freshwater crocodiles, a statistically significant trend was apparent for larger reptiles to be more likely to have BIV-reactive sera than smaller individuals. The use of adult tortoise populations as sentinels can assist in monitoring the presence of BIV in northern Australian freshwater streams, and thereby the potential dangers to native fauna from this agent.

KEY WORDS: Bohle iridovirus · Serological survey · ELISA · Reptiles · Crocodiles · Turtles · Snakes

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INTRODUCTION

Ranaviruses have been isolated in Australia on 4 occasions from 3 different orders: fish, amphibians and reptiles (Langdon et al. 1986, Speare & Smith 1992, Hyatt et al. 2002, Weir et al. 2012). Bohle iridovirus (BIV) was isolated from wild populations of newly metamorphosed ornate burrowing frogs *Limnodynastes ornatus* in northern Queensland (Speare & Smith 1992), and from magnificent and green tree frogs (*Litoria splendida* and *L. caerulea*) near Darwin in the Northern Territory 2 decades later (Weir et al. 2012).

Experimental infections revealed that BIV is a pathogen of extreme virulence to certain Australian species with a wide host range among local species of amphibians, fish and reptiles (Moody & Owens 1994, Cullen et al. 1995, Ariel et al. 2015). This contrasts with the very few records of natural outbreaks in these experimentally susceptible species, which may be due to the vastness of the Australian wilderness combined with the cryptic nature of most of these species, and the low human population density to witness any such outbreaks. Whatever the reasons for the absence of recorded outbreaks, such absence highlights the need for a survey to investigate the presence of ranavirus in the natural environment.

Most reptiles are predators and likely to preferentially select infected prey (Packer et al. 2003). Assuming this is the case for animals infected with ranavirus, then their predators would be exposed to ranavirus at a high level, and in the case of reptilian

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predators may even become infected. Fish and amphibians inhabit freshwater environments, so reptiles associated with water bodies would be at greatest risk of exposure to ranavirus due to co-habitation with prey species known to be susceptible to ranavirus. If water-associated reptiles survive an infection, they become a suitable target group for surveillance.

Previous ranaviral surveys in amphibians, fish and chelonians have largely focussed on detection of ranavirus directly in individual animals through viral isolation and PCR of non-lethal samples, with varying success rates for different tissues (Greer & Collins 2007, Allender et al. 2011). PCR-based surveys can detect a current infection and possibly carrier animals if the test is sensitive enough (Goodman et al. 2013).

An alternative survey method is to target specific antibodies. Antibody-based surveys are a common and effective tool to detect exposure in both individual animals and populations (Kitching et al. 2007). Furthermore, due to the relative difficulties of capturing and re-capturing wild reptiles, surveys for the presence of anti-ranaviral antibodies increase the chances of detecting exposure to ranavirus in a population, as elevated antibodies titres are known to persist beyond the presence of the antigen (Zimmerman et al. 2010). In other words, elevated serum antibody titres can provide evidence of both current and previous exposure to an infectious agent.

Such a sero-survey, using an indirect enzyme-linked immunosorbent assay (ELISA) has been successfully implemented to detect anti-ranaviral antibodies in wild chelonians and a captive colony that had previously been exposed to a ranavirus outbreak in the USA (Johnson et al. 2010). In that study, low levels of seropositive tortoises were found in the wild (1.5%), as well as in the captive collection (1.8%).

Here we describe a serological survey to assess previous exposure to BIV-like antigens in wild-living reptiles of the common freshwater and riparian fauna of northern Queensland, Australia.

MATERIALS AND METHODS

As part of this study, control reagents were produced in New Zealand white rabbits (Walter Meadow, Melbourne) and BALB-C male mice from the James Cook University mice-breeding programme (Table 1). Six species of reptiles were experimentally challenged with BIV in order to produce positive controls in the ELISA used for the sero-survey of archived samples described below. Rabbits and mice were kept in the JCU Small Animal Unit at standard operating conditions, and the reptiles were kept in the JCU Aquatic Animal Research Facility as described below. All procedures were covered under permit from the JCU Animal Ethics Committee (A302), and a Scientific Purposes Permit (H0/000096/95/SAA) from the Queensland Department of Environment and Heritage.

ELISA configuration

The technique described by Johnson et al. (2010) to prevent non-specific binding of turtle serum was not successful in our trials. However, the final configuration of the ELISA described here for ranavirus-reactive reptile serum used the adhesive strength of the immunoglobulins (Ig) by coating test serum directly to the 96-well plate, and so was able to differentiate between ranavirus reactive and non-reactive reptile serum.

The following ELISA configuration and method were found to be optimal for the detection of sero-reactivity to a BIV-like antigen in reptilian sera. Test sera were complement-inactivated at 56°C for 30 min and diluted 1:50 in carbon bicarbonate buffer (CBC), and 50 µl were coated onto 96-well microtitre plates (Sarstedt) in triplicate and incubated at room temperature (25°C) overnight. The subsequent incubation steps were for 1 h at room temperature, and all wells were washed with Tris-EDTA-NaCl–

Table 1. Reagents produced in this study, with reference to animals used for production and antigens used

Antigen	Animal used for production	Reagent produced (all polyclonal antibodies)
Bohle iridovirus (BIV)	New Zealand white rabbits	Rabbit α BIV
Saw-shelled turtle immunoglobulin	BALB-C male mice	Mouse α saw-shelled turtle immunoglobulin
Krefft's river turtle immunoglobulin	BALB-C male mice	Mouse α Krefft's river turtle immunoglobulin
Freshwater crocodile immunoglobulin	BALB-C male mice	Mouse α freshwater crocodile immunoglobulin
Brown tree snake immunoglobulin	BALB-C male mice	Mouse α brown tree snake immunoglobulin
Green tree snake immunoglobulin	BALB-C male mice	Mouse α green tree snake immunoglobulin
Keelback snake immunoglobulin	BALB-C male mice	Mouse α keelback snake immunoglobulin

Tween-20 buffer (TEN-T) between each step. The following day, 50 µl BIV antigen diluted 1/50 in Tris-EDTA-NaCl–Tween-20–Casein buffer (TEN-TC) were added to all test wells, followed by 50 µl of rabbit αBIV (1:200 in TEN-TC), 50 µl of horseradish peroxidase (HRPO)-conjugated goat αrabbit (BioRad, cat. no. 170-6515) diluted 1:1000 in TEN-TC, and lastly 100 µl of the substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS) (Peroxidase substrate, Kierkegaard and Perry Laboratories), which is converted to a green coloured product in the presence of HRPO.

Controls were included to test for attachment of reptile test serum using 50 µl of species-specific mouse αreptile Ig (1:100 in TEN-TC), which was detected by 50 µl of goat αmouse (1:1000 in TEN-TC) antisera (BioRad, cat. no. 172-1011). Negative control antigen (cell culture without virus) was included to test for non-specific reactivity of the BIV antisera, and BIV without reptile sera was included to test for non-specific binding of antigen to the wells. The optical density of the wells was measured and recorded by a microplate reader (Titretek Multiskan MCC, EFLAB) at dual wavelengths of 414 and 492 nm. ELISA results are expressed as optical density (OD) multiplied by 100. The production of reagents for the ELISA and determination of cut-off levels for each species are described below.

Virus

The BIV isolate was propagated in bluegill fry (BF-2) cells (Wolf et al. 1966) at 26°C using Dulbecco's modified Eagle's medium with 5% foetal bovine serum, 5% bovine donor serum and 1% antibiotics (benzyl penicillin, streptomycin, polymycin B and kanamycin) and fungicide (Fungizone). The virus was harvested at 90–100% cytopathic effect, freeze/thawed 3 times at –20°C and stored at –70°C. The negative control used in the ELISA consisted of un-infected cell cultures prepared with the same protocol.

Polyclonal antiserum to BIV

The polyclonal antiserum for use in the indirect detection of BIV was produced in New Zealand white rabbits (Walter Meadow). Prior to vaccination, the viral preparation was purified in a sucrose gradient (40–70%) by ultracentrifugation (150 000 × *g* at 15°C for 2 h) followed by centrifugation in a potassium tartrate gradient (25–60%) at 150 000 × *g* (4°C for 16 h). The viral preparation (250 µg protein ml^{-1}) and montanide adjuvant (Tall-Bennett) were emulsified in a 1:1 ratio and subsequently re-dispersed in equal volume in a saline phase (2% Tween-80 [BDH Chemicals] in 0.85% NaCl). Rabbits received a total of 1 ml of the multiple emulsion inoculum distributed over 4 subcutaneous sites (1 above each leg). The subsequent 3 injections at intervals of 6 wk contained only the viral preparation. Blood samples were collected 1 wk after the last injection, and rabbit gamma globulins were purified according to the manufacturer's instructions using 3 consecutive precipitations with 35% ammonium sulphate $(NH_4)_2SO_4$ (Sigma Chemical) followed by affinity purification at 4°C in an Affigel blue column (BioRad, cat. no.732-2036).

Polyclonal antisera to reptilian immunoglobulins

For each of 6 species of reptiles (saw-shelled turtle *Myuchelys latisternum*, Krefft's river turtle *Emydura macquarii krefftii*, freshwater crocodile *Crocodylus johnstoni*, brown tree snake *Boiga irregularis*, green tree snake *Dendrelaphis punctulatus*, keelback snake *Tropidonophis mairii*), 1 ml of serum from 3 individuals was pooled and Affi-T (Kem-En-Tec, cat. no. 1340 D) purified at 4°C to separate the gamma-globulins. For each type of reptile serum, we vaccinated 5 BALB-C male mice with 100 µg $mouse^{-1}$ of reptile Ig, mixed in equal volume with Freund's complete adjuvant initially, and with Freund's incomplete adjuvant in subsequent vaccinations at 3-weekly intervals for a total of 4 vaccinations. Serum antibody levels of the mice were checked 3 wk after the final vaccination for reactivity to the specific gamma globulin and cross reactivity to gamma globulins from the other species in this study, before ascites fluid was induced using the method described by Harlow & Lane (1988). When ascites started to develop, the mice were anaesthetised with CO_2 and the ascites harvested with a syringe. The fluid was centrifuged at 800 × *g* and purified in an Affi-T column (Kem-En-Tec, cat. no. 1340D) according to the manufacturer's instructions.

Determination of cut-off levels

Pathogenesis trials were conducted on the 6 species tested in this study (Table 2) in order to calibrate this test to an independent standard, namely that of exposed and non-exposed animals. Turtles were

Table 2. Origin and number of reptiles in each treatment for the 6 species tested

Species	Origin	Treatment	No. of animals	Total
Krefft's river turtle *Emydura macquarii krefftii*	Ross River, Townsville, north Queensland (Qld)	Injected	6	17
		Co-habitation	6	
		Negative control	5	
Saw-shelled turtle *Myuchelys latisternum*	Ross River, Townsville, north Qld	Injected	6	16
		Co-habitation	5	
		Negative control	5	
Freshwater crocodile *Crocodylus johnstoni*	Hartley's Creek Crocodile Farm, north Qld	Injected	6	16
		Co-habitation	5	
		Negative control	5	
Brown tree snake *Boiga irregularis*	Townsville snake relocation programme	Injected	3	10
		Co-habitation	2	
		Oral exposure	3	
		Negative control	2	
Green tree snake *Dendrelaphis punctulatus*	Townsville snake relocation programme	Injected	2	8
		Co-habitation	1	
		Oral exposure	3	
		Negative control	2	
Keelback snake *Tropidonophis mairii*	Giru, 40 km south of Townsville	Injected	2	8
		Co-habitation	2	
		Oral exposure	2	
		Negative control	2	

kept in glass tanks (40 × 60 × 90 cm) with 15 cm deep tap water and access to a basking platform. The turtles were offered raw beef and frozen vegetables 3 times weekly. Crocodiles were housed in 1000 l polypropylene bins (RELN™) and fed a diet of kangaroo and chicken meat 4 times weekly. Snakes were housed in polystyrene boxes (35 × 60 × 20 cm), on bricks in 10 cm deep water in RELN™ bins to prevent ants from entering the boxes. Snakes were fed 2 newly metamorphosed *Lymnodynastes ornatus* froglets twice weekly. The froglets were raised from local tadpoles maintained on a diet of boiled lettuce. Pens were cleaned daily and snakes had access to water at all times. The animals were kept at approximately 25°C.

Stock with a known history of exposure to ranavirus was not available, and antibody levels of experimentally infected animals was therefore assessed in relation to their initial levels and to that of non-exposed animals. Experimental challenge was performed as described by Ariel et al. (2015). Briefly, turtles and crocodiles were assigned to 1 of 3 treatments: (1) a single intra-coelomic injection with 500 µl BIV ($10^{4.5}$ $TCID_{50}ml^{-1}$); (2) co-habitation (intra-coelomic injection with phosphate buffered saline [PBS]) housed with BIV-injected animals and (3) negative control (placebo as for the co-habitation animals). In addition to these 3 treatments, snakes in the oral exposure group were fed 2 infected froglets inoculated intraperitoneally with 50 µl live BIV ($10^{3.5}$ $TCID_{50}$) at 4 d post inoculation. At this dose rate, frogs died between 6 and 10 d after inoculation, and virus was isolated in cell culture from inoculated frogs, while samples from non-inoculated frogs did not produce cytopathic effect in cell culture via the viral isolation technique described by Ariel et al. (2015). The day the infected froglets were eaten by the snakes was considered the first day of the challenge.

Twice weekly for 4 wk after exposure, blood was collected from the femoral vein on alternating sides of adult turtles (Dessauer 1970) and the ventral caudal vein of crocodiles and snakes, using a 25 gauge needle and a 1 ml syringe. Blood was also collected during scheduled necropsy at the end of the trials. Animals were humanely euthanised with an overdose of pentabarbitol (Lethabarb, 2 ml kg^{-1} body weight). The blood sample was left to clot at room temperature for 1 h, and then the clot was dislodged from the tube and left at 4°C overnight. The following day the serum was separated and clarified by centrifugation at 900 × *g* (10 min at 4°C) and stored at –20°C until tested in ELISA.

The OD cut-off levels for non-reactive samples were selected for each species as the 95% confidence limit based on the mean OD of all non-inoculated animals + 2 standard deviations. The maximum level for non-reactive serum was determined to be OD 110 for adults of both species of turtles, OD 100

for freshwater crocodiles and OD 70 for the snakes tested. Sera below the cut-off level were considered to be non-reactive.

Immunisations of adult *E. macquarii krefftii*

In an attempt to produce hyper-immune serum as a positive control for the ELISA, 1 adult male *E. m. krefftii* was repeatedly vaccinated during the winter months of May to September and another male during summer from December to April, as follows: the first inoculum was prepared as described earlier for polyclonal antisera production in rabbits. The turtles were injected in the thigh muscle, intra-coelomic and subcutaneously with 250 µl of the inoculum site^{-1}. The subsequent 5 vaccinations were administered 3 wk apart in the same manner and consisted of virus only.

Sero-survey

The turtles and crocodiles sampled in this study were captured from wild populations during 1994 and 1995 in the Townsville (19° 18′ S, 146° 45′ E) and Mount Surprise (18° 12′ S, 144° 21′ E) regions of northern Queensland and released on site after blood sampling. Samples were also obtained from snakes temporarily held under the Queensland Department of Environment and Heritage snake relocation programme for Townsville. Blood samples were collected and processed as described above. Morphometric measurements for turtles included straight carapace length from anterior border of the nuchal scale to the notch between the 2 most posterior marginal scales (Peters 1963), while the crocodiles were measured from snout to vent and the total length of snakes was measured from snout to tail tip. In total, 86 *M. latisternum*, 52 *E. m. krefftii*, 47 *C. johnstoni* and 76 snakes (19 *B. irregularis*, 18 *D. punctulatus*, 9 *T. mairii*, 21 *M. spilota*, 7 *L. childreni* and 2 *L. fuscus*) were tested.

The proportion of BIV-reactive and non-reactive sera was graphed according to location and year of sampling for the turtles and crocodiles (see Fig. 1) and for each of the snake species tested (see Fig. 2). Two-way ANOVAs without replication were performed between animal size and OD values for *E. m. krefftii* and *C. johnstoni* using Microsoft Excel. The proportion of BIV-reactive and non-reactive sera in each size class of *E. m. krefftii* and *C. johnstoni* was also graphed (see Fig. 3).

RESULTS

Serum levels of experimentally infected animals

The experimental challenge trials did not produce a consistent and measurable sero-conversion result for exposed individuals of the 6 reptile species tested, although certain individuals appeared to sero-convert.

Three of 6 BIV-injected adult *Emydura macquarii krefftii* turtles produced a detectable increase in serum antibody against BIV during the trials. The increase was first detected on Day 14 post inoculation (pi) for 2 of these turtles, and reactivity of subsequently sampled sera persisted in the BIV-reactive spectrum for the remainder of the experiment. The samples from the third turtle did not show sero-conversion until the final sampling on Day 26 pi. None of the other turtles appeared to sero-convert after inoculation with BIV or co-habitation exposure to BIV-inoculated turtles during the course of this experiment.

All serum samples collected from the 2 adult male *E. m. krefftii* turtles that were repeatedly injected with BIV remained sero-negative using the ELISA described here.

The only snake treatment group that showed a detectable increase in serum antibodies against BIV was *Dendrelaphis punctulatus*, which was fed with infected frogs: 3 out of 3 snakes had an increase in antibody by >50 OD, which was first detected on Day 15 pi and remained elevated for the following 2 wk. No other snakes, nor crocodiles in challenged or negative control groups, produced a detectable increase in serum antibody against BIV over the course of the trials.

Serum survey

Turtles

Of the *E. m. krefftii* turtles sampled (n = 15) from the Ross River in 1994, 27 % had BIV-reactive serum. In 1995, more sites and turtles (n = 37) along the Ross River were sampled, and the prevalence of BIV-reactive serum was 41 % (Fig. 1). Only 2 turtles were recaptured in 1995, and their sera were non-reactive in both years.

Serum antibody reactivity to BIV was detected in 50 % of individual *Myuchelys latisternum* turtles (n = 6) in the Lynd River in 1994 and 59 % of turtles sampled in 1995 (n = 80; Fig. 1). None

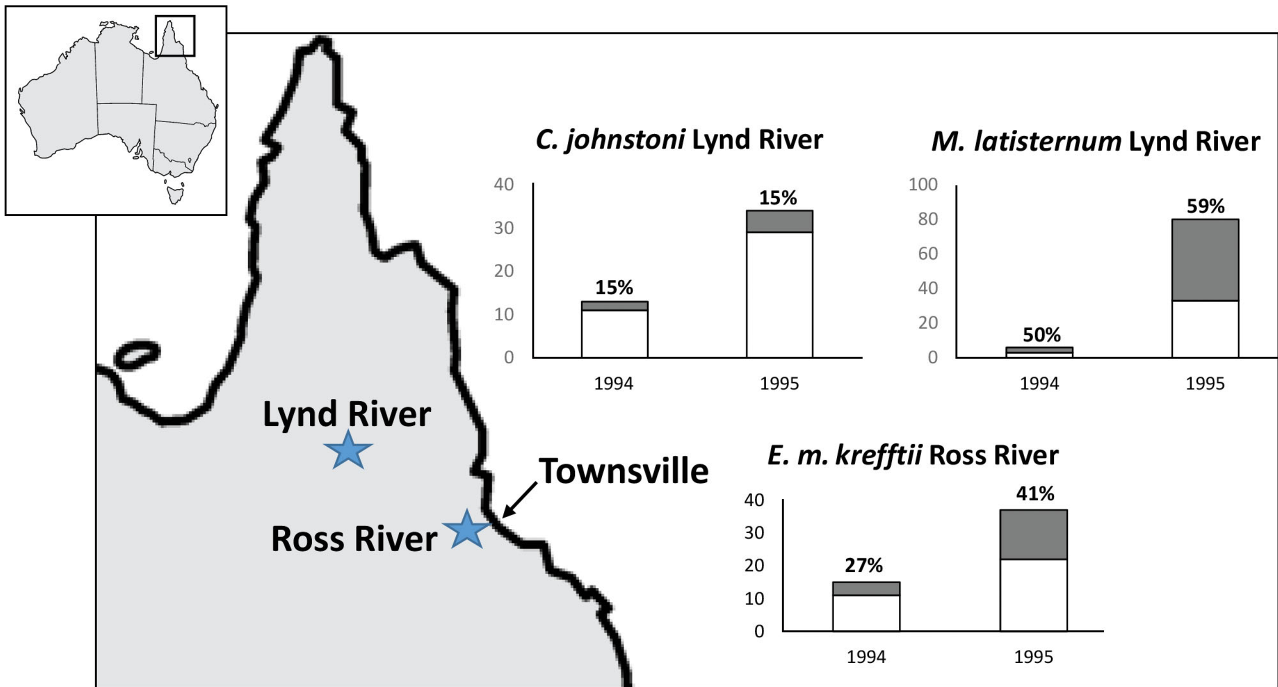

Fig. 1. Distribution of samples with Bohle iridovirus (BIV)-reactive (dark grey) and non-reactive sera (white) in freshwater crocodiles *Crocodylus johnstoni* (n = 47), saw-shelled turtles *Myuchelys latisternum* (n = 86) and Krefft's river turtles *Emydura macquarii krefftii* (n = 52) captured in the Lynd and Ross Rivers during 1994 and 1995. Percentages refer to the proportion of reactive sera in the sampled population in each year and location

of the turtles in 1995 were recaptured from the previous year.

Crocodiles

From the Lynd River, 15% of *Crocodylus johnstoni* sampled had BIV-reactive serum in both years (n = 13 and 34, respectively); however, the majority of the sera were non-reactive (Fig. 1). No crocodiles were recaptured in 1995. The Hartley's Creek crocodiles were of known age and classified as yearlings, and approximately half of the samples (44%) were BIV-reactive.

Snakes

A proportion of sera were reactive to a BIV-like antigen in most of the snake species tested (Fig. 2). The exception was the water python *Liasis fuscus* with 2 non-reactive individuals. *Tropidonophis mairii* snakes had the highest prevalence of BIV-reactive sera at 78%, followed by *L. childreni* with 57%, *Morelia spilota* with 33%, *Boiga irregularis* with 26% and lastly *D. punctulatus* with 22%.

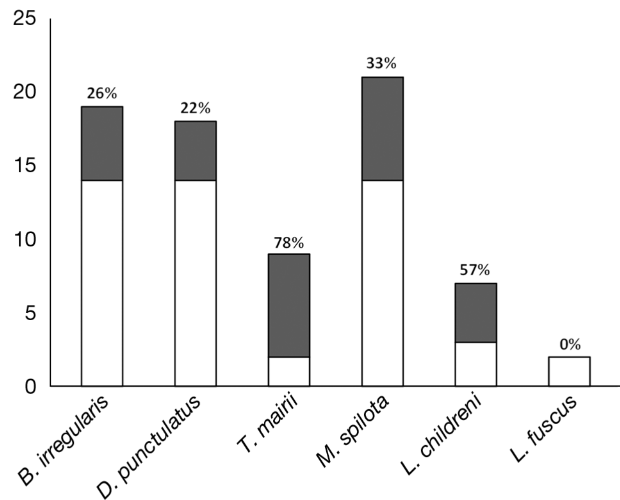

Fig. 2. Distribution of samples with Bohle iridovirus (BIV)-reactive (dark grey) and non-reactive sera (white) in snakes captured in the Townsville region during 1995: *Boiga irregularis* (n = 19), *Dendrelaphis punctulatus* (n = 18), *Tropidonophis mairii* (n = 9), *Morelia spilota* (n = 21), *Liasis childreni* (n = 17) and *L. fuscus* (n = 2)

Size of animals and sero-reactivity

Sufficient data were available for *E. m. krefftii* and *C. johnstoni* to statistically analyse reptile size and

corresponding OD. For *E. m. krefftii*, immune sera were more commonly encountered in larger turtles (Fig. 3A), whereas none of the juveniles (<12 cm SCL) had BIV-reactive sera. In the sub-adult category (12–16 cm SCL, n = 10), only a single individual had reactive serum. In the small adult category (16–20 cm SCL, n = 16), 25% had reactive sera, and in the largest size class (>20 cm SCL, n = 21), approximately half the serum samples (48%) were BIV-reactive. A 2-way ANOVA without replication showed a significant difference in OD titres of BIV-reactive serum proteins among size classes ($F_{4,12}$ = 7.89, p = 0.0023).

A similar pattern was evident for *C. johnstoni*, where sera were non-reactive in the smaller size classes (20–60 cm snout–vent), and the proportion of reactive sera increased over the 2 larger size classes (60–80 and 80+ cm) from 5 to 43% respectively. Furthermore, the reactive samples all had very high titres (250–300 OD × 100). A 2-way ANOVA without replication showed a significant difference in OD titres of BIV-reactive serum between size classes ($F_{1,3}$ = 72, p = 0.0034).

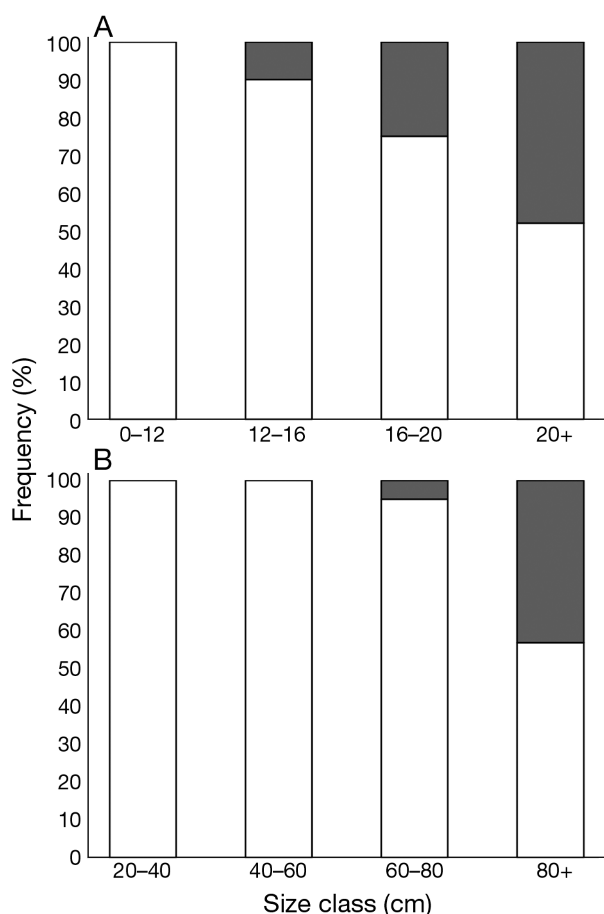

Fig. 3. Proportion with Bohle iridovirus (BIV)-reactive (dark grey) and nonreactive sera (white) of each of 4 size classes of (A) *Emydura macquarii krefftii* (n = 52; straight carapace length) and (B) *Crocodylus johnstoni* (n = 47; snout–vent length)

DISCUSSION

Only a few outbreaks of ranavirus in indigenous Australian species have been reported, despite evidence of high virulence of infection under experimental conditions to a wide range of local species of fish, amphibians and reptiles (Moody & Owens 1994, Cullen et al. 1995, Ariel et al. 2015). The sero-survey described here revealed that parts of the wild reptile populations in northern Queensland tested positive for reactivity to BIV, indicating that ranavirus is regularly circulating in the reptile populations.

The measurable antibody response in sera from reptiles challenged with BIV was highly variable in this study. A proportion of *Emydura macquarii krefftii* turtles sero-converted after 1 intracoelomic exposure, while other individuals failed to convert after a single or repeated injections. None of the *Myuchelys latisternum* turtles, 3 species of snakes and freshwater crocodiles produced a detectable antibody response to IC exposure in this study, while sera from all 3 *Tropidonophis mairii* snakes exposed *per os* had an increase in titre over the course of the experiment. None of the experimental animals discussed here showed clinical signs or evidence of an infection during post mortem investigation (Ariel et al. 2015).

Although the ELISA did not consistently detect an increase in BIV-reactivity in the sera of the experimentally exposed reptiles, an alternative immune response cannot be ruled out. The reptilian innate immune system is known to respond quickly as a non-specific first line of defence against a range of pathogens (Zimmerman et al. 2010), and this could well have been the case for these animals.

Furthermore, the experimental design may not have been conducive to eliciting an antibody response in these reptiles, as many factors can influence this. The snakes which were fed infected prey were more likely to have an antibody response than animals exposed via other routes, indicating that the route of exposure and therefore choice of food could have an influence on pathogenesis and the immune response.

Despite the subdued adaptive response of reptiles in these trials, the wild-caught snakes, crocodiles and turtles had anti-BIV immunity ranging from 15 to 59% of the population surveyed according to species,

location and year of sampling. The relatively high percentage of reactive sera in the samples suggests that individuals are repetitively exposed, able to survive an infection and produce an adaptive immune response. However, it is unknown what the duration of the antibody response and survival rate is, as is the infectious dose, frequency and route of exposure.

Although ranavirus infection of turtles have regularly been reported in case studies (Westhouse et al. 1996, Chen et al. 1999, Marschang et al. 1999), few serological surveys have been conducted that define the occurrence of immunological memory to ranavirus in the wild or in captive collections. One such study of sero-prevalence to ranavirus in free-ranging gopher tortoises *Gopherus polyphemus* in several states in the USA found low sero-prevalence in adjacent populations in Florida (1.2%) and Georgia (3.1%) (Johnson et al. 2010). The study also reported low sero-prevalence (1 of 55 turtles tested) in a captive population where 16 eastern box turtles had died due to ranaviral infection the previous year. The authors noted that the prevalence in the wild populations was likely underestimated if ranavirus was as virulent to these wild species as it was under experimental conditions to red-eared sliders *Trachemys scripta elegans* described by Johnson et al. (2007). In these cases, the relatively low sero-prevalence in highly susceptible species indicates that the majority of individuals tested were not exposed as their chance of survival was low and hence the results of a sero-survey of survivors will not accurately reflect past exposure in the population.

In several species of Australian turtles and frogs, the young age-classes appear to be highly susceptible in trials, while the older age-classes of reptiles are refractory to infection under the experimental conditions tested (Cullen & Owens 2002, Ariel et al. 2015). If ranavirus is persisting in the environment and susceptible individuals become infected, amplify the virus and then fall prey to predators that are resistant to or can survive an infection, then those predators are likely to develop antibodies against ranavirus.

A serum survey of cane toads *Rhinella marina* for ranavirus-specific antibodies in Queensland and New South Wales (NSW) found that the overall prevalence of reactive sera in the surveyed populations was 2.7%, although it ranged from 0 to 18% at different locations sampled (Zupanovic et al. 1998). The cane toad is an introduced species in Australia, and it is suggested that it may be responsible for the spread of ranavirus among the native Australian fauna as the toads advance across the Australian continent from east to west (Henderson 2006).

In south-eastern Australia, Whittington et al. (1994, 1999) likewise documented the widespread occurrence of epizootic haematopoietic necrosis virus (EHNV)-like antibodies in rainbow trout across NSW and Victoria, prior to an invasion of cane toads in those areas. Both EHNV and BIV are ranaviruses and show high cross reactivity in assays using polyclonal antisera (Hengstberger et al. 1993) which was used in both surveys. This suggests that ranaviruses could have been present in Australia before the cane toads and that the native fauna may be relatively resistant to ranavirus, which would explain the high level of sero-reactivity in certain populations. The high proportion of sero-positive animals could also be attributed to reptiles being predators of both amphibians and fish and therefore accumulating more exposure via the food, than, for example, a herbivore.

The proportion of reactive sera in the crocodile and tortoise populations from the Lynd River did not change greatly between years for any of those species. The proportion of individual crocodiles with reactive sera was consistently at 15% and much lower than the turtles inhabiting the same river (50 and 59%), which could indicate that they generally occupy different ecological niches and are differentially exposed to ranavirus. Furthermore, it appeared that individual reptiles were exposed, while others either did not raise an immune response or were not exposed via a suitable route. The proportion of turtles surveyed in the Ross River with reactive sera did increase from one year to the next, but the range of locations where turtles were captured were also expanded, so this is not necessarily a reflection of increased exposure of the population, but it does confirm the presence of ranavirus in the Ross River that runs through Townsville.

E. m. krefftii turtles and freshwater crocodiles showed a trend for larger reptiles to be more likely to have BIV-immune sera than the smaller reptiles. This size differentiation could be caused by a number of factors. Ariel et al. (2015) reported that hatchling turtles died after challenge with live ranavirus, while the adults lived, which could have been caused by a relatively immature immune response in the hatchlings. This seems to support the hypothesis of differential age susceptibility, where the gradual maturation of the immune system could result in increasing numbers of resistant individuals with age (Borysenko 1978). An ontogenic diet shift from non-infected prey to potentially BIV-infected prey (Kennett & Tory 1996, Tucker et al. 1996) may equally account for the pattern of a higher proportion of larger reptiles with BIV-reactive sera. Alternatively, larger animals

may simply have accumulated more exposure than smaller animals. A fourth factor is the difference in behaviour of small and large animals in these ecosystems, which could influence both exposure to ranavirus and the ability to control an infection.

The efficiency of the reptilian immune system is directly related to temperature and decreases with a reduction in ambient temperature (Mondal & Rai 2001, Kurath & Adams 2011), while many ranaviral isolates show optimal growth patterns at around 28°C (Ariel et al. 2009). Body core temperature of turtles could therefore be pivotal in the balance between the immune system and BIV replication, thereby influencing the virulence of an infection as previously reported for turtles, amphibians and fish (Whittington & Reddacliff 1995, Rojas et al. 2005, Allender et al. 2013).

Reptiles have the possibility of increasing their body temperature in a process called behavioural fever (Zimmerman et al. 2010). Basking in the sunlight can increase the body temperature of reptiles temporarily by as much as 10°C above surface water temperatures and would influence the kinetics of the immune response (Auth 1975, Merchant et al. 2007). Basking is more common in adult turtles and crocodiles (Seebacher & Grigg 1997, Dreslik & Kuhns 2000), which could further increase their chances of surviving an infection. In contrast, juveniles tend to be cryptic and hide among the weeds in what is assumed to be a cooler environment (Tucker 1997).

The snake survey did not target species associated with water, but merely tested all non- or mildly venomous snakes available in the Townsville relocation programme. Information regarding prey items and exact location of capture was not available and this may have varied greatly between individuals of the same species. The species of snake with the highest proportion of sero-reactive samples was the keelback snake (78%), which is an excellent swimmer due to the keels on individual scales and it is well known for eating fish and frogs in and near water (Llewelyn et al. 2010). The green tree snake eats predominantly frogs and lizards, while the other snake species may opportunistically eat frogs, but mainly target non-aquatic prey species (Shine 1991). In each of these species, a proportion of snakes was seropositive among those sampled, supporting the notion that ranavirus regular occurs in and around Townsville.

Amphibians, fish and reptiles are at risk of ranavirus infection, and as a result of co-habitation and predator–prey relationships, these groups could potentially provide reservoirs of infection for one another (Mao et al. 1999, Bayley et al. 2013, Brenes et al. 2014), so any surveillance and management program would have to address multiple species and orders.

Adult turtles are abundant across the Australian continent (Georges & Thomson 2010). They are easily captured and may survive exposure to BIV while producing antibodies at levels detectable by ELISA. They are therefore a potential indicator species for a past presence of BIV in a particular freshwater environment. The use of such sentinel turtles can facilitate a survey for the presence of ranavirus in a location and help to clarify the geographical distribution of ranavirus and the potential threat to other native fauna from this agent.

Although the duration of an antibody response and natural route of exposure for ranavirus in reptiles require further studies, the results reported here indicate that the freshwater and riparian reptiles of northern Queensland are regularly exposed to ranavirus and that larger animals and individuals eating infected prey are more likely to have a high titre of serum antibodies against ranavirus.

Acknowledgements. We gratefully acknowledge the following people: Dr. G. Hutchinson for scientific advice, Dr. C. Limpus for access to samples during the Mt. Surprise freshwater crocodile study, Dr. M. Hero for help in catching keelback snakes, M. Pople for access to snakes in the Queensland Department of Environment and Heritage snake relocation programme for blood sampling, Dr. N. Moody for purification of BIV antigen for polyclonal antiserum production and Professor Jacque Robert for comments on an earlier draft of the manuscript. E.A. was supported by an Australian Postgraduate Research Award.

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Editorial responsibility: James Jancovich, San Marcos, California, USA

Submitted: March 2, 2017; Accepted: August 31, 2017
Proofs received from author(s): October 29, 2017