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Giant toads *Bufo marinus* in Australia and Venezuela have antibodies against 'ranaviruses'

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ABSTRACT: A serological survey was conducted for antibodies against 'ranaviruses' in the giant toad *Bufo marinus* in Australia and Venezuela. Sera containing antibodies against 'ranaviruses' were found in both countries. In Australia positive antibodies were identified in populations throughout most of the known range of *B. marinus*. Results were confirmed by immunofluorescence and immunoelectron microscopy where a characteristic staining pattern of 'ranaviruses' in infected cells was observed. Whilst a 'ranavirus(es)' has been isolated from populations of *B. marinus* in Venezuela, no virus has been isolated from Australian *B. marinus* populations. The significance of 'ranavirus' sero-positive *B. marinus* in Australia is discussed.

KEY WORDS: Giant toads · *Bufo marinus* Australia · Venezuela · Ranaviruses

INTRODUCTION

The family *Iridoviridae* encompasses 5 genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and the 'goldfish virus 1-like viruses' (Goorha 1995).

Ranaviruses have been isolated from fish and amphibians (Ahne et al. 1997, Zupanovic et al. 1998). Affected species include the common frog *Rana temporaria* and toad *Bufo bufo* in the United Kingdom (Drury et al. 1995, Cunningham 1996), the leopard frog *R. pipiens* and the American bullfrog *R. catesbeiana* in North America (Granoff et al. 1966, Wolf et al. 1968), *R. esculenta* in Croatia (Fijan et al. 1991) and the giant toad *B. marinus* in Venezuela (Zupanovic et al. 1998). In Australia, 2 ranaviruses have been identified. Bohle iridovirus (BIV) was isolated from the ornate burrowing frog *Limnodynastes ornatus* (Speare & Smith 1992, Hengstberger et al. 1993) and epizootic haematopoietic necrosis virus (EHNV) (Langdon et al. 1986) from redfin perch *Perca fluviatilis*. The significance of ranaviruses has been recognised by Hengstberger et al. (1993), Cunningham (1996), Zupanovic et al. (1998), and Whittington & Hyatt (1997) in areas of ornamental fish trade, impact on wild and commercial fish populations and the international trade in amphibians. Inherent in these areas is the concern about the unregulated transport of ranaviruses and the possible associated spread of disease (Laurance et al. 1996, Ahne et al. 1997).

In 1935 *Bufo marinus*, known in Australia as the cane toad, was imported from Hawaii in an attempt to control insect pests of sugar cane. The toads were imported to Gordonvale (Queensland), bred at Meringa (Queensland) and the progeny released in areas around Cairns, Gordonvale and Innisfail (Tyler 1994). The toad's range expanded rapidly through north and east Queensland (Easteal 1983) and into New South Wales and the Northern Territory (Fig. 1). The dense populations of toads and their rapidly expanding range

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1 Normanton
2 Richmond
3 Charters Towers
4 Bowen
5 Marlborough
6 Emerald
7 Chinchilla
8 Kingaroy
9 Miriam Vale
10 Lennox Head
11 Boonah
12 Dotswood Station
13 Mt Isa
14 Borroloola
15 Mareeba
16 Gympie
17 Mossman
18 Ingham
19 Mackay
20 Tiaro
21 Tilpal Station
22 Townsville

Fig. 1. Distribution of *Bufo marinus* in Australia and sites of serum collection. Shaded area represents known distribution of *B. marinus*. Stars represent locations where positive sera were identified

are attributed to abundant food and the lack of co-evolved predators and pathogens. Within Australia the toad is considered a threat to indigenous vertebrates (Covacevich & Archer 1975, Freeland 1985, 1986, 1987). Toads are toxic to predators at all stages of their life cycle; they may compete for food and also act as predators on native fauna. Until recently the transmission of infectious agents from toads was not considered a major threat to Australia's vertebrates.

The significance of the toad's expanding geographical range in respect to the transmission of disease(s) was noted by Hengstberger et al. (1993). These authors speculated that if cane toads are, or have the potential to behave as, vectors for ranaviruses then there is potential for a significant environmental impact. This speculation was supported by subsequent work which showed that BIV was pathogenic to barramundi *Lates calcarifer* and 2 native Australian frog species, *Limnodynastes terraereginae* and *Litoria latopalmata* (Moody & Owens 1994, Cullen et al. 1995). Collectively, the transmission work involving BIV has shown that this ranavirus can infect a range of species and is pathogenic to both amphibians and fish. As BIV is a pathogenic virus to a broad range of species, and as *B. marinus* is expanding its distribution, it is important to know if populations of free-ranging *B. marinus* have been exposed to any 'ranaviruses'.

Work by Whittington & Speare (unpubl. data 1993) indicated that free-ranging cane toads in Townsville (Queensland) had serological evidence of infection with a ranavirus. In this paper we present data on the prevalence of antibodies to 'ranaviruses' in the sera of free-ranging *Bufo marinus* in Australia and Venezuela, and show that infection with 'ranaviruses' is widespread in both countries. The possible implications of these results are discussed.

MATERIALS AND METHODS

Collection of sera. For Australian toads, there were a total of 1115 samples collected from 22 locations throughout the known range of *Bufo marinus* during 1993, 1994, 1996 and 1997. A total of 240 sera from *B. marinus* in Venezuela were also examined. Blood was collected by cardiac puncture from free-ranging *B. marinus* in Australia and Venezuela (Figs. 1 & 2), placed into sterile tubes and allowed to clot at room temperature (RT). Serum was separated by centrifugation (800 × *g* for 10 min) and stored at –20°C.

Detection of antibodies. *ELISA for detection of anti-ranavirus antibody in toad sera:* Sera collected from both Australia and Venezuela were tested at the Australian Animal Health Laboratory (AAHL). Sera collected from the Townsville area of Queensland, Australia, were tested at the Elizabeth Macarther Agricultural Institute (EMAI). The ELISA assays used at the respective laboratories are described below.

At AAHL, the antibody capture ELISA was performed as described below. The toad antibodies used in the ELISA react against EHNV, BIV and Guatopo virus (GV) (unpubl. data). Microtitre plates (Dynatech) were coated with sheep anti-EHNV serum in coating buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 9), incubated

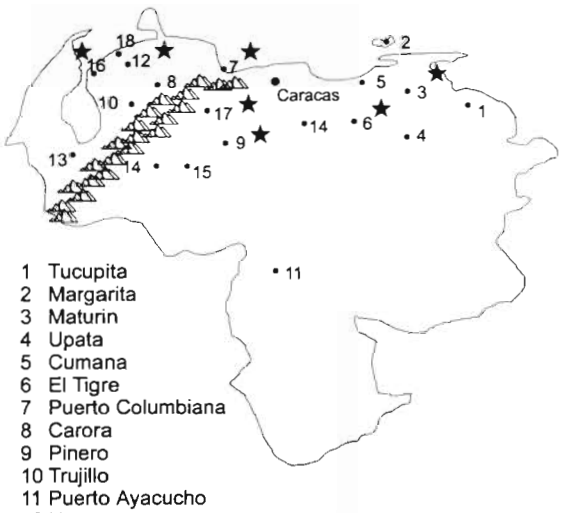

Fig. 2. Sampling sites for the collection of sera from *Bufo marinus* in Venezuela. Stars represent locations where positive sera were identified. The Andes mountain range is indicated

overnight at 4°C and washed (× 3) in phosphate-buffered saline with 0.05% v/v Tween 20 (PBST). Free-binding sites were blocked with 2% gelatin in PBST (30 min, 37°C), washed in PBST, and then incubated with EHNV (60 min, 37°C). Following incubation, the plates were washed (× 3) in PBST and incubated toad sera added for 60 min at 37°C. Following a further 3 washes in PBST, rabbit antisera against IgG were added (60 min, 37°C). Plates were again washed in PBST and incubated with toad anti-rabbit IgG serum conjugated to horseradish peroxidase (Silenus) in PBST with 2% gelatine (60 min, 37°C). After a final wash 42 mmol l^{-1} 3,3',5,5' tetramethyl benzidine in DMSO, citrate acetate buffer pH 5.9 and 3% H_2O_2 was added. The reaction was stopped after 10 min (RT) by the addition of 1 M H_2SO_4 (50 µl $well^{-1}$). Optical densities (OD) were measured at 450 nm (Labsystems Multiscan MS plate reader).

At EMAI the ELISAs were performed as described by Whittington et al. (1997). Reagent volumes were 100 µl per well unless otherwise stated. Purified rabbit antibodies against EHNV were diluted in borate buffer (100 mM boric acid, 25 mM disodium tetraborate, 75 mM NaCl, pH 8.4), added to polystyrene microtitre plates (Linbro, Flow ICN) and incubated overnight at 4°C. Free-binding sites were then blocked using PBST and 1% w/v ovalbumin, incubated for 30 min at RT. After washing plates (× 5) in distilled water with 0.05% v/v Tween 20, cell culture supernatant containing 104.5 to 106.1 $TCID_{50}$ ml^{-1} of either EHNV or BIV was added to each well and incubated for 60 min at RT. After washing plates as above, serum of *Bufo marinus*, which had been diluted in PBST with 0.1% w/v ovalbumin (PBSTO) and stored overnight at 4°C, was added and incubated for 60 min at RT. After washing as above, sheep antiserum against *B. marinus* IgG, diluted in PBSTO, was added and incubated for 60 min at RT. This was followed after washing by rabbit anti-sheep-immunoglobulin horse radish peroxidase conjugate (KPL Laboratories) diluted 1:3000 in PBSTO and incubated for 60 min at RT. After washing, the chromogen 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) was added and the plate was incubated at RT on a shaker. The reaction was stopped after 13 min by the addition of 0.01% w/v sodium azide in 0.1 M citric acid. Absorbance was read at 450 nm without blanking the plate.

All sera were tested with and without ranavirus antigen to confirm the specificity of the reaction.

Immunofluorescence and immunoelectron microscopy: A random sample of toad field sera was tested by immunofluorescence to confirm the validity of the ELISA results. Confluent monolayers of Chinook salmon embryo cells (CHSE-214, ATCC CRL 1681) were infected with BIV (106.5 $TCID_{50}$ ml^{-1}). At 48 h post infection, when approximately 50% of cells showed a cytopathic effect, cells were fixed in cold (–20°C) absolute methanol (3 min) followed by cold (–20°C) acetone (3 min). Cells were rehydrated in PBSA with 1% BSA and Australian field serum (Table 1) added for 45 min at 37°C. Following incubation, the cells were washed (× 3) in PBSA with 1% BSA, incubated with (1:100) rabbit anti-toad IgG serum (45 min, 37°C), washed (× 3) in PBSA with 1% BSA and then incubated with (1:100) sheep anti-rabbit Ig-FITC conjugate (45 min at 37°C). After a final wash (× 3) the cells were covered with 50% glycerol in PBSA/1% BSA and viewed with a Leica Reichert Polyvar light microscope.

BIV was purified as described by Hengstberger et al. (1993) and adsorbed to carbon-coated, parlodion filmed, 400 mesh gold grids. The grids were washed in 1% cold water fish gelatin (Sigma) in PBS for 10 min. Field serum from *Bufo marinus* (Marlborough) were added to the grids (1:1000) for 60 min at RT. Grids were washed (× 6) in PBS, incubated in rabbit anti-toad IgG (60 min, RT), washed (× 6) in PBS and then incubated with 10 nm protein A-gold (60 min, RT). Grids were then washed (× 6) in PBS, stained with 2% phosphotungstic acid (pH 6.8) and viewed in a Hitachi scanning transmission electron microscope at 75 kV.

RESULTS

Antibody capture ELISA

The overall prevalence of antibodies to 'ranaviruses' in Australia was low (2.7%) (Table 1). However, in the

Table 1. Serological survey of Australian cane toads. FAT: fluorescent antibody tests. Numbers are no. positive/no. tested

Region	Location	Location prevalence (ELISA)	Location numbers (ELISA)	FAT	Total regional numbers (ELISA)	Total regional prevalence (ELISA)
Northern NSW	Lenox Head (28.47°S, 153.35°E)	0.4%	1/255	–	1/255	0.4%
S-E Queensland	Boonah (28.00°S, 152.36°E)	18%	6/33	1/1		
	Gympie (26.10°S, 152.35°E)	0%	0/23	–		
	Kingaroy (26.32°S, 151.50°E)	0%	0/29	0/1	6/85	7.1%
Central Queensland	Chinchilla (26.42°S, 150.35°E)	0%	0/2	–		
	Emerald (23.30°E, 148.08°E)	0%	0/27	–		
	Marlborough (22.51°S, 159.50°E)	8%	9/116	1/1		
	Miriam Vale (24.17°S, 151.34°E)	0%	0/33	–		
	Tilpal Station (22.50°S, 150.10°E	0%	0/47	0/1		
	Tiaro (25.43°S, 152.34°E)	2%	2/86	2/2	11/311	3.5%
Northern Queensland (coast)	Bowen (20.00°S, 148.10°E)	0%	0/200	1/1		
	Charters Towers (20.02°S, 146.20°E)	9%	1/11	–		
	Dotswood Station (19.50°S, 146.55°E)	0%	0/22	–		
	Ingham (18.36°S, 146.12°E)	0%	0/38	–		
	Mackay (21.10°S, 149.10°E)	0%	0/20	–		
	Mossman (16.21°S, 145.15°E)	0%	0/23	–		
	Townsville (19.13°S, 146.48°E)	14%	3/21	–	4/335	1.2%
Northern Queensland (inland)	Mareeba (17.00°S, 145.28°E)	17%	4/24	1/1		
	Mt Isa (20.50°S, 139.29°E)	0%	0/27	–		
	Richmond (20.45°S, 143.05°E)	4%	1/24	–	5/75	6.7%
Gulf country	Normanton (17.40°S, 141.05°E)	0%	0/31	–	0/31	0%
Northern Territory	Borroloola (16.00°S, 136.15°E)	13%	3/23	1/1	3/23	13%

locations where sero-positive samples were identified, prevalence varied from 0.4% at Lennox Head (New South Wales) to 18% at Boonah (Queensland). The locations where sera were collected were divided into 7 broad regions, northern New South Wales (Nth NSW), south-east Queensland (S-E Qld), central Queensland (C. Qld), northern Queensland (coastal and inland), Gulf country, Northern Territory (NT), and (Table 1). Antibody-positive sera to 'ranaviruses' were identified in all regions except the Gulf country (0/31). Fig. 1 shows the geographical location of sero-positive samples. The distribution extends from Lennox Head (NSW) to Borroloola (Northern Territory).

A total of 240 sera from *Bufo marinus* in Venezuela were examined with an overall antibody prevalence of 8.3%. The samples came from 18 different localities. The prevalence of positive sera varied from 6 to 27% (Table 2). Positive sera were identified in the northern regions of Venezuela, both east and west of the Andes (Fig. 2).

Immunofluorescence and immunoelectron microscopy

A small number of field sera was examined by immunofluorescence; the number was limited by the availability of sera. This procedure served as a positive control for the ELISA assays. The ELISA positive samples returned positive immunofluorescence results. Intracellular fluorescent foci and aggregates were observed within the cytoplasm; extracellular foci were also observed (Fig. 3A). Infected cells incubated with

Table 2. Serological survey of Venezuelan cane toads. na: sample size too small for valid interpretation (percent prevalence). Numbers are no. positive/no. tested

Location	Total numbers (ELISA)	Total prevalence
Cumana (10.29°N, 64.12°W)	0/16	0%
Puerto Columbiana (10.55°N, 67.65°W)	5/22	23%
Maturin (9.45°N, 63.10°W)	3/11	27%
Guatopo (10.12°N, 66.80°W)	0/23	0%
Altagracia (10.44°N, 71.30°W)	1/3	na
Carora (10.12°N, 70.30°W)	0/3	0%
Puerte Ayacucho (5.39°N, 67.32°W)	0/22	0%
Quibor (9.55°N, 69.35°W)	1/3	na
Quisira (10.54°N, 71.17°W)	0/2	0%
Upata (8.02°N, 62.25°W)	0/4	0%
Trujilo (9.20°N, 70.38°W)	1/16	6%
El Vigia (8.44°N, 64.18°W)	0/2	0%
Pinero (8.90°N, 68.12°W)	2/22	9%
Urumaco (11.21°N, 71.20°W)	3/20	15%
Bruzual (8.03°N, 69.20°W)	0/21	0%
Tucupita (9.02°N, 62.04°W)	0/19	0%
El Tigre (8.44°N, 64.18°W)	4/22	18%
Margarita (11°N, 64°W)	0/9	0%

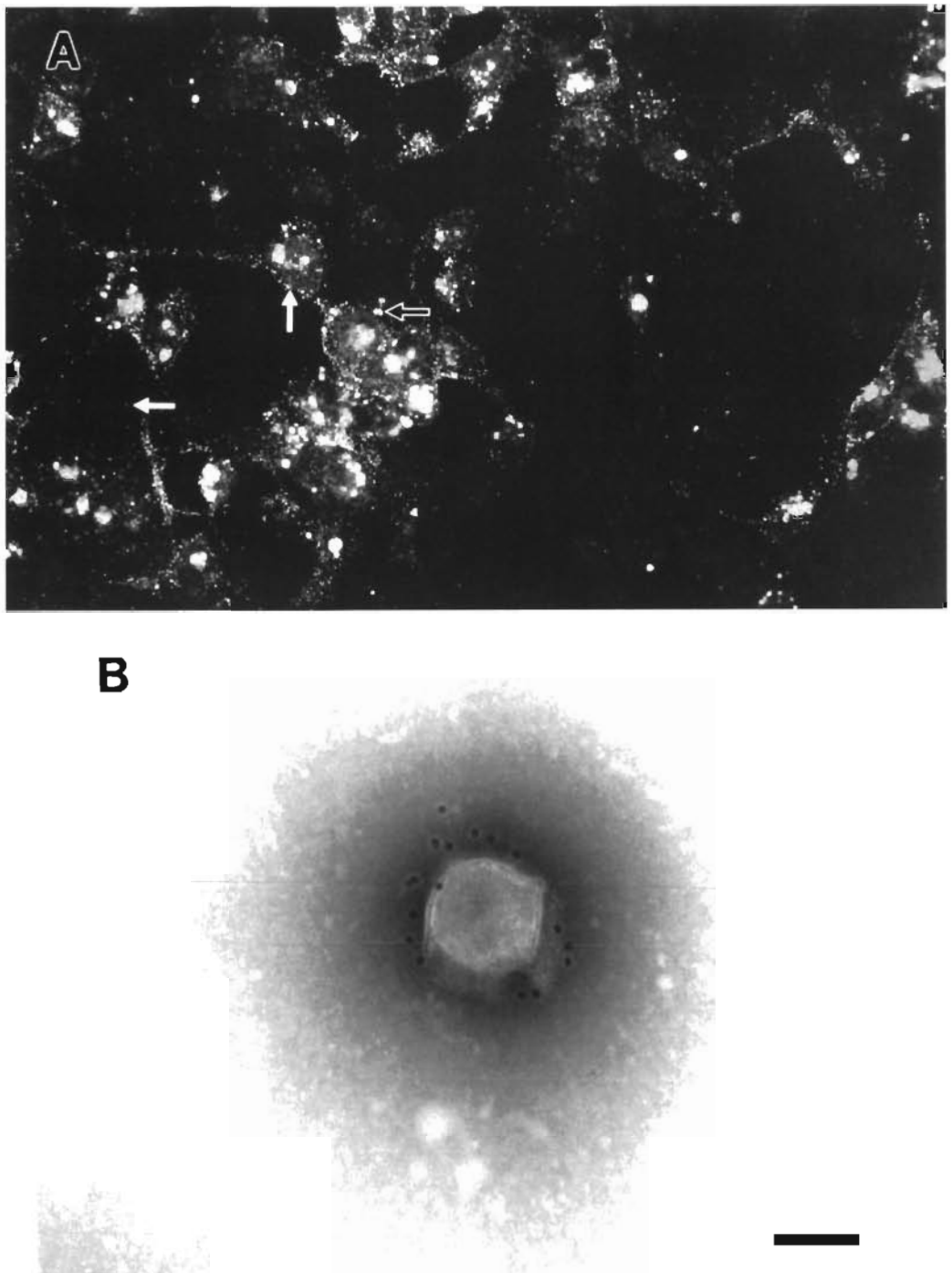

Fig. 3. (A) Representative immunofluoresence of BIV-infected cells incubated with toad serum (Marlborough) that was positive by antibody capture ELISA. White arrows indicate intra- and extracellular viruses. Open arrow indicates virus aggregates/ matrix. (B) Transmission electron micrograph of BIV negatively stained with phosphotungstic acid. The virus had been incubated with toad sera from a free-ranging toad (Marlborough), rabbit anti-toad IgG and protein A-gold. Scale bar = 100 nm

ELISA negative sera were negative, i.e. no fluorescent patterns were observed. When field serum were incubated with purified BIV, viruses were specifically gold-labelled. Very little, if any, background labelling was observed (Fig. 3B).

DISCUSSION

The tests used in this study detected antibodies in toad sera against viruses that have been tentatively assigned to the genus *Ranavirus*. To date, toad sera

from 22 different locations have been tested for the presence of antibodies. Of these, a total of 30 sera from 9 locations, representing 6 regions of north-eastern Australia, have been identified as positive. With the exception of the Gulf country, these geographical locations represent most of the known range of *Bufo marinus* in Australia. As the prevalence of positive antibodies was generally low, it was important to confirm the status of the ELISA positive sera. A small number of sera were tested by immunofluorescence. The fluorescent patterns were similar to those described for EHNV (Hyatt et al. 1991) and correspond to intracellular and extracellular viruses in addition to intracellular virus aggregates (data not shown). Immunoelectron microscopy also confirmed that the antibodies were virus specific. These results confirm that ELISA positive sera have antibodies specific to 'ranaviruses'. Collectively, the data presented in this paper show that Australian populations of *B. marinus* are infected with a 'ranavirus'. It should be noted that polyclonal antibodies against ranaviruses do not contain significant neutralising characteristics; this prevents the use of classical neutralisation assays for the identification of the infectious agent(s).

In Venezuela, 240 sera were found to be 'ranavirus' antibody-positive. These were collected from toads both east and west of the Andes. Whilst no positive sera were detected in southern Venezuela, it should be noted that very few samples were collected in this area. The isolation of a virus(es) (Guatopo virus, GV), from Venezuelan toads (Zupanovic et al. 1998) and the broad distribution of sero-positive animals within the country are significant findings. How long the toad populations have been infected is not known. It is not known if other populations of *Bufo marinus* in Texas, Mexico, Brazil, Peru, Guyana, Philippines, Papua New Guinea, Hawaii, Barbados, Jamaica, Puerto Rico and Fiji are also infected. Unpublished data from the Australian Animal Health Laboratory has indicated that GV can cause haematopoietic disease in tadpoles of *B. marinus* and in at least one species of Australian frog *Litoria infrafrenata* (Parkes & Hyatt pers. comm.). In addition GV is different to BIV at the genomic and polypeptide level (Zupanovic et al. 1998).

The question therefore arises as to the identity of the virus that induces the antibody response in Australian *Bufo marinus*. The virus may be an endemic ranavirus such as BIV. BIV was isolated in a suburb of Townsville called Bohle. A serological survey in Townsville detected antibodies against ranaviruses (3/21 or 14%). However there may also be an 'exotic' virus that was imported with the original toads (e.g. GV) or imported by an unknown host. If the virus had been imported with the original toads, then the virus would have survived a complex set of events. The original toads (101) came indirectly from South America by a process of 'island hopping', i.e. Guyana to Barbados, Barbados to Puerto Rico, Puerto Rico to Hawaii and then from Hawaii to Australia. Within Australia the toads, which showed no signs of disease, were bred and the progeny released along the north-east coast of Australia (Easteal 1983, Tyler 1994). If the virus had been released into the Australian environment, then it would have been introduced into the above countries, been carried in healthy toads and then transmitted to the progeny.

As *Bufo marinus* extends its range into the Northern Territory and further south into New South Wales, it has the potential to carry a broad range of infectious agents. Known infectious agents associated with *B. marinus* in other countries include viruses, bacteria, fungi, protozoa and helminths (Speare 1990). The viruses include an erythrocytic iridovirus (an unclassified group of iridoviruses) from Costa Rica (Speare et al. 1991) and a *Toddia* spp. (Pereira et al. 1973) that, although described as a protozoan, is probably a virus (Speare et al. 1991). Of the remaining agents, *Aeromonas hydrophila*, *Salmonella* spp., *Bacillus* spp., 2 *Mycobacterium* spp., *Fusobacterium necrophorum*, *Fonsecaea pedrosoi*, *Spirametra mansoni*, *Trichodina* spp. and *Rhabdias spherocephala* have been identified as causing pathological changes in *B. marinus* or diseases in other amphibians, fish or mammals (Hird et al. 1981, Speare 1990, Hau et al. 1992, Subasinghe 1992, Twiddy & Reilly 1994, Prasad & Rao 1995). Infectious agents which have been identified in/with *B. marinus* within Australia include species of protozoa, helminths and fungi (Delvinquier 1986, Freeland et al. 1986, Delvinquier & Freeland 1988a, b, Speare 1990, Speare et al. 1994). A notable pathogen is *Mucor amphibiorum* (Speare et al. 1997). *M. amphibiorum* can cause ulcerative dermatitis and septicemia in free-ranging platypus *Ornithorhynchus anatinus* (Obendorf et al. 1993) and it is also recognised as a possible pathogen of most Australian anurans (Speare et al. 1997). Whilst Australian *B. marinus* has not been reported to be infected with any protozoan of known pathogenicity, *Trichodina* spp. is identified as a pathogen of fish (Avault 1996). The above list of potentially pathogenic organisms illustrates the potential for a free-ranging species such as *B. marinus* to be a vector for disease. It is, however, the presence of a 'ranavirus(es)' within Australian *B. marinus* populations that could be of major environmental concern.

This concern is based on the pathogenic characteristics of 'ranaviruses' (Speare & Smith 1992, Cullen et al. 1995, Drury et al. 1995, Ahne et al. 1997). The significance of the data presented in this paper will be known only when the epidemiology of the 'ranaviruses' infecting cane toads is understood. If the

native aquatic fauna (within and outside) the current range of *Bufo marinus* is sero-negative to 'ranaviruses', then the transmission of a pathogenic virus(es) could signal a significant environmental problem.

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