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Susceptibility of eastern water dragons *Intellagama lesueurii lesueurii* to Bohle iridovirus

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ABSTRACT: Ranaviruses infect and have been associated with mass mortality events in fish, amphibians and reptiles and are capable of interclass transmission. Eastern water dragons (EWDs), a semi-aquatic squamate, have an overlapping distribution with several species shown to be susceptible to Bohle iridovirus (BIV). However, this species has not been previously investigated, and no known mass mortalities have occurred in wild populations. Here we report the experimental infection of juvenile EWDs with BIV to investigate a water-dwelling lizards' susceptibility to a ranaviral strain present in northern Queensland, Australia. Lizards were exposed via oral inoculation, intramuscular injection, or cohabitation with orally infected lizards. All exposure methods were effective in establishing an infection as demonstrated by skin lesions and pathological changes in the internal organs. Necrosis, haemorrhage and inflammation were observed histologically in the pancreas, liver, spleen, kidney and submucosa of the gastrointestinal tract of BIV-exposed lizards. Variably sized basophilic intracytoplasmic inclusion bodies were observed in the liver of 6/14 BIV-exposed lizards. Virus was isolated from the liver and kidney of all BIV-infected lizards and confirmed with quantitative PCR (qPCR). The outcome of this study demonstrates that juvenile EWDs are susceptible to BIV, thereby adding Australian lizards to the broad host range of ranaviruses. Furthermore, this study provides additional evidence of BIV's ability to infect different classes of ecothermic vertebrates.

KEY WORDS: Ranavirus · Bohle iridovirus · Experimental infection · Reptiles · Lizards

INTRODUCTION

Ranaviruses (family *Iridoviridae*) are large double-stranded DNA viruses that infect wild and captive amphibian, reptilian, and fish populations. They have been associated with mass mortality events worldwide and are considered emerging pathogens of significant ecological importance (Bigarré et al. 2008, Miller et al. 2011, Price et al. 2014, Tamukai et al. 2016). The first reported ranaviral infection in lizards was in a captive-bred leaf-tailed gecko *Uroplatus fimbriatus* from Germany in 2005 (Marschang et al. 2005). A further 7 lizard species with ranaviral infection have been described, commonly associated with skin lesions and liver necrosis (Alves de Matos et al. 2011, Behncke et al. 2013, Stöhr et al. 2013, Tamukai et al. 2016). Ranaviruses have not been detected in wild or captive lizards in Australia but have been reported overseas in captive bearded dragons *Pogona vitticeps*, a species that is endemic to Australia (Stöhr et al. 2013, Tamukai et al. 2016).

In Australia, 2 ranaviruses have been isolated and characterised: *Epizootic haematopoietic necrosis virus* (EHNV) associated with mortality in wild redfin perch *Perca fluviatilis* and farmed rainbow trout *Oncorphynchus mykiss* in Victoria (Langdon et al. 1986, 1988, Langdon & Humphrey 1987, Whittington et al. 1996), and Bohle iridovirus (BIV) isolated from ornate burrowing frogs *Limnodynastes ornatus* that died during or soon after metamorphosis in Townsville, Queensland (Speare & Smith 1992). More recently, a BIV-like virus was isolated from magnificent

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tree frogs *Litoria splendida* and green tree frogs *Litoria caerulea* in a captive population suffering mortalities (Weir et al. 2012).

Fish, amphibians and hatchling tortoises native to the Townsville region have been shown to be susceptible to BIV under experimental conditions (Moody & Owens 1994, Cullen et al. 1995, Ariel & Owens 1997, Cullen & Owens 2002, Ariel et al. 2015), providing further evidence of interclass transmission of ranaviruses and their ability to infect a wide range of ectothermic vertebrates (Bayley et al. 2013, Brenes et al. 2014b). The eastern water dragon (EWD) *Intellagama lesueurii lesueurii* is a semi-aquatic aboreal squamate that has an overlapping distribution with several of these susceptible fish, amphibian and tortoise species. Their natural range extends down the east coast of Australia from Cooktown, Queensland, to Kangraoo Valley, New South Wales (Brown 2002). Juveniles often occur at high density on rocks and overhanging branches along margins of freshwater creeks, rivers and lakes (Brown 2002). They are considered strong swimmers and are capable of submersion in water for more than 60 min (Brown 2002). The EWD has a preferred body temperature range of 23.75–36.0°C (Wilson 1974). This species has not previously been investigated, and it is important to determine if it has the ability to contribute to the spread of BIV in the wild. In order to further the understanding of the complex ecology of BIV, this study aims to determine if a water-dwelling lizard, the EWD, is susceptible to BIV under experimental conditions.

MATERIALS AND METHODS

Virus

The BIV isolate, sourced from Speare & Smith (1992), was propagated in fathead minnow (FHM) cells at 25°C, in Dulbecco's Modified Eagle Medium (Gibco®) (DMEM), supplemented with 10% fetal bovine serum (Bovogen Biologicals) and 100× Antibiotic-Antimycotic (Gibco®). The virus was harvested at complete cytopathic effect (CPE) on Day 3 and frozen at −20°C, thawed 3 times and stored at −20°C. The virus was vortexed each time before refreezing and on the final thaw cycle was centrifuged at 13523 × *g* for 5 min then titrated. The titres of the viral stock used in the infection trials were $10^{5.33}$ $TCID_{50}$ ml^{-1}. Aliquots of the virus were frozen at −80°C until the day of inoculation, when they were thawed at room temperature.

Animals

Nineteen juvenile EWDs were obtained from a private breeder under permit (Scientific Purposes Permit No. WISP15053914) from the Queensland Department of Environment and Heritage Protection. The lizards were housed individually for 16 wk prior to commencement of the experimental infection trial. The virulence of the viral stock was tested by exposure of 5 juvenile barramundi *Lates calcarifer*, a species known to be extremely susceptible to BIV (Moody & Owens 1994). Juvenile barramundi were obtained from the Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University. The animals were kept at 28.8°C (±2.5°C) and housed at the experimental facilities at the College of Public Health, Medical and Veterinary Sciences, James Cook University. Fluorescent room lights were kept on a 12 h light:12 h dark cycle to mimic environmental conditions. All animal experiments were carried out with the approval of the James Cook University Animal Ethics Committee (Ethics Approval No. A2277).

Infection trials

Eastern water dragons

The lizards were randomly assigned to 1 of 4 treatments: (1) oral dose (OR) of 100 µl ($10^{4.33}$ $TCID_{50}$) BIV viral stock given by syringe at the back of the mouth slowly in an upright position to prevent re-gurgitation; (2) intramuscular injection (IM) of 100 µl ($10^{4.33}$ $TCID_{50}$) BIV viral stock given on the proximal aspect of the lateral hind limb; (3) cohabitation (CH) with orally infected lizards from another experiment for 10 d; and (4) negative control (NC). The orally infected lizards from the CH treatment were removed from the group, infected, mouths wiped and then returned to the group after 10 min. Lizards in the CH and NC treatments received an oral placebo of 100 µl phosphate-buffered saline (PBS). Five lizards were randomly assigned to each of the OR, IM and NC treatments, while 4 lizards were assigned to the CH treatment.

All BIV-infected animals were housed in the Aquatic Animal Infection Facility at James Cook University while the NC animals were housed in an adjoining clean room. Juvenile EWDs, infected with BIV or used as sentinels (CH) or NC, were observed for 23 d after initial exposure, and clinical signs as well as behaviour were recorded daily. Animals in the OR, IM and NC treatments were kept individu-

ally in 5 l plastic vivariums with a 10 cm plastic pipe hide and water dish.

Lizards in the CH treatment were kept in 20 l plastic vivariums with multiple plastic pipe hides and a large water dish. Lizards in the CH treatment were housed across 3 groups: 2 groups of 2 lizards and 1 group of 3 lizards. Each group contained only 1 orally infected lizard. Animals housed within these groups had a 2 wk acclimation period.

All lizards were fed a diet of 3–4 small crickets 3 times weekly where 2 out of the 3 feeds were dusted with a calcium, vitamin and mineral supplement. Enclosures were cleaned on alternate days. Clinical signs, behaviour, and mortality were recorded twice daily until the point of euthanasia. The lizards were humanely euthanised using the 2-stage euthanasia method with tricaine methanesulfonate (MS-222), as described by Conroy (2009), at the point when they had lost the ability to reorientate themselves when placed on their backs or showed reduced activity and/or a fright response. Changes in activity levels were determined by observing locomotion, foraging and feeding behaviours as well as social interaction between lizards. Fright response was determined by observing the lizard's reaction to opening, moving or touching the outside of the vivarium.

Barramundi

Four juvenile barramundi were intramuscularly injected with 100 µl ($10^{4.33}$ $TCID_{50}$) BIV viral stock, and 5 negative controls were intramuscularly injected with 100 µl PBS as placebo treatment. The fish were housed in freshwater in the Aquatic Animal Infection Facility at James Cook University in 2 separate 600 l tanks. The barramundi were fed an ad libitum diet of 6 mm fish pellets daily. Behaviour and clinical signs were recorded daily for 7 d. Fish were humanely euthanised with an overdose of 2-phenoxyethanol when they lost the ability to swim normally and orientate themselves in the water. NC fish were euthanised at the same time as the BIV-injected fish to provide a time-equivalent reference for necropsy and histological purposes.

Gross pathology and histopathology

All lizards underwent post-mortem examination. Pathological changes were recorded, and a range of tissues (lung, liver, pancreas, spleen, kidney, digestive tract, heart, tongue, brain, hind leg) and any skin or internal lesions were preserved in 10% neutral-buffered formaldehyde. Fixed tissues were processed and embedded in paraffin wax. Sections were cut at a thickness of 5 µm, stained with haematoxylin and eosin, and mounted using routine methods (Bancroft & Gamble 2008).

Viral isolation

Samples from selected organs (liver and kidney) of juvenile EWDs, and from the spleen of juvenile barramundi, were stored at −80°C until examined by viral isolation (VI). Samples were homogenised with 1 ml DMEM supplemented with 100× Antibiotic-Antimycotic and subjected to 3 freeze/thaw cycles at −20°C before clarification by centrifugation at 13 523 × *g* for 5 min. Ten-fold serial dilutions were prepared from each pooled sample, and a total of 500 µl was added in duplicate to 80% confluent monolayers of FHM cells in a 24-well tissue culture plate (SARST-EDT®). The plates were incubated at 25°C and checked daily for CPE. For each sample that did not cause CPE during 1 wk incubation, a blind passage was performed by transferring 1 ml cell culture supernatant from inoculated wells to wells with newly sub-cultivated 80% confluent FHM cells in a separate 24-well plate. Supernatant from the positive wells in the 24-well plate were confirmed by quantitative PCR (qPCR) as described below.

Molecular confirmation

Samples from selected organs (liver and kidney) were collected aseptically during necropsy and stored separately at −80°C. DNA was extracted using a Bioline ISOLATE II Genomic DNA Kit according to the manufacturer's instructions. PCR amplification targeting the major capsid protein (MCP) region of the EHNV genome was performed using primers previously described by Jaramillo et al. (2012). The reaction mixture contained 1× GoTaq® qPCR Mastermix (Promega), 0.8 µM of each primer (forward primer 5′-GAC TGA CCA ACG CCA GCC TTA ACG-3′, reverse primer 5′-GCG GTG GTG TAC CCA GAG TTG TCG-3′), and ~80 ng of template DNA, and nuclease-free water was added to a final concentration of 20 µl. Thermocycling was performed on a Rotor-Gene 6000 Real-Time PCR Machine with reaction conditions: 95°C for 2 min followed by 40 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 15 s, with a final extension at 95°C for 2 min.

These generated an amplicon of 94 nucleotides in the presence of the target viral sequence. Each run contained a positive (BIV DNA) and negative control. Three products at random were sequenced (Macrogen) and confirmed to be BIV.

RESULTS

Clinical signs

Eastern water dragons

Lizards in the IM, OR, and CH treatments had similar clinical signs. These included swollen abdomen, loss of appetite, decreased activity, decreased alertness, loss of equilibrium, and focal areas of skin ulceration or pustules (Table 1). The mean interval between experimental infection and the onset of clinical signs was 3 d in the IM treatment, 9 d in the OR treatment, and 9.5 d in the CH treatment. No clinical signs were observed in the NC treatment.

Skin lesions were observed 8 d p.e. in the IM treatment, 7 to 10 d p.e. in the OR treatment, and 9–10 d p.e. in the CH treatment. A single lizard in the IM treatment had erythema and multifocal ulceration on the plantar aspect of the metacarpals and phalanges (Fig. 1A). Three lizards in the OR treatment had pustules on the distal phalanges, carpals and metacarpals. Another 3 lizards in the CH treatment had ulcerative lesions on the forelimbs, similar to that described in the IM treatment (Fig. 1B).

Lizards in the IM treatment appeared to have high activity levels up until 12 h prior to the loss of both startle and rollover reflexes. The activity level in the OR lizards appeared to decrease over 2 d before the loss of both startle and rollover reflexes. Lizards in the CH treatment were euthanised at the conclusion of the 23 d trial.

Barramundi

Barramundi injected intramuscularly with BIV stopped feeding 1 d post exposure (p.e.) and began to lose their ability to orientate themselves in the water by 6 d p.e. No clinical signs were observed in barramundi injected with placebo. NC and BIV-injected barramundi were euthanised 7 d p.e.

Gross pathology

Lizards in the IM treatment had multifocal ecchymotic haemorrhages on the serosal surface of the intestines, colon and stomach. An individual in this treatment also had ulcerative lesions in the buccal cheek and in the caudal pharynx ventral to the skull. The liver was diffusely mottled and kidneys mildly haemorrhagic. Haemorrhage was also observed on the epicardial surface of the heart. The spleen was mildly enlarged in 2 individuals, and all lizards had severe focally extensive intramuscular haemorrhage at the injection site in both hind legs (Table 2).

Lizards in the OR treatment had diffusely mottled livers and mottled splenic pallor. The kidneys were pale in appearance and had minimal or no haemorrhage. One animal had intracoelemic haemorrhage which was visible as a focal dark area on the dorsal skin of the lizard prior to euthanasia (Table 2). CH lizards had diffusely mottled livers, mildly haemorrhagic kidneys and mottled splenic pallor (Table 2). One animal had a lesion in the throat alongside the trachea.

All BIV-infected lizards were severely anaemic upon euthanasia or death. Control animals had normal livers, kidneys, spleens, digestive tracts and had stomachs full of digesta. No mortalities were observed in the NC treatment. Fat bodies were present in all lizards upon euthanasia.

Table 1. Number of eastern water dragons *Intellagama lesueurii lesueurii* in each treatment with observed clinical signs versus number of animals in each treatment, and average time (days post-exposure, p.e.) to the onset of clinical signs, death, and interval between onset of clinical signs and death. IM: intramuscular injection; OR: oral dose; CH: cohabitation; NC: negative control. DAB: distended abdomen; LOA: loss of appetite; DAC: decreased activity levels; LEQ: loss of equilibrium; EXL: skin lesion. na: not applicable

	Clinical signs (n)					Time (d p.e.)		
	DAB	LOA	DAC	LEQ	EXL	Onset	Death	Interval
IM	4/5	5/5	4/5	2/5	1/5	3 (1–5)	6.9[a] (5–8)	3.9 (2.5–7)
OR	3/5	2/5	5/5	1/5	3/5	9 (8–11)	12 (9–14)	3 (1–5)
CH	1/4	1/4	4/4	0/4	3/4	9.5 (9–10)	19.9[a] (15.5–23)	10.4 (5.5–14)
NC	0/5	0/5	0/5	0/5	0/5	na	na	na

[a]Death was recorded as day of euthanasia except when a lizard died overnight, in which case 0.5 d was deducted

Histopathology

Histopathological changes in the liver (dilation of sinusoids, necrosis, hepatocyte atrophy, congestion, intracytoplasmic inclusion bodies) were ob-

Table 2. Number of eastern water dragons *Intellagama lesueurii lesueurii* in each treatment with observed gross pathology versus number of animals in each treatment. EXL: skin lesion; INL: internal lesion; HAS: haemorrhaging at site of injection in hind leg; HAL: haemorrhagic liver; HAK: haemorrhagic kidney; HGIT: haemorrhaging along surface of gastrointestinal tract; ENS: enlarged spleen; SPL: splenic pallor; na: not applicable. Other abbreviations as in Table 1

	EXL	INL	HAS	HAL	HAK	HGIT	ENS	SPL
IM	1/5	1/5	5/5	4/5	4/5	3/5	2/5	0/5
OR	3/5	0/5	na	3/5	1/5	0/5	0/5	3/5
CH	3/4	1/4	na	3/4	2/4	0/4	0/4	2/4
NC	0/5	0/5	na	0/5	0/5	0/5	0/5	0/5

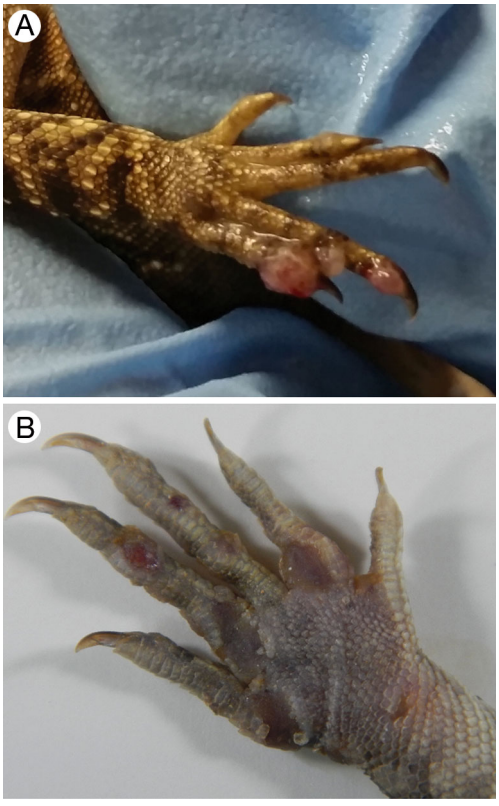

Fig. 1. Bohle iridovirus (BIV)-infected eastern water dragon *Intellagama lesueurii lesueurii* with (A) pustular and (B) ulcerative skin lesions

served in 10 animals from the OR and IM treatments and in 2 CH lizards (Table 3). Diffuse variably sized basophilic intracytoplasmic inclusion bodies were detected in the liver of 2 IM, 3 OR, and 1 CH lizard. In one case, these inclusion bodies were detected near a focal area of necrosis in the liver (Fig. 2A). Mild multifocal renal cortical and tubular necrosis was detected in 7 animals. In addition, multifocal sub-capsular haemorrhage was observed in 2 animals and a focal granuloma in the renal cortex of another animal (Table 3). Moderate to severe diffuse splenic necrosis was observed in 10 animals (Table 3), with mild splenic inflammation detected in 1 lizard and focal splenic haemorrhage in 2 lizards. Two lizards in the IM treatment were detected with peri-pancreatic haemorrhage (Table 3). The same 2 lizards had focal submucosal haemorrhage in the colon (Fig. 2B).

Large numbers of melanomacrophages were observed in the vasculature of the heart and lungs of 5 lizards across the 3 treatments. A pharyngal submucosal intramuscular haemorrhage with lymphocytic infiltration was observed in a CH lizard, while mild focal sub-mucosal lymphocytic infiltration was observed in another CH lizard. IM lizards had focally extensive severe intramuscular haemorrhage at the site of injection in the hind legs (Fig. 2C). The toes and limbs of the lizards in both the OR and CH treatments had focally extensive severe epidermal and dermal necrosis (Fig. 2D). Bacteria were present on the surface and in the superficial keratin in some cases. No abnormalities were detected in the brain of BIV-infected or NC lizards.

Viral isolation

Virus was isolated from the liver and kidney samples from all infected EWDs and from spleen samples collected from 4 infected juvenile barramundi. CPE was confirmed to be due to BIV by qPCR on tissue culture supernatant (Jaramillo et al. 2012). No virus was isolated from the same tissue samples collected from the lizards and barramundi in the NC treatment.

Table 3. Number of eastern water dragons *Intellagama lesueurii lesueurii* in each treatment with observed histopathological changes in examined organs versus number of animals in each treatment. GI: gastrointestinal. Other abbreviations as in Table 1

	Liver	Kidney	Spleen	Pancreas	GI tract	Heart	Lung	Tongue	Skin & muscle
IM	5/5	4/5	4/5	2/5	2/5	2/5	1/5	0/5	5/5
OR	5/5	3/5	4/5	0/5	0/5	2/5	1/5	0/5	3/5
CH	2/4	3/4	2/4	1/4	0/4	1/4	1/4	1/4	3/4
NC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

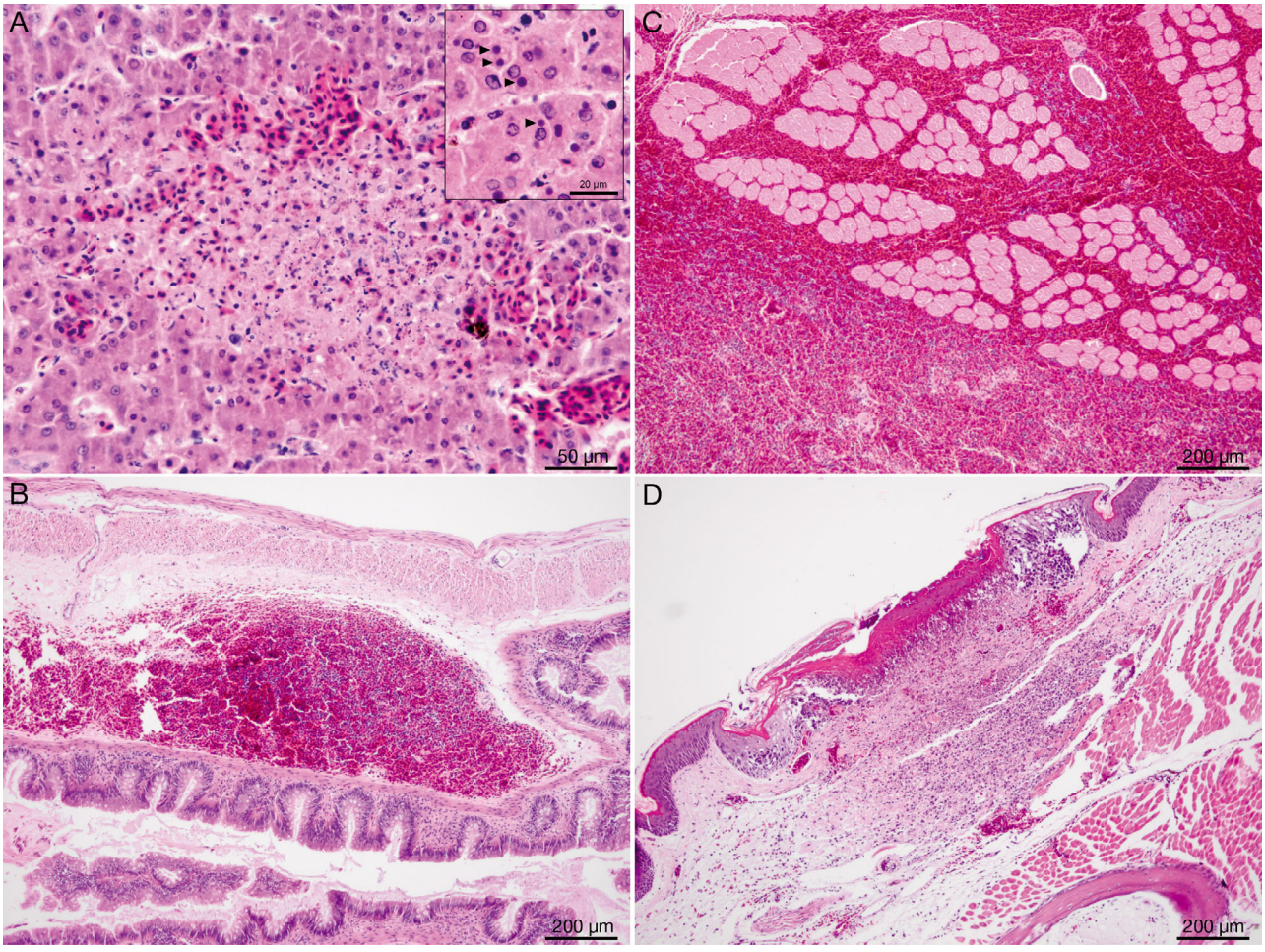

Fig. 2. Histological sections of (A) liver, (B) colon, (C) injection site in the hind leg, and (D) skin lesion from Bohle iridovirus (BIV)-infected eastern water dragons *Intellagama lesueurii lesueurii* stained with haematoxylin and eosin. (A) Focal hepatic necrosis with variably sized basophilic intracytoplasmic inclusion bodies in the liver (arrowheads in the insert). (B) Submucosal haemorrhage. (C) Focally extensive intramuscular haemorrhage. (D) Focally extensive severe epidermal and dermal necrosis

Molecular confirmation

BIV was confirmed in the liver and kidney samples collected from all infected lizards using the above-described qPCR method. Tissue samples from lizards in the NC treatment were negative.

DISCUSSION

This study has shown that an Australian native water-dwelling lizard, the EWD, is susceptible to BIV under experimental conditions. BIV infection trials have been conducted at a range of temperatures (21–29°C) in fish, amphibians, and reptiles with low (25%) to high (100%) mortality outcomes (Moody & Owens 1994, Cullen et al. 1995, Ariel & Owens 1997, Cullen & Owens 2002, Ariel et al. 2015). This experiment was conducted at 28.8°C (±2.5°C) and was effective at establishing infection in the three different exposure treatments (OR, IM and CH) with varying severity of clinical signs and histopathological changes. Environmental temperature has been shown to affect the survival and disease progression in ranaviral-infected turtles, fish and amphibians (Rojas et al. 2005, Jun et al. 2009, Allender et al. 2013).

Australian ectotherms shown to be susceptible to BIV include tilapia fry *Oreochromis mossambicus*, barramundi fingerlings, juvenile green tree frogs, striped burrowing frogs *Cyclorana alboguttata*, short-footed frogs *Cyclorana brevipes*, red-backed toadlets *Pseudophryne coriacea*, northern bango frogs *Limnodynastes terraereginae*, broad palmed frogs *Litoria*

latopalmata, hatchling saw-shell turtles *Myuchelys latisternum* and Krefft's turtles *Emydura macquarii krefftii* (Moody & Owens 1994, Cullen et al. 1995, Ariel & Owens 1997, Cullen & Owens 2002, Ariel et al. 2015). All these susceptible species are associated with freshwater, as is the EWD, which is semi-aquatic in nature, often seen diving into freshwater creeks, rivers and lakes (Brown 2002). EWDs interact with central bearded dragons in their natural environment. The central bearded dragon is also endemic to Australia but in overseas collections has been reported to be infected with ranavirus in previous studies (Stöhr et al. 2013, Tamukai et al. 2016). EWDs often move between freshwater environments and are therefore a potential source of ranaviral transmission between separate populations of fish, amphibians, and reptiles. Juvenile lizards were chosen for this study, as previous studies with BIV have shown juvenile amphibians and reptiles to be more susceptible to infection than adults, which are often reported as resistant (Ariel & Owens 1997, Cullen & Owens 2002, Ariel et al. 2015).

EWDs were exposed to BIV via the OR, IM, and CH treatments. Previous ranaviral infection trials in fish, amphibians, and tortoises have used direct exposure to cultivated virus via bath-exposure, oral inoculation, intramuscular injection, and intraperitoneal or intracoelomic injection, as well as horizontal transmission via direct contact or cohabitation with infected individuals (Johnson et al. 2007, Allender et al. 2013, Brenes et al. 2014a, Ariel et al. 2015, Forzán et al. 2015). Clinical signs and histopathological changes in these studies differed depending on the route of exposure, as seen here, but all routes seemed effective in establishing an infection.

Clinical signs observed in this study correspond to those described in the literature for ranavirus-infected lizards, and include lethargy, inappetence, and incoordination (Marschang et al. 2005, Behncke et al. 2013, Stöhr et al. 2013). The interval between infection and onset of clinical signs was shortest in the IM treatment (3 d), followed by the OR (9 d) and CH (9.5 d) treatments. Lizards in the OR treatment exhibited a slow decline in activity levels over the 2 d prior to loss of both startle and rollover reflexes. Inappetence was described in 2 of the 5 individuals within this group occurring 1 d prior to death. The decline in activity levels was sudden (<12 h) in lizards in the IM treatment, which demonstrated a faster onset of clinical signs and time to death than the other 2 treatments. A decline in activity levels was observed in all CH lizards with 3 developing skin lesions. Two of the 4 lizards in the CH treatment did not exhibit loss of startle or rollover reflex during the 23 d trial. These two were housed in different groups and had either no or only mild histological changes. The mild histological changes were observed in the individual that had developed skin lesions and included a focal granuloma in the kidney. Liver and kidney samples collected from these 2 animals were qPCR positive, and BIV was successfully isolated from these tissues, suggesting the possibility of asymptomatic carriers. It is possible that these 2 CH lizards were still in the incubation period; however, as all other BIV-infected lizards had exhibited clinical signs by Day 11 p.e., we believe that these individuals were either unaffected or beginning to clear the virus. Similar findings have been described in brown tree snakes, where 3 BIV-inoculated individuals remained asymptomatic with no observable histopathological changes, while virus was re-isolated from 1 orally infected individual (Ariel et al. 2015).

Skin lesions were observed in 1 IM lizard, in 3 OR lizards, and 3 CH lizards. These lesions were observed on average 9 d p.e. and were ulcerative, erythematous and pustular. The appearance of skin lesions in this study differ from previous reports of skin lesions in lizards, which described them as grayish skin alterations, brown-crusted or dark skin lesions (Stöhr et al. 2015). Throughout the duration of the study individual lizards were often seen swimming in and resting completely submerged on the bottom of the water dishes. Possibly, the semi-aquatic nature of the EWD, which is unlike previously reported lizard species, could have contributed to the different appearance of skin lesions, or perhaps it is due to host or viral strain characteristics. Histological investigation revealed focally extensive severe epidermal and dermal necrosis. Gram-negative bacteria were found in association with some of the skin lesions, similar to reports for ranaviral infections in Asian glass lizards and bearded dragons (Stöhr et al. 2013, Tamukai et al. 2016). Histologically the skin lesions observed in this study are similar to previous reports.

Histopathological changes in this study were seen across all treatments in the examined tissues except the brain, indicating systemic infection in all infected lizards. Histopathological changes seen in the liver (haemorrhage and multifocal hepatic necrosis) of infected EWDs were similar to those observed in green striped tree dragons and a leaf-tailed gecko (Marschang et al. 2005, Behncke et al. 2013), but with varying degrees of severity between individuals and within treatments. Other histopathological

changes included dilation of hepatic sinusoids with either scattered necrotic cells or severe multifocal hepatic necrosis. Multifocal submucosal haemorrhages and luminal haemorrhages similar to that reported by Behncke et al. (2013) were observed in 2 lizards in the IM treatment. Histopathological changes were seen across most of the internal organs in the IM treatment. However, the most severe lesions were observed in lizards belonging to the OR group. We believe the difference is due to the route of infection and individual variation.

Variably sized basophilic intracytoplasmic inclusion bodies were observed in the liver of 6 infected EWDs. These 6 lizards represented all the treatments: 3 OR, 2 IM, and 1 CH-exposed lizard. Intracytoplasmic inclusion bodies have previously been described in a range of ranavirus-infected tissues of fish, amphibians, tortoises, and lizards (Reddacliff & Whittington 1996, Marschang et al. 1999, Behncke et al. 2013, Forzán et al. 2015). Although ranaviral infection is often associated with the presence of inclusion bodies, this is not consistently reported. Intracytoplasmic inclusion bodies could not be identified in histological sections from 8 infected EWDs despite confirmation of infection via qPCR and VI. Similarly, inclusions were not found in Australian freshwater turtles infected with BIV or in several other species of tortoises and lizards infected with different ranaviruses (Allender et al. 2013, Ariel et al. 2015, Tamukai et al. 2016). This could be a strain or host characteristic, or simply due to low level or patchy occurrence which was not detected in the histological sections examined. Further studies on the sequential spread of BIV in lizards infected via different routes would provide additional information on pathogenesis and natural transmission.

EWDs inhabit the environment where BIV was first isolated, and their distribution overlaps with several species shown to be susceptible to this lethal pathogen (Speare & Smith 1992, Moody & Owens 1994, Cullen & Owens 2002); however, this species has not previously been investigated, and no known mass mortalities have occurred in wild populations. This study demonstrated that EWDs are severely affected by a BIV infection and may amplify and contribute to the spread of the virus in the wild, thereby adding an Australian squamate to the broad host range of ranavirus.

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