CHARACTERISATION OF RANAVIRAL INFECTION AND ITS MANAGEMENT IN AUSTRALIAN LIZARDS

A thesis submitted by

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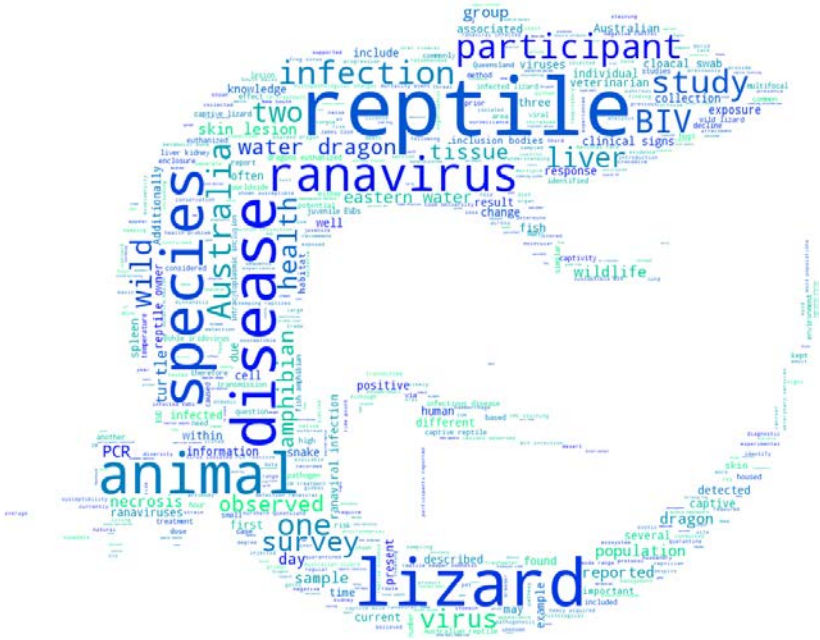

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The research presented and reported in this thesis was conducted with the approval of the James Cook University Research Ethics Committee and in accordance with the National Statement on Ethical Conduct in Human Research, 2007; Australian Code for the Care and Use of Animals for Scientific Purposes, 2007; and the Queensland Animal Care and Protection Act, 2001.

The proposed research methodology received clearance from the James Cook University Animal Ethics Committee (A2087 & A2277) and Human Research Ethics Committee (H6574). This research was conducted under permits WISP15053914 and WITK18689817 granted by Department of Environment and Heritage Protection, Queensland Government, and Department of National Parks, Sport and Racing, Queensland Government, respectively.

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SUMMARY

Background

Reptiles are considered to be one of the most evolutionary and ecologically remarkable groups of living organisms, having successfully inhabited most of the planet including the oceans. Despite this, reptile species worldwide are on the decline due to threats such as residential and commercial development, agriculture and aquaculture, climate change, and introduction of invasive species and diseases. Approximately 19% of all assessed reptiles globally are listed as 'threatened' by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. Infectious diseases are listed as one of the top five causes of global species extinctions and one of the biggest causes of morbidity and mortality in reptiles. Ranaviruses (family *Iridoviridae*) have been identified as emerging pathogens of ecological significance in ectothermic vertebrates due to their expanding host and geographic range. This group of viruses infects over 175 species of ectothermic vertebrates worldwide and is listed as notifiable to the The World Organization for Animal Health (Office International des Epizootics, OIE) in amphibians and fish. The majority of ranaviral research has been conducted in amphibians with only a few surveys targeting wild reptiles despite several reported mortality events in captive lizards and turtles. Hence the aims of this thesis were to investigate the susceptibility and pathogenesis of *Ranavirus* sp. in juvenile eastern water dragons (*Intellagama lesueurii lesueurii*); determine if *Ranavirus* sp. is present in Australian lizards; and to identify and understand Australian reptile owners experience and management of disease in captive reptile collections.

The susceptibility of an Australian semi-aquatic lizard to Bohle iridovirus

In Chapter 2 we investigated the susceptibility of juvenile eastern water dragons to a local ranavirus isolate (Bohle iridovirus, BIV) via oral inoculation, intramuscular injection, and cohabitation with orally infected lizards. This lizard species was investigated as they share habitat with several fish, amphibians and reptiles shown to be susceptible to BIV. A range of tissues (spleen, kidney, lung, liver, kidney, gastrointestinal tract, heart, tongue, brain, and bone marrow) were collected for histopathology, and liver and kidney samples were also collected for viral isolation and polymerase chain reaction (PCR). The outcome of this study demonstrated that juvenile eastern water dragons are susceptible to BIV via all exposure methods and have the ability to infect naïve individuals. These findings add another ectotherm to the list of species susceptible to ranavirus.

The pathogenesis of Bohle iridovirus infection in juvenile eastern water dragons

In order to investigate the pathogenesis of BIV in this host, juvenile eastern water dragons were orally infected with BIV and euthanized at pre-determined time-points (Chapter 3). Tissue samples were collected for histopathology, immunohistochemistry (ISH), in-situ hybridization (ISH), viral isolation and PCR. The findings from this study identified the progression of BIV infection which appeared to start in the spleen, followed by the liver, then the other organs. Ranaviral DNA was detected by PCR in liver, kidney and cloacal swabs at 3 days post infection, suggesting cloacal swabs could be a reliable source of diagnostic sampling in BIV-infected lizards. Histopathology changes were observed in the liver and tongue at 3 days post infection and IHC identified viral antigen in the spleen at 6 days post infection. The ISH labelling of skin, bone marrow, liver, pancreas, stomach, intestine and spleen matched

the location and pattern detected by IHC. Infection was well underway before clinical signs were observed.

Molecular detection of *Ranavirus* sp. in captive and wild Australian lizards

Wild and captive Australian lizards from northern Queensland, New South Wales and Australian Capital Territory were surveyed for ranaviral DNA using combined oral-cloacal swabs and PCR (Chapter 4). Ranaviral DNA was detected in samples from 4/123 asymptomatic captive lizards and 5/63 asymptomatic wild lizards. These PCR-positive samples belonged to three central bearded dragons (*Pogona vitticeps*) and one frilled neck lizard (*Chlamydosaurus kingii*) from two different captive collections, and five wild eastern water dragons from Paluma Range National Park, Queensland. Amplicons from this study shared 100% nucleotide identity with the cognate regions of BIV and four other ranaviruses and were only one base different to the cognate region of epizootic haematopoietic necrosis virus, an Australian ranavirus that affects fish and is listed as notifiable to the OIE. The detection of ranavirus in asymptomatic lizards in both captivity and in the wild introduces the possibility of carrier lizards and highlights importance of disease management strategies (e.g. quarantine).

The health and wellbeing of Australian pet reptiles

An online survey (SurveyMonkey®) of Australian reptile owners was conducted between November and December 2017 (Chapter 5). This cross-sectional study consisted of multiple choice and open-ended questions. Quantitative data were analysed descriptively using frequencies, mean, median, standard deviation, range, and interquartile range. Open-ended question responses were analysed thematically and grouped into themes. The average age of participants was 34 years old with snakes and lizards the most popular reptile kept in captivity. Most participants cleaned enclosures weekly, disinfected enclosures monthly, and used

UVA/UVB lights, heat lamps and multivitamin supplements to prevent health problems within their collection. Quarantine periods were employed by 72% of participants for an average of 4 weeks, with only 30% physically isolating the animal. Disease knowledge was limited to non-infectious diseases such as metabolic bone disease. Barriers to seeking veterinary assistance for unwell reptiles included cost and perceived lack of knowledge/experience on the veterinarians' part. Findings from this survey identified the need for more readily available resources for Australian reptile keepers including access to information on diseases and experienced veterinarians.

Outcomes

This research has identified eastern water dragons as a susceptible species to ranaviral infection and provides further evidence of the ability of ranaviruses to infect a wide range of ectothermic vertebrates. The detection of ranavirus in asymptomatic wild and captive lizards suggests the possibility that ranavirus is circulating in the wild and is part of the normal microflora of Australian lizards. This also identifies lizards as a potential host that can spread and amplify ranaviruses in the wild. Further investigation is required to characterize the ranavirus found in this study, and molecular and serum surveys of wild and captive populations. Furthermore, the detection of ranavirus in captive lizards combined with the results from the survey of Australian reptile owners highlights the need for more readily available resources on disease identification, prevention, and treatment.

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LIST OF ABBREVIATIONS

ATV: Ambystoma tigrinum virus

BF-2: Bluegill fry cells (ATCC CCL-91)

BIV: Bohle iridovirus

BLAST: Basic Local Alignment Search Tool

CANV: *Chrysosporium* anamorph of *Nannizziopsis vriesii*

CDC: Centers for Disease Control and Prevention

CH: Cohabitation

CMTV: Common midwife toad virus

CPE: Cytopathic effect

CSIRO: Commonwealth Scientific and Industrial Research Organisation

d.p.e: Days post exposure

DAB: Distended abdomen

DAC: Decreased activity levels

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

dpi: Days post infection

E: Endothelium

ECV: European catfish virus

EHNV: Epizootic haematopoietic necrosis virus

EID: Emerging infectious disease

ELISA: Enzyme-linked immunosorbent assays

ENS: Enlarged spleen

EPBC: Environment Protection and Biodiversity Conservation

EWD: Eastern water dragon

EXL: Skin lesion

FHM: Fathead minnow cells

FV3: Frog virus 3

GI: Gastrointestinal tract

HAK: Haemorrhagic kidney

HAL: Haemorrhagic liver

HAS: Haemorrhaging at site of injection in hind leg

HE: Hematoxylin and eosin

HGIT: Haemorrhaging along surface of gastrointestinal tract

IC: Intracoelomically

IHC: Immunohistochemistry

IM: Intramuscular injection

INL: Internal lesion

IQR: Interquartile range

ISH: In-situ hybridization

IUCN: International Union for Conservation of Nature

JCU: James Cook University

LEQ: Loss of equilibrium

LOA: Loss of appetite

M: Mesothelium

MCP: Major capsid protein

MS-222: Tricaine methanesulfonate

N: Number

NA: Not applicable

NC: Negative control

NE: Not examined

OIE: The World Organization for Animal Health (Office International des Epizootics)

OR: Oral inoculation

P1: Passage 1

P2: Passage 2

P3: Passage 3

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

SARS: Severe acute respiratory syndrome

SCRV: Santee-Cooper ranavirus

SD: Standard deviation

SGIV: Singapore grouper iridovirus

SPL: Splenic pallor

SVL: Snout to vent length

$TCID_{50}$: Tissue culture infective dose

US: United States

UVA/UVB: Ultraviolet A/B

WHA: Wildlife Health Australia

WHO: World Health Organization

WNV: West Nile virus

WWF: World Wide Fund for Nature

LIST OF APPENDICES

CHAPTER 1 – Positioning the research

The world's biodiversity is in decline as humans increasingly use the planet's natural resources and modify its environments through processes such as unsustainable hunting, land clearing for agriculture or urban development, and damming of waterways (McCartney, 2009; Pereira, Navarro, & Martins, 2012; Woinarski, Burbidge, & Harrison, 2015). The Living Planet Index, "*a measurement of the state of the world's biological diversity based on population trends of vertebrate species from terrestrial, freshwater and marine habitats"*, indicates that global populations of vertebrate species have, on average, declined in size by 60% in the last 45 years (WWF, 2018; Zoological Society of London & WWF, 2014). This is directly linked to a continually increasing human population which within the same time period has doubled from around 3.7 billion (in 1970) to 7.6 billion (in 2018) (Worldometers.info, 2018).

Humanity's consumption of the resources and services that nature provides is estimated to be worth more than US$125trillion annually, with overharvest of wild populations and destruction of habitats for agriculture two of the biggest drivers of current biodiversity loss (WWF, 2018). These ongoing global changes have profound implications for wildlife health and conservation such as shifts in wildlife populations dynamics and changes in disease ecology (Daszak, Cunningham, & Hyatt, 2000; Deem, Karesh, & Weisman, 2001). Physical changes to the environment (i.e. habit degradation) can result in population declines and changes in population dynamics by impairing nutritional status, restricting movement and limiting gene flow, reducing reproduction rates, and potentially enhancing disease transmission rates (Acevedo-Whitehouse & Duffus, 2009; Deem et al., 2001). At population level, these environmental changes can have significant ecological but also physiological

effects. Environmental stressors can, for example, result in sex-ratio changes, decreased reproductive parameters, and immunosuppression, either leading directly to disease or increasing the populations risk of acquiring diseases (Acevedo-Whitehouse & Duffus, 2009). Furthermore, some environmental stressors can directly compromise health by inducing genotoxicity (i.e. cancer, mutations) or developmental abnormalities (Acevedo-Whitehouse & Duffus, 2009; Hinton et al., 2005). The effects of these environmental stressors remain mostly unexplored in wildlife species.

Animals have long served as sentinels or surveillance tools for monitoring environmental health hazards often providing advanced warning of a danger (Fox, 2001; Neo & Tan, 2017). A classic example is the 'canary in the coal mine' where canaries were used in coal mines as an early warning signal for toxic gases such as carbon monoxide (Neo & Tan, 2017). Their increased respiratory rate, small size, and high metabolism led them to succumb before the miners giving them time to act. Avian species have also acted as a sentinel for disease outbreaks. For example, the discovery of West Nile virus (WNV), a zoonosis that can cause neurological disease and death in humans, in the western hemisphere was signaled by an outbreak of disease in crows and other wild birds (Eidson et al., 2001). The dead crow reports preceded confirmation of viral activity by several months, and WNV-positive birds were found >3 months before the onset of human cases (Eidson et al., 2001). This highlights how awareness and knowledge of animal health can be used to discover, monitor and predict environmental and human health hazards, as well as prepare us to manage these events (Stephen, 2016).

1.1. Emerging infectious diseases

Environmental stressors have led to the emergence of over 40 infectious diseases since 1970 that affect humans, domestic animals and wildlife (e.g. Ebola virus, antibiotic-resistant tuberculosis, and ranavirus and chytridmycosis in amphibians) (Acevedo-Whitehouse & Duffus, 2009; Deem et al., 2001; Ryser-Degiorgis, 2013). These emerging infections can be classed as either newly emerging (e.g. Hendra and Ebola virus) or re-emerging/resurging (e.g. dengue and drug-resistant malaria) (Morens, Folkers, & Fauci, 2004). Over 60% of emerging infectious disease (EID) events are caused by zoonotic pathogens, with most EIDs originating from wildlife animal reservoirs (e.g. Severe acute respiratory syndrome (SARS), and Hendra virus) and spilling over to humans either directly (e.g. rabies) or via an intermediate animal or vector (e.g. Zika or Nipah virus) (Jones et al., 2008; Morens et al., 2004; World Health Organization, 2018). Additionally, the reverse is also possible whereby human diseases, such as Influenza A and methicillin-resistant *Staphylococcus aureus* can be transmitted to companion animals, livestock and wildlife (Messenger, Barnes, & Gray, 2014).

In animals, the transmission of infectious pathogens from reservoir animal populations (often domestic animals) to wildlife underpins the emergence of a range of wildlife EIDs, posing a particular threat to endangered species and biodiversity (Daszak et al., 2000). One such example is canine distemper, which was transmitted from sympatric domestic dogs to free-living African wild dogs (*Lycaon pictus*) resulting in high mortality and extinction of several populations (Alexander, Kat, Munson, Kalake, & Appel, 1996; Daszak et al., 2000). The cause of this disease spill-over is believed to be a result of the expansion of human populations and the encroachment of domestic dog carriers into areas previously not inhabited by these species (Daszak et al., 2000). However, some pathogens circulate between domestic and

wildlife hosts causing disease in both, such as Brucellosis. This zoonotic bacterial infection is believed to have been introduced to America with cattle and now affects wild elk and bison residing in Yellowstone National Park posing a potential threat to domesticated cattle that graze at the park boundaries (Daszak et al., 2000; Dobson & Meagher, 1996). The increased emergence of zoonotic EIDs demonstrates the importance of understanding transmission pathways (human-domestic-wildlife) and wildlife health status highlighting the need for further understanding of the effects that increased contact between wildlife, domestic animals and humans can have.

1.2. One Health

Wildlife health surveillance has become an integral component in the identification and management of potential threats to human and animal health (Ryser-Degiorgis, 2013). In the past, wildlife health and diseases were only considered important if they threatened human health, agriculture or production animals. However, the threat of wildlife disease is now taken more seriously because of outbreaks in endangered species, increasing veterinary involvement, and advances in host-parasite population biology (Daszak et al., 2000). The public interest in wildlife-associated disease has grown in the wake of evidence that over 60% of all emerging infectious diseases are zoonotic, with most (>75%) originating from wildlife such as West Nile virus, avian influenzas, and Lyme disease (Buttke, Decker, & Wild, 2015; Jones et al., 2008; Kahn, 2006). Furthermore, the increased recognition of the interconnectedness between human, wild and domestic animals and the environment has given rise to the 'One Health paradigm'.

The One Health concept recognizes that the health status of humans, animals and ecosystems are closely linked, and their management requires a coordinated, collaborative, interdisciplinary and cross-sectoral approach to addressing a wide range of risks at the animal-human-ecosystem interface (Zinsstag, 2012). Currently, this approach predominately involves the fields of veterinary medicine and public health, which has led to a focus on disease transmission at the animal/human interface (Jenkins, Simon, Bachand, & Stephen, 2015). The One Health concept is recognized nationally and globally by organizations such as Centers for Disease Control and Prevention (CDC), World Health Organisation (WHO), and The World Organisation for Animal Health (Office International des Epizooties, OIE). Each of these organizations have differing roles in promoting One Health. The WHO, for example, works closely with the Food and Agriculture Organization of the United Nations and the OIE to promote multi-sectoral responses to food safety hazards, risks from zoonoses, and other public health threats at the human-animal-ecosystem interface and provide guidance on how to reduce these risks (World Health Organization, 2019a).

1.3. Disease management

The OIE is an intergovernmental organization specifically responsible for improving animal health worldwide. Their purpose is to support effective decision-making responses to outbreaks, approval of trade movement, information management, and disease surveillance. Additionally, the OIE has contributed to capacity building by publishing manuals such as "Quarantine and Health Screening Protocols for Wildlife prior to Translocation and Release into the Wild", and "Training Manual on Wildlife Diseases and Surveillance" (OIE, 2010; Woodford, 2000). The OIE produces a list of notifiable terrestrial and aquatic animal diseases which include several zoonotic diseases and diseases known to cause mass mortality in animal

species. The purpose of this list is to ensure transparency in and enhance knowledge of the worldwide animal health situation. For the year 2018, this list includes 117 animal diseases, infections and infestations such as anthrax, bovine tuberculosis, epizootic haematopoietic necrosis disease in fish, and infections with *Ranavirus* species in amphibians (OIE, 2018).

In Australia, the coordinating body for wildlife health is Wildlife Health Australia (WHA) who works nationally with over 40 agencies and organizations, and over 700 wildlife health professionals to better manage the adverse effects of wildlife diseases. Their principal objectives are the protection and enhancement of the natural environment. Additionally, WHA undertake research, investigate and monitor wildlife, and promote capacity building through communication, education and training. An example of one such group supported by WHA is The Bat Health Focus Group, which consists of members from organizations such as CSIRO Australian Animal Health Laboratory and universities, and professionals such as veterinarians, epidemiologists and wildlife carers. This group are using a collaborative One Health approach to consider bat health issues in relation to the broader context of biosecurity, public health, livestock health and environmental impacts. The WHA Bat Health Focus Group prepares reports presenting information on Australian bat lyssavirus testing of bats and Hendra virus testing of flying foxes. The information gained through such linkages with WHA assists in limiting deleterious impact of wildlife diseases on Australia's natural ecosystems and environment, biodiversity, animal and human health, and trade and tourism.

Surveillance programs are important in tracking and controlling zoonoses, reducing mortality events and production losses, and establishing baselines for wildlife (e.g. normal blood parameters). Additionally, by monitoring wildlife health we can potentially predict environmental, human health, and disease hazards. Once baseline data is available for

wildlife, further research can evaluate a species role in disease transmission (human-to-animal or animal-to-animal), identify habitats of importance for conservation, examine responses to human activity (i.e. development, pollution or habitat destruction) and contribute to wildlife conservation and biodiversity.

Due to the zoonotic potential of diseases carried by animals, surveillance of wildlife has primarily been limited to primates (Zika and Ebola virus), birds (Newcastle disease, avian influenza), bats (Hendra virus, Australian bat lyssavirus) and rodents (haemorrhagic fever, rat-bite fever) (World Health Organization, 2019b). However, there has been minimal disease monitoring of reptiles despite several species being implicated in human zoonoses such as Salmonella and West Nile virus (Corrente et al., 2017; Jacobson et al., 2005; Whiley, Gardner, & Ross, 2017). Furthermore, reptiles have been identified as potential vectors and hosts of pathogens that affect other wildlife such as *Batrachochytrium dendrobatidis,* the fungus responsible for mass mortalities in amphibians worldwide, and amphibian and fish ranaviruses, both of which are listed as notifiable by the OIE (Daszak et al., 1999; Kilburn, Ibáñez, & Green, 2011; OIE, 2018).

1.4. Reptiles

Reptiles are considered one of the most ecologically and evolutionarily remarkable groups of living organisms, having successfully populated most of the planet, including the oceans and some of the harshest and more environmentally unstable ecosystems on earth (Pincheira-Donoso, Bauer, Meiri, & Uetz, 2013). Almost 11,000 recognised species of reptiles can be found worldwide inhabiting all continents except Antarctica. Terrestrial reptiles, which make up over 85% of all reptiles, occupy a wide range of habitats including forests, grass lands,

middle elevations in mountainous habitats and deserts (Reptile Database, 2018; Zug & Dowling, 2018). Reptiles have persisted over hundreds of millions of years through big ecological changes and extinction events in other species by accumulating a vast diversity of morphological, behavioral, ecological, reproductive, and defensive strategies in order to survive (Pincheira-Donoso et al., 2013). For example, one evolutionary adaptation is the acquisition of water-independent reproduction, shifting from moisture dependent eggs to a terrestrial egg (Pincheira-Donoso et al., 2013; Zug & Dowling, 2018). Furthermore, the evolutionary adaption of reptiles has given rise to asymmetric species richness among phylogenetic groups, with squamate reptiles (amphisbaenians, lizards, snakes) diversifying into more than 10,400 species and accounting for 97% of all reptile diversity (Losos, 2011; Pincheira-Donoso et al., 2013; Reptile Database, 2018). This order is mostly responsible for the prominent global diversity of reptiles (Pincheira-Donoso et al., 2013).

Morphologically, reptiles vary in size and shape. The body size of reptiles varies widely with the Virgin Islands dwarf gecko (*Sphaerodactylus partenopion*) and Jaragua dwarf gecko (*Sphaerodactylus ariasae*) considered the smallest reptiles worldwide with a snout-to-vent length of 16-18mm (Penn State, 2001). In contrast, the total carapace length of a leatherback sea turtle (*Dermochelys coriacea*) can reach up to 2 m, while the reticulated python (*Python reticulatus*) and saltwater crocodile (*Crocodylus porosus*) can grow to over 6 m in length (Britton, Whitaker, & Whitaker, 2012; Zug & Dowling, 2018). All reptiles have a continuous external covering of epidermal scales from microscopic tubular scales seen in the *Sphaerodactylus* geckos to the large body scales in lizards and snakes (Zug & Dowling, 2018). In turtles these scales, referred to as scutes, can be found covering the shell while in crocodiles these are referred to as plates (Zug & Dowling, 2018). The shape of reptiles differs

between classes. For example, crocodiles and lizards possess four limbs, while the limbs of sea turtles have developed into flippers and disappeared in snakes (Zug & Dowling, 2018).

Physiologically, the ectothermic nature of reptiles and their dependence on the environment means that the metabolic rate of reptiles is approximately one tenth of that of a similar-sized endotherm thus reducing their energy needs ("Ectotherm", 2017). This, along with their high resistance to evaporative water loss, enables reptiles to inhabit harsh ecosystems, such as those found in central Australia, thriving where food supply is low and sporadic (Dawson & Dawson, 2006). The diversity between reptiles is also evident regarding their dietary requirements. For example, crocodiles, alligators and snakes are strictly carnivorous, while green sea turtles (*Chelonia mydas*) are omnivores as juveniles and herbivorous as adult. Similarly, lizards can be herbivorous (e.g. green iguanas, *Iguana iguana*), omnivorous (e.g. bobtail lizard, *Tiliqua rugosa*), or carnivorous (e.g. Gila monster, *Heloderma suspectum*) depending on the species and/or life stage.

Reptiles play an essential role in the balance of the ecosystems they live in and are excellent ecological indicators due to their high degree of sensitivity to changes in the environment (Rajpoot, 2016). One of the many roles' reptiles play in ecosystems is that of bio-monitors controlling pests. For example, the Asian house gecko (*Hemidactylus frenatus*) and the dubious four-clawed gecko (*Gehydra dubia*) have been identified as potentially useful predators of pest and vector mosquitos, while the Indian spiny-tailed lizard (*Sara hardwickii*) eats locusts which are pests of crops (Canyon & HII, 1997; Rajpoot, 2016). The consumption of rodents and insect pests are not only beneficial for the agricultural industry but also for disease control by helping reduce potential zoonotic vectors (e.g. yellow fever carrying mosquitos, *Aedes aegypti*) (Endangered Species International, 2011; Valencia-Aguilar, Cortés-

Gómez, & Ruiz-Agudelo, 2013). Reptiles are also a vital part of the food chain in many ecosystems acting as both predator and prey which prevents overpopulation and provides food for species higher in the food chain (Endangered Species International, 2011; "Importance of reptiles in the ecosystem," 2017). They help to maintain the balance in the food chain and provide a clean healthy environment by eating dead animals as seen with the example of the Komodo dragon (*Varanus komodoensis*) (Palmer, 2017). Seed dispersal and pollination in some ecosystems, particularly island habitats, is primarily mediated by reptile species (lizards and turtles) that feed on pollen, nectar and fruit (Olesen & Valido, 2003; Valido & Olesen, 2007). For example, the blue-tailed day gecko (*Phelsuma cepediana*) is the only pollinator for the rare plant *Trochetia blackhumiana* on the island of Mauritius (Palmer, 2017; Rajpoot, 2016).

Despite their diversity and success in most terrestrial and aquatic environments, reptile species are declining on a global scale. These declines are due to physical changes made to the environment such as land clearing and farming, introduced invasive species and disease, environmental pollution, unsustainable use, and global climate change (Gibbons et al., 2000; IUCN, 2018). Approximately 19% of the 6723 reptile species assessed by the International Union for Conservation of Nature (IUCN) are listed as 'threatened' (IUCN, 2018). This includes 296 species listed as Critically Endangered, 505 species as Endangered and 475 species as Vulnerable. Examples include the Critically Endangered Orinoco crocodile (*Crocodylus intermedius*) of Colombia and Venezuela under threat from livestock and unsustainable harvesting of aquatic resources, and the Endangered Blue Mountain water skink (*Eulamprus leuraensis*) found in southeastern Australia under threat from residential and commercial development, mining, pollution, and fire regimes (Balaguera-Reina, Espinosa-Blanco, Antelo,

Morales-Betancourt, & Seijas, 2018; Shea, Cogger, & Greenless, 2018). Identifying the potential threats to reptile species is key to formulating mitigating directives and strategies to ensure conservation of rare, threatened or endemic reptile species which are essential parts of many terrestrial and aquatic ecosystems.

1.5. Australian reptiles

Australia is home to a large diversity of reptiles with over 1000 described species widely distributed across the continent and its wide range of habitats (tropical rainforests, sand ridge deserts, alpine areas, freshwater wetlands, arid stony plains) each of which is favoured by its own distinctive reptile species (Cogger, 2014; S. K. Wilson & Swan, 2017). This species richness is due to the unique shape, size, geographical isolation, and habitat that Australia offers (S. K. Wilson & Swan, 2017). One of such unique habitats is found in the arid regions of Australia and is characterised by a group of spinifex or porcupine grasses. These grasses provide humid shelter sites and are believed to support more species of reptiles than an area of comparable size anywhere else in the world (S. K. Wilson & Swan, 2017). Examples of reptiles found in such environments include the great desert skink (*Egernia kintorei*), desert death adder (*Acanthophis pyrrhus*) and black-collared dragon (*Ctenophorus clayi*) (S. K. Wilson & Swan, 2017).

Additionally, these arid regions are the main hotspot of lizard richness which runs from central Australia west to the Hamersley Range and Pilbara coast in Western Australia, with subsidiary hotspots to the north of the Great Dividing Range around the Atherton Tablelands on the east coast of Queensland, and in the Kimberley Plateau in northern Western Australia (Powney, Grenyer, Orme, Owens, & Meiri, 2010). The rich distribution of lizards in these arid and semi-

arid habitats is strikingly different from the distributions of amphibians, birds and mammals (Powney et al., 2010; Roll et al., 2017). Australian deserts support more lizard diversity than other deserts worldwide (Webb, Harlow, & Pike, 2015). One sand ridge site in the Great Victoria Desert, South Australia is home to 47 species of lizards coexisting, while only 12 lizard species are recorded in a similar habitat in the North American desert (Webb et al., 2015).

Of the 1000+ described reptile species found in the wild in Australia, approximately 6% are listed as 'threatened' under the Environment Protection and Biodiversity Conservation (EPBC) Act 1999 (Australian Government, 2018; Chapman, 2009). This includes 10 species listed as Critically Endangered, 20 species as Endangered and 33 species as Vulnerable. The decline of Australian reptiles is directly linked to human activity and its increased use of the environment with key threatening processes including habitat loss and degradation, poaching for illegal wildlife trade, introduction of invasive species, and infectious disease and parasitism (Gibbons et al., 2000; Webb et al., 2015).

Habitat destruction and degradation have been identified as the key contributing factors to the decline of almost all threatened species on the EPBC list, including reptiles, with nearly 40% of Australian forests cleared since 1788 (Bradshaw, 2012). The most vegetation clearing has occurred in Queensland, a state that supports over half of Australia's terrestrial endemic reptile species and is a recognised hotspot for diverse reptile groups (Bradshaw, 2012; Cogger, Cameron, Sadlier, & Eggler, 1993). It is estimated that 89 million individual reptiles died per year between 1997-1999 as a result of vegetation clearing in Queensland (Cogger, Ford, Johnson, Holman, & Butler, 2003). Habitat fragmentation also poses a serious threat to reptiles due to their poor dispersal abilities (Williams, Driscoll, & Bull, 2012).

Another threat to Australian herpetofauna is wildlife trade with the exotic reptile trade considered to be the main pathway for introduction and establishment of invasive reptiles globally (Kraus, 2009). The size of the illegal wildlife trade in Australia is unknown but increasing, with reptiles accounting for 43% of Australian Customs prosecution cases for attempted export and import between 1994-2007 (Alacs & Georges, 2008; *Illegal trade in fauna and flora and harms to biodiversity*, 2017). Introduction and establishment of invasive species in Australia through this illegal trade could pose a threat to native reptiles as seen in the example of the red-eared slider (*Trachemys scripta elegans*). This exotic (non-native) species has established wild populations in Australia competing with endemic turtle species for food, habitat and basking spots (Robey, Burgin, Hitchen, & Ross, 2011). The common ecological effects from invasive reptile species derive from food-web disruptions such as the removal of native prey species or native predators through predation from the introduced predator or via introduction of species that bare novel defensive mechanisms (e.g. toxic skin, eggs or larvae as seen in cane toads) (Kraus, 2009). Additionally, invasive reptiles also compete with autochthonous species for burrow use and basking sites and have the potential to genetically contaminate native species through the introgression of 'alien' genes (Kraus, 2009). However, there is evidence of native species adapting to established invasive species through evolutionary morphological and physiological changes. One example is the red-bellied snake (*Pseudechis porphyriacus*) which has evolved to have a reduced gape size, increased body size, and developed a degree of toxin resistance in response to the introduction of cane toads in Australia in 1935 (Phillips & Shine, 2004, 2006).

Furthermore, the introduction of exotic reptiles into Australia has the potential to cause the dispersal of novel infectious diseases that could pose a risk to Australia's biodiversity and

native wildlife. Such novel diseases can result in high rates of mortality in naïve native species. The introduction of novel pathogens may also have more far-reaching and subtle effects than die-offs of reptiles, with knock-on effects permeating throughout the ecosystem (Daszak et al., 1999). For example, amphibian chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis*, has been linked to dramatic population declines and extinction of many amphibian species globally (O'Hanlon et al., 2018). One of the knock-on effects caused by this disease is the decline in snake species that prey exclusively on amphibians. It is believed that amphibian chytridiomycosis has spread to geographically isolated regions because of the international trade of amphibians for exotic pets, medical and food purposes (O'Hanlon et al., 2018). It is likely that other disease organisms such as *Ranavirus,* protozoan and helminths have been introduced to native hosts through similar pathways (Daszak et al., 1999; Kraus, 2009). This emphasizes the disastrous effect that introduced diseases can have at the individual, population and/or species levels and the need to increase our knowledge base of existing endemic diseases so these can be easily differentiated from potentially emerging infectious diseases.

1.6. Infectious diseases of reptiles

Infectious diseases, as a group, are one of the largest causes of morbidity and mortality in reptiles (Paré, Sigler, Rosenthal, & Mader, 2006). In wild reptiles, increased susceptibility to infection and emergence of new diseases is believed to be facilitated by immune suppression caused by environmental pressures (e.g. habitat loss or change), exposure to pollutants, changes in environmental conditions (e.g. increased exposure to ultraviolet irradiation and acid rain), and introduction of non-native species with associated novel diseases (Schumacher, 2006).

In captive reptiles, diseases such as metabolic bone disease (non-infectious) are commonly associated with the stress of captivity caused by inappropriate temperatures or humidity, or poor enclosure hygiene (Chinnadurai & DeVoe, 2009; Paré et al., 2006). Bacterial and fungal diseases in reptiles are occasionally caused by primary pathogens but often occur as a result of animals being immunocompromised (Paré et al., 2006). For example, *Salmonella* sp. are a commensal bacteria of reptilian gut flora that usually causes no ill effect. However, there have been several reports of these bacteria causing disease in captive snakes (Ramsay et al., 2002; Souza et al., 2014). *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV), an obligate fungal pathogen of reptiles, has been shown to be an emerging pathogen in captive reptiles and recently identified in wild eastern massasauga rattlesnakes (*Sistrurus catenatus*) (Allender et al., 2011; Mitchell & Walden, 2013). This fungal disease has been diagnosed in over 11 lizard species (e.g. central bearded dragon, *Pogona vitticeps*), several snake species (e.g. corn snake, *Pantherophis guttatus*) and in saltwater crocodiles (*Crocodylus prosus*) (Mitchell & Walden, 2013; Paré et al., 2006; Thomas, Sigler, Peucker, Norton, & Nielan, 2002). Additionally, both *Salmonella* sp. and *Chrysosporium* sp. are pathogens of public health significance as they are zoonotic in nature and can be transmitted to humans (Whiley et al., 2017).

Reptilian viruses (e.g. adenovirus, paramyxovirus and herpesvirus) have been described in many different reptile species, however our knowledge of these viruses is often limited to reports of disease in captive reptiles (Doneley, Buckle, & Hulse, 2014; Hyndman, Marschang, Wellehan, & Nicholls, 2012; Stacy et al., 2008). The impact of these viruses on wild reptiles is often unknown due to the infrequent recovery of remains, or undiscovered due to the lack of surveillance. With reptiles serving as vectors for zoonotic diseases, such as West Nile virus

and reptile-associated salmonellosis, it is important for conservation, wildlife and public health that we learn more about infectious diseases that may affect reptiles (Corrente et al., 2017; Mitchell, 2011).

1.7. Reptile virology

Reptile virology is a relatively young field that has undergone rapid development over the past few decades primarily thanks to rapid advances in diagnostic technology (Marschang, 2011). The early study of reptile viruses focused mainly on their zoonotic potential with reptiles acting as hosts for arboviruses that also infect humans and birds (Ariel, 2011). This interest increased at the end of the 20th century with the emergence of West Nile virus and the identification of crocodiles and alligators as reservoir hosts (D. L. Miller et al., 2003; Steinman et al., 2003). Other driving forces behind the study of reptilian viruses include the decline of species, disease and mortality events in captive reptiles, and zoonotic risk posed by reptiles (Behncke, Stöhr, Heckers, Ball, & Marschang, 2013; Johnson-Delaney, 2006). There is growing interest in this field with the discovery of novel viruses and expanding host range of existing viruses (Hyatt et al., 2002; Mashkour, Maclaine, Burgess, & Ariel, 2018; Pénzes, Pham, Benkő, & Tijssen, 2015; Szirovicza et al., 2016). This has acted as a driving force for studying reptilian hosts and the effect of reptilian viruses, as well as leading to the establishment of more reliable diagnostic tools (Forzán et al., 2017; Fredholm, Coleman, Childress, & Wellehan, 2015; Pallister et al., 2007).

Presently the detection and study of viruses relies on a wide range of tools, including molecular methods such as polymerase chain reaction (PCR) and next generation sequencing,

and classical virological methods such as cell culture (Marschang, 2011). The early stages of reptilian virology were based on histopathology and cell culture (Ariel, 2011).

Viruses that have shown to be significant pathogens in reptiles include ranaviruses (family *Iridoviridae*) and herpesvirus (family *Herpesviridae*) in chelonians, adenoviruses (family *Adenoviridae*) in lizards and snakes, and paramyxoviruses in snakes (Ariel, 2011; Marschang, 2011). Of these, herpesvirus and adenovirus have long been detected histologically based on associated inclusion bodies in tissues from infected reptiles, while ranaviruses have been regularly isolated in cell culture from reptiles since the late 1990's (Doneley et al., 2014; Hughes-Hanks, Schommer, Mitchell, & Shaw, 2010; Johnson et al., 2008; Marschang, 2011). Many of these viruses have been associated with morbidity and mortality events in captive and wild reptiles with ranaviruses, for example, infecting reptile species from at least 12 different families of the orders Testudines (turtles, tortoises and terrapins) and Squamata (lizards and snakes) (Duffus et al., 2015). This group of viruses infects at least 175 species across 52 families of ectothermic vertebrates including amphibians and fish where ranaviral infection in these hosts is listed as notifiable by the World Organization of Animal Health (OIE) (Duffus et al., 2015; OIE, 2018).

1.8. Ranavirus

Ranaviruses are large (~150 nm) double stranded DNA viruses that infect wild and captive amphibian, fish and reptilian populations on all continents except Antarctica (Duffus et al., 2015). This group of viruses have been associated with mass mortality events and are considered emerging pathogens of significant ecological importance due to their expanding host range and geographical distribution (Bigarré, Cabon, Baud, Pozet, & Castric, 2008; Daszak

et al., 1999; D. L. Miller, Gray, & Storfer, 2011; Price et al., 2014; Tamukai, Tokiwa, Kobayashi, & Une, 2016). Their ability to infect a wide range of ectothermic hosts from different vertebrate classes, global distribution, and high virulence establish them as a global threat to ectothermic populations worldwide (Daszak et al., 1999).

The International Committee on Taxonomy of Viruses currently recognises six species in the genus *Ranavirus*: *Ambystoma tigrinum virus* (ATV), *Common midwife toad virus* (CMTV), *Epizootic haematopoietic necrosis virus* (EHNV), *Frog virus 3* (FV3), *Santee-Cooper ranavirus* (SCRV), and *Singapore grouper iridovirus* (SGIV) (Chinchar et al., 2017). Ranaviruses were first discovered in 1965 in infected northern leopard frogs (*Lithobates pipiens*) in the United States of America (later designated FV3) (Granoff, Came, & Rafferty, 1965). Following this discovery, ranaviral infections were reported in Hermann's tortoises (*Testudo hermanni*) in Switzerland during the 1980s and associated with fish die-offs in wild redfin perch (*Perca fluviatilis*) in Australia (later designated EHNV) (Heldstab & Bestetti, 1982; Langdon, Humphrey, Williams, Hyatt, & Westbury, 1986).

Mortality in reptiles infected with ranaviruses varies greatly (0-100%) with lethargy and inappetence the two most commonly reported clinical signs (D. L. Miller, Pessier, Hick, & Whittington, 2015). Clinical signs in reptiles can be extremely diverse, where for example, turtles often present with nasal and oral discharge, and oedema of the eyes or neck, lizards display signs of central nervous disorders and skin lesions or ulceration, and snakes present with ulcerations of the oral cavity (Allender, Mitchell, Torres, Sekowska, & Driskell, 2013; Hyatt et al., 2002; Stöhr et al., 2013). The natural transmission route of ranaviral infection is unknown and highly debated, however experimental data suggests that multiple transmission routes are possible (e.g. direct contact, ingestion of infected tissue, or by indirect waterborne

contact) (Brenes, Gray, Waltzek, Wilkes, & Miller, 2014; Brunner, Storfer, Gray, & Hoverman, 2015). The most commonly used methods to diagnose ranaviruses in blood, tissue or oral-cloacal samples are electron microscopy, enzyme-linked immunosorbent assays (ELISAs), viral isolation, immunohistochemistry (IHC), DNA amplification using PCR, sanger sequencing and next generation sequencing (D. L. Miller et al., 2015).

Ranaviral infections have been increasingly reported in snakes and lizards, particularly from individuals held in captivity. The first reported *Ranavirus* infection in lizards was in a captive-bred leaf-tailed gecko (*Uroplatus fimbriatus*) from Germany (Marschang, Braun, & Becher, 2005). A further seven lizard species with ranaviral infection have been described (Alves de Matos et al., 2011; Behncke et al., 2013; Stöhr et al., 2013; Tamukai et al., 2016). Two species of *Ranavirus*, EHNV and BIV, have been described in Australian fish and amphibians (Langdon, Humphrey, & Williams, 1988; Langdon et al., 1986; Speare & Smith, 1992; Whittington, Kearns, Hyatt, Hengstberger, & Rutzou, 1996). To date, no ranaviral infections have been described in wild or captive lizards in Australia. However, ranaviral infection was identified in several green tree pythons (*Chondropython viridis*) seized during an attempt to illegally import them into Australia from Indonesia, and in central bearded dragons (a species originating in Australia) held in captivity in Germany and Japan (Hyatt et al., 2002; Stöhr et al., 2013; Tamukai et al., 2016). Although the source of infection could not be identified in the case from Germany, it is hypothesised that since there is a close clustering of reptilian and amphibian ranaviruses the Japanese outbreak was horizontally transmitted from amphibians kept within the same facility (Tamukai et al., 2016). Additionally, ranaviruses are capable of transmission between different ectothermic vertebrate classes, each with high variation in host susceptibility (Brenes et al., 2014). Bohle iridovirus and FV3 are two ranaviruses that can

cause infection and mortality in fish, amphibian and reptile species (Allender, Barthel, Rayl, & Terio, 2018; Ariel, Wirth, Burgess, Scott, & Owens, 2015; Cullen & Owens, 2002; Forzán et al., 2015; Moody & Owens, 1994).

1.9. Bohle iridovirus

Bohle iridovirus was the first *Ranavirus* described in amphibians in Australia and appears to be primarily geographically isolated to Australia. This virus was isolated from ornate burrowing frogs (*Limnodynastes ornatus*) (Figure 1.1A) that died during or soon after metamorphosis in Townsville, North Queensland (Speare & Smith, 1992). BIV-like infections have been described in captive magnificent tree frogs (*Litoria splendida*) and Australian green tree frogs (*Litoria caerulea*) in the Northern Territory, Australia, and in boreal toads (*Anaxyrus boreas boreas*) held in captivity in Iowa, USA (Cheng et al., 2014; Weir et al., 2012).

Experimental infection studies have shown that several Australian amphibians, fish and turtles are susceptible to BIV (Figure 1.1B-D). These include barramundi (*Lates calcarifer*), tilapia (*Oreochromis mossambicus*), Northern bango frog (*Limnodynastes terraereginae*), broad-palmed frog (*Litoria latopalmata*), Australian green tree frog, striped burrowing frog (Cyclorana *alboguttata*), short-footed frogs (*Cyclorana brevipes*), red-backed frog (*Pseudophryne coriacea*), saw-shell turtle (*Myuchelys latisternum*), and Krefft's turtle (*Emydura macquarii krefftii*) (Ariel & Owens, 1997; Ariel et al., 2015; Cullen & Owens, 2002; Cullen, Owens, & Whittington, 1995; Moody & Owens, 1994). Clinical signs of BIV infection include spiral or corkscrew-like swimming (fish), failure to respond to stimuli (amphibians), and lack of appetite and lethargy (turtles) (Ariel & Owens, 1997; Ariel et al., 2015; Cullen & Owens, 2002). However, several reptiles native to northern Queensland have been shown to

be refractory to BIV infection under experimental conditions (Ariel et al., 2015). This includes adult brown tree snakes (*Boiga irregularis*), common tree snakes (*Dendrelaphis punctulatus*), common keelbacks (*Tropidonophis mairii*), and yearling freshwater crocodiles (*Crocodylus johnstoni*) (Ariel et al., 2015).

Figure 1.1 Australian ectotherms with demonstrated susceptibility (A-D) or sera-reactivity (E-F) to Bohle iridovirus: A) ornate burrowing frog (*Limnodynastes ornatus*), B) Krefft's turtle (*Emydura macquarii krefftii*), C) barramundi fingerling (*Lates calcarifer*), D) Australian green tree frog (*Litoria caerulea*), E) common keelback (*Tropidonophis mairii*), F) freshwater crocodile (*Crocodylus johnstoni*) (Freeman, 2007; "Freshwater crocodile", 2018; Hines, 1998; Hoye, 2018; "Keelback snake", 2019; McCormack, 2017).

Several studies focusing on this pathogen have shown that age can play a role in a species susceptibility to infection (Ariel & Owens, 1997; Ariel et al., 2015; Cullen et al., 1995). For example, BIV appears to be extremely virulent in hatchling Krefft's turtles whereas adults of the same species are not adversely affected (Ariel et al., 2015). The same appears true for fish and frogs where the susceptibility of juveniles is higher than in more mature animals (Ariel & Owens, 1997; Cullen et al., 1995). The resistance of adult snakes and crocodiles to BIV doesn't mean that these species are unaffected by ranavirus but simply that perhaps only the young

are susceptible to infection. This could potentially result in population declines and changes in population dynamics such as an aging population or poor population turnover.

The detection of ranaviral antibodies in wild turtles, freshwater crocodiles and snakes surveyed in northern Queensland over a two-year period indicates that BIV is regularly circulating in wild reptile populations (Ariel et al., 2017) (Figure 1.1E, F). This is further supported by the detection of ranaviral antibodies in wild cane toads in the same geographic region (Whittington, Kearns, & Speare, 1997; Zupanovic et al., 1998). However, the distribution of the virus, its host range and potential effect on naïve reptile populations on the Australian continent are currently unknown. To date all Australian ectotherms shown to be susceptible or sero-reactive to BIV have an association with freshwater, either through their habitat or diet. This suggests that unexplored species with similar characteristics, such as eastern water dragons (*Intellagama lesueurii lesueurii*), could also be susceptible to BIV infection. While ranaviral infection has not been reported in captive or wild lizards in Australia, there have been reports of ranaviral mortalities in captive central bearded dragons kept overseas (Stöhr et al., 2013; Tamukai et al., 2016). Therefore, it is likely that lizards in Australia are susceptible to ranavirus and may even contribute to the spread and amplification of this virus in the wild. Unlike other Australian reptiles, lizards are yet to be investigated with respect to susceptibility to this pathogen.

1.10. Eastern water dragons

The eastern water dragon (*Intellgama lesueurii lesueurii*) is a semi-aquatic arboreal Squamate that is distributed down the eastern coast of Australia from Cooktown, Queensland, to Kangaroo Valley, New South Wales (Brown, 2002). Due to their wide distribution they occupy

a range of different habitats from tropical rainforests in the north to alpine streams in the south. Their natural range includes the area where BIV was first isolated, and overlaps with several turtle, fish and amphibian species shown to be susceptible to this pathogen (Brown, 2002; Speare & Smith, 1992). Additionally, eastern water dragons are often found in areas where human activity is high (e.g. parks and zoos) and many are also kept in captivity in both private and zoological collections (Figure 1.2).

In the wild, eastern water dragons can often be found perching on rocks and overhanging branches along margins of creeks, rivers, lakes and other bodies of freshwater. They are well adapted to swimming, can rest on the bottom of shallow waterways for up to 90 minutes and are equipped with a long muscular laterally-compressed tail that comprises of about two-thirds their total length (Brown, 2002). In comparison to other large dragons, eastern water dragons have a lower preferred body temperature (23.75−36.0°C) allowing them to remain in the water or in the shade on hot days (Hoskin, 2017; K. J. Wilson, 1974). These lizards have ontogenetic diet-shift and while juveniles and sub-adults are completely insectivorous feeding on ants, crickets, spiders, and worms, the diet of an adult dragon is omnivorous and includes vegetation, fruit, molluscs, insects, frogs, and small reptiles and mammals (Cogger, 2014). Their total body length can be up to 60 cm for females and 1 m for males, making them the largest member of the Agamidae family. They reproduce in early October laying between 6-18 eggs with the sex of the hatchlings determined by the temperature of the nest which is usually in sandy or soft soil, in an area open to the sun (Doody et al., 2006). In captivity, they are long lived (up to 25 years) and require the appropriate diet, environmental conditions and enclosure size to meet their needs (Harlow & Harlow, 1997). There are Codes of Practice available in each state/territory which outline the minimum acceptable standards for keeping

reptiles in captivity (e.g. *Code of practice for the private keeping of reptiles* 2013). This includes information on spatial requirements for enclosures, diet information, and husbandry.

The abundance of eastern water dragons in the wild and captivity, as well as their overlapping distribution with several ectothermic species shown to be susceptible to BIV, makes them an ideal study species. The field of ranavirus research is currently dominated by studies on fish and amphibians, despite increasing reports of ranavirus infection in captive reptiles. It is important that research expands our knowledge of ranaviruses in reptiles considering the global decline and ecological significance of reptiles. It is in this context that the work presented herein was undertaken with the overarching aim of investigating the susceptibility of a selection of Australian lizards to *Ranavirus* sp. infection.

The aims of this thesis are as follows:

1. Investigate the susceptibility and pathogenesis of Bohle iridovirus (*Ranavirus* sp.) in juvenile eastern water dragons.
    - Experimental infection of juvenile eastern water dragons is described in Chapter 2 and 3

2. Determine if ranaviruses are present in wild and/or captive Australian lizards.
    - The findings of a molecular survey for ranaviral DNA in wild and captive Australian lizards are presented and discussed in Chapter 4

3. Identify and understand Australian reptile owners experience and management of disease in captive reptile collections.
    - The findings of a questionnaire answered by 179 Australian reptile owners are presented and discussed in Chapter 5

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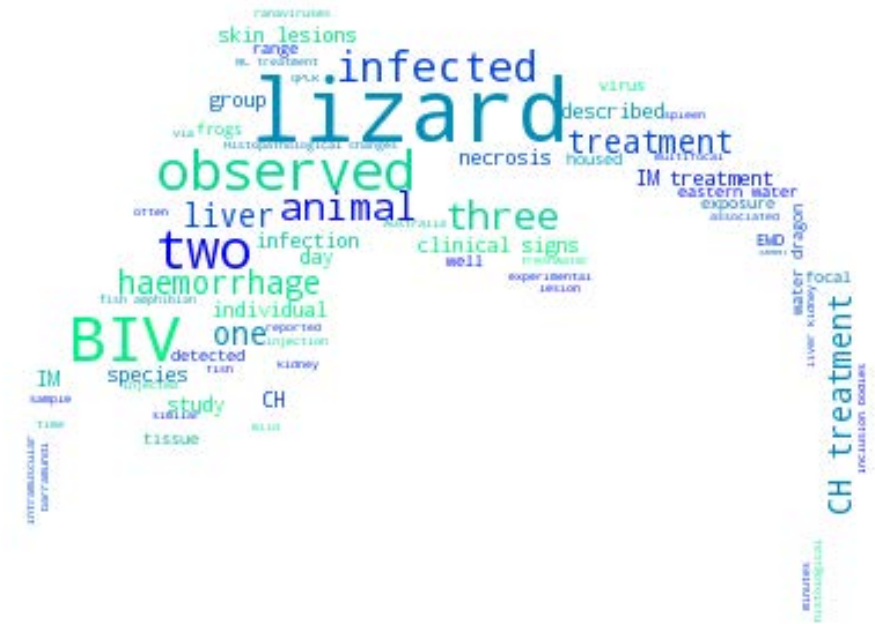
CHAPTER 2 – The susceptibility of an Australian semi-aquatic lizard to Bohle iridovirus (*Ranavirus* sp.)

2.1. Aims of this chapter

1. Determine if eastern water dragons are susceptible to Bohle iridovirus
2. Explore different routes of infection and identify those that cause disease
3. Describe the clinical signs, pathology and mortality of a ranaviral infection in eastern water dragons



2.2. Introduction

Ranaviral infections in lizards are being detected at an increasing frequency and are often associated with sudden death, skin lesions, lethargy, and inappetence. The first report of a ranaviral infection in lizards was in a captive-bred leaf-tailed gecko (*Uroplatus fimbriatus*) that died suddenly in Germany (Marschang, Braun, & Becher, 2005). Another seven lizard species held in captivity in Germany and Japan, including a species endemic to Australia, have been reported with ranaviral infections (Alves de Matos et al., 2011; Behncke, Stöhr, Heckers, Ball, & Marschang, 2013; Stöhr et al., 2013; Tamukai, Tokiwa, Kobayashi, & Une, 2016). While ranaviral infections have not been reported in wild or captive lizards in Australia, they have been detected in Australian fish and amphibians, and illegally imported green pythons (*Morelia viridis*) (Hyatt et al., 2002; Langdon, Humphrey, & Williams, 1988; Langdon, Humphrey, Williams, Hyatt, & Westbury, 1986; Speare & Smith, 1992; Weir et al., 2012; Whittington, Kearns, Hyatt, Hengstberger, & Rutzou, 1996).

The effect of ranaviral infections on Australian fish, amphibian and turtle species has been explored through challenge studies conducted under experimental conditions (Ariel & Owens, 1997; Ariel, Wirth, Burgess, Scott, & Owens, 2015; Cullen & Owens, 2002; Cullen, Owens, & Whittington, 1995; Langdon, 1989; Moody & Owens, 1994). Due to the ability of ranaviral infections to be transmitted between different classes of ectotherms (e.g. frog to turtle), it is important to identify susceptible species. An isolate of ranavirus, Bohle iridovirus (BIV), discovered in Townsville Australia, and shown to be infectious to fish, amphibians, and hatchling turtles native to the region, was used to infect a semi-aquatic lizard that has an overlapping distribution with these susceptible species.

2.3. Publication and outputs

This chapter includes results presented as a peer-reviewed paper and a conference poster. I was the first author of the peer-reviewed paper. My overall contribution to this study and subsequent outputs were as follows:

- I designed the study in collaboration with my primary advisor;
- I prepared the James Cook University animal ethics application and Department of Heritage and Protection Scientific Purposes permit for this study;
- I sourced, quarantined, monitored and completed daily husbandry for the lizards prior to and during the study;
- Using cell culture methods, I cultivated and titrated the viral stock used in this study;
- With the help of a research assistant, I inoculated the lizards used in this study;
- I conducted the necropsies of lizards and undertook sample collection for viral isolation, PCR and histology;
- I prepared the initial histology slides and analysed them with the help of Dr. Jennifer Scott;
- I prepared the cells and tissue samples for viral isolation and analysed the results with the help of another PhD student;
- I extracted the DNA from samples for analysis by PCR and prepared samples for sequencing;
- I was the lead author of the manuscript, which I drafted. It was later accepted for publication in a peer-reviewed journal (see below); and
- I prepared the poster that I presented at the 10th International Symposium on Viruses of Lower Vertebrates held in June 2017 in Budapest, Hungary.

Results from this study are included in the following peer-reviewed publication and conference poster:

- **Maclaine, A.**, Mashkour, N., Scott, J., & Ariel, E. (2018). Susceptibility of eastern water dragons Intellagama lesueurii lesueurii to Bohle iridovirus. *Disease of Aquatic Organisms, 127*(2), 97-105. doi:10.3354/dao03193
- **Maclaine, A.,** Scott J., Mashkour, N., & Ariel, E. Susceptibility of eastern water dragons, *Intellagama lesueurii lesueurii*, to Bohle iridovirus. Poster presented at: The 10th International Symposium on Viruses of Lower Vertebrates; 4-7 June 2017; Budapest, Hungary

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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

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2.4. Conclusion

The aims of this chapter were met in the following manner:

1. Determine if eastern water dragons are susceptible to Bohle iridovirus

- Male and female juvenile eastern water dragons are susceptible to Bohle iridovirus using the routes and dose described in this experiment.

2. Explore different routes of infection and identify those that cause disease

- Juvenile eastern water dragons were exposed to BIV via oral inoculation (OR), intramuscular injection (IM), or via cohabitation (CH) with orally infected lizards (Figure 2.1).
- All exposure methods were effective in establishing infection.

3. Describe the clinical signs, histopathology and mortality of a ranaviral infection in eastern water dragons

- Severity of clinical signs and histopathology, and the time between exposure and death, varied depending on exposure method.
- Clinical signs included inappetence, lethargy, swollen abdomen, loss of equilibrium, and skin lesions on the digits and lower limbs.

- Histopathological changes included liver necrosis, variably sized intracytoplasmic inclusion bodies in the liver, necrosis and haemorrhaging in the kidney, splenic necrosis, pancreatic haemorrhages, and epidermal and dermal necrosis with secondary fungal and bacterial invaders in association with skin lesions. Extensive intramuscular haemorrhage at the site of injection was evident in the IM group.
- Average time between exposure and death was 6.9 days in the IM group, 12 days in the OR group, and 19.9 days in the CH group.

This study has shown that a semi-aquatic Australian lizard is susceptible, under the conditions described in this chapter, to infection with BIV, a ranavirus isolated from frogs in the region that these lizards inhabit (Speare & Smith, 1992). Clinical signs and histopathology observed in BIV-infected lizards are similar to that described in other ranaviral-infected lizards, with the exception of the appearance of skin lesions. (Behncke et al., 2013; Marschang et al., 2005; Stöhr et al., 2013). This study has identified juvenile eastern water dragons as a potential source of transmission of BIV, as shown by their ability to infect naïve individuals.

However, factors such as dose, temperature and age-class, may also play a role in an individual's susceptibility to ranavirus as shown in other ranaviral studies in frogs, turtles and fish (Allender, Barthel, Rayl, & Terio, 2018; Allender, Mitchell, Torres, Sekowska, & Driskell, 2013; Ariel et al., 2015; Forzán et al., 2015; Rojas, Richards, Jancovich, & Davidson, 2005).

Further investigation on the progression of infection in this species will help clarify the pathogenesis of the disease. Additionally, molecular surveys of wild lizards will further the understanding of the importance and epidemiology of this disease in native species, particularly in areas where BIV has been identified in other ectotherms. It is recommended that future studies look at infection kinetics to identify the role that temperature and other factors, such as dose, can play in ranaviral infections in lizards.

Figure 2.1 Housing conditions for lizards in the A) oral, intramuscular, negative control, and B) cohabitation treatments

2.5. References

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CHAPTER 3 – The pathogenesis of Bohle iridovirus infection in juvenile eastern water dragons

3.1. Aims of this chapter

1. Describe the progression of a ranaviral infection in orally infected juvenile eastern water dragons
2. Identify the time-points at which:
    - histopathological changes can be observed,
    - ranaviral DNA can be detected in cloacal swabs, and liver and kidney samples via PCR

3.2. Introduction

Chapter 2 revealed that juvenile eastern water dragons (*Intellagama lesueurii lesueurii*) are susceptible to infection with Bohle iridovirus (BIV), adding this species to the list of Australian ectotherms shown to be susceptible to this ranaviral isolate (Ariel & Owens, 1997; Ariel, Wirth, Burgess, Scott, & Owens, 2015; Cullen & Owens, 2002; Cullen, Owens, & Whittington, 1995; Moody & Owens, 1994) (Table 1). The clinical signs, gross pathology and histopathology associated with a BIV infection in this species have been described in the previous chapter and are similar to that described in other ranaviral lizards (Behncke, Stöhr, Heckers, Ball, & Marschang, 2013; Marschang, Braun, & Becher, 2005; Stöhr et al., 2013).

Table 3.1: List of Australian species shown to be susceptible to Bohle iridovirus via experimental challenge trials.

Species	Reference
Eastern water dragon (*Intellagama lesueurii lesueurii*)	(Maclaine, Mashkour, Scott, & Ariel, 2018)
Saw-shelled turtle (*Elseya latisternum*)	(Ariel et al., 2015)
Krefft's river turtle (*Emydura macquarii krefftii*)	
Green tree frog (*Litoria caerulea*)	(Cullen & Owens, 2002)
Striped burrowing frog (*Cyclorana alboguttata*)	
Shortfooted frog (*Cyclorana brevipes*)	
Red-backed toadlet (*Pseudophryne coriacea*)	
Tilipia (*Oreochromis mossambicus*)	(Ariel & Owens, 1997)
Northern bango frog (*Limnodynastes terraereginae*)	(Cullen et al., 1995)
Broad palmed frog (*Litoria latopalmata*)	
Barramundi (*Lates calcarifer*)	(Moody & Owens, 1994)

Most of the research on ranaviruses has been conducted in amphibians and fish, with reptiles underrepresented. The studies have explored the effects of temperature, dose, and exposure route; and have looked at the development of the disease overtime (Allender, Barthel, Rayl, & Terio, 2018; Forzán et al., 2017; Forzán et al., 2015). However, none have described the pathogenesis of BIV infection in any species shown to be susceptible, or the pathogenesis of a ranaviral infection in a lizard host.

3.3. Publication and outputs

This chapter includes results presented as a conference poster and a manuscript submitted to a peer-reviewed journal: Veterinary Pathology (May 2018). I was the lead author for this manuscript and my overall contribution to this study and its outputs was as follows:

- I designed the study in collaboration with my primary advisor;
- I prepared the ethics application for the James Cook University Animal Ethics Committee and for the Department of Heritage and Protection Scientific Purposes Permit;
- I monitored and completed daily husbandry for the lizards used prior to and during the study;
- With the help of a research assistant, I orally inoculated the lizards with viral stock used in the previous study;
- I euthanised all lizards and performed the necropsies where I undertook sample collection for PCR, histopathology, and viral isolation;
- I extracted the DNA from samples for analysis by PCR and prepared samples for sequencing;
- I prepared the initial histology slides and analysed them with the help of Dr. Jennifer Scott. The IHC and ISH was done in collaboration with Dr. María Forzán, Cornell Wildlife Health Lab, Department of Population Medicine, Animal Health Diagnostic Center, Cornell University College of Veterinary Medicine, United States of America;

- I was the lead author of the manuscript, which I drafted. It was later accepted for publication in a peer-reviewed journal (see below); and
- I prepared the presentation that I presented at the 4th International Symposium on Ranaviruses held in June 2017 in Budapest, Hungary.

Results from this study are included in the following peer-reviewed publication and conference poster:

- **Maclaine, A.**, Forzán, M., Mashkour, N., Scott, J., & Ariel, E. (2019). Pathogenesis of Bohle iridovirus (Genus Ranavirus) in experimentally infected juvenile eastern water dragons (Intellagama lesueurii lesueurii). *Vet Pathol, 56*(3), 465-475 doi:10.1177/0300985818823666
- **Maclaine, A.,** Scott, J., Wirth, W., Mashkour, N., & Ariel, E. Pathogenesis of Bohle iridovirus in juvenile eastern water dragons, *Intellagama lesueurii lesueurii*. Presentation presented at: The 4th International Symposium on Ranaviruses; 7-10 June 2017; Budapest, Hungary.

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Pathogenesis of Bohle Iridovirus (Genus *Ranavirus*) in Experimentally Infected Juvenile Eastern Water Dragons (*Intellagama lesueurii lesueurii*)

Alicia Maclaine[1], María J. Forzán[2], Narges Mashkour[1], Jennifer Scott[1], and Ellen Ariel[1]

Abstract

Juvenile eastern water dragons (*Intellagama lesueurii lesueurii*) are highly susceptible to infection with Bohle iridovirus (BIV), a species of ranavirus first isolated from ornate burrowing frogs in Townsville, Australia. To investigate the progression of BIV infection in eastern water dragons, 11 captive-bred juveniles were orally inoculated with a dose of $10^{4.33}$ $TCID_{50}$ and euthanized at 3, 6, 8, 10, 12, and 14 days postinfection (dpi). Viral DNA was detected via polymerase chain reaction (PCR) in the liver, kidney, and cloacal swabs at 3 dpi. Mild lymphocytic infiltration was observed in the submucosa and mucosa of the tongue and liver at 3 dpi. Immunohistochemistry (IHC) first identified viral antigen in foci of splenic necrosis and in hepatocytes with intracytoplasmic inclusion or rare single-cell necrosis at 6 dpi. By 14 dpi, positive IHC labeling was found in association with lesions in multiple tissues. Selected tissues from an individual euthanized at 14 dpi were probed using in situ hybridization (ISH). The ISH labeling matched the location and pattern detected by IHC. The progression of BIV infection in eastern water dragons, based on lesion severity and virus detection, appears to start in the spleen, followed by the liver, then other organs such as the kidney, pancreas, oral mucosa, and skin. The early detection of ranaviral DNA in cloacal swabs and liver and kidney tissue samples suggests these to be a reliable source of diagnostic samples in the early stage of disease before the appearance of clinical signs, as well as throughout the infection.

Keywords

eastern water dragon, Bohle iridovirus, *Intellagama lesueurii lesueurii*, lizards, ranavirus, reptiles

Ranaviruses infect a wide range of ectothermic vertebrates worldwide and are considered emerging pathogens of significant ecological importance in amphibians and fish.[28] Lately, ranaviruses have been detected at an increasing frequency in reptiles and are associated with mortality events in captive lizards belonging to the Agamidae, Anguidade, and Iguanidae families.[8,33,34]

Several studies have documented the infection potential of individual ranaviral isolates to hosts in different classes of lower vertebrates.[7,9,10] Studies exploring the effects of temperature, dose, and exposure route have been conducted in amphibians and turtles using frog virus 3 (FV3),[2,17] in amphibians using *Ambystoma tigrinum* virus (ATV),[12] and in fish, amphibians, and reptiles using Bohle iridovirus (BIV).[4,5,24,27,32] Several species of fish, amphibians, and turtles shown to be susceptible to BIV have an overlapping geographic distribution with eastern water dragons (*Intellagama lesueurii lesueurii*).[11] This semiaquatic squamate is a strong swimmer often found in large numbers along freshwater rivers and creeks on the east coast of Australia.[11] Eastern water dragons (EWDs) are also common in the pet trade, and although viruses (eg, adenovirus and ranavirus) are commonly identified in captive lizards and other ectotherms held in collections all over the world,[15,34] natural ranaviral infection has not been reported in captive or wild EWD populations to date. Experimental exposure of EWDs to BIV, a ranavirus originally isolated from ornate burrowing frogs (*Limnodynastes ornatus*) in Townsville, Australia, showed that the lizards were highly susceptible to infection via intramuscular and oral exposure and developed

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histologic lesions of necrosis, hemorrhage, and inflammation in the pancreas, liver, spleen, kidney, and gastrointestinal tract.[24] However, the progression and effect of BIV in juvenile EWDs are not understood. Here we describe the pathogenesis of experimental BIV infection in juvenile EWDs via time-course sampling of individual animals to identify the progression of infection from inoculation to euthanasia.

Materials and Methods

Eleven captive-bred EWDs were purchased from a commercial breeder and kept under permit (Scientific Purposes Permit #WISP15053914) from the Queensland Department of Environment and Heritage Protection at the College of Public Health, Medical and Veterinary Sciences, James Cook University (JCU). Husbandry and experimental and sampling procedures were carried out with approval from the JCU Animal Ethics Committee (Ethics Approval #A2277). At the start of the trial, dragons were 10.5 months old and weighed 11.9 to 18.1 g (mean, 14.5 g). Sex was determined only after euthanasia, during necropsy examination (5 females, 4 males, 2 unknown). The data analyzed in this study are available upon request to the authors.

Husbandry of Experimental Dragons

Dragons were housed individually for 20 weeks in 5-L plastic vivariums with a small plastic pipe hide and a water dish. After acclimation, 13 dragons were randomly assigned to either infection groups ($n = 11$) or served as negative controls ($n = 2$) and were moved to larger 20-L group tanks (2–4 dragons per tank). Dragons were uniquely identified using a nontoxic marker (Duramark; Staples, Stuttgart, Germany) on the dorsal skin and were permitted a further 14-day acclimation period within these groups prior to inoculation. Negative control (NC) dragons were housed in a quarantine room and the BIV-infected dragons in the adjoining infection room. Room conditions were controlled with a 12-hour dark and 12-hour light cycle, and temperatures were recorded twice daily (average, 28.8°C; range, 26.3°C–30.1°C).

The dragons were fed a diet of small crickets 3 times weekly, where 2 of the 3 feeds were dusted with Multical Dust supplement (Vetafarm, New South Wales, Australia). Weight was recorded weekly to ascertain steady growth.

BIV Viral Stock

The viral strain used in this study was the 1992 BIV isolate from ornate burrowing frogs that was isolated at JCU[32] and subsequently sequenced[19] (GenBank accession number KX185156). This strain was propagated at 25°C in fathead minnow (FHM) cells, in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, NY, USA), supplemented with 100× antibiotic-antimycotic (Thermo Fisher Scientific, NY, USA) and 10% fetal bovine serum (Bovogen Biologicals, Melbourne, Australia). Titration of viral stock was performed in a 96-well tissue culture plate following the standard methods to determine the tissue culture infection dose ($TCID_{50}$).[30]

Animal Inoculation and Sampling

Time from infection to euthanasia (end point) in this experimental infection study was predetermined and ranged from 3 to 14 days postinfection (dpi). This was based on a previous infection study of BIV in juvenile EWDs, where the onset of clinical signs and death via oral exposure occurred at 8 to 11 dpi and 9 to 14 dpi, respectively.[24] The same method of exposure and viral titer was used in this study due to its effectiveness at causing acute disease, and the predetermined end points allowed us to cover the early stages of infection and euthanize the dragons at a predefined humane end point. To reduce the number of dragons needed for this study, tissues collected from 3 dragons involved in a previous study,[24] infected and housed in the same conditions as those in the present study, were included in the analysis. These 3 dragons were euthanized at corresponding time points (1 at 12 dpi and 2 at 14 dpi), received an equivalent oral dose of BIV, and were housed in similar conditions.

After the 14-day acclimation period, dragons in the infection treatments were orally inoculated with 100 µl ($10^{4.33}$ $TCID_{50}$) BIV using a syringe placed at the back of the oral cavity. NC dragons received 100 µl orally of phosphate-buffered saline. Dragons were examined twice daily for any behavioral change (eg, decreased activity) and clinical signs previously described[24] (ie, distended abdomen, loss of appetite, decreased activity, loss of startle and rollover reflexes, loss of equilibrium, and focal areas of skin ulceration or pustules). Consumption of food and changes in appetite were recorded and water was refreshed daily. A cloacal swab was collected at –1, 3, 6, 8, 10, 12, and 14 dpi; stored at –80°C in 1 ml DMEM (Thermo Fisher Scientific, United States) supplemented with 100× antibiotic-antimycotic (Thermo Fisher Scientific, United States), and later tested for ranaviral DNA by polymerase chain reaction (PCR).

The dragons were euthanized at predetermined end points: 3, 6, 8, 10, 12, and 14 dpi, to record the time-course progression of infection. Two dragons were euthanized at each of the following time points: 3, 6, 8, 10, and 12 dpi; 1 dragon was euthanized at 14 dpi. The dragon to be euthanized at each time point was determined by random selection prior to commencing the experiment. The 2 NC dragons were euthanized at the end of the trial (14 days). Euthanasia was a 2-stage method using tricaine methanesulfonate (MS222).[14] Briefly, lizards were injected intracoelomically (IC) with 250 mg/kg 1% MS222 (stage 1). Once it was confirmed that the dragon had lost its toe-pinch reflex, lizards were injected IC with 0.4 to 0.5 ml 50% (v/v) MS222 (stage 2). Immediately after euthanasia, a dorsal and ventral photograph was taken, and any lesions or abnormalities were documented. During necropsy, small tissue samples were aseptically obtained from the liver and kidney, frozen at –80°C, and later used for PCR and viral isolation.

Although no testing was performed prior to inoculation with BIV, we are confident of the naive status of all dragons since (1) all dragons originated from the same captive breeding facility where no outbreaks have ever been detected, and (2) the dragons kept as uninfected (controls) were all negative for ranavirosis based on histopathology and PCR results.

Histopathology

A cross section of the proximal limb for normal skin and skin samples with gross lesions, lung, liver, pancreas, spleen, kidney, digestive tract, heart, tongue, brain, adrenal gland, reproductive organs, and bone marrow were preserved in 10% neutral buffered formalin. Tissues were processed routinely for histological examination and stained with hematoxylin-eosin.[6] Sections that included bone (toes, feet, legs, spine, and head) were trimmed and placed in Gooding and Stewart's decalcifying fluid for 24 hours prior to processing.[6] The histopathology and immunohistochemistry (IHC) results from 3 orally exposed dragons from a previous study of BIV in juvenile EWDs were included.[24]

Immunohistochemistry and In Situ Hybridization

Formalin-fixed, paraffin-embedded tissues from individuals euthanized at 3, 6, 8, 10, 12, and 14 dpi were immunolabeled for *Ranavirus* sp antigen using a polyclonal rabbit antibody against a member of the *Ranavirus* genus: epizootic hemorrhagic necrosis virus (EHNV).[29] Anti-EHNV antibodies are known to cross-react with other viruses in the genus.[3,20] In an automated staining system (BOND-MAX; Leica Microsystems, Buffalo Grove, IL), sections were labeled using a previously validated protocol for affinity-purified rabbit anti-EHNV (lot M708, OIE Reference Laboratory for EHN Virus, University of Sydney) IHC staining. Briefly, slides were dewaxed with Bond Dewax Solution (cat. AR9222; Leica Microsystems). A Heat Epitope Retrieval with Bond Epitope Retrieval Solution 1 was applied for 30 minutes (cat. AR9961; Leica Microsystems). Rabbit anti-EHNV antibody diluted at 1:3000 was applied to slides for 15 minutes. A polymer (secondary antibody, anti-rabbit poly-HRP-IgG; cat. DS9390; Leica Microsystems) was applied for 10 minutes. Leica Bond Polymer Refine Red Detection was applied for 15 minutes (cat. DS9390; Leica Microsystems). Finally, the tissue was counterstained with hematoxylin applied for 5 minutes (cat. DS9390; Leica Microsystems). Control slides were processed with the above method but omitting the primary antibody. A tissue was considered positive if strong and distinct staining with anti-EHNV (*Ranavirus sp*) antibody was present in the cytoplasm of 1 or more cells and background staining was either absent or clearly distinct from true specific staining. Diffuse pale staining of a specific type of tissue or cell (eg, stomach glandular epithelium) was considered background artifactual staining.

Selected tissues (skin, bone marrow, liver, pancreas, stomach, intestine, and spleen) from an individual euthanized at 14 dpi were labeled with an in situ hybridization probe (ACDBio RNAscope Probe- V-FV3-orf90 R, cat. 439991) for Frog Virus 3 (GenBank KF646249.1), following the manufacturer's specifications (Advanced Cell Diagnostics, Newark, CA) and compared with the immunolabeling using anti-EHNV antibody.

Viral Isolation

Sections of the liver and kidney aseptically collected at necropsy were homogenized with 1 ml DMEM supplemented with 100× Antibiotic-Antimycotic (Thermo Fisher Scientific, NY, USA) and subjected to 3 freeze/thaw cycles at –20°C before clarification by centrifugation at 13 523 *g* for 5 minutes. Tenfold serial dilutions ($1–10^{-5}$) were prepared from each sample, and a total of 50 µl was added in duplicate to 80% confluent monolayers of FHM cells in a 96-well tissue culture plate (SARSTEDT, Nümbrecht, Germany). The plates were incubated at 25°C and checked for cytopathic effect daily for 1 week. Supernatant from each sample was preserved at –20°C for further analysis.

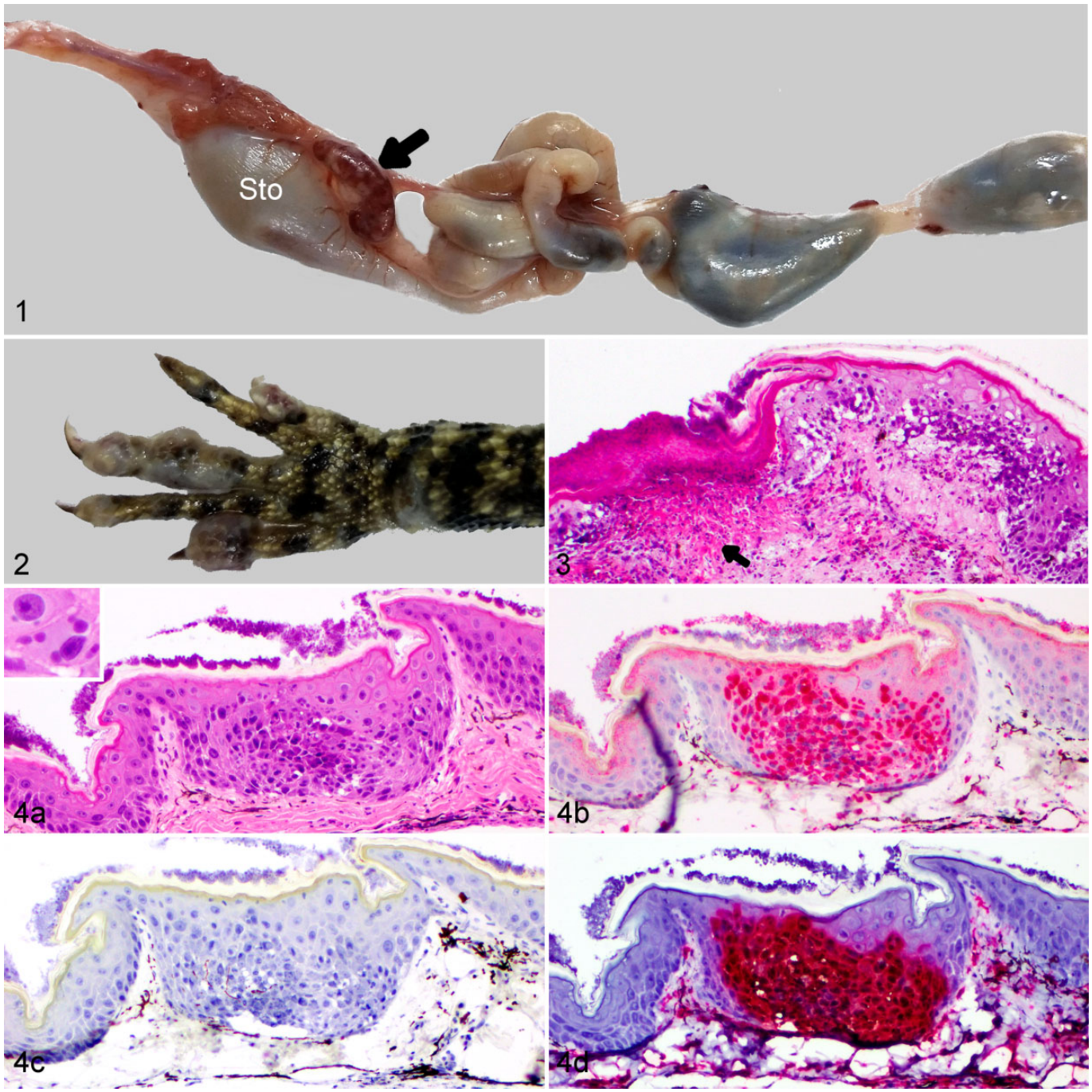
Polymerase Chain Reaction

Total DNA was extracted from a 200-µl aliquot of the media that the cloacal swab sample was stored in and from small portions of the homogenized liver and kidney samples using a spin-column DNA purification procedure (Bioline ISOLATE II Genomic DNA Kit, animal tissue protocol; Bioline, Luckenwalde, Germany). Viral isolation supernatant was prepared using the same protocol following the preparation instructions for "cultured cells." A single-round of PCR targeting the major capsid protein (MCP) region of the EHNV genome was performed using primers (forward primer, 5′- GACTGACCAA CGCCAGCCTTAACG-3′; reverse primer, 5′-GCGGTGG TGTACCCAGAGTTGTCG-3′) designed by Jaramillo et al.[21] The reaction mixture was as follows: 1× GoTaq qPCR Mastermix (Promega, Madison, WI), 0.8 µM of forward and reverse primer, 2 µl of template DNA (≈80 ng), and nuclease-free water in a 20-µl reaction. Thermocycling parameters were as follows: 95°C for 2 minutes, then 40 cycles of [95°C for 5 seconds, 58°C for 10 seconds and 72°C for 15 seconds] with a final extension at 95°C for 2 minutes. All PCR tests were run with positive (BIV DNA) and negative controls (no template control).

Results

Gross Pathology

Lesions were detected at necropsy in the skin of dragons euthanized at 10, 12, and 14 dpi (Fig. 2). The skin of the distal limbs, particularly the digits, had multifocal swelling and small ulcerations, sometimes associated with keratin layer retention (dyskeratosis). The spleens of dragons euthanized at 3 dpi were diffusely congested. The spleens of dragons euthanized at 6, 8, 10, 12, and 14 dpi had multifocal white pinpoint foci of necrosis (Fig. 1). A few petechial hemorrhages were observed in

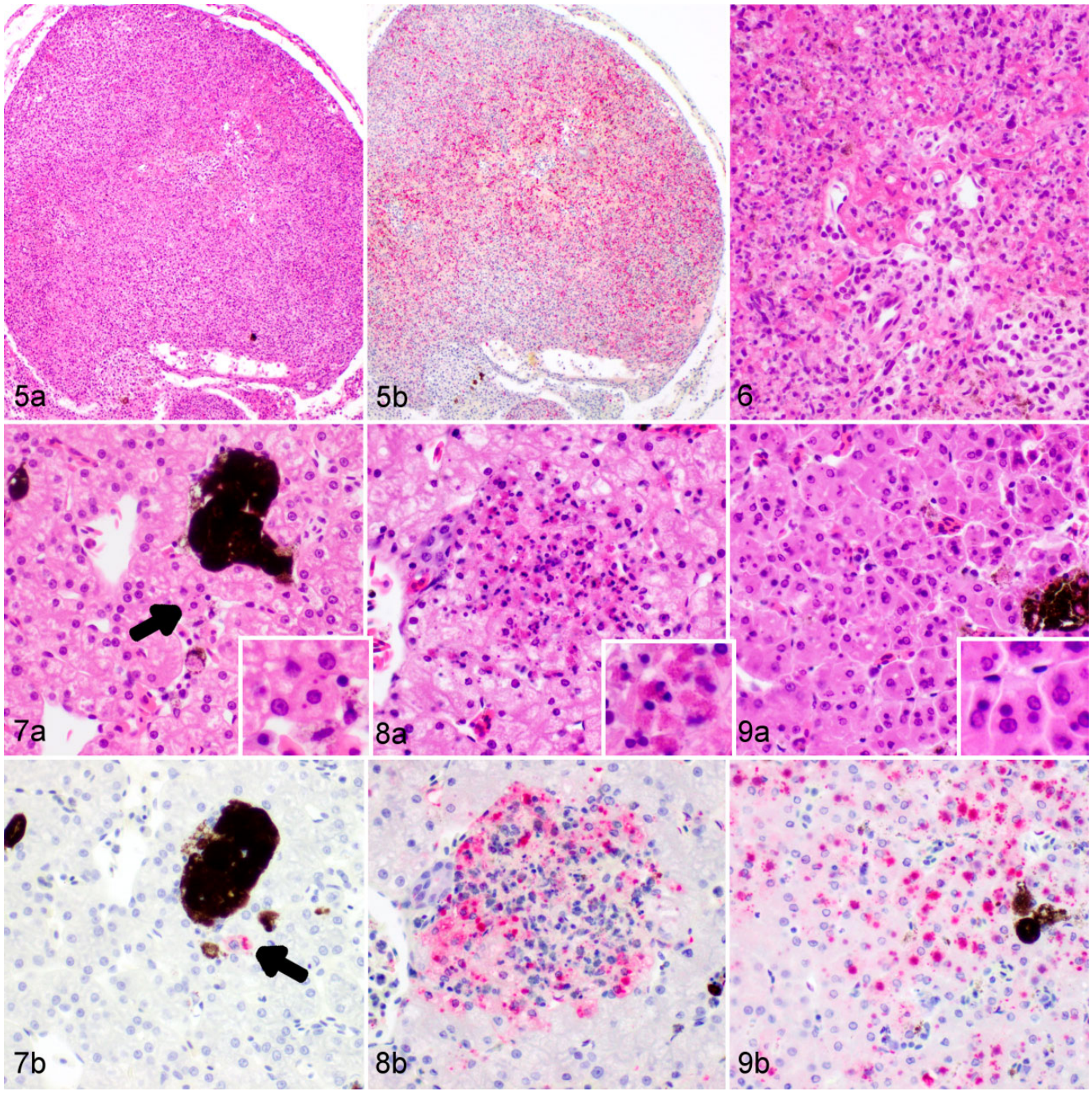


Figures 1–4. Bohle iridovirus experimental infection ($10^{4.33}$ $TCID_{50}$ orally), eastern water dragon. **Figure 1.** Multifocal pinpoint necrosis in the spleen (arrow), at 8 days postinfection (dpi). Sto, stomach. **Figure 2.** Skin of the forelimb, 14 dpi. Multifocal swelling and necrosis. **Figure 3.** Skin, 10 dpi. Focally extensive epidermal vacuolation with ulceration and collagen degeneration (arrow). Hematoxylin and eosin (HE). **Figure 4.** Skin, 14 dpi. (a) Focal epidermal necrosis with prominent intracytoplasmic inclusion bodies (inset). HE. (b–d) Strong immunolabeling for ranavirus using an antibody to epizootic hematopoietic necrosis virus (b); lack of immunolabeling when primary antibody is omitted (c); in situ hybridization for ranavirus using a frog virus 3 probe (d).

dragons euthanized at 6, 8, 10, 12, and 14 dpi. Multifocal ecchymotic hemorrhages on the serosal surface of the intestines and stomach were present in dragons euthanized at 10 and 12 dpi. One individual euthanized at 8 dpi had petechial hemorrhages in the tongue.

Histopathology

Histopathological changes are listed in Table 1. At 3 dpi, multifocal infiltration of lymphocytes was observed in the mucosa and submucosa of the tongue and in the liver. The liver had small foci of melanomacrophage aggregations and several



Figures 5–9. Bohle iridovirus infection, eastern water dragon. **Figures 5–6.** Spleen, 6 days postinfection (dpi). Multifocal to coalescing splenic necrosis (Figs. 5a and 6, hematoxylin and eosin [HE]), with positive immunohistochemical labeling for ranavirus (Fig. 5b, anti-EHNV antibody) colocalizing with the areas of necrosis. **Figures 7–9.** Liver. (a) HE. (b) Immunohistochemistry (IHC) for ranavirus using anti-EHNV antibody. **Figure 7.** 6 dpi. A melanomacrophage cluster is adjacent to a hepatocyte with small intracytoplasmic inclusion bodies (a, arrow and inset) and matching positive IHC labeling for ranavirus (b). **Figure 8.** 10 dpi. Focal heterophilic inflammation (inset) and hepatocyte necrosis (a) associated with positive immunolabeling (b). **Figure 9.** 14 dpi. Multifocal hepatocellular degeneration and single-cell necrosis with basophilic intracytoplasmic inclusion bodies (a, inset) associated with positive immunolabeling (b).

small focal areas of lymphocyte accumulation. Necrosis was not observed in any tissue. Dragons euthanized at 6 dpi had moderate splenic necrosis (Figs. 5-6) and no lesions in any other viscera except for possible rare intracytoplasmic inclusions in hepatocytes (Fig. 7). At 8 dpi, there was multifocal to diffuse necrosis in the spleen with infiltration of macrophages. Dragons euthanized at 10 dpi had severe splenic necrosis, focal lymphocyte and granulocyte infiltration in the

Table 1. Histopathological Findings in Juvenile Eastern Water Dragons Infected Orally With $10^{4.33}$ $TCID_{50}$ of Bohle Iridovirus (*Ranavirus sp*) and Euthanized at Different Days Postinfection (dpi).[a]

		dpi					
Organ	Lesion	3	6	8	10	12	14
Skin	Epidermal necrosis					2/3	3/3
	Intracytoplasmic inclusion bodies					1/3	
	Ballooning degeneration of epidermis				1/2	1/3	
	Bacteria				1/2		2/3
	Subcutaneous inflammation					1/3	1/3
	Subcutaneous hemorrhage					1/3	
	Fungal hyphae (secondary invaders)						2/3
Tongue	Infiltration of lymphocytes	1/2	2/2		1/2	1/3	
	Epithelial necrosis					1/3	2/3
Liver	Melanomacrophages	2/2	2/2			1/3	1/3
	Vacuolation				1/2	1/3	
	Lymphocyte accumulation	1/2					
	Intracytoplasmic inclusion bodies				2/2	3/3	2/3
	Hepatocyte necrosis					1/3	1/3
Spleen	Necrosis		2/2	1/2	2/2	2/3	2/3
Pancreas	Inflammation				2/2	1/3	
	Necrosis					1/3	
Gastrointestinal tract	Lymphocyte accumulation in submucosa				1/2		
	Submucosal and muscularis hemorrhage					1/3	
Kidney	Interstitial necrosis					1/3	
	Tubular necrosis					1/3	1/3
Lung	Intracytoplasmic inclusion bodies					1/3	
Heart	Necrotic cells					1/3	

[a] The data shows the number of dragons with lesions present/total number of dragons in group. Data include 3 dragons from a previous study that were euthanized at corresponding time points (1 at 12 dpi and 2 at 14 dpi), received an equivalent oral dose of Bohle iridovirus, and were housed in similar conditions.

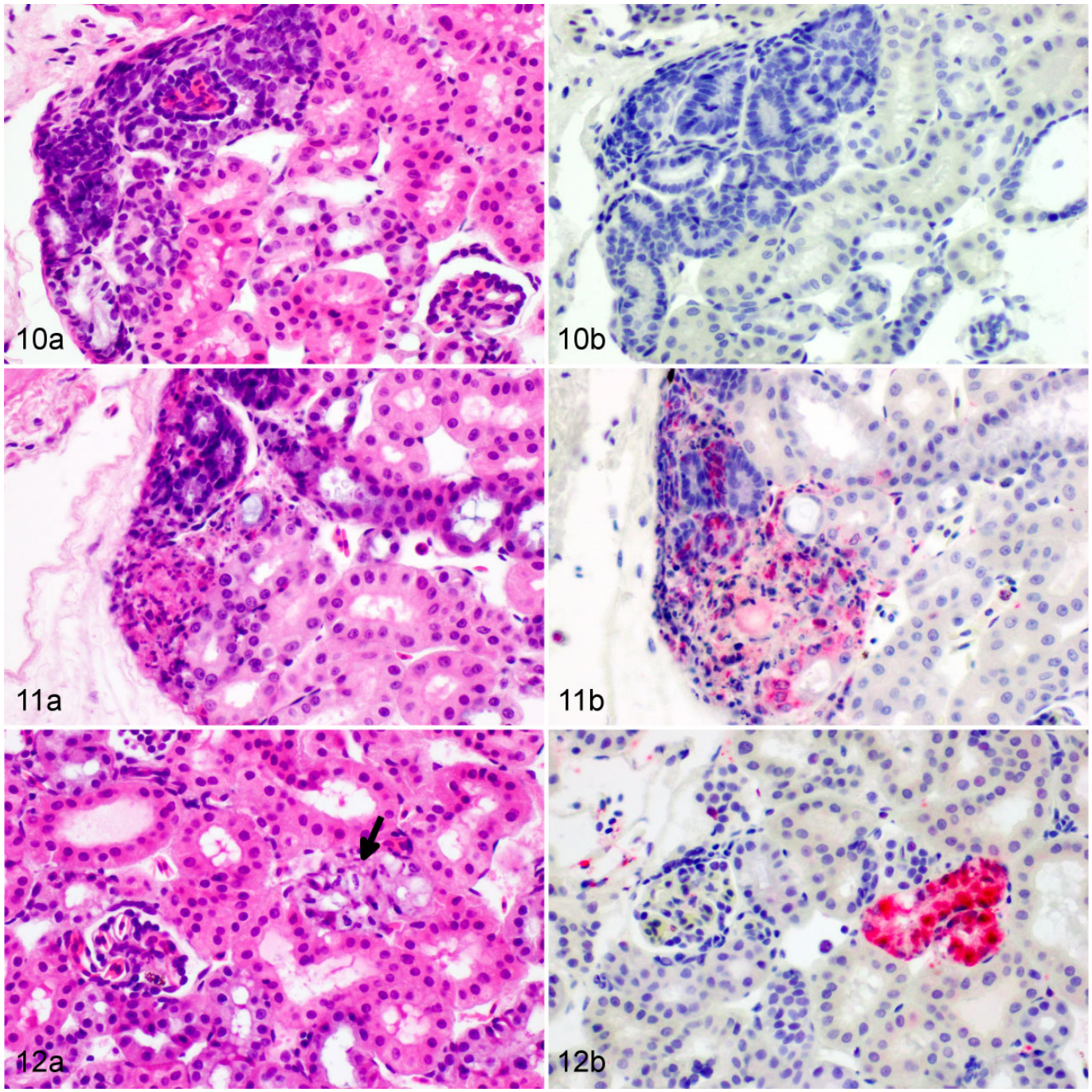
pancreatic connective tissue, and inflammation around the pancreatic duct and the submucosa of the duodenum adjacent to the pancreas. Variably sized basophilic intracytoplasmic inclusion bodies were observed in the hepatocytes and heterophils of the liver, which had mild necrosis and congestion (Fig. 8). Mild interstitial and tubular necrosis of the kidney was present (Fig. 11). Ballooning degeneration of epidermis with necrosis was associated with the skin lesions of 1 dragon (Fig. 3).

Dragons euthanized at 12 dpi had moderate to severe necrosis in the kidney, tongue, gastrointestinal tract, spleen, pancreas, and liver. The necrosis and hemorrhage in the spleen extended out through the splenic capsule and into the pancreas in 1 dragon. Intracytoplasmic inclusion bodies were observed within hepatocytes in the liver and in a consolidated section of the lung adjacent to the trachea. Multifocal subcutaneous hemorrhage and necrosis, as well as severe extensive multifocal subcutaneous necrosis with ballooning degeneration in epidermis, were observed in association with toe and leg lesions in 2 of 3 dragons euthanized at 12 dpi.

Dragons euthanized at 14 dpi had marked multifocal to coalescing necrosis in the liver, spleen, and kidney (Figs. 9, and 12). Mild to moderate necrosis of the tongue epithelium with small intracytoplasmic inclusions was present (Fig. 14). Scattered melanomacrophages were prominent in the liver, heart, and lung. Intracytoplasmic inclusion bodies were frequent in hepatocytes throughout the liver (Fig. 9). Multifocal epidermal necrotizing dermatitis and inflammation in the dermis and underlying connective tissue were observed in association with skin lesions (Fig. 4). In some areas, these lesions also had fungal hyphae extending into the keratin layer and bacteria in the superficial keratin, considered secondary invaders. No histopathological changes were observed in the brain of BIV-infected dragons or in any of the tissues of NC dragons.

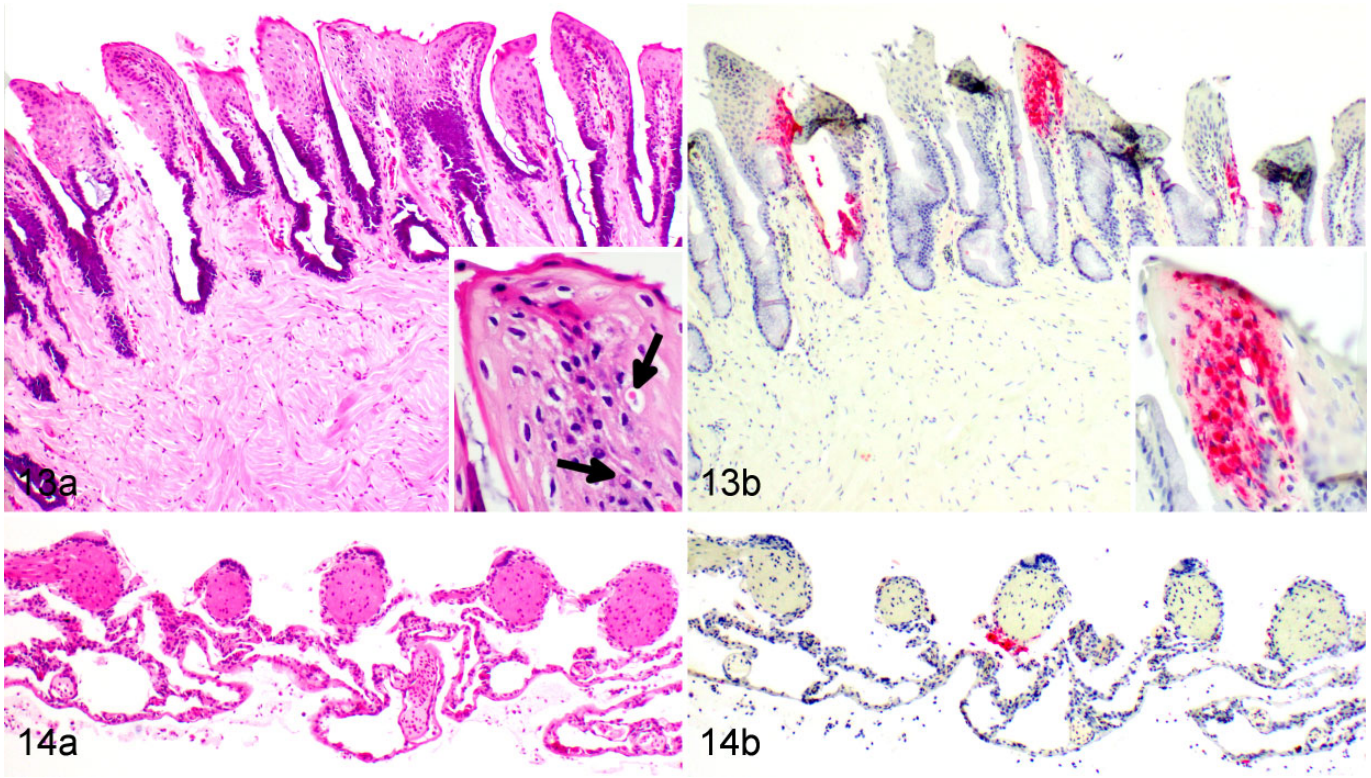
Immunohistochemistry and In Situ Hybridization

No immunolabeling was present in any tissue at 3 dpi (Table 2). Positive labeling with anti-EHNV antibody (*Ranavirus* sp) was first present in the spleen and liver at 6 dpi. The splenic staining was intense, generalized, and associated with necrosis, whereas the liver staining was sparse, often in individual or small clusters of cells, most of which showed mild degeneration or only small intracytoplasmic inclusion bodies. Rare hepatocytes stained positively at 6 dpi, usually in areas were small intracytoplasmic inclusions could be found but necrosis and inflammation were absent (Fig. 7). None of the other tissues showed any immunolabeling (eg, Fig. 10). Positive immunolabeling at 8 dpi was found only in the spleen, also in association with necrosis. At 10 dpi, positive immunolabeling in the spleen was associated with marked necrosis; immunolabeling was associated with mild to moderate necrosis in the liver (Fig. 8), skin,



Figures 10–12. Bohle iridovirus infection, kidney, eastern water dragon. **Figure 10.** 6 days postinfection (dpi). There are no histologic lesions (a) and no immunolabeling for ranavirus (b). **Figure 11.** 10 dpi. There is focal interstitial and tubular necrosis (a) and positive immunolabeling for ranavirus (b). **Figure 12.** 14 dpi. There is tubular epithelial degeneration (a, arrow) and strong positive immunolabeling for ranavirus in the affected tubule and a few endothelial cells (b).

adrenal gland, kidney (Fig. 11), and pancreas. Positive immunolabeling without lesions was present in the bone marrow, lung (Fig. 14), and the endothelium and mesothelium of the stomach. At 12 dpi, immunolabeling associated with moderate to severe necrosis was present in the spleen, liver, skin, kidney, pancreas, lung, tongue, and gastric and intestinal mucosa; positive staining restricted to the endothelium was present in the bone marrow; positive staining restricted to the mesothelium was present in the ovary. At 14 dpi, all tissues examined were positive (Figs. 4, 9, and 13) except for the heart, which had included clusters of positive melanomacrophages in 1 of 2 EWDs euthanized at 12 dpi but was consistently negative in all other individuals at all other times. Brain and spinal cord were consistently negative on dragons euthanized at 6, 10, and



Figures 13–14. Bohle iridovirus infection, eastern water dragon. **Figure 13.** Tongue, 14 days postinfection (dpi). Moderate multifocal necrosis of lingual epithelium, with intracytoplasmic inclusion bodies (a, inset) and strong positive immunolabeling for ranavirus (b). **Figure 14.** Lung, 10 dpi. No significant histologic lesions (a), but strong positive immunolabeling for ranavirus in a few cells (b) at 10 dpi.

12 dpi. No brain tissue was available for testing from individuals euthanized at 14 dpi.

Artifactual background staining was consistently present in the corneal (keratinized) layer of the epidermis, variably present in the glandular epithelium of the stomach and mucosal epithelium of the intestine. This artifactual staining was almost as intense as true immunolabeling in the skin but was present diffusely, was absent when the primary antibody was omitted from the protocol (Fig. 4c), and was found in a NC dragon known to be negative for BIV. Background staining was much paler than specific staining in the gastric and intestinal mucosa and thus easy to classify as artifact. Background staining was absent in all negative control slides (ie, those produced omitting the primary antibody).

The FV3 in situ hybridization (ISH) probe labeled skin (Fig. 4d), bone marrow, liver, pancreas, stomach, intestine, and spleen in the 1 individual tested, which was euthanized at 14 dpi. The ISH labeling matched the location and pattern detected by IHC. The background nonspecific IHC staining noted in the epidermis (Fig. 4b), gastric glandular epithelium, and intestinal mucosa did not correspond to the ISH staining, confirming it was an artifact and did not result from the presence of virus.

Viral Isolation

Virus was isolated from every liver and kidney sample from all experimentally infected EWDs at all time points tested. Cytopathic effect was observed in cell cultures after 96 hours of incubation and confirmed to be due to BIV by PCR on tissue culture supernatant.

Polymerase Chain Reaction

PCR for ranaviral DNA was positive for every sample (liver, kidney, and cloacal swab) from dragons euthanized at 3, 6, 8, 10, 12, and 14 dpi (Table 3). All positive PCR results corresponded to a positive IHC staining except for the liver from dragons euthanized at 3 and 8 dpi and the kidney from dragons euthanized at 3, 6, and 8 dpi (Table 2). All tissues from both control dragons ($n = 2$) were PCR negative for ranaviral DNA. Cloacal swabs collected prior to infection (day -1) from all experimental dragons were negative for ranaviral DNA.

Discussion

Detailed examination of samples from BIV-inoculated juvenile EWDs revealed that viral infection and inflammation of visceral organs were well under way 4 days prior to onset of clinical signs (eg, skin lesions). Lesions caused by BIV infection in juvenile EWDs in this study were consistent with what we have recently described in fatally infected EWDs,[24] where multiple organs were affected. Maclaine et al[24] reported variably sized basophilic intracytoplasmic inclusion bodies near focal areas of necrosis in the liver, splenic necrosis, and multifocal renal

Table 2. Immunohistochemical Labeling[29] of Selected Tissues From Juvenile Eastern Water Dragons Orally Infected With $10^{4.33}$ $TCID_{50}$ Bohle Iridovirus (*Ranavirus sp*) and Euthanized at Different Days Postinfection (dpi).

	dpi					
Organ	3	6	8	10	12	14
Skin	–	–	–	+	+	+
Bone marrow	–	–	–	+[a]	+	+
Tongue	–	–	–	–	+	+
Heart	–	–	–	–	+[b]	–
Lung	–	–	–	+[a]	+	+
Adrenal gland	–	–	–	+	NE	+
Kidney	–[d]	–[d]	–[d]	+[d]	+[d]	+[d]
Spleen	–	+	+	+	+	+
Liver	–[d]	+[c,d]	–[d]	+[d]	+[d]	+[d]
Pancreas	–	–	–	+	+(e)	+(e, m, ish)
Stomach	–	–	–	+(e, m)	+	+(e, m, ish)
Intestine/Colon	–	–	–	–	+	+(m, ish)
Ureter/Oviduct	–	–	–	+	+	

Abbreviations: e, endothelium; ish, indicates matching staining with frog virus 3 (FV3) probe in situ hybridization; m, mesothelium; NE, tissue not examined; +, staining present in tissue; –, no staining in examined tissue.
[a] No lesion.
[b] Clusters of melanomacrophages.
[c] Very few cells.
[d] Tissues that had a positive polymerase chain reaction signal for *Ranavirus sp* DNA.

Table 3. PCR Testing for Ranaviral DNA in the Tissues of Juvenile Eastern Water Dragons Euthanized at Different Days Postinfection (dpi) After Oral Inoculation With $10^{4.33}$ $TCID_{50}$ Bohle Iridovirus (*Ranavirus sp*).[a]

	PCR (+) (dpi)					
Organ or Sample	3	6	8	10	12	14
Liver	2/2	2/2	2/2	2/2	2/2	1/1
Kidney	2/2	2/2	2/2	2/2	2/2	1/1
Cloacal swab	2/2	2/2	2/2	2/2	2/2	1/1

[a] The data show the number of dragons with positive polymerase chain reaction (PCR) results/total number of dragons in the group.

interstitial and tubular necrosis in the terminal phase of infection. In the current study, lymphocyte accumulation was observed in the tongue and liver 72 hours after inoculation (3 dpi) but was not associated with positive IHC staining. Necrosis was not evident until 6 dpi, where it was observed in the spleen while inclusion bodies and early single-cell necrosis were found in the liver of infected dragons. Our findings suggest that BIV infection in juvenile EWDs induces cell death in the spleen between 3 dpi and 6 dpi, as evident by the positive IHC staining that was associated with mild necrosis, and, to a lesser extent, in the liver where IHC staining was often in individual cells showing early degeneration or inclusion bodies at 6 dpi. In samples collected at 10 dpi, the damage to kidney, liver, and spleen was more severe, with positive IHC staining associated with mild to moderate necrosis in the liver, and evident in the skin, adrenal gland, kidney, and pancreas. Positive IHC staining without lesions was observed in the lung, bone marrow, and endothelium and mesothelium of the stomach, indicating presence of virus in these areas, either in phagocytic or possibly antigen-presenting cells. All examined tissues from dragons euthanized on 14 dpi had positive IHC staining. The progression of infection in EWDs appears to start in the spleen, followed by the liver, then the other organs such as the kidney and pancreas, and subsequently oral mucosa and skin.

Immunolabeling with anti-EHNV antibodies was effective in demonstrating BIV presence in various tissues. However, caution should be exercised when interpreting IHC staining of sections of skin where artifactual background staining of the corneal layers was apparent, something that seems to be unavoidable in this species.

Inclusion bodies have previously been described in multiple organs of ranavirus-infected fish, tortoises, turtles, and amphibians[1,13,16,17,22,25,29,35] and, up until now, only in the liver of lizards.[8,24] While inclusion bodies are commonly associated with ranaviral infections,[8,17,18] they are not consistently reported.[2,5,34] This could be due to low numbers or sporadic occurrence of the viral inclusions, individual variation in host or strain, the viral load, duration of exposure, or a combination of these factors. In our study, variably sized intracytoplasmic inclusion bodies were observed in the liver of EWDs euthanized at 6, 10, 12, and 14 dpi but were not consistently found in all dragons euthanized at these time points. In addition, basophilic inclusion bodies were observed in a consolidated area of lung adjacent to the trachea and in association with skin lesions in dragons euthanized 10, 12, and 14 dpi (Figs. 3-4). An apparent delayed appearance of inclusion bodies in infected animals was also described in FV3-infected wood frogs (*Rana sylvatica*) euthanized at 9 and 14 dpi but was not a consistent finding across all individuals within those groups and not recorded in animals euthanized prior to this.[16] Although this is the first report of inclusion bodies in lungs of ranaviral-infected dragons, there are reports of inclusion bodies in keratinocytes adjacent to areas of necrosis in skin lesions of BIV-like infected boreal toads (*Anaxyrus boreas boreas*), in circulating leukocytes of a FV3-infected eastern box turtle (*Terrapene carolina carolina*), and in the cytoplasm of skin epidermal cells of FV3-infected wood frogs euthanized 14 dpi.[1,13,16]

To identify the progression of BIV infection in EWDs at different time points, we collected cloacal swabs and tissue samples for PCR, virus isolation, histopathology, and IHC. In our study, *Ranavirus* sp DNA was detected in all cloacal swabs, as well as liver and kidney tissue samples collected at the first time point (3 dpi) in the early stages of infection, which contrasts to an FV3 pathogenesis study in wood frogs (*Rana sylvatica*) where DNA was not detected in multiple tissues until shortly before death.[16] Although the detection of ranaviral DNA in cloacal swabs could arguably be passage of the original inoculum from the mouth to the cloaca over 3 days post-exposure, the early detection by PCR in liver and kidney

samples was accompanied by an inflammatory response in the organs as visualized by histology and by virus isolation from these organs. The results from the IHC staining from both the wood frog study[16] and the study reported here did not appear to be as sensitive as PCR, and positive immunolabeling of organs was more consistently identified later in the trials than PCR-positive results for the same organs. Alternatively, the positive virus isolation and PCR result from kidney and liver could reflect the presence of virus in blood cells (viremia), as it disseminated systemically from its site of entry in the digestive tract. The presence of nonspecific staining of the keratin layer and the absence of IHC staining in positive PCR tissues could be addressed by a more specific and sensitive technique such as ISH[16] (as seen in Fig. 4b,d).

Several factors may influence the outcome of the exposure to infection in a given host. Although infection caused acute and fatal disease affecting multiple organs in this study, it could have a different outcome with other doses, routes of infection, or environmental conditions. Environmental temperature has been shown to affect the disease progression and survival in ranavirus-infected fish, amphibians, and turtles[2,23,31] but has not been explored in ranavirus-infected lizards. This experimental infection was conducted using male and female juvenile EWDs during spring when the air temperature was within the preferred body temperature range of this species[36] and resembled temperatures in the wild and therefore reflective of the seasonal conditions. Dose-dependent studies have shown that the viral load affects the severity and type of lesions, as well as the chance of survival.[17] Dragons in this study were infected with a high dose of BIV that caused an acute infection with a sudden onset. The dose used in this study was equivalent to the dose used by Maclaine et al,[24] where it caused high mortality in EWDs exposed orally, intramuscularly, and via cohabitation. This may not reflect natural BIV infection where the infective dose is likely less and the infection is possibly protracted. Because the dragons were housed communally in small groups, we cannot rule out the possibility that reinfection from contact with shedding cohorts could have exacerbated the severity of lesions or modified the course of infection. However, cohabitation with orally infected lizards under similar experimental conditions showed a delay of 9.5 days for appearance of clinical signs, indicating that orally infected lizards were shedding virus around 9.5 dpi, and in this case, the histology, PCR, and viral isolation of animals housed together indicated that they were initially infected on 0 dpi with the massive oral dose they received.

The effect of different *Ranavirus* sp isolates and how they interact with different reptilian hosts has not been fully explored, but there appear to be similarities in the clinical signs and histopathological changes observed in lizards that were naturally infected with ranavirus. Skin lesions observed in dragons infected with BIV in this study were most commonly ulcerative and pustular and located on the distal limbs and digits, in contrast to brown-crusted skin lesions observed on the ventral abdominal surface and dorsum of ranavirus-infected Asian glass lizards (*Dopasia gracilis*).[33] In the current study, bacteria in the superficial keratin and fungal hyphae extending into the keratin layer in association with lesions were considered secondary invaders and have previously been reported in association with skin lesions of lizards infected with other *Ranavirus* sp.[24,33,34] Skin lesions are believed to be a result of the ranaviral infection, and an intersegment breach in the skin barrier allows for entry of other microorganisms such as bacteria and fungi. Liver necrosis and basophilic intracytoplasmic inclusion bodies in the liver observed in infected EWDs were similar to those observed in green striped tree dragons[8] but with the absence of bacterial colonies that were observed in a leaf tail gecko (*Uroplatus fimbriatus*).[26] Mild interstitial and tubular necrosis was observed in the kidney of infected EWDs in contrast to the vacuolar tubulonephrosis of the distal renal tubules reported in green striped dragons.[8] Another difference between lesions in the kidney of frogs infected with FV3[16] is the relatively late appearance of lesions in the kidney of our dragons infected with BIV. Although this could be associated with different affinities in each species of ranavirus, it may simply reflect different anatomical features between amphibians and reptiles. Lesions in amphibian kidneys are often associated, at least partially, with their interstitial hematopoietic tissues. The kidneys of our water dragons, like those of most reptiles, did not appear to contain hematopoietic tissues and thus lacked the tissue targeted by ranaviruses in amphibians.

Previous reports of ranavirus infection in lizards are from captive lizards that presented with skin lesions or were investigated following high mortalities or sudden death.[8,26,33,34] As with most case reports or case series, ranavirus was found to be the cause at postmortem examination, but not always considered by clinicians upon presentation. Our findings show that clinical signs of ranavirus infection in water dragons, such as inappetence and skin lesions, are nonspecific and appear in the late stages of infection. Further hampering the clinical (antemortem) diagnosis of ranavirus in skin lesions of lizards are secondary fungal or bacterial infections. We recommend that veterinarians consider ranavirus as a differential diagnosis in fatal outbreaks of skin lesions in lizards.

Maclaine et al[24] reported that naive EWDs can be infected by direct contact with BIV-infected EWDs, although it is not understood whether the transmission is caused by ingestion of infected excreta or water, or contact with skin lesions. Viral shedding from skin lesions, from gastrointestinal mucosa or from infected renal tubular cells, may lead to transmission by direct contact with skin or by ingestion or contact with infected excreta or water, respectively. In this study, ranaviral DNA was detected in cloacal swabs at all time points, as well as in all kidney and liver samples.

Here we report the progression and effect of BIV in experimentally infected juvenile EWDs. Ranavirosis became clinically evident at 7 dpi. Virus-associated histologic lesions were not observed at 3 dpi but were observed at 6 dpi and subsequent times. BIV may travel by blood within macrophages and other white blood cells, entering endothelial cells and then passing into tissue from either apoptotic endothelial cells or with migrating macrophages. The early detection of ranaviral DNA

in cloacal swabs and liver and kidney tissue samples suggests these are a reliable source of diagnostic samples in the early stage of disease before the appearance of clinical signs, as well as throughout the infection.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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3.4. Conclusion

The aims of this chapter were met in the following manner:

1. Describe the progression of a ranaviral infection in orally infected juvenile eastern water dragons

- Based on lesion severity and virus detection, ranaviral infection in orally infected juvenile eastern water dragons appears to start in the spleen, followed by the liver, then other organs such as the kidney, pancreas, oral mucosa, and skin.
- Viral infection and inflammation of organs were well underway 4 days prior to the onset of clinical signs (e.g. skin lesions)

2. Identify the time-points at which:
   - histopathological changes can be observed
   - ranaviral DNA can be detected in cloacal swabs, and liver and kidney samples via PCR

- Lymphocyte accumulation was observed in the tongue and liver 3 days post infection (dpi), but necrotic changes were not evident in examined tissues until 6 dpi.
- Immunohistochemical staining first detected viral antigens at 6 dpi in the spleen.

- Ranaviral DNA was detected via PCR in the liver, kidney, and cloacal swabs from the first sampling time-point at 3 dpi.

This study has explored the pathogenesis of BIV in orally infected juvenile eastern water dragons and has found that inflammation and infection of organs were underway 4 days prior to the onset of clinical signs (e.g. skin lesions). The progression of BIV infection in these lizards, based on lesion severity and virus detection, appears to start in the spleen, followed by the liver and then the other organs.

This study has demonstrated that ranaviral DNA can be detected in cloacal swabs from 3 dpi suggesting this is a reliable source of diagnostic sampling in BIV-infected eastern water dragons. Future studies are needed to explore the presence of ranavirus in wild and captive Australian lizards and should aim to collect cloacal swabs as well as skin and oral swabs, and blood samples for serology.

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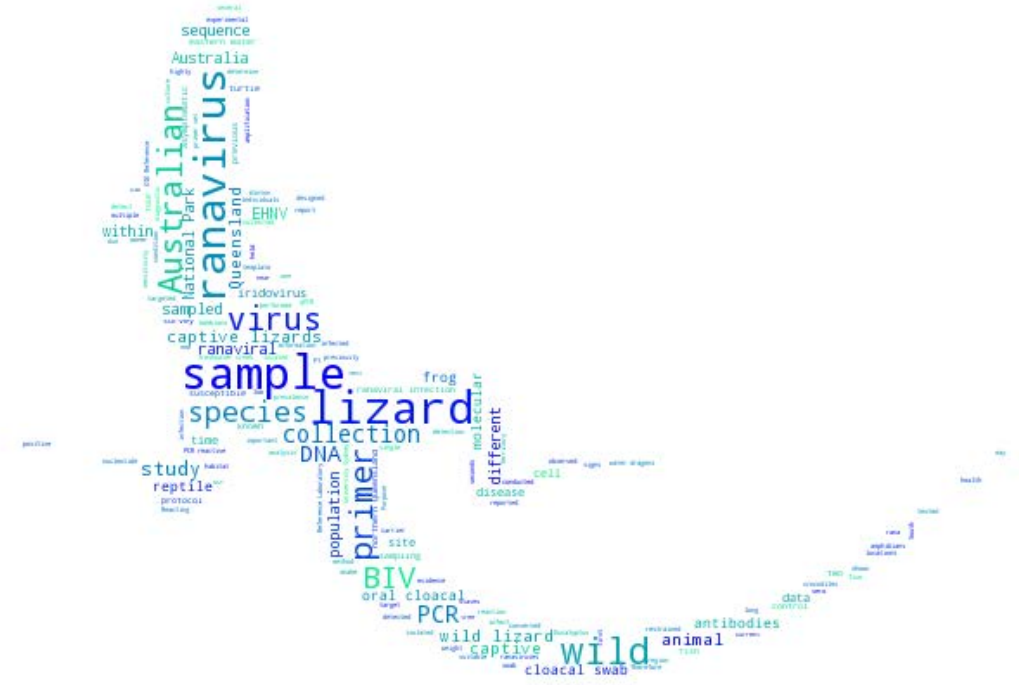
CHAPTER 4 – Molecular detection of Ranavirus sp. in captive and wild Australian lizards

4.1. Aim of this chapter

1. Determine if ranaviruses are present in wild and/or captive Australian lizards using molecular techniques



4.2. Introduction

Ranaviruses are large double-stranded DNA viruses that have the ability to infect a wide range of ectothermic vertebrates worldwide. These viruses have been associated with mass mortality events and are transmissible to different classes of lower vertebrates (Bigarré, Cabon, Baud, Pozet, & Castric, 2008; Brenes, Gray, Waltzek, Wilkes, & Miller, 2014; Butkus, Allender, Phillips, & Adamovicz, 2017; Kik et al., 2011; Miller, Gray, & Storfer, 2011). In Australia, two ranaviruses have been isolated: epizootic haematopoietic necrosis virus in fish, and Bohle iridovirus in amphibians (Langdon, Humphrey, & Williams, 1988; Langdon, Humphrey, Williams, Hyatt, & Westbury, 1986; Speare & Smith, 1992).

Epizootic haematopoietic necrosis virus (EHNV) is considered to be the most important ranavirus affecting fish and is listed as notifiable by The World Organisation for Animal Health (OIE) (OIE, 2018; Price, Ariel, et al., 2017). This ranaviral species is endemic to southern Australia and regularly reported during mortality evens in wild redfin perch (*Perca fluviatilis*) in Victoria (Langdon et al., 1986; Whittington, Becker, & Dennis, 2010). Several fish species and the European common frog (*Rana temporaria*) have also been shown to be susceptible to this *Ambystoma tigrinum virus*-like ranavirus via experimental exposure (Bayley, Hill, & Feist, 2013; Becker, Tweedie, Gilligan, Asmus, & Whittington, 2013, 2016; Jensen, Ersboll, & Ariel, 2009; Jensen, Holopainen, Tapiovaara, & Ariel, 2011; Langdon, 1989).

Bohle iridovirus (BIV) was first isolated from wild caught ornate burrowing frogs (*Limnodynastes ornatus*) in northern Queensland that died during or soon after metamorphosis (Speare & Smith, 1992). More recently, a BIV-like virus was isolated from captive magnificent tree frogs (*Litoria splendida*) and green tree frogs (*Litoria caerulea*) during

a mortality event in Darwin, Northern Territory (Weir et al., 2012). Phylogenetic analysis of the Australian BIV isolate places it in the frog virus 3 clade, but it is most closely related to the German Gecko ranavirus (Hick, Subramaniam, Thompson, Whittington, & Waltzek, 2016; Stöhr et al., 2015). Experimental studies of Australian native fish, amphibians and reptiles have shown that species within these classes are susceptible to infection with BIV (Ariel, Wirth, Burgess, Scott, & Owens, 2015; Cullen & Owens, 2002; Cullen, Owens, & Whittington, 1995; Moody & Owens, 1994). Recently, juvenile eastern water dragons, a species that shares habitat with several species shown to be susceptible to BIV, has been added to this list (Maclaine, Mashkour, Scott, & Ariel, 2018). BIV antibodies have been detected in wild populations of turtles, crocodiles, snakes and cane toads (*Bufo marinus*) in northern Queensland indicating that ranavirus is circulating in the herpetofauna in this region (Ariel et al., 2017; Whittington, Kearns, & Speare, 1997; Zupanovic et al., 1998)

Ranaviral infection in lizards have so far been limited to long term captive lizards held in collections outside of Australia that were investigated after signs of disease were observed (Behncke, Stöhr, Heckers, Ball, & Marschang, 2013; Marschang, Braun, & Becher, 2005; Stöhr et al., 2013; Tamukai, Tokiwa, Kobayashi, & Une, 2016). Outbreaks in wild Australian lizards have not been reported, which may be due to the lack of targeted surveillance and the vastness of a continent that is sparsely populated by humans. A recent systematic survey of wild eastern fence lizards (*Sceloporus undulates*) in central Virginia, United States was the first study to target and report molecular evidence of ranavirus in wild lizards (Goodman, Hargadon, & Davis Carter, 2018). This lizard species was selected because they share habitat with turtles previously diagnosed with ranaviral infection and this virus is known to cross-infect sympatric species.

The aim of this study was to determine if wild and/or captive Australian lizards are infected with Ranavirus sp. using molecular methods. The study targeted known susceptible species of lizards in captive settings and in natural areas where ranaviral antibodies have previously been detected.

4.3. Material and methods

4.3.1. Ethics statement

Sample collection from captive and wild lizards was conducted under permissions from James Cook University Animal Ethics Committee (Ethics Approval No. A2087 & A2277), Queensland Department of Environment and Heritage Protection (Scientific Purposes Permit No. WISP15053914), and Queensland Department of National Parks, Sport and Racing (Scientific Purposes Permit No. WITK18689817). Lizards were restrained without anesthesia for oral and cloacal swab collection, and collection of morphometric data (e.g. weight). None of the lizards were euthanized to collect this data.

4.3.2. Study sites

Captive lizards were sampled from four different collections held in the following Australian states/territories: New South Wales, Queensland and Australian Capital Territory. All captive-collections were chosen based on willingness of owners and availability of lizards. All lizards were held under a current reptile license at the time of sampling.

Wild lizards were sampled at six locations within northern Queensland, Australia: Paluma Range National Park (18°52’18”S, 146°07’30”E), Girringun National Park (18°05’00”S, 145°35’36”E), Tully Gorge National Park (17°35’30”S, 145°34’5”E), Wooroonooran National Park (17°08’47”S, 145°47’36”E), Wambiana Station (20°33'15.9"S, 146°06'38.7"E), and west of Winton (22°07'54.1"S 142°07'34.4"E) (Figure 4.1A). Within Paluma Range National Park sampling of wild lizards was conducted at multiple sites along the margin of freshwater creeks and streams (Figure 4.1B, Figure 4.2A).

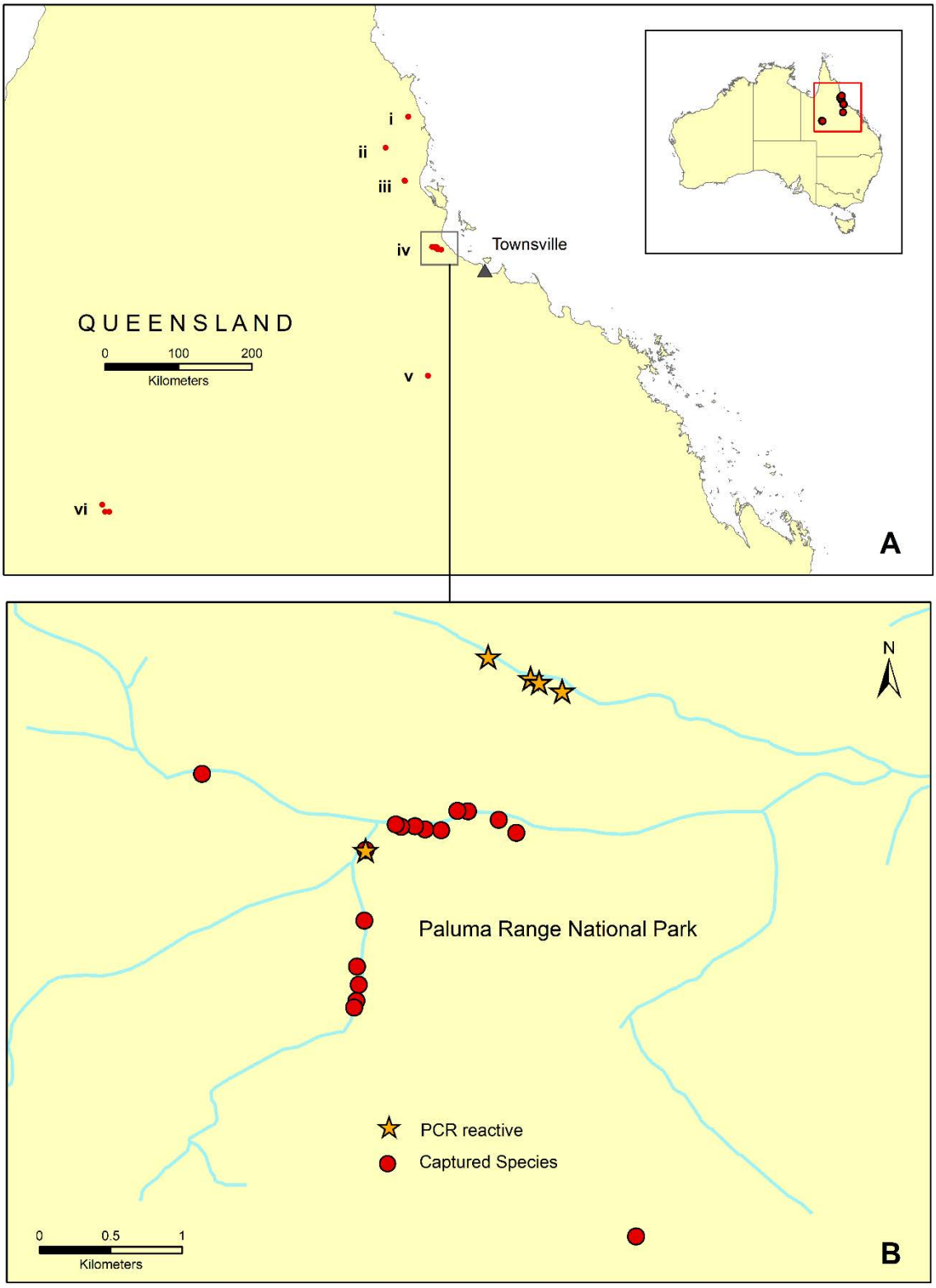


Figure 4.1. Map of northern Queensland with the six locations where wild lizards were sampled: i) Wooroonooran National Park, ii) Tully Gorge National Park, iii) Girringun National Park, iv) Paluma Range National Park, v) Wambiana Cattle Station, vi) Near Winton (Fig. 4.1A, insert – Australian continent). GPS locations of samples collected from wild lizards in Paluma Range National Park (●) with the PCR-positive samples highlighted (★) (Fig. 4.1B).

The four National Parks are located within the Wet Tropics World Heritage Area and lizards were sampled in low-elevation eucalyptus forests and dense, high elevation notophyll rainforests (Figure 4.2A, B) (Stanton & Stanton, 2005). Lizards sampled at these sites were near freshwater creeks and streams and the collection sites were remote, accessible only by foot. Wambiana Cattle Station is a working cattle property located near Charters Towers, Queensland, and is comprised of open eucalypt savanna woodlands, dominated by Reid River box (*Eucalyptus brownii*) and silver-leaf ironbark (*Eucalyptus melanophloa*) (Figure 4.2C). The sites near Winton are in central Queensland and consist of open acacia scrub that is dominated by spinifex clumps, with sandy soil and rock outcrops (Figure 4.2D). Both Wambiana and the site near Winton are exposed to human activities such as grazing, clearing and agriculture. These sites were selected as they had habitat suitable for a range of wild lizards.

Figure 4.2 Images of sites where wild lizards were sampled: A) Paluma Range National Park, B) Murray Falls, Girringun National Park, C) Wambiana Cattle Station, D) near Winton ("*Murray Falls*", 2019; "*Paluma National Park*", 2017; "*Wambiana Station*", 2018; "*Winton*", 2015)

4.3.3. Sampling

In this study, a total of 186 live lizards (123 captive, 63 wild) were sampled between April 2015 and March 2018 (Table 4.1). Captive lizards where restrained by the owner for sample collection. Wild lizards were captured by hand at night, and restrained while morphometric data (weight, snout to vent length (SVL)) and samples were collected for each animal. Where possible, information on sex, age class, body condition, SVL, weight, health history and origin were recorded for each lizard. Information for captive lizards was collected at the discretion of the owner and as a result some data were unavailable.

A combined oral and cloacal swab was taken from each lizard for molecular analysis. For this purpose, a sterile wooden-stem cotton-tipped swabs (Livingstone Pty Ltd, Australia) was inserted into the oral cavity and then into the cloaca. Swabs were then immediately placed into 1 ml of Dulbecco's Modified Eagle Medium (Gibco™) (DMEM) and transported on ice to the laboratory at James Cook University (Townsville, Queensland) within 12 hours. Samples were stored at –80°C until analysis.

Table 4.1 Number of lizards sampled for ranavirus testing with reference to Family, species, and captive/wild status

Family	Species	Captive	Wild	Total
Agamidae	Boyd's forest dragon (*Hypsilurus boydii*)	1	1	2
	Central bearded dragon (*Pogona vitticeps*)	78		78
	Dwarf bearded dragon (*P. henrylawsoni*)	2		2
	Eastern water dragon (*Intellagama lesueurii lesueurii*)	6	52	58
	Frilled neck lizard (*Chlamydosaurus kingii*)	4		4
	Gilbert's dragon (*Lophognathus gilberti*)		2	2
	Grassland earless dragon (*Tympanocryptis pinguicolla*)	7		7
	Jacky dragon (*Amphibolurus muricatus*)	7		7
	Nobbi dragon (*Diporiphora nobbi*)		5	5
	Ring-tailed dragon (*Ctenophorus slateri*)		1	1
Carpohodactylidae	Northern leaf-tailed gecko (*Saltuarius cornutus*)		1	1
	Smooth knob-tailed gecko (*Nephrurus levis*)	1		1
Scincidae	Blue-tongued skink (*Tiliqua scincoides*)	6		6
	Shingleback lizard (*T. rugosa*)	5		5
	Cunningham's skink (*Egernia cunninghami*)	1		1
	Gidgee skink (*E. stokesii*)	1		1
	Hosmer's skink (*E. hosmeri*)	1		1
	Pink-tongued skink (*Cyclodomorphus gerrardii*)	1		1
Varanidae	Black-headed monitor (*Varanis tristis*)		1	1
	Ridge-tailed monitor (*V. acanthurus*)	1		1
	Storr's monitor (*V. storii*)	1		1
TOTAL		123	63	186

4.3.4. Molecular analysis

Nucleic acid extraction of the lizard oral/cloacal swab samples was performed using a Bioline ISOLATE II Genomic DNA Kit according to the manufacturer's cultured cell protocol. Once extracted, the DNA was stored at –20°C until used.

Samples were tested for *Ranavirus* sp. DNA using a single-round of quantitative polymerase chain reaction (qPCR). Amplification was performed using primers described by Jaramillo et al. (2012) that target a 94 base nucleotide segment of the major capsid protein (MCP) region, which is a conserved region of the ranavirus genome. The reaction mixture contained 1 × GoTaq® qPCR Mastermix (Promega), 0.8 µM of forward (5'-GACTGACCAACGCCAGCCTTAACG-3') and reverse primer (5'-GCGGTGGTGTACCCAGAGTTGTCG-3'), ~80 ng of template DNA, and nuclease-free water in a 20 µl reaction. Thermocycling conditions were as follows: 95°C for 2 minutes, then 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds and 72°C for 15 seconds followed by a melt curve stage from 75°C to 95°C at 0.5°C intervals, and a final extension at 95°C for 2 minutes. All thermocycling was performed on a Rotor-Gene 6000 Real-Time PCR Machine. Each run contained a positive and a negative control (no template DNA). The positive control, BIV DNA, was obtained from The OIE Reference Laboratory for EHNV, University of Sydney, Australia, and reconstituted according to the protocol supplied. The qPCR product of samples that had an amplification and melt curve consistent with the control were sent to Macrogen Inc (Seoul, South Korea) for purification and Sanger sequencing. Additionally, confirmation of reacting samples were sought from The OIE Reference Laboratory, University of Sydney using real-time quantitative PCR (Jaramillo et al., 2012).

4.3.5. Viral isolation

Viral isolation was attempted by The OIE Reference Laboratory, University of Sydney on PCR positive samples as follows: Unfiltered material was inoculated, in duplicate, into bluegill fry (BF-2) cell suspension and incubated for nine days at 22°C during which the cells were examined for developing cytopathic effect (CPE) (P1). The cells were freeze/thawed once at –20°C. Duplicate cultures were pooled and 0.45 µM filtered before being inoculated into fresh BF-2 cell suspensions and incubated and observed for a further nine days (P2). This process was repeated in a third blind passage. An aliquot of pooled filtered P1 culture supernatant was tested by conventional PCR to detect two different regions of the MCP gene using the OIE Manual of Diagnostic Tests for Aquatic Animals. The P1 culture supernatant were tested by qPCR for ranavirus using the protocol and primers described by Jaramillo et al. (2012).

4.3.6. Phylogenetic analysis

The nucleotide sequences were assembled using Geneious 10.2.3 (Biomatters Ltd., New Zealand) and alignments were carried out using MUSCLE (Edgar, 2004), before being compared to known sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 2.99264733 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The Minimum Evolution tree was searched

using the Close-Neighbor-Interchange algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 35 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 46 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

4.4. Results

Of the 186 oral-cloacal swab samples obtained, samples from four captive lizards and five wild lizard swabs reacted in the PCR producing a single peak consistent with the positive control (BIV DNA) (Figure 4.3). Available data on each of those individuals are presented in Table 4.2.

Reacting samples from captive lizards belonged to collections in Canberra, Australian Capital Territory and Townsville, Queensland, and were from two different species (*Pogona vitticeps* and *Chlamydosaurus kingii*). All four individuals were female, aged over one year old, and were long term captive animals with no previous health issues.

Reacting samples from wild lizards were from male sub-adult and adult eastern water dragons (*Intellagama lesueurii lesueurii*) sampled in the Paluma Range National Park, Queensland. Samples from the five wild eastern water dragons that reacted in the PCR were sampled at three different locations approximately 230 – 330 m apart along the same freshwater creek, while the last lizard was sampled at another freshwater creek located approximately 1.6 km south-west (Figure 4.1B).

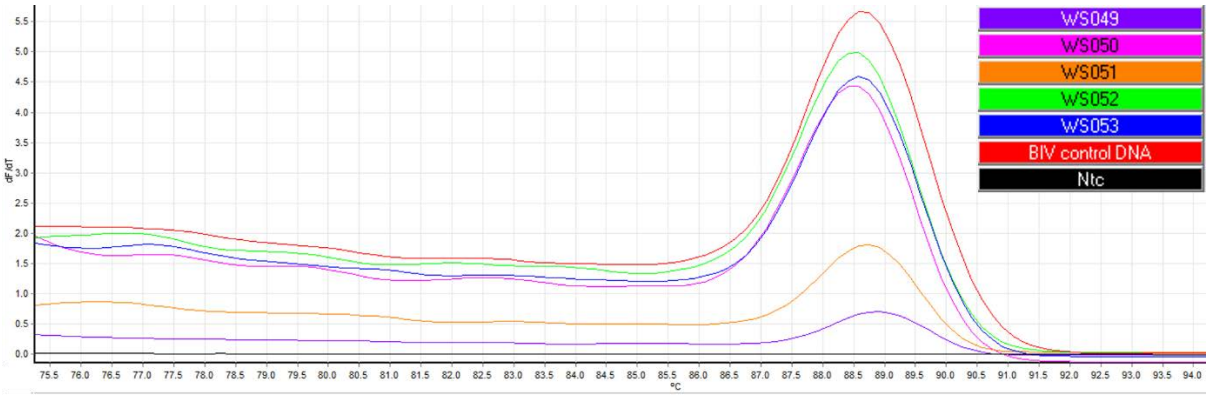


Figure 4.3. The melt curve of q PCR using primers designed by Jaramillo, 2012. Positive samples from wild lizards (purple, pink, orange, green and blue) have a single peak consistent with the BIV control DNA (red).

Table 4.2 Status, species, sex, age/age class, weight, snout to vent length (SVL), and sampling location of Australian lizards with a reacting PCR for *Ranavirus* sp. *NA = data not available

Status	Species	Sex	Age/Age Class	Weight	SVL	Sampling Location
Captive	Central bearded dragon (*Pogona vitticeps*)	Female	1 year 7 months	126g	NA	Canberra, ACT
	Central bearded dragon (*Pogona vitticeps*)	Female	1 year 8 months	166g	NA	Canberra, ACT
	Central bearded dragon (*Pogona vitticeps*)	Female	1 year 6 months	NA	NA	Canberra, ACT
	Frilled neck lizard (*Chlamydosaurus kingii*)	Female	6 years 0 months	NA	NA	Townsville, QLD
Wild	Eastern water dragon *(Intellagama lesueurii lesueurii*)	Male	Adult	450g	245mm	-18.9842, 146.21085
	Eastern water dragon *(Intellagama lesueurii lesueurii*)	Male	Adult	550g	250mm	-18.97417, 146.22324
	Eastern water dragon *(Intellagama lesueurii lesueurii*)	Male	Sub-adult	140g	163mm	-18.97337, 146.22125
	Eastern water dragon *(Intellagama lesueurii lesueurii*)	Male	Sub-adult	60g	115mm	-18.97337, 146.22125
	Eastern water dragon *(Intellagama lesueurii lesueurii*)	Male	Adult	500g	230mm	-18.97201, 146.21858

4.4.1 Phylogenetic analysis

PCR using oral-cloacal swabs from wild eastern water dragons produced amplicons that shared 100% nucleotide identity with the cognate regions of BIV, tiger frog virus, pike-perch virus, giant salamander virus, rana esculenta virus and grouper iridovirus. Amplicons from this study were only one base different to the cognate regions of EHNV, rana catesbeiana virus, hynobius nebulosus virus, European catfish virus, cod iridovirus, ranavirus maxima, soft-shelled turtle iridovirus, frog virus 3 and Korean ranavirus-1 (Figure 4.4). A phylogenetic tree

of these sequences further demonstrates the similarities between our sequences and BIV (Figure 4.5).

Ranavirus could not be confirmed by PCR in the original sample material submitted to The OIE Reference Laboratory, University of Sydney and no CPE was observed in P1-P3 in BF2 cells.

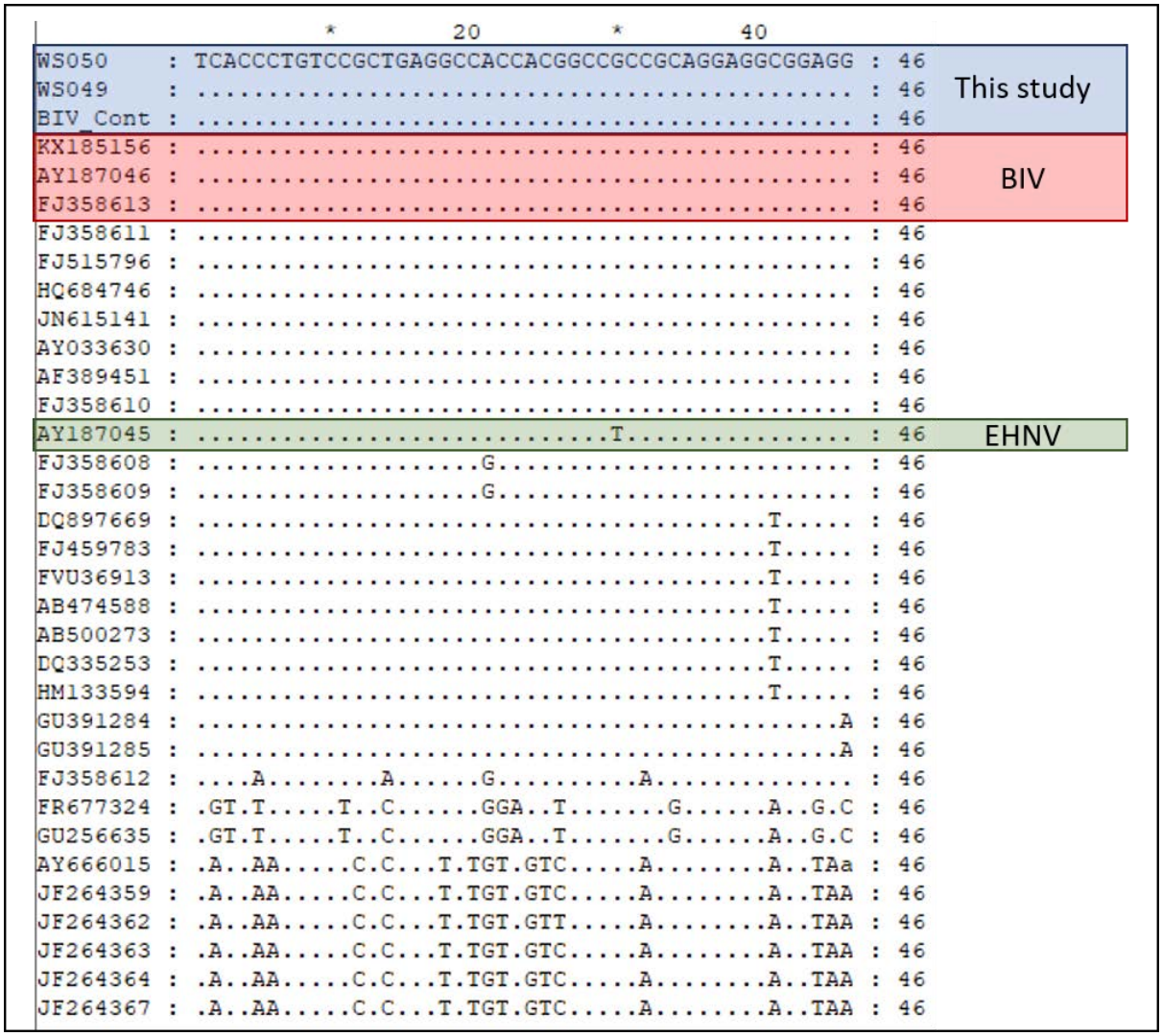


Figure 4.4. Trimmed sequence of qPCR products from two PCR-positive samples from wild eastern water dragons and the BIV control (blue) aligned with the corresponding major capsid protein region from other ranavirus sequences available in GenBank (see Figure 4.5) including BIV (red) and EHNV (green).

Figure 4.5. Alignment of trimmed sequence of qPCR products from two PCR-positive samples from wild eastern water dragons (▲) with ranavirus sequences available from GenBank including EHNV (▼) and BIV (●).

4.5. Discussion

The molecular detection of ranavirus in asymptomatic lizards supports the notion that ranavirus circulates naturally within the wild Australian herpetofauna. Although bearded dragons (*Pogona vitticeps*) are endemic to Australia, ranaviral infection in this species was first reported in Germany and Japan (Stöhr et al., 2013; Tamukai et al., 2016). Reports of ranaviral infections in Australia were limited to captive and wild amphibians, in farmed and wild fish, and in illegally imported green pythons (Hyatt et al., 2002; Langdon & Humphrey, 1987; Langdon et al., 1988; Langdon et al., 1986; Speare & Smith, 1992; Weir et al., 2012; Whittington et al., 2010; Whittington, Kearns, Hyatt, Hengstberger, & Rutzou, 1996).

Sero-surveillance for ranaviral antibodies in freshwater turtles and crocodiles, and snake populations in northern Queensland revealed evidence of previous exposure in several locations (Ariel et al., 2017). This study detected ranavirus in wild lizards in far north Queensland and in captive lizards in two states/territories.

Combined oral-cloacal swab samples collected in this study were tested using PCR. This method is commonly used to evaluate blood, oral-cloacal swabs, and tissues for ranaviral DNA (Allender et al., 2011; Butkus et al., 2017; Price, Wadia, et al., 2017). The sensitivity of PCR to detect viral DNA in these sample types allows researchers to use non-lethal sampling techniques when surveying populations for disease. This is particularly important when threatened species are involved. However, PCR-based surveys target the pathogen and therefore can only detect a current infection. An alternative method is indirect enzyme-linked immunosorbent assays (ELISA), which has previously been used in tortoises, alligators, crocodiles, turtles and snakes to detect antibodies to specific pathogens including iridovirus

(Ariel et al., 2017; Brown et al., 2001; Jacobson et al., 2005; Johnson, Wendland, Norton, Belzer, & Jacobson, 2010; Origgi et al., 2001; Schumacher, Brown, Jacobson, Collins, & Klein, 1993). The detection of ranaviral antibodies in wild Australian reptiles not previously investigated for their susceptibility to ranavirus (*Morelia spilota, Antaresia children, Liasis fuscus*) provides further evidence of ranavirus circulating in Australian native reptile populations (Ariel et al., 2017). This together with the findings from this study, suggests that ranavirus may be part of the normal microflora in Australian lizards.

Many factors must be considered when conducting molecular or sero-surveys of reptiles such as the window of detection, sensitivity of the test, carrier states, and non-converters. It is difficult to determine the true prevalence of disease in a population as the duration of the infection, antibody response and survival rate is unknown (Ariel et al., 2017; Johnson et al., 2010). Additionally, it is not known how long ranaviral antibodies or DNA remain at detectable levels, or if all infected individuals mount an adaptive immune response (Ariel et al., 2017). Prevalence in wild populations can also be underestimated if low sero-prevalence is reported in species known to be highly susceptible to ranavirus under experimental conditions as they would simply die (Ariel et al., 2017). Therefore, we recommend conducting molecular and serum surveys simultaneously and at regular intervals to determine the presence and prevalence of ranavirus in targeted populations.

The PCR primers used in this study are widely used for EHNV and were recommended for the detection of ranaviral infection in our diagnostic samples (Jaramillo et al., 2012). This primer set targets the highly conserved MCP region of the EHNV genome but when overlaid against other ranaviral isolates such as BIV, the reverse primer has mismatches on the 3-prime end suggesting that this primer set is only suitable for samples where EHNV is suspected (Figure

4.6). Stability on the 3-prime end of the primer is critical to the attachment of the enzyme with the efficiency of the primer extremely low in sequences that have this mismatch resulting in less false priming (Premier Biosoft, 2019). These primers have low sensitivity and poor specificity and did not efficiently amplify the sample. This is supported by poor quality chromatograms, the ambiguous sequence produced by these primers, and the insertion of a single nucleotide polymorphism. We are confident that our samples are PCR-positive for ranavirus as supported by the sequence data. However, due to the limitations of this primer set we are unable to identify how different the PCR-positive samples are from the control or identify which Ranavirus sp. wild and captive Australian lizards are infected with. It is recommended that significantly better primers be designed that are suitable for multiple ranaviruses, and that this current primer set be used only for diagnosis in suspected EHNV cases.

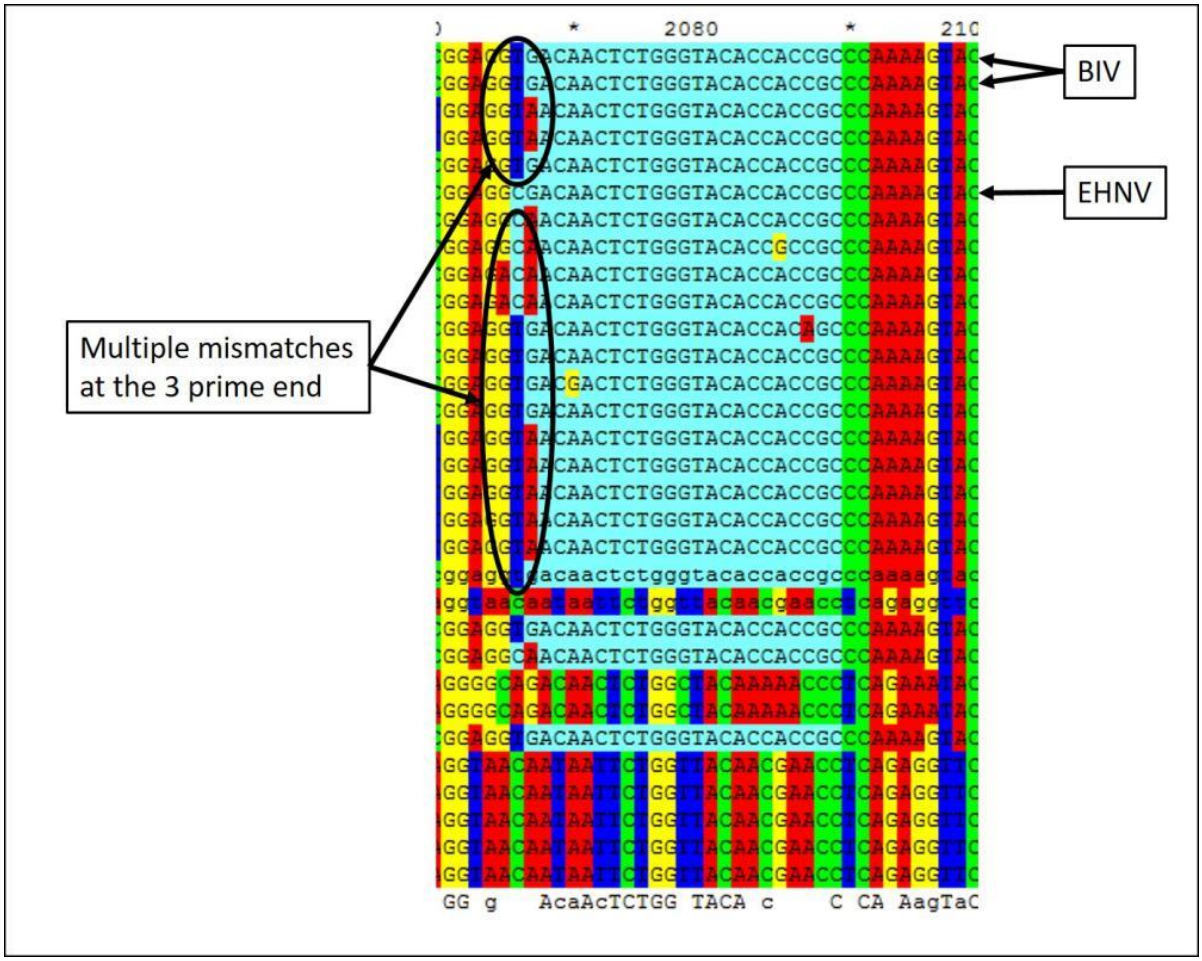


Figure 4.6. Alignment of several ranaviral sequences from GenBank trimmed at 2060-2100 bases. Sequences were aligned using MUSLCE (Edgar, 2004). The reverse primer (24 bases) is highlighted in light blue with the mismatches at the 3 prime end circled.

The failure of the OIE laboratory to confirm our results could be due to their extraction protocol, primer sets, amplification protocol, or degradation of the sample during transport. Further work with these samples will be conducted using primers that are more suited to our sample set such as those designed by Stöhr et al. (2015) or primers designed in-house using multiple sequences to ensure that the primers firstly target conserved sequences. In-house primers would conform to the PCR design guidelines outlined by Premier Biosoft (2019) for primer length, melting and annealing temperature, GC content and clamp, secondary structures, repeats, runs, 3' end stability, avoiding secondary template structures and cross

homology. Future systematic surveys of wild Australian lizards in northern Queensland should aim to collect samples for molecular analysis and serum for antibodies reactivity tests to provide additional information on the ranaviral status of wild lizard populations.

At the time of sampling all lizards were clinically healthy and had no apparent signs of disease (e.g. skin lesions or lethargy). This differs from previous reports of diagnostic cases in captive lizards where the ranaviral infection is often associated with clinical signs such as inappetence, lethargy and skin lesions (Behncke et al., 2013; Marschang et al., 2005; Stöhr et al., 2013; Tamukai et al., 2016). However, there is one previous report of ranavirus in an asymptomatic host, a wild-caught Iberian mountain lizard (*Iberolacerta monticola*) in Portugal (Alves de Matos et al., 2011; Price et al., 2014). Despite our lizards being asymptomatic at the time of sampling, the PCR-positive samples from four captive lizards that belonged to two different private collections had experienced mortality events from unknown causes within the previous five years.

The PCR-positive samples from asymptomatic captive lizards introduces the possibility of carrier lizards, unbeknown to the keeper, that can infect and kill naïve animals within the collection. A study using juvenile eastern water dragons has shown that BIV (*Ranavirus* sp.) can be transmitted to naïve animals through direct contact causing disease and death (Maclaine et al., 2018). Carrier animals may remain asymptomatic until times of stress such as breeding or introduction of new animals. Therefore, it is important to ensure that Australian reptile keepers have a knowledge of infectious diseases and can implement preventative strategies to protect their reptile collection.

4.6. Publication and outputs

The results from this study have been presented at three talks to researchers and members of the public. My overall contribution to this study was as follows:

- I designed the study in collaboration with my primary advisor;
- I prepared the ethics application for the James Cook University Animal Ethics Committee, and for the Department of Heritage and Protection Scientific Purposes Permit;
- I sampled all captive lizards and Donald McKnight sampled all wild lizards;
- I extracted DNA from all samples and performed the PCR analysis on these samples;
- I prepared the samples for sequencing and analysed all sequence data with Dr Graham Burgess;
- I processed all morphometric and location data;
- I designed the maps with the help of Edith Shum;
- I presented a summary of the results from this study at the Joint Meeting of the Australian Society of Herpetologists and the Society for Research on Amphibians and Reptiles in New Zealand held in Queensland, Australia.

Results from this study were presented at the following conference:

- **Maclaine, A.,** Burgess, G. W., McKnight, D. T., & Ariel, E. Molecular detection of *Ranavirus* sp. in wild eastern water dragons. Presentation presented at: Joint Meeting of the Australian Society of Herpetologists and the Society for Research on Amphibians and Reptiles in New Zealand; 10-13 December 2018; Queensland, Australia.

4.7. Conclusion

The aim of this chapter was met in the following manner:

1. Determine if ranaviruses are present in wild and/or captive Australian lizards using molecular techniques

- Ranavirus were identified in Australian wild and captive lizards using PCR
- Of the 123 captive lizards sampled, samples from three adult central bearded dragons (*Pogona vitticeps*) and one adult frilled neck lizard (*Chlamydosaurus kingii*) reacted to the PCR.
- PCR-positive samples from captive lizards were from two separate collections, both of which reported previous mortality events from unknown causes.
- Of the 83 wild lizards sampled, samples from five sub-adult and adult eastern water dragons (*Intellagama lesueurii lesueurii*) reacted to the PCR. These lizards were all sampled in the Paluma Range National Park, Queensland.
- All individuals at the time of sampling were clinically healthy with no signs of disease (e.g. skin lesions, lethargy, inappetence).

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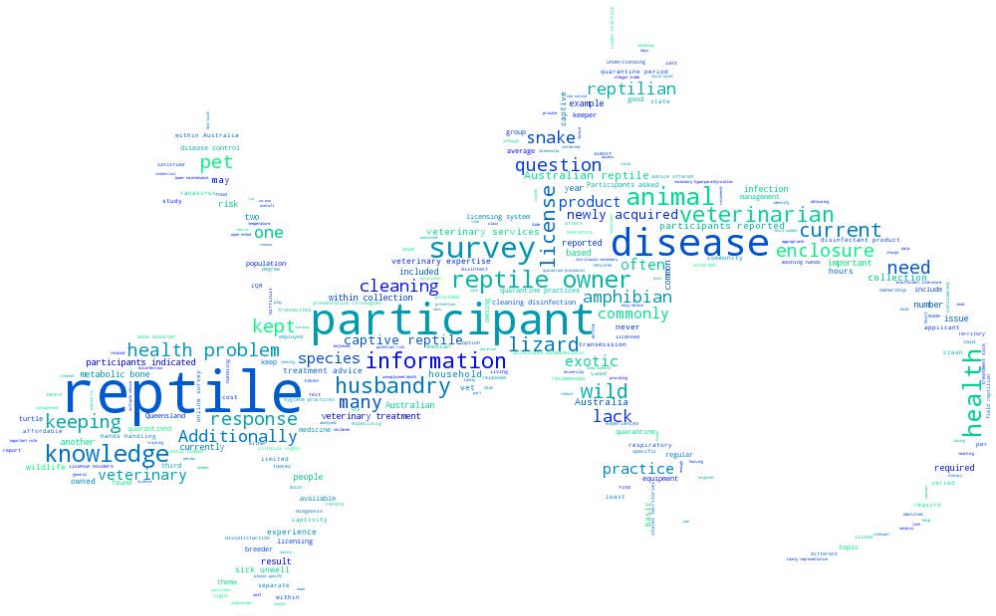
CHAPTER 5 – The health and wellbeing of Australian pet reptiles: a survey of Australian reptile owners

5.1. Aims of this chapter

1. Identify the socio-demographic profile of Australian reptile owners and their husbandry practices
2. Identify the range and provenance of captive-kept reptiles in Australia
3. Gauge the knowledge of reptile diseases amongst Australian reptile owners
4. Identify quarantine practices used by Australian reptile owners
5. Identify perceived potential barriers to seeking veterinary expertise for the management of captive-kept reptile health issues in Australia



5.2. Introduction

Reptile ownership across the globe is increasing in popularity with many people choosing to keep a pet snake, lizard or turtle (O'Malley, 2005). A survey of Australian pet owners conducted by Australia Animal Medicines in 2016 reported that almost 3% of participants kept a reptile as a pet, with an average of 1.7 reptiles owned per household (Australia Animal Medicines, 2016). This report estimated the total number of households owning reptiles to be 250,000 and the total number of captive-kept reptile population to be 415,000 nationally (Australia Animal Medicines, 2016). The main reason for acquiring a reptile, according to participants, was for companionship (34%); although, 10% of participants said the main reason for obtaining a reptile was that they were lower maintenance than other pets such as cats and dogs (Australia Animal Medicines, 2016). However, keeping reptiles in captivity requires good husbandry practices that provide adequate living conditions and meet the animals nutritional and environmental needs.

Inadequate nutrition and environmental conditions are believed to be responsible for more than 90% of illnesses in captive reptiles (Rossi, 2006). The two key basics of reptile husbandry that are most frequently misunderstood are: feeding a balanced diet of appropriate size and providing the correct thermal environment that meets the animals required preferred optimal temperature zone (Currumbin Valley Vet, 2018a). A misunderstanding of these two basics can cause health problems such as nutritional secondary hyperparathyroidism and obesity or lead to the impairment of essential functions such as digestion and shedding (Currumbin Valley Vet, 2018b). The most common health problem diagnosed in captive reptiles, particularly lizards, is metabolic bone disease, which is caused by dietary and/or husbandry mismanagement (Mader, 2006). Metabolic bone disease refers to a group of

disorders, including nutritional secondary hyperparathyroidism, that affect the integrity and function of bones (Mader, 2006). Amphibians and reptiles affected by metabolic bone disease are often found to have irregular, deformed, soft or rubbery bones during post-mortem examinations (Mader, 2006; Oppenheimer, 2012; Pessier & Pinkerton, 2003). In order to prevent such health problems, reptiles need to be kept in appropriate housing, climatic conditions, and fed a nutritious diet that contains all the required nutrients, including minerals and vitamins. These key husbandry requirements for captive reptiles are based on their basic needs in nature and are species specific.

In addition to meeting the basic husbandry and dietary needs for a captive reptile, one must also employ strategies, such as quarantine, to limit the risk of disease transmission within the collection. It is recommended that a minimum quarantine period be implemented for all newly acquired specimens, and a quarantine area physically separated from other reptiles, fish and amphibians be established (Gillespie, 2006). Many reptile-specific diseases have long incubation times and are difficult to diagnose (e.g. inclusion body disease in pythons). Additionally, some viral diseases, such as ranavirus, can be transmitted between different classes of ectotherms (e.g. an amphibian ranavirus can be transmitted to a lizard) (Brenes, Gray, Waltzek, Wilkes, & Miller, 2014). Other disease preventative strategies include, but are not limited to, regular cleaning and disinfection of enclosures and equipment, and washing hands before and after handling, feeding or cleaning.

Hygiene practices within the reptile collection are necessary for disease control but are also important for the prevention of transmission of diseases from reptile to human. The most recognised reptilian zoonosis is salmonellosis, commonly referred to as reptile-associated salmonellosis (Johnson-Delaney, 2006). In the United States of America, it has been estimated

that 6% of all the sporadic salmonellosis cases, and 11% of cases in people aged under 21, can be attributed to contact with amphibians or reptiles (Mermin et al., 2004; Pedersen, Lassen-Nielsen, Nordentoft, & Hammer, 2009). There have been numerous studies that have identified the presence of *Salmonella* sp. in the faeces of captive and wild reptiles (Briones et al., 2004; Bull, Godfrey, & Gordon, 2012; Cheng, Wong, & Dykes, 2014). Many reptiles carry *Salmonella* sp. in their intestinal tract as part of their normal flora and may intermittently shed these bacteria in their faeces, highlighting the potential risk of acquiring reptile-associated salmonellosis from handling reptiles (Pedersen et al., 2009). It is important that reptile owners be aware of the potential risks associated with keeping reptiles and be familiar with ways to reduce these risks such as employing good hygiene practices.

Veterinarians also play an important role in educating reptile owners about these risks and providing information on how to mitigate them. In Australia there are eight universities that offer veterinary science degrees with varied amounts of contact hours for teaching in wildlife, avian, herpetofauna, aquarium fish, and exotic pet medicine. Undergraduate non-clinical teaching hours in these areas for the duration of the degree (excluding final year rotations) average 72.5 hours ranging from 27.6 hours at University of Queensland, Queensland, to 157 hours at Murdoch University, Western Australia (Broadman, Warren, Jackson, & Hufschmid, 2016; Pratt, 2016). As reptile ownership increases in popularity, veterinarians experienced in reptilian medicine will be required to meet the needs of the Australian reptile owners.

The overall aim of this study is to explore Australian reptile keepers and breeders' experiences with disease, heath preventative and quarantine practices, and barriers to seeking veterinarian advice/treatment for their reptiles.

5.3. Methodology

5.3.1. Study design

A cross-sectional study of Australian reptile owners was designed to identify the socio-demographic profile of Australian reptile owners, their husbandry and quarantine practices, and their knowledge of reptile diseases. This study also aimed to identify potential barriers to seeking veterinary treatment for sick/unwell reptiles, and to identify if reptiles were being taken from or released into the wild.

This study was approved by James Cook University Human Research Ethics Committee (Approval Number H6574; Appendix A3).

5.3.2. Data collection tool

A trial paper-based survey was developed and distributed at the Townsville Pet Expo in June 2016 to establish face-validity. This survey contained multiple choice questions and questions that required a response, even if the 'other' option was selected. Based on these responses, survey questions were refined and further developed into an online survey that was conducted during November-December 2017 (Appendix B).

The online survey contained multiple choice and open-ended questions that asked reptile keepers about their: 1) demographics, license status, collection profile; 2) husbandry, health records and quarantine practices; 3) disease knowledge and prevention; 4) use of veterinary services; and 5) knowledge about reptiles being taken from or released into the wild. Participants had the option to give further information about some of their responses to the

multiple choice questions, and were also provided with a comment box at the conclusion of the survey. The following questions are examples of those that required further information:

- Where do you usually obtain your reptile(s) from? ('A licensed breeder', 'An unlicensed breeder', 'A pet shop', 'From the wild', 'Inherited from a friend or family member') ***Other (please specify below)***
- Have you had any of the following health problems within your reptile collection? ('Gut impaction', 'Intestinal worms', 'Metabolic bone disease', 'Mites', 'Ticks', 'Respiratory disease', 'Never had a health problem', 'Unexplained death') ***Other (please specify below)***
- Were you satisfied with the veterinary treatment/advice offered? (Yes/No/Never sought veterinary treatment and/or advice) ***If you were not satisfied with the treatment/advice offered, please specify below why?***

Participants were unable to skip questions and were required to give a response if the 'other' option was selected. To test the validity of participants responses, a fictional disease 'Falling fatigue syndrome' was included in the question regarding diseases that may affect reptiles.

5.3.3. Target population and recruitment

Participants had to currently own at least one reptile, be aged over 18 years old, and presently live in Australia. Participants were invited to participate via social media or email, where a link to a Survey Monkey® site was made available. This link was posted on Australian-based reptile-focused Facebook® groups, emailed to members of herpetological and wildlife health societies, and shared on Twitter®. The invitation to participate in this survey included

information about the study, and participation was on a voluntary basis. Participants were asked to consent to taking the survey at the start of the online questionnaire. No identifiable information was collected from participants.

5.3.4. Data collection and analysis

Participants responses to the survey questions were collected between the 13th of November and the 5th December 2017, and stored in Excel datasheets by Survey Monkey®, which were downloaded at the completion of the survey. Answers to the question about 'common name of lizard/s participant currently owned' were recategorised into Family groups: Agamidae, Anguidae, Carphodactylidae, Diplodactilidae, Gekkonidae, Scincidae, and Varandiae. Similarly, the results from the questions about the use of cleaning and disinfectant products were recategorised into 'household cleaning product', 'household disinfectant product', 'reptile-specific product', 'removal/change of substrate', and 'veterinary grade disinfectant'. Quantitative data were analysed descriptively: frequencies (%) were used for categorical variables; and mean, standard deviation (SD), and range were used to describe numerical variables, unless the data were skewed in which case median and interquartile range (IQR) were used. Open-ended question responses were analysed thematically and grouped into themes which are presented below in the results section. Only completed surveys were analysed.

5.4. Results

There were 275 participants, of which 179 completed the online survey. The response rate could not be calculated as the denominator (total number of Australian reptile owners who are a member of herpetological or wildlife societies and/or pertaining to online reptile focused groups) was unknown. The mean age of participants was 34 ([18-70], SD=11.20), with most participants residing in Queensland (45.25%) (Table 5.1). Two thirds of all participants (67.60%) indicated that they did not breed reptiles. Almost all participants had a current/valid reptile license (94.41%) with most of their reptiles listed on this license (84.36%). A small number of participants knew of someone who had released a captive-bred reptile into the wild (13.97%), while a larger number knew of someone who had taken a reptile from the wild (39.66%).

Table 5.1 – Profile of 179 Australian reptile owners who participated in the online reptile health survey (2017).

Characteristic	**Frequencies** (N=179, unless otherwise stated)
Age (years)	
	Mean = 34 [18-70] †
	SD* = 11.20
State of residency	
Queensland	81 (45.25%)
New South Wales	43 (24.02%)
Victoria	39 (21.79%)
South Australia	10 (5.59%)
Western Australia	4 (2.23%)
Australian Capital Territory	2 (1.12%)
North Territory	0 (0%)
Tasmania	0 (0%)
Do you own and/or breed reptiles	
Own and do not breed	121 (67.60%)
Own and breed	58 (32.40%)
Possess current/valid reptile license	
Yes	169 (94.41%)
No	10 (5.59%)
Any reptiles not listed on license	
No	151 (84.36%)
Yes	17 (9.50%)
I don't have a license	8 (4.47%)
I don't know	3 (1.68%)
Length participant has owned a reptile in months (N=178)	
	Median = 118
	IQR = 67.5
Age of participant when they got their first reptile (years)	
	Mean = 24 [4-55] †
	SD* = 10.92
Know of someone that has released a reptile into the wild	
No	154 (86.03%)
Yes	25 (13.97%)
Know of someone that has taken a reptile from the wild	
No	108 (60.34%)
Yes	71 (39.66%)

† *Range,* **SD = standard deviation*

A large majority of participants (83.24%; 149/179) had obtained their reptiles from a licensed breeder, while 28 participants (15.64%; 28/179) obtained their reptiles from 'other' sources (Table 5.2). These 'other' sources included reptile expos, another licensed person, adoption/rescue agencies, via social media, and from the wild with scientific permits. The most commonly owned type of reptile were snakes (69.83%; 125/179) and lizards (63.13%; 113/179). No participants reported owning exotic (non-native to Australia) reptiles at the time they took the survey. The median total number of reptiles owned by participants was 4 [IQR = 13] and the median number of lizards owned was 1 [IQR = 3]. The most commonly kept lizards belonged to the Scincidae (39.84%) and Agamidae (30.42%) families. Reptiles were most commonly kept in a glass enclosure, tank or vivarium (72.63%; 130/179) located in the living area of the home (58.66%; 105/179).

Table 5.2 – Reptile collection profile of 179 reptile owners who participated in the online reptile health survey (2017).

Characteristic	**Frequencies** (N=179, unless otherwise stated)
Source of reptile	
A licensed breeder	149 (83.24%)
A pet shop	40 (22.35%)
Inherited from a friend or family member	21 (11.73%)
From the wild	4 (2.23%)
An unlicensed breeder	2 (1.12%)
Other	28 (15.64%)
Type of reptile owned	
Snake	125 (69.83%)
Lizard	113 (63.13%)
Freshwater turtle	36 (20.11%)
Crocodile	2 (1.12%)
Other	6 (3.35%)
Own exotic/non-native reptile	
No	179 (100%)
Yes	0 (0%)

Total number of reptiles owned	
	Median = 4 IQR^ = 13
Number of lizards owned (N=957)	
	Median = 1 IQR^ = 3
Number of lizards owned categorised by their taxonomic Family (N=871)	
Scincidae	347 (39.84%)
Agamidae	265 (30.42%)
Carphodactylidae	95 (10.91%)
Diplodactilidae	86 (9.87%)
Varandiae	64 (7.35%)
Gekkonidae	9 (1.03%)
Anguidae	5 (0.57%)
Type of enclosure reptile housed in	
Glass enclosure, tank or vivarium	130 (72.63%)
Wooden enclosure	86 (48.04%)
Tubs or racks	41 (22.91%)
Pit or pond	27 (15.08%)
Other	20 (11.17%)
Location of enclosure	
Living area	105 (58.66%)
Bedroom	51 (28.49%)
Dining or Kitchen area	42 (23.46%)
Outdoors (e.g. patio, veranda, etc.)	37 (20.67%)
Garage	19 (10.61%)
Other	33 (18.44%)

^*IQR = interquartile range*

Participants indicated that they kept regular health records for their reptiles (65.36%; 117/179) that most commonly included 'information about food intake' (51.40%; 92/179), 'shedding dates and details' (48.04%; 86/179), and 'information about the overall health of the animal' (42.46%; 76/179) (Table 5.3). A small number of participants (3.91%; 7/179) included 'other' details such as temperature, age of reptile, visits to veterinarian, and test results. More than two thirds of participants (72.63%; 130/179) indicated that they were not the only person to handle reptiles within their collection. Other adults living with participants (61.45%; 110/179) were the most common group to also handle the captive-kept reptiles.

Hands were often washed after handling a reptile (81.01%; 145/179), while a small number (10.06%; 18/179) did not wash their hands before, between or after handling a reptile. Reptile enclosures were often cleaned weekly (49.72%; 89/179) and disinfected monthly (44.69%; 80/179). The most common products used to clean enclosures were household cleaning products (46.93%). These included commercial (e.g. Ajax®) and non-commercial products (e.g. white vinegar and water). The most commonly used product to disinfect enclosures was veterinary grade disinfectant (51.96%) such as F10™SC.

Newly acquired reptiles were often quarantined (72.07%; 129/179) and the median duration of the quarantine period was 4 weeks [IQR = 24]. The top three quarantine procedures followed with newly acquired reptiles were 'washing hands' (67.04%; 120/179), 'handling the quarantined animal last' (52.51%; 94/179), and 'using separate cleaning equipment' (43.58%; 78/179). A fifth of participants did not follow any quarantine procedures (20.11%; 36/179). Other quarantine procedures included use of disposable equipment such as gloves, housing animals individually, limited handling of quarantined animals, and preemptive mite and worming treatments. Half of the participants indicated in this 'other' response that they had never acquired a second reptile.

Table 5.3 – Record-keeping, handling, husbandry, and quarantine practices of 179 reptile owners who participated in the online reptile health survey (2017).

Characteristic	**Frequencies** (N=179)
Keep regular health records	
Yes	117 (65.36%)
No	62 (34.64%)
Information included in health record	
Information about food items	92 (51.40%)
Shedding dates and details	86 (48.04%)
Information about health	76 (42.46%)
Weight	70 (39.11%)
Information about medical treatment	66 (36.87%)
Breeding and mating details	50 (27.93%)
Measurements	47 (26.26%)
Cleaning/disinfection dates	45 (25.14%)
Details/dates about faeces	33 (18.44%)
I do not keep any health records	57 (31.84%)
Other	7 (3.91%)
Owner the only person handling collection	
No	130 (72.63%)
Yes	49 (27.37%)
Who else handles reptile collection	
Adults living with you	110 (61.45%)
Relative/friend adult not living with you	83 (46.37%)
Children living with you	56 (31.28%)
Relative/friend child not living with you	53 (29.61%)
Other adults	48 (26.82%)
I do not let anyone else handle my reptile(s)	33 (18.44%)
Other children	32 (17.88%)
When handling how often do you wash hands	
After handling a reptile	145 (81.01%)
Before handling a reptile	117 (65.36%)
Between reptiles	84 (46.93%)
I don’t wash my hands	18 (10.06%)
How often do you clean the reptile enclosure	
Weekly	89 (49.72%)
Monthly	35 (19.55%)
Daily	26 (14.53%)
Other	29 (16.20%)
How often do you disinfect the reptile enclosure	
Monthly	80 (44.69%)
Weekly	49 (27.37%)
Daily	1 (0.56%)
Other	49 (27.37%)

Product used to clean enclosure	
Household cleaning product	84 (46.93%)
Veterinary grade disinfectant	35 (19.55%)
Reptile-specific product	13 (7.26%)
Removal/change of substrate	13 (7.26%)
Household grade disinfectant	9 (5.03%)
None reported	25 (13.97%)
Product used to disinfect enclosure	
Veterinary grade disinfectant	93 (51.96%)
Household grade disinfectant	22 (12.29%)
Reptile-specific product	20 (11.17%)
Household cleaning product	18 (10.06%)
None reported	26 (14.53%)
Are newly acquired reptiles quarantined	
Yes	129 (72.07%)
No	50 (27.93%)
Length of quarantine period in weeks	
	Median = 4 IQR^ = 24
Quarantine procedures	
Wash hands	120 (67.04%)
Handle quarantined animals last	94 (52.51%)
Use separate cleaning equipment	78 (43.58%)
Regular disinfection of enclosure and furnishings	68 (37.99%)
Separate room in the same dwelling	57 (31.84%)
Same room, separate enclosure	55 (30.73%)
Separate room in a different dwelling	16 (8.94%)
Disinfectant footbaths	7 (3.91%)
I do not follow any quarantine procedures	36 (20.11%)
Other	36 (20.11%)

^*IQR = interquartile range*

When questioned about their knowledge of reptile diseases that may affect their reptiles, most participants had heard of respiratory disease (94.41%; 169/179), salmonella (88.27%; 158/179), parasitic disease (82.68%, 148/179), and metabolic bone disease (81.01%; 145/179) (Table 5.4). Participants monitored their reptile collections for signs of disease such as 'abnormal skin shedding' (93.30%; 167/179), 'wounds' (84.36%; 151/179), 'skin changes, lesions or ulcers' (82.68%; 148/179), and 'weight gain or loss' (80.45%; 144/179). Over one third of participants (39.66%; 71/179) reported to have never had a health problem within their

collection. The most commonly reported health problems were mites (26.26%; 47/179), respiratory disease (26.26%; 47/179), and unexplained death (21.23%; 38/179). A quarter of participants (23.46%; 42/179) indicated that they had had 'other' health problems within their collection. These included cancer, constipation, ear infection, eye infection, coccidia infection, fungal infection, sunshine virus, burns, spinal abscess, enlarged heart, seizure, spinal disease, and neurological disorder.

In order to reduce the risk of their reptile developing a health issues, participants employed numerous preventative strategies such as 'cleaning and/or disinfection' (92.74%; 166/179), 'use of heat cords, lamps or mats' (79.89%; 143/179), and 'use of UVA/UVB lighting' (71.51%; 128/179). Some participants (15.08%; 27/179) employed 'other' management strategies such as monitoring of temperatures, UV and humidity, regular exposure to unfiltered sunlight, and providing a varied diet.

Veterinary treatment was sought at least once by 62.57% (112/179) of participants. Of these participants, 59.29% (67/113) found it affordable and 84.82% (95/112) were satisfied with the treatment/advice offered. Participants were asked to elaborate on why they were not satisfied with the treatment/advice offered. The analysis of the open-ended responses provided by participants revealed two main themes: 1) Accessibility to veterinary expertise; 2) Dissatisfaction with veterinary services. These themes are supported by illustrative direct quotes from participants responses.

Accessibility to veterinary expertise

Many participants experienced difficulties accessing veterinary experts in reptilian health because many private veterinarians lack knowledge, and those who had the relevant knowledge were scarce. Almost half of the participants who provided further information about their experience of veterinary services reported that the veterinarian who had treated their reptile had limited knowledge about or experience with treating reptiles. For example, one participant wrote:

> *"I was passed to another vet as the first didn't know what a blue tongue was."* (Participant #6)

Another said:

> *"Had one vet ask me where the cloaca is on a snake and genuinely told me they GOOGLED for a diagnosis."* (Participant #17)

Additionally, some participants reported that there were not enough veterinary experts able to treat their sick/unwell reptiles in the area they lived:

> *"Very hard to find vets with enough experience on reptiles."* (Participant #7)

> *"They were not specialised enough in a country town. Had to send information to a zoo in Sydney for feedback."* (Participant #13)

Dissatisfaction with veterinary services

Participants expressed their dissatisfaction with veterinary services, which in some cases led to the prescription of inadequate and/or ineffective treatment. A few participants reported that their reptile had not responded to the treatment prescribed by the veterinarian. One participant wrote:

> *"[My] snake has respiratory infection and vet supplied incorrect needles for injections. Snake is now on third lot of antibiotics and still no signs of improvement."* (Participant #19)

Another said:

> *"I have a turtle that has been fighting a chronic respiratory infection for over 3 years. He has been seen at multiple clinics with varied success."* (Participant #87)

Participants dissatisfaction was often compounded with a perceived high cost for services provided. Participants felt that in many cases veterinary services were not affordable.

> *"I find veterinary help affordable to a certain degree. Operations are very expensive."* (Participant #48)

> *"Cost and availability of suitably experienced vets are MAJOR issues."* (Participant #87)

However, for other participants, affordability was not perceived as an issue because they had been the recipient of *pro bono* veterinary services or because they prioritised the cost incurred by managing the health of their animals. One participant reported:

> *"Have also had an excellent experience with a vet doing a FREE surgery to put a snake's tongue back in its mouth after it has been severely damaged."* (Participant #17)

While another declared:

> *"I don't consider veterinary treatment as either 'affordable' or 'not affordable'. If an animal in my care is unwell and requires veterinary treatment, he/she gets it, irrespective of the cost!"* (Participant #14)

The participants who had not sought veterinary treatment/advice for their reptile indicated that they had never had a sick/unwell reptile, or that they had asked for advice from another reptile keeper.

Table 5.4 – Disease knowledge, experience with disease, and disease management of 179 reptile owners who participated in the online reptile health survey (2017).

Characteristics	**Frequencies** (N=179, unless otherwise stated)
Have heard of the following disease that may affect reptiles	
Respiratory disease	169 (94.41%)
Salmonella	158 (88.27%)
Parasitic disease	148 (82.68%)
Metabolic bone disease	145 (81.01%)
Parvovirus	111 (62.01%)
Sunshine virus	102 (56.98%)
Herpesvirus	86 (48.04%)
Adenovirus	75 (41.90%)
Inclusion body disease	71 (39.66%)
Retrovirus	66 (36.87%)
Papillomavirus	64 (35.75%)
Ranavirus or Iridovirus	53 (29.61%)
Yellow fungus disease	50 (27.93%)
Paramyxovirus	45 (25.14%)
West Nile virus	37 (20.67%)
Reovirus	26 (14.53%)
Falling fatigue syndrome[#]	23 (12.85%)
Collection monitored for following signs of disease	
Abnormal skin shedding	167 (93.30%)
Wounds	151 (84.36%)
Skin changes, lesions or ulcers	148 (82.68%)
Weight gain or loss	144 (80.45%)
Lumps	140 (78.21%)
Conjunctivitis or nasal discharge	139 (77.65%)
Constipation or diarrhoea	138 (77.09%)
Dehydration	134 (74.86%)
Inflammation of the mouth or gums	126 (70.39%)
Neurological signs (e.g. tremors, star-gazing)	116 (64.80%)
Mouth deformities	114 (63.69%)
Weakness or partial paralysis	112 (62.57%)
Prolapse of the cloaca, hemipenes, etc.	108 (60.34%)
Nose-rubbing	106 (59.22%)
Swelling of the ears, eyes or face	104 (58.10%)
Missing digits, toes or limbs	101 (56.42%)
Eye colour changes	88 (49.16%)
Fractures	81 (45.25%)
Egg bound	67 (37.43%)
Birthing difficulty	53 (29.61%)
Other	23 (12.85%)

Health problems within collection	
Never had a health problem	71 (39.66%)
Mites	47 (26.26%)
Respiratory disease	47 (26.26%)
Unexplained death	38 (21.23%)
Metabolic bone disease	16 (8.94%)
Intestinal worms	14 (7.82%)
Gut impaction	12 (6.70%)
Ticks	9 (5.03%)
Other	42 (23.46%)
Preventative strategies currently in place to reduce risk of health issue	
Cleaning and/or disinfection	166 (92.74%)
Use of heat cord, lamps or mats	143 (79.89%)
Use UVA/UVB lighting	128 (71.51%)
Quarantine newly acquired animals	112 (62.57%)
Regular use of a calcium supplement	112 (62.57%)
Regular use of a multivitamin supplement	78 (43.58%)
Regular mite treatment	34 (18.99%)
Regular worming	23 (12.85%)
I do not implement any preventative strategies	1 (0.56%)
Other	27 (15.08%)
Sought veterinary treatment/advice	
Yes	112 (62.57%)
No	67 (37.43%)
Veterinary treatment/advice affordable (N=113)*	
Yes	67 (59.29%)
No	46 (40.71%)
Satisfied with veterinary treatment/advice (N=112)	
Yes	95 (84.82%)
No	17 (15.18%)
Why have you never sought veterinary treatment/advice	
Have sought veterinary treatment and/or advice	101 (56.42%)
Never had a sick/unwell reptile	54 (30.17%)
Asked for advice from another reptile keeper	35 (19.55%)
Able to treat without seeking treatment/advice	13 (7.26%)
Animal died	7 (3.91%)
Consultation and/or treatment not affordable	6 (3.35%)
Had a sick/unwell reptile but did not seek treatment/advice	2 (1.12%)
Live too far away from a veterinarian	1 (0.56%)
Other	21 (11.73%)

*[#]Falling fatigue syndrome is a fictitious disease implemented into this survey to test validity; *One participant responded to the affordability question despite indicating in the previous question that they had not sought veterinary treatment*

Participants were asked at the conclusion of the survey to provide any additional information they felt relevant to the topic of the health management of captive-kept reptiles. The thematic analysis of these responses revealed two additional themes: 1) Short comings of the current reptile licensing system; and 2) Insufficient literature; and a third theme, which converged with the previous theme related to accessing veterinary services: 3) Lack of veterinary expertise.

Short comings of the current reptile licensing system

Some participants reported that obtaining a reptile license was too easy, requiring no evaluation of the applicant's knowledge about keeping or owning a reptile, and that the current licensing system should be strengthened and include mandatory training in reptile husbandry. For example, one participant wrote:

> *"I believe it is too easy to obtain a reptile license. I did not have to do any test or questionnaire or anything. I have taken in reptiles in terrible states because of other people's lack of care/knowledge."* (Participant #79)

While another participant said:

> *"I firmly believe that the process for obtaining a reptile license is far too easy. The online application takes only a few minutes to complete, and requires the applicant to show minimal understanding of the requirements for such a varied species of animals. The licensing process should include a mandatory training class and examination on the topic of captive reptile health management."* (Participant #59)

Insufficient literature

A number of participants found it difficult to find information relevant to maintaining reptiles in captivity. Participants reported that there was insufficient literature available about the reptile species they kept or on the general management of reptiles. One participant reported:

> *"I didn't find good information online regarding the practicalities of cleaning and disease control when starting out. Care sheets etc. tend to ignore it. How often to clean? Disinfect? What with?"* (Participant #75)

Lack of veterinary expertise

A few participants reported that when it came to managing health issues within their reptile collection, consulting with a veterinarian was often not prioritised and was only considered as a last resort due to expense and/or the lack availability of veterinary expertise. For example, one participant wrote:

> *"I'm more inclined to check forums and other reptile keepers for health advice before taking the turtle to a vet. Once I can determine whether there is a risk of a significant issue which I can't treat with change/improvements of environment, then I'll seek veterinary help."* (Participant #10)

5.5. Discussion

Most Australian reptile owners and breeders who took part in the 2017 survey had a valid reptile license and predominately owned snakes and/or lizards. However, participants indicated that they knew of someone releasing and/or taking reptiles from the wild. Additionally, many participants indicated that they thought the current reptile licensing system needed to be changed as it was too easy to obtain a license. Despite employing seemingly good husbandry practices and health preventative strategies, some participants failed to quarantine newly acquired animals and seemed confused about the difference between cleaning and disinfection. Additionally, participants level of knowledge about reptilian diseases was limited to common husbandry diseases such as metabolic bone disease. Veterinary services for sick/unwell reptiles were considered by some participants to be unaffordable, and the treatment/advice to be unsatisfactory because the evident lack of veterinary expertise in the field of reptilian health. Overall, this survey has highlighted the inconsistent and insufficient implementation of quarantine periods, confusion about cleaning and disinfection protocols and products amongst Australian reptile owners, and the need for more accessible evidence-based information and resources about reptile health and husbandry (e.g. veterinarians and books).

Throughout Australia many people are choosing to keep a snake, turtle or lizard as a pet. The reason for this was not explored in this survey, although other surveys have shown that people choose to keep reptiles for companionship and because these animals are considered lower maintenance than other more traditional pets (Australia Animal Medicines, 2016). This perception of reptiles being lower maintenance pets may be a contributing factor to the number of dietary and husbandry related disorders seen by veterinarians (Currumbin Valley

Vet, 2018a; Rossi, 2006). The results from this survey found that the median number of reptiles owned per household was 4 [IQR=13], with snakes and lizards the most popular type of reptile kept. This is more than double the 2016 triannual nationwide survey that reported an average of 1.7 reptiles per household (Australia Animal Medicines, 2016). The 2016 survey was targeted towards the 'traditional' pet owner compared to our survey, which specifically targeted reptile owners and breeders. Therefore, the data collected in our survey are likely over-representative of the 'average' reptile owner as participants were invested in the topic and targeted in reptile keeping and breeding groups. Based on the survey results, the typical Australian reptile owner is one that is licensed, does not own exotic (non-native to Australia) reptiles, and has all reptiles listed on their license. However, this is likely not representative of all the Australian reptile owning community, with cases of reptiles being seized due to being kept by an unlicensed person or because they were an exotic species (Australian Border Force, 2012; Gartry, 2016; Victoria State Government, 2018).

Participants of this survey voiced their concerns over the current reptile licensing system. Participants thought reptile licenses were too easy to obtain and that licensing authorities should be evaluating the applicant's knowledge about keeping or owning a captive reptile. The current reptile licensing system within Australia varies between states and territories. In the Northern Territory and Australian Capital Territory a license is not required to keep some species of reptiles that are listed as exempt, such as the blue tongued lizard (*Tiliqua scincoides*) (*Keeping protected and prohibited wildlife*, 2017; *Reptile policy*, 2016). States such as New South Wales, classify reptiles into different categories based on how difficult the species is to keep in captivity (see Table 5.5). They also implement minimum licensing periods for each category that must be met before you can obtain a reptile from the next category up (*Reptile keeper licences* 2018). For example, a basic reptile class 1 (R1) license must be held

for two years before upgrading to a class R2 advanced license, which then must be held for at least 1 year to upgrade to R3, and so on up to R5 (*Reptile keeper licences* 2018) (Table 5.5). All states and territories, except Northern Territory, require a current license prior to the acquisition of a new reptile. Currently all licensing bodies only screen applicants for wildlife convictions/animal welfare offences, but do not ask applicants about their knowledge about keeping and housing reptiles. However, most state/territory governments do supply a link to their Code of Practice for keeping reptiles on their licensing application page. Additionally, there is a lack of physical inspections and annual reporting in some states/territories. In Queensland alone there are currently 33,721 *recreational wildlife license holders* (as at 30 June 2017), with an average of 7,698 licenses issued per year (Department of Environment and Science, 2018). However, only 300 reptile license holders are physically inspected per year due to costs (Department of Environment and Science, 2018). Additionally, Queensland *recreational wildlife license* holders are not currently required to submit a 'return of operations', which usually includes information about the reptiles they are keeping such as births, deaths, purchases, and sales (Department of Environment and Science, 2018). The current record-keeping system in some states/territories that do not require annual reporting could be creating loopholes that could allow license holders to engage undetected in illegal trade of both native and non-native species within Australia, which could represent a biosecurity and conservation issue.

Table 5.5 – New South Wales Native Animal Keeper License Species List. Adapted from (Reptile keeper licences 2018).

Common name (scientific name)	Species group	Current class
Children's python (*Antaresia childreni*)	Python	R1
Murray short-necked turtle (*Emydura macquarii*)	Turtle/tortoise	R1
Eastern bearded dragon (*Pogona barbata*)	Dragon	R1
Green python (*Morelia viridis*)	Python	R2
Lace monitor (*Varanus varius*)	Monitor	R2
Frilled lizard (*Chlamydosaurus kingii*)	Dragon	R2
Red-bellied snake (*Pseudechis porphyriacus*)	Elapidae	R3
Pilbara death adder (*Acanthophis wellsei*)	Elapdiae	R4
Eastern brown snake (*Pseudonaja textilis*)	Elapdiae	R5

The exotic reptile trade industry is considered to be the main pathway for introduction and establishment of invasive reptiles globally (Kraus, 2009). The introduction and illegal trade of these exotic reptiles are two of the processes most threatening Australia's biodiversity (Diaz, Ross, Woolnough, & Cassey, 2017). Australian Customs have detected illegally imported reptiles in baggage and in the post (e.g. iguanas), and there are reports of some exotic species, such as the red-eared slider (*Trachemys scripta elegans*), establishing populations within Australia (*Illegal trade in fauna and flora and harms to biodiversity*, 2017; Robey, Burgin, Hitchen, & Ross, 2011). The establishment of wild populations is believed to be due to the escape or deliberate release of captive reptiles into the wild. Other exotic species, such as the American corn snake (*Elaphe guttata*), have been detected in the wild but are believed to not yet have established a population (McFadden, Topham, & S. Harlow, 2017). Results from this survey indicated that several participants knew of people deliberately releasing reptiles into the wild. This survey did not quantify this, or establish what species were being released. However, the implications of the movement of animals between captivity and the wild populations raises questions about disease dispersal and biosecurity. Amphibian

chytridiomycosis is an example of a disease that has spread via the international trade of amphibians for exotic pets, medical and food purposes to geographically isolated regions, and has caused native wild populations of Australian amphibians to either go extinct or dramatically decline (Laurance, McDonald, & Speare, 1996; O'Hanlon et al., 2018; Skerratt et al., 2007). This infectious disease has caused mass mortality and extinction events in amphibians worldwide. Illegal trade and movement of reptiles into and within Australia could explain the first detection of a ranavirus in Australian captive and wild lizards (see Chapter 4).

Participants were also asked about their knowledge of diseases that affect reptiles. Viral diseases were less frequently known by participants compared to common husbandry-related diseases, such as metabolic bone disease. Participants were only asked if they had heard of a selection of reptilian diseases, therefore we are unable to comment on the depth of their knowledge and understanding of each of these diseases. However, there may have been some response bias, with some participants who may have responded 'yes' to knowing some of the listed diseases when they had never heard of them. This is supported by the 23 participants who reported that they had heard of the fictitious disease 'Falling fatigue syndrome'. Additionally, 40% of participants indicated that they had never had a health problem within their collection. This is unlikely given participants poor knowledge of reptilian diseases. It is more likely that some health problems either went undetected or undiagnosed. The limited knowledge of reptilian diseases could play a part in the lack of understanding of how diseases are transmitted and the importance of quarantine and disease control. Additionally, this could reflect the lack of available resources and evidence-based literature, or access to this kind of information for the general public. It is recommended that literature on these topics be regularly updated to reflect current knowledge and made readily available to all reptile owners.

Most reptile owners indicated that they were keeping regular health records, monitoring their collection for signs of disease, and undertaking good husbandry practices (e.g. regular cleaning, use of UVA/UVB). However, only one third of participants were quarantining newly acquired reptiles in either a separate room or separate enclosure. Many participants employed quarantine practices such as washing hands, handling the new animal last, and using separate equipment. However, while it is important to have good quarantine practices in place, it is more important to isolate newly acquired reptiles as infectious agents do not respect terrarium boundaries (Pasmans, Blahak, Martel, & Pantchev, 2008). Quarantined animals should be monitored for signs of disease during their quarantine period as several reptile-specific diseases have long incubation times, are difficult to diagnose, and are capable of interclass transmission (e.g. transmission of ranavirus from frogs to lizards) (Brenes et al., 2014). The risk of diseases spreading in private and zoological collections that fail to isolate and follow basic quarantine practices is high, especially if the reptile is not from a disease-free source. Generally, quarantine should last for at least 90 days except for pythons, which should be quarantined for at least six months (Jacobson, Morris, Norton, Wright, & Nathan, 2001). The current Codes of Practice for the Private Keeping of Reptiles for New South Wales and Victoria recommend a quarantine period not less than 30 days for a newly acquired lizard or turtle, while newly acquired snakes should be kept in a separate enclosure away from existing reptiles for 6-12 months (*Code of practice for the private keeping of reptiles*, 2013; *Code of practice for the welfare of animals - private keeping of reptiles*, 2017). In contrast, the Queensland Code of Practice for Captive Reptile and Amphibian Husbandry recommends that a newly acquired specimen be quarantined from other captive reptiles or amphibians for a minimum of seven days (*Code of practice captive reptile and amphibian husbandry*, 2010). A consistent approach to quarantine duration and practices needs to be outlined in all Codes of

Practice, especially for those who move interstate with their reptiles. These Codes of Practice should be readily available to all future and current reptile owners, and include recommendations on the management of reptile husbandry, diseases, and zoonoses.

Another important disease control practice is the regular cleaning and disinfection of reptile enclosures and equipment. Participants failed to understand the difference between cleaning and disinfecting, and often used disinfectant products to clean and vice versa. Although often used interchangeably, disinfecting and cleaning have a different purpose. Cleaning refers to the physical act of removing soil and organic contamination from surfaces (e.g. faeces, blood) but does not eliminate pathogens such as bacteria and other microorganisms (Slomka-Mcfarland, 2006). Disinfection of surfaces reduces the pathogen load by using commercial products, such as F10™SC, but does not eliminate it (Slomka-Mcfarland, 2006). Cleaning should always precede disinfection as many disinfectant products will not work if organic matter is present (faeces, urates, food). Therefore, it is recommended that enclosures be cleaned prior to disinfection, and that disinfectant products are not used to clean enclosures. Reptile-safe products should always be used to clean or disinfect enclosures as some products, such as phenols, formalin and formaldehyde, are highly toxic to reptiles (Wissman, 2018). The New South Wales Code of Practice recommends that the enclosure and food and water containers be cleaned immediately if they become contaminated with waste (*Code of practice for the private keeping of reptiles*, 2013). There are no guidelines on how often to disinfect enclosures. Disease control does not just apply to the reptile collection, but also to humans who interact with these animals. An example of a disease that can be transmitted from infected reptiles to people when common hygiene practices, such as washing hands after handing reptiles, are not employed is reptile-associated salmonellosis (Pedersen et al., 2009). This zoonotic disease is commonly reported in children and young infants. It is

therefore important that reptile owners be made aware of the potential risks associated with keeping reptiles and be informed about appropriate hygiene practices. Our survey found that almost 20% of participants did not wash their hands after handling reptiles, which could potentially pose a health risk to the keeper or other children/adults residing in the same household. This survey found that reptiles were most commonly handled by adults living with the participant, whereas children who handled the participants reptiles were most likely to reside within the same household or be a relative or friend's child. These results may not capture the handling practices of all reptile owners but are likely representative of the average reptile owner.

With the rising popularity of reptile ownership, comes the need for more veterinarians who have experience in the field of reptilian medicine. Additionally, the veterinarian can play an important role in educating reptile owners about reptile keeping, husbandry practices, disease control and the risk of zoonotic diseases such as reptile-associated salmonellosis. As identified in this online-survey, many participants felt that their veterinarian lacked experience and/or knowledge when it came to the treatment of their sick/unwell reptile. Currently, Australian veterinary science degrees offer differing numbers of teaching hours on the basic principles of avian, wildlife and exotic pet medicine (Broadman et al., 2016; Pratt, 2016). With veterinarians potentially playing an important role in reptilian health and disease management, it is important that the current curriculum addresses this, and that contact hours across all Australian universities be consistent on the topic of reptilian health. Additionally, continuing professional development of existing veterinarians is needed to meet the needs of current and future reptile owners. The lack of veterinary expertise in the field of reptilian health may lead to some reptile keepers self-diagnosing and treating illnesses. A few participants reported that they reached out to the reptile community for diagnosis and

treatment options rather than seeking veterinary assistance. Self-diagnosis and treatment of sick/unwell animals is not recommended, especially when a set of clinical signs can be explained by several different diseases. For example, lethargy and anorexia are common clinical signs for reptiles infected with adenovirus, herpesvirus or ranavirus (Marschang, 2011). The mismanagement of the health of reptiles kept in captivity could constitute an animal welfare issue and/or lead to disease outbreaks. Additionally, many participants felt that veterinary treatment for a sick/unwell reptile was unaffordable. This is not dissimilar to reports about expensive and widely varied prices for standard veterinary services for domestic dogs and cats (Coe, Adams, & Bonnett, 2007; Kollmorgen, 2014). Participants were also asked to self-report health problems that they had observed within their collection. However, we do not know if these health problems were verified by veterinary diagnosis. Many participants had experienced an unexplained death within their collection, with the cause likely not explored. It is possible that the lack of knowledge about reptilian diseases could mean that clinical signs of infection went undetected and that animals died as a result of an undiagnosed disease. An undiagnosed sudden death within a captive collection could have implications for other reptiles, current and future. This could include the spread of disease between reptiles currently held within the collection or reinfection of a newly acquired reptile if the terrarium and equipment is not correctly disinfected.

Additionally, other health issues such as organ failure, neurological disorder and abscesses were reported. It is possible that several of these could be explained by viral infections such as adenovirus. This online-survey identified the need for more accessible information for the general reptile community, especially on the topics of disease and preventative strategies. This could help decrease health problems commonly diagnosed in captive reptiles, such as metabolic bone disease. Currently the reptile community has access to books, state

government codes of practice, websites and forums, open-access journals (e.g. Amphibian & Reptile Conservation), and older published articles (e.g. Diseases of Aquatic Organisms articles published 5 years ago or may be accessed freely). It is important that new information, such as emerging diseases, be made available to the reptile community to enhance their understanding of owning and keeping reptiles.

Reptile ownership within Australia is becoming increasingly popular as people choose to keep a snake, turtle or lizard as a pet. A survey of Australian reptile owners has shown the need for education on the importance of quarantining animals, cleaning and disinfection protocols, and knowledge about the clinical signs, transmission and prevention of reptilian diseases. This survey has also highlighted the need for further training in the field of reptilian medicine for veterinarians, and the need for a consistent approach to reptile licensing in Australia.

5.6. Publication and outputs

The results from this study have been presented at three talks to researchers and members of the general public, and as a conference poster. My overall contribution to this study was as follows:

- I designed the study in collaboration with Dr. Diana Mendez;
- I prepared the ethics application for the James Cook University Human Research Ethics Committee;
- I distributed the trial paper-based survey at the Townsville Pet Expo in June 2016 to establish face-validity;
- I amended the survey according to the trial paper-based survey;
- I designed and managed the online survey;
- I analysed the quantitative and qualitative data generated from this study and undertook descriptive and thematic analyses of these data in consultation with Dr. Daniel Lindsay and Dr. Diana Mendez;
- I presented a summary of the results from this study to the North Queensland Herpetological Society, James Cook University Turtle Health Research group, and to the Turtle Love and Conservation Society, Townsville; and
- I prepared the poster that I presented at Joint Meeting of the Australian Society of Herpetologists and the Society for Research on Amphibians and Reptiles in New Zealand held in Queensland, Australia.

Results from this study are included in the following conference poster:

- **Maclaine, A.,** & Mendez, D. Experiences with disease and preventative strategies: A survey of Australian reptile owners. Poster presented at: Joint Meeting of the Australian Society of Herpetologists and the Society for Research on Amphibians and Reptiles in New Zealand; 10-13 December 2018; Queensland, Australia

5.7. Conclusion

The aims of this chapter were met in the following manner:

1. Identify the socio-demographic profile of Australian reptile owners and their husbandry practices

- Australian reptiles' owners are on mean 34 years old [18-70], with almost half of participants residing in Queensland.
- Most Australian reptile owners do not breed reptiles and have a current/valid reptile license.
- Common husbandry practices included: keeping regular health records, cleaning and disinfecting enclosures, use of heat and UV lighting, and use of dietary supplements.

2. Identify the range and provenance of captive-kept reptiles in Australia

- The most commonly owned type of reptile were snakes and lizards.
- The most commonly kept lizards belonged to the Scincidae and Agamidae families.
- Captive reptiles were most commonly obtained from licensed breeders.
- Other sources of reptiles included: pet shops, friend or family member, reptile expos, adoption/rescue agencies, from the wild, and from unlicensed breeders.

3. Gauge the knowledge of reptile diseases amongst Australian reptile owners

- Viral diseases, such as adenovirus, were less frequently heard of compared to common husbandry-related diseases, such as metabolic bone disease.

4. Identify quarantine practices used by Australian reptile owners

- The top three quarantine practices employed by reptile owners were: 'washing hands', 'handling the quarantined animal last' and 'using separate cleaning equipment'.
- Other quarantine practices included: use of disposable equipment such as gloves, housing animals individually, limited handling of quarantined animals, and preemptive mite and worming treatments.
- 20% of participants had not employed any quarantine practices. Viral diseases, such as adenovirus, were less frequently heard of compared to common husbandry-related diseases, such as metabolic bone disease.
- Only 72% of newly acquired reptiles were quarantined. The median duration of the quarantine period was 4 weeks [IQR = 24].

5. Identify perceived potential barriers to seeking veterinary expertise for the management of captive-kept reptile health issues in Australia

- Of participants who sought veterinary treatment/advice for their reptile, 40% did not find it affordable and 15% were not satisfied due to the lack of knowledge and/or experience on the veterinarians' part or because the treatment was ineffective.

This study has provided a snapshot of the socio-demographic profile of Australian reptile owners and has identified the range of reptiles held in captivity, common husbandry and quarantine practices, disease knowledge, and barriers to seeking veterinary treatment/advice.

This study has identified the need for more education on minimum quarantine periods and practices, cleaning and disinfection protocols, and general information on diseases that affect reptiles. Additionally, a need for more veterinarians experienced in the field of reptilian medicine is essential to meet the growing needs of Australian reptile owners. Further investigation on the number of unlicensed reptiles and reptile owners in Australia, and movement of animals between captivity and the wild, will help to identify possible sources of disease dispersal and risks to biosecurity and conservation. Future questionnaires should aim to explore what other ectotherms (fish and amphibians) are kept by reptile owners, in order

to highlight potential disease pathways, as well as be coupled with a molecular survey to test for selected reptilian viruses such as adenovirus and ranavirus.

Current Australian reptile owners and the animals held in their care could benefit from:

- A consistent licensing system across all Australian state and territories to ensure all animals are kept by appropriately experienced keepers;
- A record-keeping book where keepers can record details on births, deaths, purchases or sales, which could then be returned to the state/territory licensing authority annually (this practice is already employed in some states/territories while others like Queensland have plans to add this to their current licensing system);
- Provision of a booklet that contains information on minimum quarantine periods, cleaning/disinfecting products and protocols, and how to prevent common health problems such as metabolic bone disease;
- Readily accessible up-to-date evidence-based resources that contain information about reptilian diseases and the clinical signs, diagnosis, and treatment associated with each.

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CHAPTER 6 – General discussion

The overarching aims of this thesis were answered in the following manner:

1. Investigate the susceptibility and pathogenesis of Bohle iridovirus (*Ranavirus* sp.) in juvenile eastern water dragons

Experimental trials demonstrated that juvenile eastern water dragons are susceptible to Bohle iridovirus via several different routes of exposure including co-habitation under the conditions described in Chapter 2 and 3. The clinical signs and histopathology associated with the infection differed depending on the route of exposure. Further investigation of the pathogenesis of the virus in orally infected juvenile eastern water dragons revealed that the infection is established in the internal organs four days before clinical signs become evident. Detection of ranaviral DNA in cloacal swabs was concurrent with histopathological changes and viral isolation from the liver of exposed animals and was therefore considered a reliable diagnostic sample for early detection.

2. Determine if ranaviruses are present in wild and/or captive Australian lizards

A molecular survey of wild and captive Australian lizards found evidence of *Ranavirus* sp. in wild asymptomatic eastern water dragons, captive central bearded dragons and a frilled neck lizard. The isolate is similar to ranaviruses known to infect other Australian ectotherms.

3. Identify and understand Australian reptile owners experience and management of disease in captive reptile collections

A survey of Australian reptile owners identified the key health problems experienced in captive reptile collections. Diagnosis and treatment of these health problems were often employed without consulting a veterinarian. There was a perceived lack of experience of veterinarians in the field of reptilian medicine and participants considered treatment too expensive. Knowledge of reptilian diseases among reptile owners were limited to non-infectious diseases. Strategies employed by reptile owners to protect their reptiles from health problems included cleaning and/or disinfection of enclosures and quarantine of newly acquired animals.

Australia is home to a large diversity of reptiles widely distributed across the continent (Cogger, 2014; Wilson & Swan, 2017). This vast number of reptiles is found both living naturally in the wild and kept in captivity by reptile enthusiasts and zoological parks. Keeping reptiles in captivity is becoming increasingly popular in Australia and around the world (Australia Animal Medicines, 2016; O'Malley, 2005). The interest in reptiles by enthusiasts has opened up a pathway for illegal trade with reptiles accounting for 43% of Australian Customs prosecution cases for attempted export and import between 1994 and 2007 (Alacs & Georges, 2008). The illegal movement of animals has been identified as a potential pathway for the spread of pathogens. An example is amphibian chytridiomycosis, an infectious disease that has caused mass mortalities in amphibian populations worldwide, believed to be spread by international trade (O'Hanlon et al., 2018). It is likely that other pathogens, such as ranavirus, have been introduced to native hosts through similar pathways emphasizing the need to increase our knowledge base of existing endemic diseases, susceptible species, and to develop reliable diagnostic techniques (Daszak et al., 1999; Kraus, 2009).

Ranaviruses have been associated with morbidity and mortality events in wild and captive amphibian, fish and reptile species worldwide, and are considered emerging pathogens of significant ecological importance due to their expanding host range and geographical distribution (Bigarré, Cabon, Baud, Pozet, & Castric, 2008; Daszak et al., 1999; Miller, Gray, & Storfer, 2011; Price et al., 2014; Tamukai, Tokiwa, Kobayashi, & Une, 2016). The majority of research into this virus has been conducted in fish, amphibians and testudines (turtles, tortoises and terrapins), with reports of ranaviral infections in squamates (amphisbaenians, lizards, snakes) limited mostly to groups of captive lizards (Behncke, Stöhr, Heckers, Ball, & Marschang, 2013; Miller et al., 2011; Stöhr et al., 2013; Tamukai et al., 2016). The geographic and taxonomic distribution of ranaviruses in wild reptiles is understudied despite many

reptiles sharing habitat with susceptible fish and amphibian species. There has only been one systematic screening for ranavirus in wild terrestrial squamate populations (Goodman, Hargadon, & Davis Carter, 2018).

The overall aims of this study were to investigate a species of Australian lizard's susceptibility to a local species of ranavirus, to determine if ranaviruses are present in Australian captive and wild lizards, and to identify and understand Australian reptile owners experience and management of disease in captive reptile collections.

Eastern water dragons (*Intellagama lesueurii lesueurii*) were chosen for the challenge trials based on their semi aquatic nature and overlapping distribution with several fish, amphibian and turtle species shown to be susceptible to a ranavirus isolate (Bohle iridovirus) that was detected in amphibians in the same region (Speare & Smith, 1992). Juvenile eastern water dragons were exposed to Bohle iridovirus via oral inoculation, intramuscular injection and cohabitation with an infected lizard (Chapter 2), all of which were effective at establishing infection under the experimental conditions described in this chapter. Clinical signs observed in this study (distended abdomen, inappetence, lethargy, incoordination and skin lesions) were similar to those previously described in other ranaviral infected lizards (Behncke et al., 2013; Marschang, Braun, & Becher, 2005; Stöhr et al., 2013). However, the appearance of skin lesions observed in this study were pustular and ulcerative in appearance as opposed to previous reports of brown-crusted or dark-skinned lesions (Stöhr et al., 2013). This finding could be attributed to the semi-aquatic nature of eastern water dragons which differs from other reported terrestrial lizard species. One lizard in the cohabitation treatment remained asymptomatic with no observed histopathological changes while another in the same treatment had a single skin lesion and a focal granuloma in the kidney. Liver and kidney

samples from both animals were PCR-positive and virus was successfully isolated from these tissues, suggesting the possibility of asymptomatic carriers. Similar findings have been described in brown tree snakes (*Boiga irregularis*) that remained asymptomatic with no observable histopathological changes, while virus was isolated from the liver (Ariel, Wirth, Burgess, Scott, & Owens, 2015). The exposure of juvenile eastern water dragons to ranavirus has identified this species susceptibility to infection and the potential for infected individuals to amplify and contribute to the spread of this virus as demonstrated by naïve individuals becoming infected while cohabiting with infected lizards. As an animal ethics requirement, lizards were euthanized when they lost the ability to reorientate themselves when placed on their back or showed reduced activity and/or flight response. Disease progression in these experimental animals was likely advanced and pathology comparable to other reports of ranaviral infected lizards that were investigated after a period of illness or death (Behncke et al., 2013; Marschang et al., 2005; Stöhr et al., 2013). This design did not elucidate the pathogenesis of ranaviral infection in eastern water dragons and another experiment was therefore designed to address this and to determine the type of samples that would be suitable to diagnose early infection or identify carrier animals.

Chapter 3 describes how juvenile eastern water dragons were infected orally with Bohle iridovirus and sampled at pre-determined time points (days 3, 6, 8, 10, 12 and 14) to explore the progression of this infection over two weeks. The study design was similar to that of a pathogenesis study of fatal frog virus 3 infection in adult wood frogs (*Rana sylvatica*), where regular sampling allowed to establish the time-points at which histological changes occurred and when immunohistochemical staining and PCR detection of viral DNA in tissues was positive (Forzán et al., 2017). If we are to determine the current and future impact of ranaviral infection on wild lizard populations, we must first describe their pathogenesis in a lizard

model species. Based on lesion severity, it appears that Bohle iridovirus infection in eastern water dragons is first established in the spleen, followed by the liver, then the other internal organs. This was supported by immunohistochemical staining with anti-*epizootic haematopoietic necrosis virus* polyclonal antibodies, which cross reacts with BIV and was effective in demonstrating ranaviral presence associated with marked necrosis in the liver, kidney and spleen, as well as without lesions in the bone marrow and lung. *In situ* hybridization (ISH) with a frog virus 3 probe supported the findings in most organs, however, it did not stain the gastric and intestinal mucosa and keratinized layer of the epidermis which suggests that those were non-specific staining caused by the polyclonal antisera. The results of the ISH indicates that this technique is both more sensitive and specific for ranaviral detection in PCR-positive reptilian tissues and is therefore recommended for future studies. This new diagnostic approach for ranaviral infected reptilian tissues has been described for the first time in Chapter 3. The detection of ranaviral DNA in cloacal swabs, and liver and kidney samples in the early stages of infection contrasts to the frog virus 3 pathogenesis study in wood frogs where DNA was not detected in multiple organs until shortly before death (Forzán et al., 2017). While it could be argued in this study that this was the original dose passing through the digestive tract, the early detection by PCR was accompanied by histopathological changes of the organs, and positive viral isolation. The detection of ranaviral DNA in cloacal swabs concurrently with viral isolation and development of lesions in internal organs suggests that this is a reliable source of diagnostic sampling in ranaviral infected eastern water dragons. Oral swabs were not collected in this study as it would have been difficult to differentiate the inoculum from new virus. However, future studies should consider sampling the oral cavity, as well as the cloaca, to ascertain if ranaviral DNA could be detected in the mouths of infected animals. This sampling method would provide a useful

tool when conducting health surveys of eastern water dragons and other lizards with the aim to better understand the ecology of ranavirus in Australia.

To determine if Australian lizards are infected with ranaviruses, wild lizards in northern Queensland and captive lizards in eastern Australia were sampled with a combined oral-cloacal swab (Chapter 4). A survey of 123 captive lizards and 63 wild lizards found that nine samples reacting in the PCR produced a single peak consistent with the positive control (Bohle iridovirus DNA). This is the first-time molecular evidence of *Ranavirus* sp. has been reported in Australian lizards in Australia with all previous ranaviral infections in Australia limited to captive and wild amphibians, farmed and wild fish, and in illegal imported pythons (Hyatt et al., 2002; Langdon & Humphrey, 1987; Langdon, Humphrey, & Williams, 1988; Langdon, Humphrey, Williams, Hyatt, & Westbury, 1986; Speare & Smith, 1992; Weir et al., 2012; Whittington, Becker, & Dennis, 2010; Whittington, Kearns, Hyatt, Hengstberger, & Rutzou, 1996). The PCR-positive samples from wild lizards belonged to five male eastern water dragons residing in the Paluma Range National Park located approximated 65 km north west of Townsville, Queensland, thereby adding another species to the list of reptiles susceptible to ranavirus. Additionally, samples from three captive central bearded dragons (*Pogona vitticeps*) from Canberra, Australian Capital Territory and one captive frilled neck lizard (*Chlamydosaurus kingii*) from Townsville, Queensland were PCR-positive. While ranaviral infections have previously been reported in captive bearded dragons held in collections overseas this is the first report in captive central bearded dragons in Australia, and the first report worldwide in a frilled neck lizard (Stöhr et al., 2013; Tamukai et al., 2016). Other viruses have been detected in reptiles in Australia such as adenovirus in captive lizards, paramyxovirus in captive pythons, and herpesviruses in farmed saltwater crocodiles (*Crocodylus porosus*) and captive freshwater crocodiles (*Crocodylus johnstoni*) (Doneley,

Buckle, & Hulse, 2014; Hyndman, Marschang, Wellehan, & Nicholls, 2012; Hyndman & Shilton, 2011; Hyndman et al., 2015). Future work aims to characterize the *Ranavirus* sp. detected in this study.

All PCR-positive samples were from clinically healthy lizards with no apparent signs of disease. There are two additional reports of ranaviruses in seemingly healthy lizards, a wild-caught Iberian mountain lizard (Iberolacerta monticola) in Portugal and wild-caught eastern fence lizards (*Sceloporus undulatus*) in the United States (Alves de Matos et al., 2011; Goodman et al., 2018). This contrasts with other reports of ranaviral infected lizards that presented with inappetence, lethargy and skin lesions (Behncke et al., 2013; Marschang et al., 2005; Stöhr et al., 2013; Tamukai et al., 2016). Usually these lizards were only investigated following high mortalities or sudden death with ranavirus not considered the primary pathogen at the time of presentation to a veterinarian. The detection of ranavirus in asymptomatic animals during the survey, confirms the findings of the infection trials and introduce the possibility of carrier lizards which can infect naïve animals through direct contact causing morbidity and mortality. Carrier animals may remain asymptomatic until times of stress such as inadequate thermogradient in a captive setting or environmental stressors (e.g. habitat destruction) in the wild. With many reptilian viruses having non-specific clinical signs it is recommended that veterinarians add ranavirus (and possibly other viruses) as a differential diagnosis in fatal outbreaks of lizards that have experienced periods of inappetence, lethargy or present with skin lesions. As a result of the molecular and serological evidence of ranavirus in wild reptiles in northern Queensland (Ariel et al., 2017), it is hypothesised that ranavirus may be part of the normal microflora in Australian lizards with clinical signs only present in times of stress such as those associated with captivity The prevalence of ranavirus in Australian reptiles is unknown and there is evidence that reptiles are illegally moved between captivity and the

wild as well as illegal import from overseas (Alacs & Georges, 2008). While serological surveys may cast light on the presence of ranaviruses in wild and captive lizard, it is also important to identify preventative strategies that Australian reptile keepers can employ to protect their reptile collection and wild animals alike.

The overall aim of Chapter 5 was to explore Australian reptile keepers' experiences with disease, health management and quarantine practices, and barriers to seeking veterinarian advice/treatment for their reptiles. A survey of 179 Australian reptile owners found that their level of knowledge about reptilian diseases was limited to non-infectious diseases such as metabolic bone disease, the most commonly diagnosed problem in captive lizards (Mader, 2006). Infectious diseases like ranavirus, for example, were only heard of by less than 30% of participants despite infectious diseases being considered one of the largest causes of morbidity and mortality in reptiles (Paré, Sigler, Rosenthal, & Mader, 2006). Over 60% of participants self-reported health problems that they had experienced within their reptile collection. The most commonly reported problems were mites, respiratory disease and unexplained death. Some participants reported that the lack of veterinary expertise in the field of reptilian health and the expense of seeking treatment had led to self-diagnosing and treating of reptilian illnesses.

An undiagnosed illness or sudden death within a captive collection could have implications for other reptiles, current and future. Diseases infecting reptiles, including those of viral origin, have an incubation period, often cause non-pathognomonic clinical signs, or animals remain asymptomatic. Such diseases are therefore hard to recognise on symptomatology alone. Consequently, reptile keepers could unknowingly spread such diseases to other reptiles held in the collection, newly acquired reptiles, terrarium and other equipment if not

correctly disinfected, or to other collections when animals are sold on. This issue is concerning and also compounded by the lack of knowledge about infectious diseases amongst reptile keepers, many of which are unlikely to identify these diseases and/or employ appropriate strategies to prevent and limit transmission pathways to other animals. It is therefore important that information about reptilian diseases, especially emerging diseases, is updated regularly and readily available to reptile keepers to enhance their understanding of keeping reptiles as many freely available resources are either outdated (e.g. text books), inconsistent (e.g. state/territory Codes of Practice quarantine periods), or unreliable (e.g. anecdotal information on blogs).

The most popular disease preventative strategy employed by participants was cleaning and/or disinfecting, with quarantine listed as the fourth most implemented measure. However, the roles of cleaning and disinfection protocols and products were poorly understood and incorrectly used in many cases, and quarantine periods were frequently not employed for newly acquired reptiles. Only 72% of participants quarantined newly acquired animals (median duration: 28 days) and only 32% of these physically isolated the animal. This is concerning as several reptile-specific diseases have long incubation times (e.g. sunshine virus in pythons), are difficult to diagnose and are capable of interclass transmission (e.g. transmission of ranavirus from frogs to lizards) (Brenes, Gray, Waltzek, Wilkes, & Miller, 2014). Generally, quarantine should last for at least 90 days with the exception of pythons that should be quarantined for at least six months (Jacobson, Morris, Norton, Wright, & Nathan, 2001). It is recommended that current state and territory Codes of Practices for keeping reptiles reflect this and be consistent as currently Queensland, for example, recommends a quarantine period of 7 days while New South Wales recommends 30 days (*Code of practice captive reptile and amphibian husbandry*, 2010; *Code of practice for the*

private keeping of reptiles, 2013). Participants voiced concerns over the current reptile licensing system which varies between states and territories with only some requiring annual reporting, and not all reptile species requiring a current licence to be kept in captivity. Additionally, a few believed reptile licenses were too easy to obtain and should require some form of assessment of competency in reptile husbandry and captive reptile health management. Another concern raised by this survey was the number of participants who knew of someone deliberately releasing reptiles into the wild. While this survey did not quantify this, or establish what species were involved, the implications of the movement of these animals raises questions about disease dispersal and biosecurity. Illegal movement of reptiles within Australia could help to explain the first detection of a *Ranavirus* sp. in wild Australian eastern water dragons.

This study has demonstrated the susceptibility of eastern water dragons to Bohle iridovirus and has described the pathogenesis of this infection in this host. It is the first study to explore the effects of ranavirus in lizards, as well as the first to describe the use of *in situ* hybridization as a more sensitive and specific staining technique in tissues from ranaviral infected reptiles. Additionally, this study describes the first detection of *Ranavirus* sp. in clinically healthy Australian captive and wild lizards. Future work is needed to characterise the *Ranavirus* sp. found in this study and to determine the prevalence of ranavirus in Australian lizards. We recommend that future surveys include both oral-cloacal swabs and serum samples for identification of current and previous exposure to ranaviral infection. This study has also identified several key areas that need attention to better equip reptile owners and help protect captive and wild reptiles from infectious diseases that may be unknowingly passed on by reptile keepers, such as up-to-date easily accessible resources and experienced veterinarians.

Reptiles are considered one of the most ecologically and evolutionarily remarkable groups of living organisms (Pincheira-Donoso, Bauer, Meiri, & Uetz, 2013). They play an essential role in the balance of the ecosystem and are excellent ecological indicators because of their high degree of sensitivity to changes in the environment (Rajpoot, 2016). However, little is known about their health or the diseases they are susceptible to. It is through the work presented in this study that we can remove something from the unknown list and open up new research avenues for future investigators.

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APPENDICES

Appendix A1 - Ethics Approval for Chapters 2, 3, and 4

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Appendix B - Raw data (weight, snout to vent length, sex) for lizards experimentally infected with Bohle iridovirus (*Ranavirus* sp.)

Lizard ID#	Weight (g)	Snout to vent length (mm)	Sex
A1	14.5	65	Unsexed
A2	16.4	76	Unsexed
A3	12.6	59	Male
A4	17.7	69	Female
A5	15.2	64	Female
A6	13.5	63	Female
A7	14.4	64	Male
A8	14.1	67	Unsexed
A9	12.7	60	Unsexed
A10	15.8	70	Unsexed
B1	13.8	69	Unsexed
B2	19.3	76	Female
B3	12.7	65	Female
B4	13.9	70	Unsexed
B5	14.4	69	Unsexed
B6	12.1	66	Unsexed
B7	15.5	75	Male
B8	17.6	74	Unsexed
B9	18.1	70	Male
B10	11.9	56	Male
C1	14.0	70	Unsexed
C2	13.1	58	Female
C3	14.0	62	Unsexed
C4	7.7	49	Unsexed
C5	12.5	65	Unsexed
C6	13.1	61	Female
C7	14.6	63	Male
C8	14.0	61	Male
C9	6.0	56	Male
C10	11.4	58	Female

Health and Wellbeing of Australian Reptiles

Dear Reptile Enthusiast,

My name is Alicia Maclaine. I am a student from James Cook University where I am currently doing my PhD exploring the presence and impact of DNA viruses on Australian reptiles in North Queensland.

As part of my PhD I am conducting a study titled: **"*Health and Wellbeing of Australian Reptiles*"**. The aim of this study is to explore reptile keepers and breeders' experiences with disease, health preventative and quarantine practices, and barriers to seeking veterinarian advice/treatment.

As part of this study you are invited to participate in an online survey. Taking part in this study is completely voluntary and you can withdraw at anytime. If you agree to be involved in this study, you will be required to complete an online survey. The survey should only take **15 minutes** to complete. Before you participate in the survey you will be requested to give participation consent and confirm that you currently own a reptile, over 18 years old and reside in Australia.

Your responses will remain unidentified and confidential.

The data from the study will be used in research publications and reports to the James Cook University Ethics Committee. You will not be identified in any way in these publications.

Thank you very much for your cooperation in this important study about reptile health management.

1. By checking the box below, you acknowledge that you have read the information provided about this study, are 18 years or older, reside in Australia, and agree to participate in the study.

☐ I agree

2. What is your age (in years)?

3. What is your postcode?

4. Which of the following best describes you?

☐ I own reptiles and breed them

☐ I own reptiles but do not breed them

5. Do you hold a current/valid reptile license?

☐ Yes

☐ No

☐ I don't know

6. Are any of your reptiles not currently listed on your license?

☐ Yes

☐ No

☐ I don't know

☐ I don't have a reptile license

7. For how long have you owned reptiles? (Please enter a number into both boxes)

Years

Months

8. At what age did you get your first reptile?

9. Where do you usually obtain your reptile(s) from? [Tick all that apply]

☐ A licensed breeder

☐ An unlicensed breeder

☐ A pet shop

☐ From the wild

☐ Inherited from a friend or family member

☐ Other (please specify below)

10. Which of the following types of reptiles do you currently own? [Tick all that apply]

☐ Crocodile

☐ Freshwater turtle

☐ Lizard

☐ Snake

☐ Other (please specify below)

11. Do you own any exotic/non-native reptiles? (For example: corn snake, red-eared slider turtle, green iguana, etc.)

☐ Yes

☐ No

12. How many reptiles, in total, do you currently owns?

13. How many of these are lizards?

14. Please list the number and common name of lizards you currently own. (For example: 1 central bearded dragon, 2 leaf-tailed geckos, 3 shinglebacks, etc.) If you do not currently own any lizards, please enter '0'.

15. In what type of enclosure(s) do you keep your reptile/s? [Tick all that apply]

☐ Glass enclosure, tank or vivarium

☐ Pit or pond

☐ Tubs or racks

☐ Wooden enclosure

☐ Other (please specify below)

16. Where do you keep your enclosure(s)? [Tick all that apply]

☐ Bedroom

☐ Dining or kitchen area

☐ Garage

☐ Living area

☐ Outdoors (e.g. patio, veranda, etc.)

☐ Other (please specify below)

17. Do you keep regular health records for your reptile(s)?

☐ Yes

☐ No

18. What information do you include in these health records? [Tick all that apply]

☐ I do not keep any health records

☐ Breeding and mating details (e.g. number of eggs laid)

☐ Cleaning/disinfection dates

☐ Details/dates about faeces

☐ Information about food items (e.g. date and type of food offered)

☐ Information about health (e.g. signs of illness or disease)

☐ Information about medical treatment (e.g. type of medication)

☐ Measurements (e.g. nose to vet length)

☐ Shedding dates and details

☐ Weight

☐ Other (please specify below)

19. Are you the only person who handles your reptile(s)?

☐ Yes

☐ No

20. Who else do you let handle you reptile(s)? [Tick all that apply] (In this survey, an adult is someone who is at least 18 years old)

☐ Adults living with you

☐ Children living with you

☐ Relative/friend adult not living with you

☐ Relative/friend child not living with you

☐ Other adults (e.g. visitors or strangers)

☐ Other children (e.g. visitors or strangers)

☐ I do not let anyone else handle my reptile(s)

21. When handling reptile(s), how often do you wash your hands? [Tick all that apply]

☐ I don't wash my hands

☐ Before handing a reptile

☐ Between reptiles

☐ After handing a reptile

22. How often do you **clean** your reptile's enclosure(s)?

☐ Daily

☐ Weekly

☐ Monthly

☐ Other (please specify below)

23. How often do you **disinfect** your reptile's enclosure(s)?

☐ Daily

☐ Weekly

☐ Monthly

☐ Other (please specify below)

24. What products do you use to clean and/or disinfect your reptile's enclosure(s)? 9If you don't use any products to clean and/or disinfect please enter 'N/A')

To clean:

To disinfect:

25. Do you quarantine/isolate newly acquired animals?

☐ Yes

☐ No

26. For how long do you quarantine/isolate a newly acquire reptile? (All boxes must be completed. If you do not quarantine/isolate new reptiles please enter '0' into each box)

Years

Months

Weeks

27. What kind of quarantine procedures do you follow when looking after a newly acquired reptile? [Tick all that apply]

☐ I do not follow any quarantine procedures

☐ Disinfectant footbaths

☐ Handle quarantined animals last

☐ Regular disinfection of enclosure and furnishing

☐ Same room, separate enclosure

☐ Separate room in a different dwelling

☐ Separate room in the same dwelling

☐ Use separate cleaning equipment

☐ Wash hands

☐ Other (please specify below)

28. Have you heard about any of the following diseases that may affect reptiles?

Adenovirus	☐ Yes	☐ No
Falling fatigue syndrome	☐ Yes	☐ No
Herpesvirus	☐ Yes	☐ No
Inclusion body disease	☐ Yes	☐ No
Metabolic bone disease	☐ Yes	☐ No
Papillomavirus	☐ Yes	☐ No
Paramyxovirus	☐ Yes	☐ No
Parasitic disease	☐ Yes	☐ No
Parvovirus	☐ Yes	☐ No
Ranavirus or Iridovirus	☐ Yes	☐ No
Reovirus	☐ Yes	☐ No
Respiratory disease	☐ Yes	☐ No
Retrovirus	☐ Yes	☐ No
Salmonella	☐ Yes	☐ No
Sunshine virus	☐ Yes	☐ No
West Nile virus	☐ Yes	☐ No
Yellow fungus disease	☐ Yes	☐ No

29. Do you monitor your reptile/s for any of the following signs of disease? [Tick all that apply]

☐ Abnormal skin shedding
☐ Birthing difficulty
☐ Conjunctivitis or nasal discharge
☐ Constipation or diarrhoea
☐ Dehydration
☐ Egg bound
☐ Eye colour changes
☐ Fractures
☐ Inflammation of the mouth or gums
☐ Lumps
☐ Missing digits, toes or limbs
☐ Mouth deformities
☐ Neurological signs (e.g. tremors, star-gazing)
☐ Nose rubbing
☐ Prolapse of the cloaca, hemipenes, etc.
☐ Skin changes, lesions or ulcers
☐ Swelling of the ears, eyes or face
☐ Weakness or partial paralysis
☐ Weight gain or loss
☐ Wounds
☐ Other (please specify below)

30. Have you had any of the following health problems within your reptile collection? [Tick all that apply]

☐ Gut impaction
☐ Intestinal worms
☐ Metabolic bone disease
☐ Mites
☐ Ticks
☐ Respiratory disease
☐ Never had a health problem
☐ Unexplained death
☐ Other (please specify below)

31. What preventative strategies do you currently implement to reduce the risk of your reptile(s) developing a health issue? [Tick all that apply]

☐ Cleaning and/or disinfection

☐ Quarantine newly acquired animals

☐ Regular mite treatment

☐ Regular use of a calcium supplement

☐ Regular use of a multivitamin supplement

☐ Regular worming

☐ Use of heat cord, lamps or mats

☐ Use UVA/UVB lighting

☐ I do not implement any preventative strategies

☐ Other (please specify below)

32. Have you ever sought veterinary treatment and/or advice for your reptile(s)?

☐ Yes

☐ No

33. Did you find it affordable?

☐ Yes

☐ No

☐ I have never sought veterinary treatment and/or advice for my reptile(s)

34. Were you satisfied with the treatment/advice offered?

☐ Yes

☐ No

☐ I have never sought veterinary treatment and/or advice for my reptile(s)

If you were not satisfied with treatment/advice offered, please specify below why?

35. If you have never sought veterinary treatment and/or advice for your reptile(s), why not? [Tick all that apply]

☐ I have sought veterinary treatment and/or advice for my reptile(s)

☐ Animal died

☐ Asked for advice from another reptile keeper

☐ Consultation and/or treatment not affordable

☐ Had a sick/unwell reptile but did not seek treatment/advice

☐ Had a sick/unwell reptile but was able to treat without seeking treatment/advice

☐ Never had a sick/unwell reptile

☐ Live too far away from a veterinarian

☐ Other (please specify below)

36. Do you know of anyone that has released a captive-bred reptile into the wild?

☐ Yes

☐ No

37. Do you know of anyone that has taken a reptile from the wild?

☐ Yes

☐ No

38. Would you like to add anything else on the topic of captive reptile health management?

Thank you very much for your cooperation in this important study about reptile health management.

If you have any questions about the study, please contact Alicia Maclaine or Dr Diana Mendez.

Alicia Maclaine (Prinicpal Investigator)
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If you have any concerns regarding the ethical conduct of the study, please contact: Human Ethics, Research Office James Cook University, Townsville, Qld, 4811. Phone: (07) 4781 5011. Email: ethics@jcu.edu.au

Appendix X – Co-authors consent for the inclusion of published and submitted articles into the following doctoral thesis:
"Pathology of ranavirus in eastern water dragons (*Intellagama lesueurii lesueurii*) and survey of ranavirus in Australian lizards"
Presented by Alicia Maclaine

Thesis section	Publications or manuscripts	Nature and extent of the intellectual input of each author, including the candidate	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis
Chapter 2	**Maclaine, A.,** Mashkour, N., Scott, J., & Ariel, E. (2018). **Susceptibility of eastern water dragons Intellagama lesueurii lesueurii to Bohle iridovirus.** *Disease of Aquatic Organisms, 127*(2), 97-105. doi:10.3354/dao03193	EA and AM conceived the concept of the study and drafted the ethics and permit applications to conduct the study. NM did the cell culture aspect of the experiment. JS examined the histopathology slides and prepared reports with AM. Results were reviewed by EA. AM prepared the manuscript (drafts were reviewed by EA and JS) and managed the submission to peer-reviewed journal.	Name: Narges Mashkour Signature:
			Name: Jennifer Scott Signature:
			Name: Ellen **Ariel** Signature:
Chapter 3	**Maclaine, A.,** Forzán, M. J, Mashkour, N., Scott, J., & Ariel, E. **Pathogenesis of Bohle iridovirus (genus *Ranavirus*) in experimentally infected juvenile eastern water dragons (*Intellagama lesueurii lesueurii*).** *Veterinary Pathology*	EA and AM conceived the concept of the study and drafted the ethics and permit applications to conduct the study. NM did the cell culture aspect of the experiment. AM prepared histopathology blocks and H&E sections. JS examined the H&E slides and prepared reports with AM. MF did the IHC and ISH, and prepared the relevant sections for inclusion in the manuscript. AM prepared the manuscript (drafts were reviewed by EA and MF). MF submitted the manuscript to a peer-reviewed journal.	Name: María J. Forzán Signature:
			Name: Narges Mashkour Signature:
			Name: Jennifer Scott Signature:
			Name: Ellen Ariel Signature: