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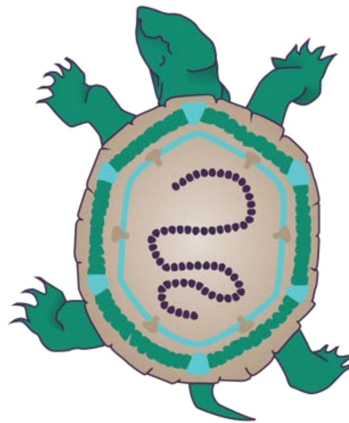
Ranaviral Infection in Australian Freshwater Turtles

Wytamma Wirth

BSc & BBiomedSc (Hons)



Thesis submitted to

James Cook University
College of Public Health, Medical and Veterinary Sciences

in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
August 2020

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Declaration of Ethics

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 James Cook University Animal Ethics Committee (A1943). This research was conducted under a permit granted by the Department of Environment and Heritage Protection (WISP13270413).

Wytamma Wirth
20 August 2020

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And to all the rest, all the best,

Wytamma

Statement of the Contribution of Others

The following table shows the persons and organisations who made substantial contributions to this thesis.

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Summary

Ranaviruses are pathogens of ectothermic vertebrates, including turtles. The distribution of ranaviruses and experimentally susceptible turtle species overlap in Australia; however, no epizootics have been documented. So, why don't we see ranaviral epizootics in Australian freshwater turtle populations? This thesis aimed to determine factors that influence ranaviral disease and to describe the distribution of ranaviral infections in Australian freshwater turtles. Results were obtained through experimental infection studies and a molecular survey. Ranaviral inoculation route and dose both influenced the time to disease development, the number of animals infected, and the type and severity of clinical signs that develop (Chapter 3 & Chapter 4). Environmental temperature influences the number of animals that develop ranaviral infections infected (Chapter 5) and the duration of infection influences ranaviral disease presentation with implications for best sampling protocols (Chapter 6). While Australian turtles are susceptible to ranaviral infection, the prevalence in wild turtle populations in North Queensland is low (Chapter 7). This thesis significantly increases our understanding of ranaviral disease in Australian reptiles and provides valuable insights into ranavirology in general. It also leads to many new questions for future scientific research. Understanding the role of diseases in Australian wildlife, like ranaviral infection in freshwater turtles, will ultimately help us to manage and preserve life on this planet.

Publications and Presentations throughout doctoral studies

Publications

- **Wirth**, Lesbarrères, Ariel. Ten years of ranavirus research (2010-2019): An analysis of global research trends. FACETS - 2020 (Accepted)
- **Wirth** and Ariel. Temperature-dependent infection of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with a ranavirus isolate (Bohle iridovirus, *Iridoviridae*). FACETS - 2020 (Accepted)
- Maclaine, **Wirth**, McKnight, Burgess, Ariel. Ranaviruses in captive and wild Australian lizards. FACETS - 2020 (Accepted)
- **Wirth**, Elliott, Rudd, Hayes, Maclaine, Mashkour, Ahasan, Dahl, Drane, Ariel. Cutaneous Lesions in Freshwater Turtles (*Emydura macquarii krefftii* and *Myuchelys latisternum*) in a Rainforest Creek in North Queensland, Australia. Frontiers in Veterinary Science - 2020
- **Wirth**, Schwarzkopf, Skerratt, Tzamouzaki, Ariel. Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with a Ranavirus isolate (Bohle iridovirus, *Iridoviridae*). Journal of General Virology - 2019
- **Wirth**, Schwarzkopf, Skerratt, Ariel. Ranaviruses and reptiles. PeerJ - 2018
- Ariel, Freeman, Elliott, **Wirth**, Mashkour, Scott. An unusual mortality event in Johnstone River snapping turtles *Elseya irwini (Johnstone)* in Far North Queensland, Australia. Australian Veterinary Journal - 2017

Presentations

Conference Presentations

- **Wirth,** et al. Dose dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with *Bohle iridovirus* (*Ranavirus sp*, *Iridoviridae*). 4th International Symposium on Ranaviruses - 2017

Conference Posters

- **Wirth**, et al. Pathogenesis of a Ranaviral Infection in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*). Wildlife Disease Association Australasian Section Conference - 2019
- **Wirth**, et al. Pathogenesis of Bohle Iridovirus (Genus *Ranavirus*) in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*). 5th International Symposium on Ranaviruses - 2019
- **Wirth** and Steinig. Auto-scope: a cheap optical microscope modification for whole slide scanning. JCU Research Festival – 2018
- **Wirth** and Ariel. Clinical signs and their time to development vary with infection route in Australian freshwater turtle species: *Emydura macquarii krefftii*. 10th international Symposium on Viruses of Lower Vertebrates – 2017

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for those who hum or whistle and are generally content while pipetting

Chapter 1

POSITIONING THE RESEARCH

We are in the middle of the sixth mass extinction event (Barnosky et al., 2011; Ceballos et al., 2015; Ceballos, Ehrlich & Dirzo, 2017). Through processes such as pollution, globalisation, and urbanisation, we are rapidly changing the face of our planet. This rapid change is not without consequence. Species are disappearing at rates up to 100 times higher than the expected background extinction rates (Ceballos et al., 2015). Because of the direct role of human activity in the current extinction crisis, this period has been labelled 'the Anthropocene extinction' (Ceballos et al., 2015; Ceballos, Ehrlich & Dirzo, 2017). The emergence of infectious diseases is one of the factors driving the Anthropocene extinction (Wake & Vredenburg, 2009). Through climate change, animal trade, and habitat destruction, we are contributing to the global emergence of infectious diseases (Wilson, 1995; Butler, 2012; Zohdy, Schwartz & Oaks, 2019). We are reducing the health of the environment, moving disease into populations that have never had them, and increasing the animal-human interface. As custodians of the Earth, we have a duty of care to minimise the impact of infectious diseases on our wildlife. But to prevent disease, we need first to understand it. We need detailed knowledge of the factors that influence the transmission, pathogenicity, and distribution of infectious diseases. With an adequate understanding of the factors that control infectious disease emergence, we could identify and manage at-risk populations, and potentially help to reduce the role of infectious diseases in the Anthropocene extinction.

Ranaviruses are a group of globally distributed pathogens responsible for disease in many species of ectothermic vertebrates including fish, amphibians, and reptiles (Gray & Chinchar, 2015). Many of these viruses are emerging and significant threats to wildlife, and humans are contributing to their global spread via trade in live animals (Storfer et al., 2007; Kolby et al., 2014; Duffus et al., 2015; Stöhr et al., 2015). Ranaviruses were first discovered in the 1960s, but major epizootics (in wild and captive populations) were not recorded until the 1980s, while the vast majority of cases have been recorded in the last decade (Duffus et al., 2015), some of which have contributed to population declines (Price et al., 2014, 2017). While no species extinctions have been directly attributed to ranaviruses, many possess the characteristics to cause population extinctions (Gray & Chinchar, 2015). Due to the fact that ranaviruses are understudied, have a vast host range, and are spread by humans, ranaviruses may emerge as important causes of extinction in the future, and they were therefore selected as the focus of this thesis.

To get a broad understanding of research in the field of ranavirology and to identify important areas that would benefit from further research, a bibliometric analysis was performed using the last ten years of ranaviral research literature. This analysis has since been accepted for publication and can be found in full in Appendix 1 (Wirth, Lesbarrères & Ariel, 2020). Among other things, this analysis identified reptiles as an important and trending topic in ranaviral research. Focussing on ranavirus research papers that used 'reptile(s)' as an author keyword, I found that ranaviral research in reptiles is recent: the median year of publication of papers from the last 10 years was 2017. In addition, reptile research was an important component of the ranaviral field, as indicated by a co-occurrence analysis of all keywords from the last 10 years. This analysis identified a cluster

of keywords containing terms such as: reptiles, chelonians, box turtles, etc. This cluster of reptile-like keywords was distinct from other keyword clusters in the literature on ranaviruses.

To get a better understanding of the research on ranaviruses infection in reptiles, a literature review was performed on this topic. This review has since been published (Wirth et al., 2018) and can be found in full in Appendix 2. Some of the important and relevant findings are summarised as:

1. For the most part, ranaviral disease research has focused on fish and amphibians, whereas the other extant class of ectothermic vertebrates, the Reptilia, has received less attention from the scientific community, although research in this area is increasing.
2. Members of the order Testudines (i.e. turtles) are the reptiles most commonly reported with ranaviral infection.
3. Ranaviral disease in turtles is primarily reported in animals from North America, despite ranaviruses and susceptible turtle species coexisting on other continents. However, it is unclear if the concentration of reports from North America is related to the generally high research output of the USA or whether the impact of the disease is actually greater.
4. Diagnosis of ranaviral infection in reptiles is often difficult and diagnostic methods commonly produce inconsistent results.
5. Ranaviruses cause disease in wild and captive reptiles, sometimes producing high levels of mortality. Despite the disease potential in reptiles, we know little about the factors that influence ranaviral disease and have a limited understanding of the immunological and pathological responses of reptiles to ranaviral infection.

6. Complex presentations and inconsistency in reptile ranaviral pathogenesis may occur because of the influence of various factors in reported cases and experiments. The host physiology and life history, and varying degrees of virulence, stressors, coinfections, exposure routes and doses, environmental temperatures, and the stage of ranavirosis (disease caused by ranaviral infection) at which the animal presents could all be acting on the outcome of an infection.
7. Differences in susceptibility via different routes of exposure may reflect real differences in natural transmission routes in reptile ranaviral epizootics. Although some reptile species appear to be highly susceptible to ranaviral infection, no studies have quantified ranaviral susceptibly (in terms of inoculation dose) for any reptile species. Environmental temperature appears to have an influence on susceptibility of turtles to ranaviral infection, however, studies have had inconsistent results.
8. Few, if any, effective treatments exist for ranaviral infection in reptiles.

There are several species of ranaviruses found in Australia (Duffus et al., 2015). Generally, the distribution of ranaviruses in Australia appears to be split latitudinally (**Figure 1.1**). Bohle iridovirus-like viruses (a *Frog virus 3* isolate) are typically found in the north of the continent and *epizootic hematopoietic necrosis virus*-like (EHNV) viruses are found in the south (Langdon et al., 1986; Speare & Smith, 1992; Whittington et al., 1996; Hyatt et al., 2002; Maclaine et al., 2020; Weir et al., 2012; Wynne, 2020). Bohle iridovirus-like viruses have a broad host range and are capable of transmission across classes of ectothermic vertebrates (fish, amphibians, and reptiles), while EHNV appears limited to fish (Price et al., 2017). Interestingly, the majority of ranaviral reports are in close proximity to the universities or laboratories studying them, i.e. the University

of Sydney and the Australian Animal Health Laboratory in the south and James Cook University in the north. This suggests that the distribution of viruses may be highly biased by where researchers are looking. It is likely that ranaviruses are more widely spread in Australia than reported.

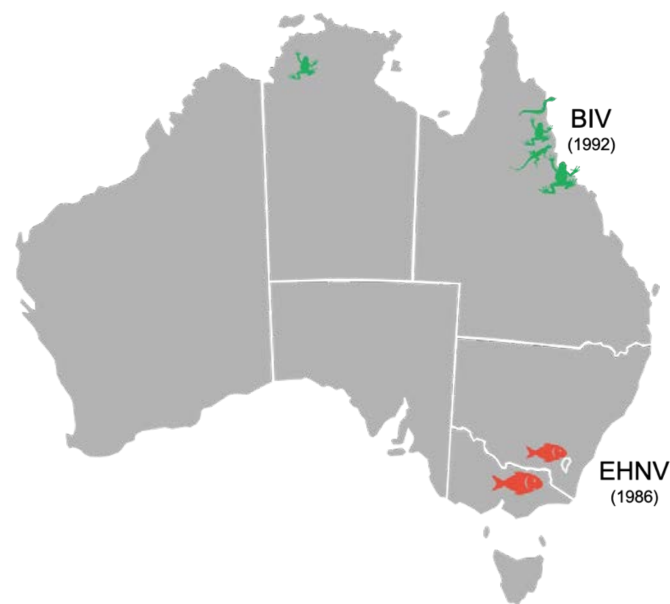

Figure 1.1 The general distribution of ranaviruses in Australia. Bohle iridovirus (BIV, green) was first reported in ornate burrowing frogs (*Platyplectrum ornatum*) in 1992 (Speare & Smith, 1992). BIV-like viruses have since been reported in other frog (Weir et al., 2012; Wynne, 2020), snake (imported, Hyatt et al., 2002), and lizard (Maclaine et al., 2020) species in Northern Australia. *Epizootic hematopoietic necrosis virus* (EHNV, red) was first reported in redfin perch (*Perca fluviatilis*) in 1986 in southern Australia (Langdon et al., 1986; Whittington et al., 1996).

Australia has a large diversity of native reptiles (the Australian Reptile Online Database, http://www.arod.com.au/), including several species that are susceptible to ranaviral infection. The green python (*Morelia viridis*), eastern water dragon (*Intellagama lesueurii lesueurii*), and two

freshwater turtles species (*Myuchelys latisternum* and *Emydura macquarii krefftii*) have succumbed to ranaviral infections in the past (Hyatt et al., 2002; Ariel et al., 2015; Maclaine et al., 2018).

Australia is home to many turtle species, primarily side-necked turtles from the suborder Pleurodira (family Chelidae) (Wilson & Swan, 2003). More than 40% of the freshwater turtle species found in Australia have a conservation status of threatened or worse (Van Dyke, Ferronato & Spencer, 2018). Because they have small geographic ranges, extirpation of Australian freshwater turtle populations can result in significant loss to the species as a whole. In 2015, a nidovirus disease outbreak killed almost all adult Bellinger River snapping turtles (*Myuchelys georgesi*), which were restricted to a small coastal catchment in Australia, the Bellinger River (Spencer et al., 2018; Zhang et al., 2018). As a result of this single disease outbreak, the Bellinger River snapping turtle is now listed as critically endangered and is one of the top 25 most threatened freshwater turtle species in the world, exemplifying the urgent need to have a better understanding of disease in Australian freshwater turtles (Stanford et al., 2018).

Most Australian freshwater turtles belong to the suborder Pleurodira, this is one of two extant suborders of turtles, the other being the Cryptodira (Cann & Sadlier, 2017). These two suborders probably split evolutionarily more than 150 million years ago (Chiari et al., 2012). This deep evolutionary divide makes it difficult to apply the results from one turtle suborder to the other. Similarly, we are cautious when extrapolating the results of studies on mice to primates and their evolutionary divide is only ~ 90 million years (**Figure 1.2**). Unfortunately, the majority of ranaviral research in turtles has been performed on members of the Cryptodira (Wirth et al., 2018).

Thus, to understand ranaviral infection in the Pleurodira, we need better (i.e. evolutionarily closer) models of ranaviral disease in these animals.

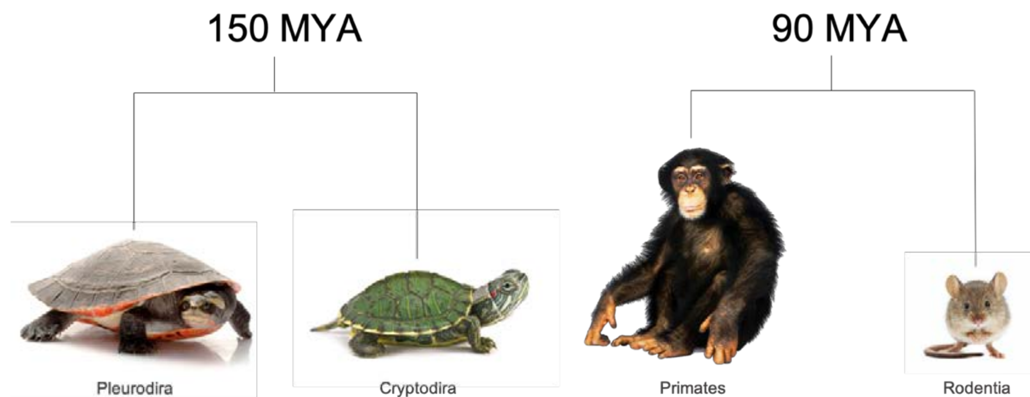

Figure 1.2 The estimated time, millions of years ago (MYA), of the last common ancestor of the Pleurodira and Cryptodira (the two surviving suborders of the Testudines) and Primates and Rodentia (two orders of the mammalian superorder Euarchontoglires).

Despite the significant impact of these viruses on other ectothermic vertebrates around the globe little is known about ranaviral infection in Australian freshwater turtles. Ranaviruses are present in Australia in environments where freshwater turtles are found (Speare & Smith, 1992) and serological surveys of Australian reptiles (including freshwater turtles) suggest that they are exposed to ranaviruses (Ariel et al., 2017a). While at least two species of Australian freshwater turtle are susceptible to ranaviral infection via experimental challenge (Speare & Smith, 1992; Ariel et al., 2015), reports of ranaviral disease in wild Australian freshwater turtles are conspicuously absent. It is unclear if ranaviral infection has not been reported in wild Australian freshwater turtle because it is not occurring in wild populations, or if this is result the result of a lack of surveillance. Due to the vastness of the Australian wilderness, the low density of humans

and the almost non-existence of surveillance, it is entirely possible that outbreaks are not registered. If, however, ranaviral disease is truly not occurring, there must be factors controlling the spread or pathogenicity of ranaviruses in Australian freshwater turtle populations.

Overall Aims

The distribution of ranaviruses and experimentally susceptible turtle species overlap in Australia, leading to the central question of this thesis: why don't we see ranaviral epizootics in Australian freshwater turtle populations? Two possible answers to this question are: epizootics are occurring in wild turtle populations, but they are not detected and reported, or unknown factors are limiting ranaviral infections in wild Australian freshwater turtle populations. I developed two aims to test these hypotheses:

- Determine factors that influence ranaviral disease in Australian freshwater turtles
- Describe the distribution of ranaviral infection in wild Australian freshwater turtle population

I address these aims through five experimental and observational chapters in this thesis, entitled:

- THE INFLUENCE OF INOCULATION ROUTE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS
- THE INFLUENCE OF VIRAL DOSE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS
- THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON RANAVIRAL INFECTION IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS
- THE INFLUENCE OF INFECTION DURATION ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS
- A MOLECULAR SURVEY FOR RANAVIRUSES IN WILD AUSTRALIAN FRESHWATER TURTLES

Chapter 2

METHODS FOR THE SAFE COLLECTION AND EFFECTIVE INCUBATION OF EGGS FROM THE KREFFT'S RIVER TURTLE (*EMYDURA MACQUARII KREFFTII*)

Aims

1. Obtain hatchlings for use in an animal model of ranaviral disease in Australian freshwater turtles
    a. Capture wild gravid Krefft's river turtles
    b. Induce the Krefft's river turtles to lay their eggs in a controlled setting
    c. Incubate the Krefft's river turtle eggs under controlled laboratory conditions until they hatch

Introduction

To determine factors that influence ranaviral disease in Australian freshwater turtles (and by extension those of the suborder Pleurodira) we need to establish a model in which to test these various factors. Ideally, this model should be representative of the system we are trying to understand, i.e. ranaviral disease in Australian freshwater turtles. The Krefft's river turtle (*Emydura macquarii krefftii* or *Emydura krefftii krefftii*) is an Australian freshwater turtle (suborder Pleurodira) found in North Queensland (Cann & Sadlier, 2017). Krefft's river turtles are the most abundant freshwater turtle species in Townsville (where this research was conducted) and

these turtles are commonly sold as pets in Queensland. Krefft's river turtle hatchlings are susceptible to infection with Bohle iridovirus, an Australian *Ranavirus* isolate (Ariel et al., 2015). Thus, I chose to use Bohle iridovirus infection in Krefft's river turtle hatchlings as a model of ranaviral disease in Australian freshwater turtles.

Hatchlings are an elusive life stage, and they are difficult to capture in the wild. It is easier to capture gravid (egg carrying) adult turtles and incubate their eggs to obtain hatchlings. Using captive hatched hatchlings from wild adult turtles as experimental animals has several benefits including: ensuring the animals are still representative of wild genetic stock, reduced husbandry overheads when compared with adult turtles, controlled antigen exposure history, partial knowledge of genealogy (mother), and the age of clutches can be synchronised (if egg laying is induced at the same time).

This chapter details the methods I used to obtain Krefft's river turtle hatchlings; the experimental animal subjects that form the basis of most of this thesis. I demonstrate the success and discuss the value of these methods in the development of an animal model for ranaviral disease in Australian freshwater turtles.

Methods

All procedures described were carried out under permits from the James Cook University Animal Ethics Committee (A1943) and the Department of Environment and Heritage Protection (WISP13270413).

Trapping

Cathedral traps (aka drum traps, **Figure 2.1**) were baited with sardines and set at locations where Krefft's river turtles had been observed basking. Traps were suspended in water (from an overhanging branch or bridge) ensuring that the trap was fully extended below water with a sufficient clearance above the water for turtles to surface once inside (**Figure 2.1**). Turtle traps were checked every 1-2 hours or left overnight, depending on turtle abundance; they were checked less frequently in areas where turtles were less abundant.

Captured turtles were removed one by one from the traps and placed headfirst into open-weave cloth bags with drawstrings and external labels (**Figure 2.2**). Bagged turtles were placed into plastic tubs out of direct sunlight. Female Krefft's river turtles were identified by their short tail compared with similar sized males (Carmel & Johnson, 2014) (**Figure 2.3**). Gravid female turtles were identified by gently palpating the body cavity via the inguinal fossae. Two opposing fingers were used to feel for hard-shelled eggs, the size of grapes (**Figure 2.4**). Gravid turtles were marked with a unique identifier and set aside for transport. All field equipment was cleaned and disinfected with a 5% bleach solution when moving between sites.

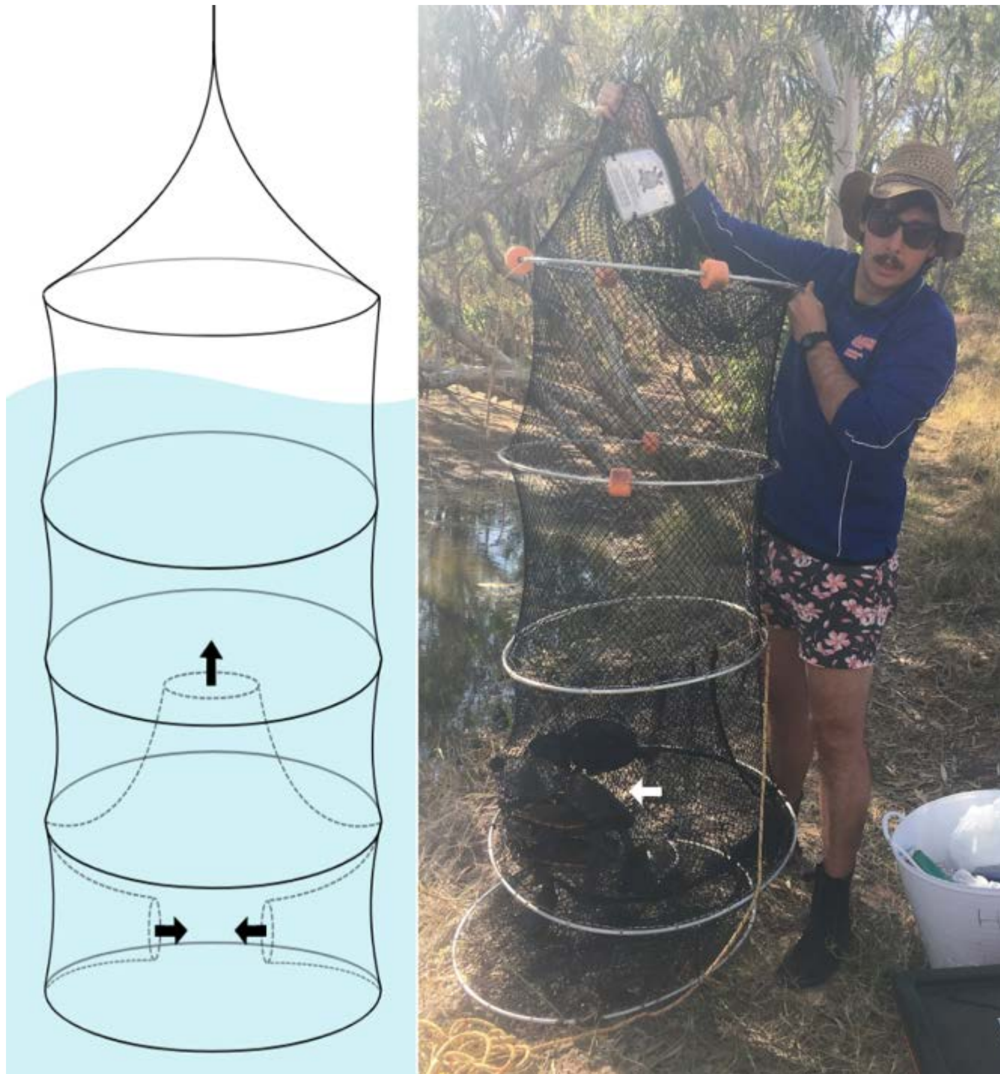

Figure 2.1 Cathedral trap for capturing turtles. Cathedral traps allow turtles to surface for air while remaining inside the trap. Black arrows in the schematic on the left panel indicate the unidirectional movement of turtles into the baited chamber at the bottom of the trap and then into the main chamber that extends above the water surface (shaded area). Several trapped turtles can be seen in the main chamber of the trap in the photo on the right (white arrow). These traps can be purchased from commercial suppliers or built from crab traps and netting. Horizontal traps such as hoopnets or collapsible minnow traps (see Howell et al., 2016) are also viable options but require shallow water for effective use.

Figure 2.2 Turtle bags. Bags help to keep turtles (dotted outline) calm and cool while waiting for processing and during transport. External label (green tag, 27) enables turtle identification without disturbing the animal by opening the bag.

Figure 2.3 Sex is identified via tail length in Krefft's river turtles. Adult female turtles (left) can be distinguished from male turtles (right) of a similar size using tail length. Note the longer thicker tail of the male turtle.

Figure 2.4 Assessing the breeding status of turtles**.** Using the thumb and index or middle finger it is possible to feel the hard-shelled grape-sized eggs within the body cavity of the turtle.

Induction of oviposition

Gravid turtles were kept in fresh tap water at ambient temperatures under quarantine in an outdoor shelter at James Cook University for up to 24 hours prior to induction. Oxytocin was injected intramuscularly into the hind leg using a 25-gauge needle (**Figure 2.5**). The dose of oxytocin was 10 IU kg^{-1}. Following oxytocin injection, turtles were placed into a 50L plastic tub with the water level just above the depth of the carapace and monitored for egg laying. A second and equal dose of oxytocin was given if the turtle had not started egg laying after 1.5 hours.

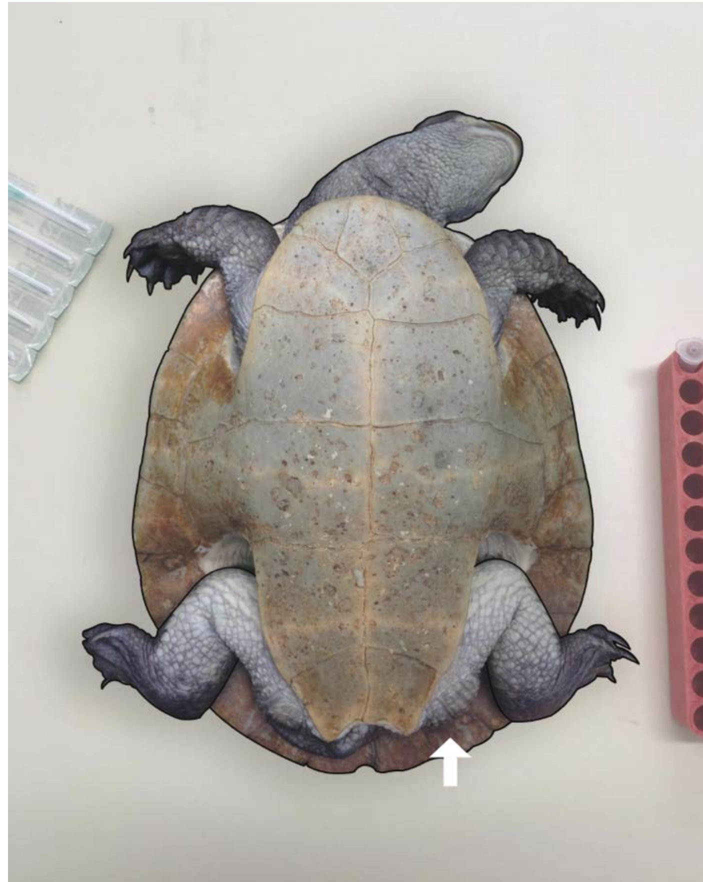

Figure 2.5 Site of oxytocin injection. Place the turtle on its carapace in an open container, keeping limbs off the table surface and thus preventing the turtle from rolling over. Inject the oxytocin into the muscle of the hind leg (white arrow). The injection volume can be halved and administered in both legs.

Eggs were removed as they were laid; they were then dried, weighed, measured (longest and widest points), and labelled using a graphite pencil before placing them on a bed of vermiculite in the incubation container (**Figure 2.6**). Incubation containers were prepared by combining autoclaved vermiculite with 1:1 sterile and deionised water by weight in a 500 mL plastic container with an easy-to-open lid. Turtles were observed for a minimum of 1 hour after the final egg was laid and palpated to check for more hard-shelled eggs. Turtles were kept in captivity in quarantine for a post-induction monitoring period to ensure that the turtle was eating and active. After the post-

induction monitoring period and confirmation of normal active behaviour the turtles were returned to the location of initial capture.

Figure 2.6 Incubation of freshwater turtle eggs in a cell culture incubator. Eggs are labelled with a soft graphite pencil and incubated at 29˚C. To ensure a sterile environment and accurate control of the temperature; eggs are incubated in a cell culture incubator. Note the spacing between eggs to allow easy access.

Incubation of eggs

Lids were placed loosely on the incubation containers, ensuring the lid could be removed without disturbing the eggs. Eggs were carefully placed into a cell culture incubator at 29˚C. Every 1-2

days the containers were opened for 5 min to facilitate gas exchange. Throughout incubation, eggs were monitored externally for signs of development (**Figure 2.7**). Eggs that were not developing (e.g. failed to develop chalky white areas) were removed to reduce potential microbial contamination. Internal development of the embryo was monitored using a process called candling (Doneley et al., 2017), by gently placing the eggs in a cradle created with modelling clay (Blu Tack™) on the lens of an upright torch. Eggs were candled weekly after 4 weeks of incubation to assess their internal development (**Figure 2.8**).

Figure 2.7 External appearance of eggs in the incubation container on a bed of vermiculite. Immediately following oviposition (day 0). Chalking starts during the first few days of incubation (day 2). The spot expands into a circum-equatorial band which gradually extends toward the polar regions of the egg until the surface is completely chalked at approximately one-month post incubation. Moisture at the leading edge of the band is normal and a sign of growth (day 16). At the end of the incubation period (day 50), the hatchling uses its egg tooth to break through the shell.

Healthy developing embryos were identified by reddish light, blood vessels, and movement during candling (Doneley et al., 2017). Eggs that did not contain a visible embryo were also removed to prevent microbial contamination (**Figure 2.8**, A). Eggs were checked daily after 40 days of

incubation. Most hatchlings were left to escape the egg by themselves. If the hatchlings were not making progress after initially cracking the egg, then small portions of the shell were carefully broken off, ensuring that yolk sac and blood vessels were not damaged.

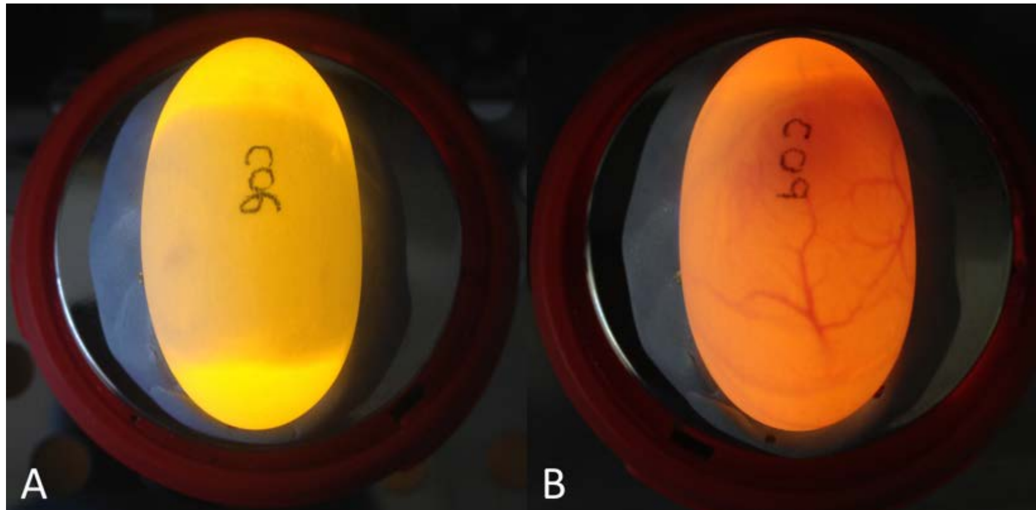

Figure 2.8 Candling of *E. m. Krefftii* eggs after 21 days of incubation**.** Egg (A, C06) shows no signs of developing embryo, note the consistent yellow colouration from the unused yolk. Fertile egg (B, C09) with developing embryo visible (dark area under label). Blood vessels can be clearly seen.

Results

Sardine-baited cathedral traps were effective for capturing gravid Krefft's river turtles. A total of 29 gravid turtles were caught and weighed an average of 1.72 kg (1.29-2.13, n=28). The cloth bags kept the turtles calm and reduced damage during transport (pers. obs.). The average time from injection of initial oxytocin to the commencement of egg laying was 18 minutes (7-28, n=12). The average number of eggs laid after oxytocin injection was 12.55 per turtle (9-19, n=29). There was a positive correlation between the number of eggs laid and the mass of the turtle (**Figure 2.9**).

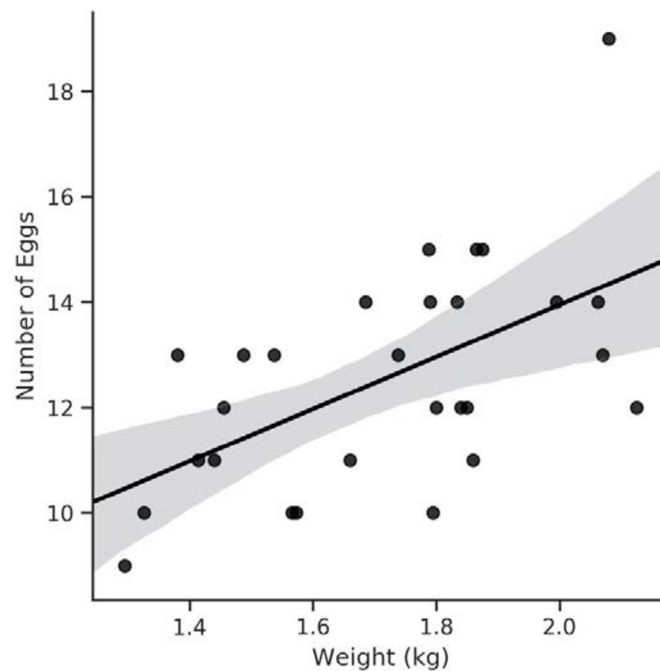

Figure 2.9 Weight of turtle vs the number of eggs laid. There is a positive correlation between mass of gravid female Krefft's river turtles (n=26) and number of eggs in a clutch ($R=0.56$, $p < 0.01$). Shaded area represents the 95% confidence interval.

The mean egg morphometrics i.e. mass, length and width, immediately following oviposition, were 9.29 g (7-12, n=311), 35.45 mm (31.6-39.9, n=312), and 20.87 mm (18.3-22.9, n=310), respectively (**Figure 2.10**). There was no significant correlation between the number of eggs laid and the size of the eggs, or between the mass of the mother and the size of the eggs. Average length of incubation per clutch was 51.9 days (48-55, n=17). The mean hatching success rate (the number of eggs that hatched after the incubation period) was 87.5 %. However, the hatching success rate ranged from 7.7-100 % depending on the clutch.

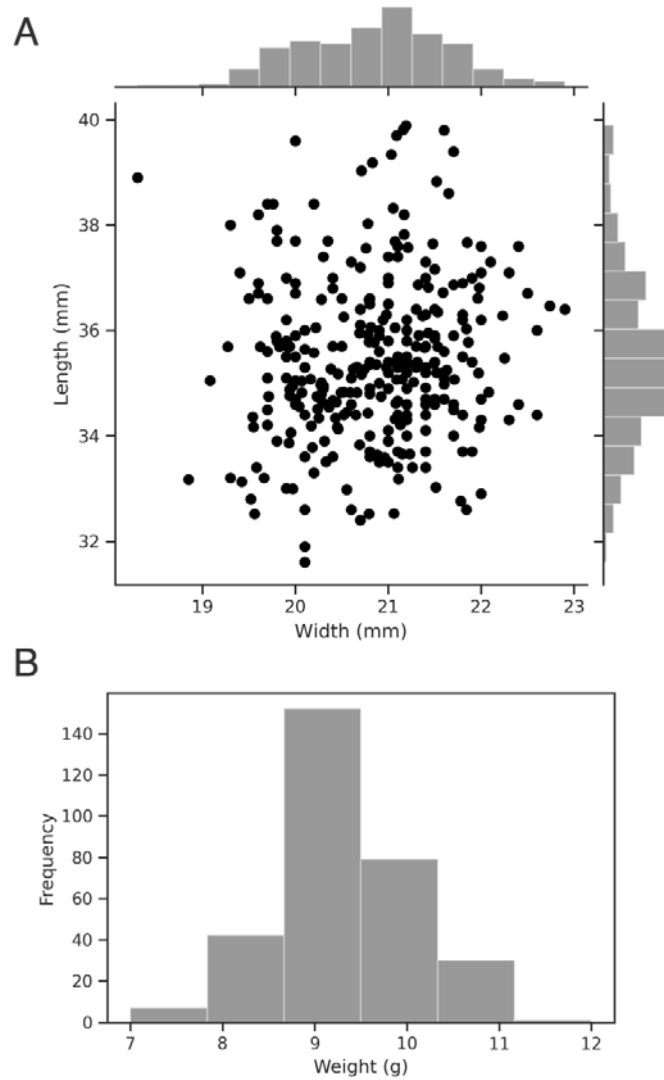

Figure 2.10 Morphometric data from eggs immediately following oviposition**.** Scatterplot showing no linear relationship between egg length and width, edge histograms show the variation and frequency of each of the measurements (A). Distribution and counts of the weight of eggs collected during the study (B).

Discussion

In this chapter I present methods that are effective for obtaining Krefft's river turtle hatchlings. These methods were used to establish a reproducible and applicable model of ranaviral disease in Australian freshwater turtles. This model is more applicable to Australia then others used to study ranaviral infection in turtles thus far e.g. those using turtles from the suborder Cryptodira.

Several studies have described methods for the capture of freshwater turtles (Spencer, 2001; Bury et al., 2012). As the majority of species in the genus *Emydura* nest between October-January (Cann & Sadlier, 2017), it is therefore advisable to set traps during this period when looking for gravid females. If a suitable location for setting cathedral traps is not available (e.g., the water is too shallow), different style traps (e.g., hoop nets) or snorkelling (hand capture) can be employed (Howell, McKnight & Seigel, 2016). It is important that the cathedral trap is fully extended to ensure effective operation. If the trap is not fully extended, turtles may not be able to enter or may get caught in the lower chamber of the trap.

Palpation of the body cavity via the femoral fossa is an effective method for detecting eggs. However, accurate enumeration of eggs requires a diagnostic method such as X-ray imaging (Gibbons & Greene, 1979; Ariel, 1997). As with other freshwater turtle species, there was a positive correlation between the mass of the turtle and the number of eggs in the clutch, so targeting larger females may be preferable. The use of cloth bags, such as those described in this chapter, provided a useful means of keeping turtles restrained while waiting for processing or during transport. However, these bags appear very effective at reducing damage such as scratches and broken claws.

Oxytocin, the mammalian analogue to the reptilian hormone mesotocin, can be used to induced oviposition in gravid Krefft's river turtles with no ill effect (Ewert & Legler, 1978; Perry & Mitchell, 2017). A range of oxytocin doses (5-40 IU kg^{-1}) have been used to induce oviposition in a variety of freshwater turtle species (Ewert & Legler, 1978; Thompson et al., 1999; Fordham, Georges & Corey, 2006; Rafferty et al., 2013; McGlashan et al., 2017; Perry & Mitchell, 2017), however, an intramuscular injection of 10 IU kg^{-1} appears effective for Krefft's river turtles. Concentrated oxytocin (10 IU mL^{-1}) reduces the volume required for injection. Following the dose of oxytocin, egg laying begins within 7-28 minutes. The water in the oviposition container prevented damage to the eggs occurring as they were laid. Obtaining eggs from a gravid female in a controlled environment may reduce the amount of microbial contamination associated with collecting eggs from wild nests in the soil.

Egg morphometrics recorded in this study were similar to those reported in other studies of Emydurid turtles (Legler & Cann, 1980; Banks, 1987; Georges, 2009). Classic optimal egg size theory predicts that egg size will be optimised to increase maternal fitness and egg number will vary to increase reproductive success (Smith & Fretwell, 1974). We found that large turtles produced more eggs, but they did not produce larger eggs, these findings fit within the predictions of optimal egg size theory. Spencer (2001) also found that *Emydura macquarii* clutch size was correlated with the size of the mother, however, the mean clutch size was much larger in their study. Some studies have found that egg size may be constrained by the size of the mother, and thus smaller turtles will produce small eggs (Schwarzkopf & Brooks, 1986). However, it did not appear to be the case for the Krefft's river turtles in this study.

Turtles produce eggs with extra-embryonic membranes for gas exchange, nutrient acquisition from the yolk, and waste disposal (Kohring, 1995). The development of the chorioallantoic membrane can be monitored externally by looking for chalking. The amniotic egg allows turtles to construct their nests out of water, although they still require a warm and moist environment during incubation for maximum success (Thompson, 1988; Rafferty & Reina, 2014; Cann & Sadlier, 2017).

The temperature inside the incubator should be monitored daily for several weeks before and during incubation of eggs, to ensure a correct and stable incubation temperature. Temperature has an effect on the development and characteristics of the embryo, and so downstream use of the turtle should be considered when choosing an incubation temperature (Booth et al., 2004; Eiby & Booth, 2011; Spencer & Janzen, 2011). Published incubation temperatures for Emydurid turtles vary greatly (16-32°C), however 28-30°C appears to be the range producing the greatest hatching success (Banks, 1987; Thompson, 1988; Booth et al., 2004; Rafferty & Reina, 2014). Although temperature can control the sex in some species of turtle (via temperature dependent sex determination) this does not occur in Emydurid turtles, and so an even sex ratio should be expected (Thompson, 1988). Eggs will 'sweat' as the embryo grows, this moisture is normal and does not need to be wiped off.

Because of the need to incubate turtle eggs at a relatively high temperature and humidity it is also important to consider measures to reduce microbial growth, especially fungal contamination (Phillott, 2002). This can be achieved by using sterile technique at all times when handling the

incubation media and turtle eggs. If fungal infection does occur, affected eggs should be quarantined to prevent spread. Antifungal compounds such as clotrimazole have been used with variable success. It is also possible to dip the eggs in a 1% iodine solution to inhibit fungal infection before they are placed in the incubation chamber (Ariel, 1997).

Publication and outputs

My overall contribution to this study 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, with the help of volunteers, set traps to capture the gravid turtles;
- I administered the oxytocin to induce oviposition;
- I, with the help of a volunteer, collected, measured, and incubated the eggs;
- I developed a temperature monitoring system to remotely track the temperature during incubation;
- I performed the analysis presented in this chapter; and
- I produced all the figures and wrote the chapter presented here.

Conclusions

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

1. Obtain hatchlings for use in an animal model of ranaviral disease in Australian freshwater turtles
    a. Capture wild gravid Krefft's river turtles
    b. Induce the Krefft's river turtles to lay their eggs in a controlled setting
    c. Incubate the Krefft's river turtle eggs under controlled laboratory conditions until they hatch

The methods presented here were sufficient for obtaining hatchlings from wild Krefft's river turtles. Baited cathedral traps were effective at capturing gravid turtles. An intramuscular injection of oxytocin was successfully used to induce oviposition in all the gravid turtles captured. Eggs were successfully incubated in a laboratory cell culture incubator, resulting in a high hatching success rate. The hatchlings produced by the methods presented in this chapter were used to model the influence of different factors on ranaviral disease in Australian freshwater turtles. The work utilising these hatchlings is presented in the following four chapters of this thesis (Chapter 3, Chapter 4, Chapter 5, and Chapter 6).

Chapter 3

THE INFLUENCE OF INOCULATION ROUTE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

Aims

1. Determine the susceptibility of hatchlings to ranaviral infection via different routes
2. Describe the clinical signs and changes in pathogenesis produced by alternative routes of infection
3. Determine an infection route for use in future experimental challenge studies

Introduction

Having obtained hatchlings using the methods described in the previous chapter, this chapter begins to examine the factors controlling ranaviral disease in Australian freshwater turtles. Inoculation route is an important factor to consider in infection models as different inoculation routes may result in different disease presentations, some more accurately reflecting the natural course of infection than others (Wirth et al., 2019). While Koch's postulates have been confirmed for ranaviral infection in several turtle species, the natural route of infection remains unknown (Johnson, Pessier & Jacobson, 2007; Allender, 2012; Allender et al., 2018). Because of the varied lifestyle of susceptible turtle species (e.g. aquatic, semi-aquatic, terrestrial), there maybe be several

infection routes involved in the transmission of ranaviruses in these animals, some more viable than others.

Krefft's river turtle (*Emydura macquarii krefftii*) hatchlings are known to be susceptible to ranaviral infection via intracoelomic inoculation (Ariel et al., 2015). The intracoelomic route is typically choose in experimental infection to elicit the maximum infection rate. Virus, injected into the body cavity, is inoculated on to the internal organs, sidestepping innate barriers, eliminating the requirements of dissemination, and maximising the likelihood that the virus will encounter a permissive tissue type. As multiple internal organs are exposed simultaneously, it is impossible to make accurate interpretations of the pathogenesis i.e. to determine the natural progression of disease from organ to organ. Thus, while intracoelomic inoculation serves as a useful model for susceptibility testing, it is not very useful for understanding the pathology of natural ranaviral infections.

This chapter describes an experimental ranaviral infection in an Australian freshwater turtle species. This experiment is designed to determine a pragmatic route of infection for use in future experimental challenge studies and to describe the influence of alternative routes of inoculation on susceptibility and the course of ranaviral disease.

Methods

All experiments were carried out under a James Cook University (JCU) Animal Ethics permit (A2344) and a Department of Environment and Science Scientific Research Permit (WISP13270413).

Animals

The hatchlings used in this study were obtained following the methods described in Chapter 2. Newly emerged hatchlings were moved to the JCU Turtle Health Research Facility where they were housed in tanks with water between 24 and 28 ˚C and given access to dry areas for basking. They were fed commercial turtle pellets (Exo Terra, aquatic turtle food) *ad libitum* for 10-15 minutes per day. Daily feeding observations and weekly morphometric data were collected from each individual during this husbandry phase.

Source of virus

The BIV isolate used in this study was produced according to the methods of (Maclaine et al., 2018). Briefly, fathead minnow (FHM) cells were used to propagate the isolate at 25 °C. The FHM cells were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, NY, USA), supplemented with 100× antibiotic/antimycotic (Thermo Fisher Scientific, NY, USA) and 10 % foetal bovine serum (Bovogen Biologicals, Melbourne, Australia). The final viral stock was titrated ($TCID_{50}$) and frozen at −80 °C until inoculation (Reed & Muench, 1938). The titre of the stock solution of BIV used in this study was $10^{5.33}$ $TCID_{50}$ mL^{-1}.

Experimental design

Two weeks prior to the start of the study, nine hatchlings were selected and assigned to either the control (n=3) or infection (n=6) group. On day 0 the turtles in the infection groups were inoculated with 20 µL of $10^{5.33}$ $TCID_{50}$ BIV via intramuscular (upper left thigh muscle) or intracoelomic injection or orally with a micro- pipette. Control animals received a placebo inoculation of DMEM

via one of the equivalent routes. The mean weight of the hatchlings at the start of the trial was 8 g (8.5–12.6). All animals in the trial were housed in individual 1 L containers with 500 mL of water that was changed daily (after feeding). Room temperature was maintained at 28 ˚C (26-30) by air conditioning. The controls and infected groups were housed in separate rooms to prevent cross-contamination. The hatchlings were examined daily for signs of disease.

Clinical signs

Each day, starting with the control group, animals were fed and assessed for signs of disease or any mortalities. The clinical signs and behaviours monitored and recorded during the trial included: movement in an open field, rollover reflex, startle reflex, basking, defaecation, oedema, skin damage (erosion, peeling, bruising), eye disorders (e.g. conjunctivitis) and oral or nasal discharge. Dietary intake and morphometric measurements i.e. weight, straight carapace length (SCL), and straight carapace width (SCW), were recorded at the start and end of the trial or in conjunction with a clinical observation or death.

Endpoint

The hatchlings were euthanased, at the onset of severe clinical signs, with a 200 µL intracoelomic injection of 1 % (v/v) neutral-buffered tricaine methanesulfonate (MS-222) followed by a intracoelomic injection of 50 % (v/v) MS-222 (Conroy et al., 2009).

Gross pathology and sample collection

Final morphometrics were collected immediately prior to euthanasia. Following euthanasia, photographs were taken of any lesions, or other clinically relevant observations. Sterile surgical

tools were used for necropsy and any gross pathological changes were noted. A liver sample was collected from each animal for subsequent PCR analysis and stored at −20 ˚C. The remaining viscera, head and left hind leg (injection site) were preserved in 10 % buffered formalin for histological analysis.

PCR

DNA was extracted from the thawed liver samples using an ISOLATE II Genomic DNA kit (Bioline) following the manufacturer's protocol for DNA extraction from tissue samples. PCR amplification was performed using the primers described by Jaramillo et al. (2012), targeting a 94 bp region of the Ranavirus major capsid protein gene (F: 5′- GACT GACC AACG CCAG CCTT AACG, R: 5′- GCGG TGGT GTAC CCAG AGTT GTCG) with GoTaq qPCR Master Mix (Promega). Thermocycling was performed in real time on a Mic qPCR Real Time Cycler (Biomolecular Systems) with the following reaction conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Samples that crossed the threshold with a sigmoidal amplification curve before 30 cycles were considered to be positive. A 10-fold dilution of BIV DNA was used as a positive control. No-template (H_2O) and negative extraction controls were also included.

Histology

Formalin-fixed samples of all infected animals and controls were processed for histological examination according to standard operating procedures (Bancroft & Gamble, 2008). Tissues were sectioned (5 µm) and stained with haematoxylin and eosin before microscopic examination.

Results

Clinical signs

All routes of infection induced severe clinical signs in at least one animal (intramuscular = 2/2, intracoelomic = 2/2, oral = 1/2), although the clinical signs varied with the inoculation route. The average time to the development of clinical signs was shorter in the intramuscular and intracoelomic groups (10 and 11.5 days) when compared with the diseased individual in the oral group (16 days). One hatchling in the oral group was euthanased due to severe clinical signs (20 days post-inoculation), which included: necrotic oral plaques, inappetence, lethargy and soft tissue oedema. The other hatchling in this group showed no signs of disease and was euthanased at the end of the trial. Both hatchlings in the intramuscular group developed severe clinical signs more rapidly than the hatchlings in any other group; one hatchling died, and one was euthanased 17 and 18 days post-inoculation, respectively. Severe clinical signs in the intramuscular group included lethargy, inappetence, oral haemorrhaging, periocular and soft tissue oedema, swelling at the injection site and increased basking behaviour. One hatchling in the intramuscular group also developed skin lesions at and around the site of injection and produced a bloody discharge from the cloaca. Both hatchlings in the intracoelomic group developed severe clinical signs; one hatchling died, and one was euthanased at 18 and 20 days post-inoculation, respectively. Severe clinical signs in the intracoelomic group included: lethargy, inappetence, petechial haemorrhage of the tongue, soft tissue oedema and increased basking behaviour.

PCR

The liver samples of both the intracoelomic and intramuscular-inoculated animals reacted in the ranaviral PCR assay. Only one of the orally infected animal samples reacted in the PCR assay (the

same animal that developed clinical signs). No control animals reacted in the PCR for ranaviral DNA in the liver.

Histopathology

Histopathological changes were observed in all animals to various degrees. The diseased animal in the oral group had an influx of granulocytes into the liver and gastrointestinal tract (GIT), necrotising splenitis and necrosuppurative stomatitis and glossitis. The orally inoculated hatchling with no clinical signs had a slight increase in hepatic granulocytes, but no other histological changes were evident. The intramuscular inoculated animals had an influx of eosinophilic granulocytes into the liver and multifocal epidermal necrosis around the injection site. The animals in the intracoelomic group had large necrotic areas in the liver and increased eosinophilic granulocytes. One intracoelomic-inoculated animal developed necrotising splenitis and the other developed necrosis and inflammation of the intestinal submucosa.

Discussion

Inoculation route contributes to the infection dynamics of diseases. The results of this chapter indicated that Krefft's river turtle hatchlings are susceptible to multiple routes of ranaviral infection. Clinical signs and histological lesions developed in turtles inoculated via both intramuscular and intracoelomic and in one of the turtles inoculated via the oral route. These results suggest that Krefft's river turtle hatchlings might be less susceptible via the oral route, but a larger study would be required to determine statistical significance. Similarly, a previous study found that red-eared sliders (*Trachemys scripta elegans*, suborder Cryptodira) were refractory to *Frog virus 3* (FV3; same species, different isolate) infection via the oral route, but susceptible to

infection via intramuscular inoculation (Johnson, Pessier & Jacobson, 2007). Although this study was too small to assess statistical differences in clinical signs, we only observed the development of skin lesions in intramuscularly inoculated turtles, suggesting that different infection routes may result in different disease presentations.

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 and poster. 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 and completed daily husbandry for the turtle hatchlings prior to and during the study;
- I developed the web-based husbandry tracker that was used to keep records of the health, diet, and morphometrics of the animals in this study;
- I prepared the viral inoculum and inoculated the hatchlings used in this study;
- I conducted the necropsies of turtle hatchlings and undertook sample collection for, PCR and histology;
- I prepared and analysed the histological slides;
- I extracted the DNA from samples for analysis by PCR;
- I performed the PCR on the samples;

- The results of this chapter were combined with the results of Chapter 4 and published in a peer-reviewed journal, I was the lead author on this publication and drafted the manuscript; 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 (found in the appendix of this thesis):

- Appendix 3 - **Wirth, W**., Schwarzkopf, L., Skerratt, L. F., Tzamouzaki, A., & Ariel, E. (2019). Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with *Ranavirus* isolate (Bohle iridovirus, *Iridoviridae*). Journal of General Virology, jgv001324.
- Appendix 6 - **Wirth, W**. & Ariel, E. (2017). Clinical signs and their time to development vary with infection route in Australian freshwater turtle species: *Emydura macquarii krefftii*. The 10th international Symposium on Viruses of Lower Vertebrates. Budapest, Hungary.

Conclusions

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

1. Determine the susceptibility of hatchlings to ranaviral infection via different routes

Krefft's river turtle hatchlings were susceptible to ranaviral infection via all inoculation routes tested (oral, intracelomic, and intramuscular), however, oral inoculation only produced disease in 50% (1/2) of the inoculated hatchlings.

2. Describe the clinical signs and changes in pathogenesis produced by alternative routes of infection

Time to development of clinical signs varied with inoculation route, however, larger studies will be required to determine statistical significance. Lethargy, inappetence and soft tissue oedema where common to all groups. The disease was generally systemic with multiple tissues affected. Group specific pathogenesis appeared to relate to the site of inoculations e.g. the oral and intramuscular groups developed lesions at the site of inoculation. These results enabled more informed euthanasia decisions for animals with ranaviral infections in the following chapters.

3. To determine an infection route for use in future experimental challenge studies

Intramuscular inoculation was selected for the future models of ranaviral disease as it is easily performed, was repeatable, has been used in other ranaviral challenge studies of turtles, and is conceivable as a natural infection route.

We now know that Krefft's river turtles are susceptible to ranaviral infection via multiple inoculation routes including: intracoelomic, oral, and intramuscular. This information is useful as

it provides more plausible routes of infection that are readily applicable to natural transmission i.e. ingestion (oral), or wounds or vectors (intramuscular).

This chapter confirms and extends the evidence for the susceptibility of Krefft's river turtles to ranaviral infections. This new knowledge strengthens the central question of this thesis; why don't we see ranaviral epizootics in Australian freshwater turtle populations? The virus has been found in environments inhabited by freshwater turtles, and these turtles are susceptible to infection via multiple routes; however, there are still no reported ranaviral outbreaks in Australian freshwater turtles. This leads to the hypothesis that there are some other factors influencing ranaviral disease in these animals. The titre of viral inoculum used in this study was $10^{5.33}$ $TCID_{50}$ mL^{-1}, this is a relatively high viral dose that may not reflect the dose encounter in a wild disease outbreak. Perhaps Australian freshwater turtles are resistant to ranaviral infection at lower, more epidemiologically relevant, doses. In the following chapter I investigate the influence of viral dose on ranaviral disease in Krefft's river turtle hatchlings.

Chapter 4

THE INFLUENCE OF VIRAL DOSE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

Aims

1. Determine influence of viral dose on ranaviral disease development in Krefft's river turtle hatchlings including:
    a. The effect of viral dose on the incubation period
    b. The effect of viral dose on clinical presentations
    c. The effect of viral dose on histopathology
2. Determine the viral dose for the tipping point of ranaviral infection and disease in Krefft's river turtle hatchlings i.e.
    a. The dose that causes severe clinical signs in 50% of animals (LD_{50})
    b. The dose at which 50% of animals become infected i.e. the 50% infectious dose (ID_{50})

Introduction

The major aim of this chapter is to understand how ranaviral disease development is influenced by viral dose. Consider an infinite range of viral doses e.g. from zero to in infinity. There is a viral dose, so low that it will not cause ranaviral disease in any animals that are exposed i.e. as viral

dose approaches zero. On the other end of that range is a dose that will kill all susceptible animals exposed i.e. as viral dose approaches infinity. While probably factual, knowledge of this infinite range is not very useful. However, if it is possible to narrow the range to something biologically relevant then it becomes useful for studying and understanding ranaviral disease in the wild.

Previous ranaviral challenge studies in turtles (including the one presented in the previous chapter) have been conducted with a single high dose of viral inoculum (Johnson, Pessier & Jacobson, 2007; Allender et al., 2013b, 2018; Brenes et al., 2014; Ariel et al., 2015; Wirth et al., 2018). These high doses complicate the interpretation of results in the context of natural infections, where inoculation doses are likely variable. Viral dose is important for epidemiological modelling of diseases as it provides a method with which to quantify susceptibility. Understanding how viral dose can influence disease allows us to better understand how a virus will spread through a given population (Dushoff, 1996; Regoes, Ebert & Bonhoeffer, 2002). Ranaviral susceptibility, in terms of viral dose, has not been quantified for any turtle species. In this chapter, I attempted to define the distribution of ranaviral disease as a function of viral dose and to find critical values within this dose range, such as the LD_{50}.

Methods

All experiments were carried out under a JCU Animal Ethics permit (A2344) and a Department of Environment and Science Scientific Research Permit (WISP13270413).

Animals

The hatchlings used in this study were obtained following the methods described in Chapter 2 and husbandry followed the methods of Chapter 3.

Source of virus

The BIV isolate used in this study was the same as that described in Chapter 3.

Experimental design

Two weeks prior to the experimental infection, 53 hatchlings were randomly selected and assigned to either the control (N=3) or infection (N=50) group. Animals in the infected group were further divided into five dose groups and their boxes were randomly assigned positions on shelves to prevent bias. The mean weight of the hatchlings at the start of the trial was 9.8 g (8.5-12.5). At the start of the infection trial (day 0) animals received an injection, with a 26G needle in the proximal left thigh muscle, of DMEM containing 0 (control group, n=3), $10^{1.33}$, $10^{2.33}$, $10^{3.33}$, $10^{4.33}$, $10^{5.33}$ $TCID_{50}$ of BIV (infection groups n=10 per group). Injection volume was determined by multiplying the individual's weight (g) by 2.5 (mean 24.4 µL; range 21-31 µL).

Clinical signs

Each day, starting with the control group, animals were fed and assessed for signs of disease or any mortalities. Clinical signs and behaviours monitored and recorded during the trial included: movement in an open field, roll-over reflex, startle reflex, basking, defaecation, oedema, skin damage (erosion, peeling, bruising), eye disorders (e.g. ocular oedema, conjunctivitis), and oral or

nasal discharge. Dietary intake and morphometric measurements (weight, SCL, SCW) were recorded at the start and end of the trial or in conjunction with a clinical observation or death.

Endpoint

The hatchlings were euthanased with a 200ul intracoelomic injection of 1% (v/v) natural buffered tricaine methanesulfonate (MS-222) followed by a 50% (v/v) MS-222 overdose (Conroy et al., 2009). Experimental hatchlings were euthanased when one of the following severe clinical signs were observed (based on the results of the Chapter 3): the hatchlings developed large lesions, extensive oedema, complete loss of appetite, or extreme lethargy. Death is here defined as hatchlings that were euthanased due to severe clinical signs. Hatchlings that did not develop severe clinical signs in the dose study were randomly selected for euthanasia at the conclusion of the trial over a six-day period (days 28-34). Clinical signs were recorded if they developed during this end-of-trial phase.

Gross pathology and sample collection

Necropsy and sample collection followed the methods described in Chapter 3.

PCR

PCR analysis followed the methods described in Chapter 3.

Histology

Histological examination followed the methods described in Chapter 3.

Statistical analysis

All statistical analysis was performed using the Statsmodels module (version 0.9.0) in Python 3.6 (Seabold & Perktold, 2010). The proportion of animals in each group euthanased due to severe clinical signs was recorded and used to calculate the median probability of death i.e. euthanasia (LD_{50}) by logistic regression. The number of samples from each dose group that reacted in the PCR assay (i.e., had ranaviral DNA in the liver) or developed clinical signs, was also recorded and used to calculate the median probability of infection (ID_{50}) by logistic regression. The 95% confidence intervals (CI) of the LD_{50} and ID_{50} were determined by bootstrapping (n=1000). The median time to the onset of clinical signs (CS_{50}) and the median time to onset of severe clinical signs (ST_{50}) were calculated. The association of inoculation dose with probability of developing clinical and severe clinical signs (odds-ratio) was calculated using logistic regression. Pearson correlation was used to determine the relationship between inoculation dose and the time to the onset of clinical signs and severe clinical signs.

Results

The LD_{50} and ID_{50} were $10^{4.43}$ $TCID_{50}$ (95% CI: $10^{3.81} – 10^{5.19}$ $TCID_{50}$) and $10^{2.52}$ $TCID_{50}$ (95% CI: $10^{1.98} – 10^{2.93}$ $TCID_{50}$) respectively for Krefft's river turtle hatchlings inoculated with BIV via the intramuscular route.

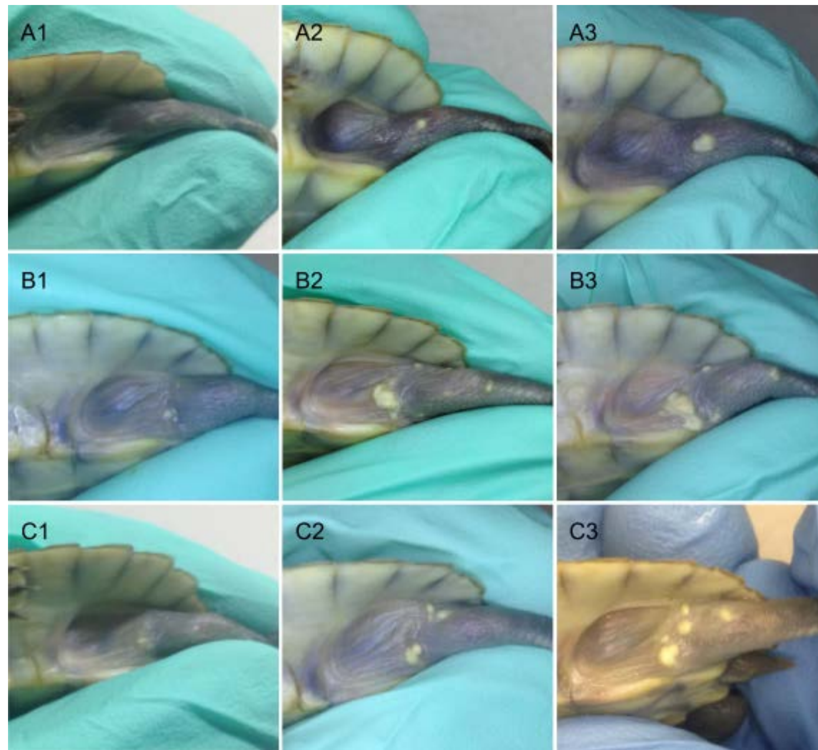

Figure 4.1 Development of skin lesions in turtle hatchlings with intramuscular injection of Bohle iridovirus. Each row represents an individual hatchling. Hatchling A from dose group $10^{5.33}$ mL^{-1} on days 14 (A1), 16 (A2), and 20 (A3) days post inoculation. Hatchling B from dose group $10^{4.33}$ mL^{-1} on days 14 (B1), 16 (B2), and 20 (B3) days post inoculation. Hatchling C from dose group $10^{3.33}$ mL^{-1} on days 17 (C1), 20 (C2), and 23 (C3) days post inoculation.

Clinical signs

Clinical signs were observed in 30 of the 50 inoculated turtles and none of the negative controls. Clinical signs were observed in some individuals from all dose groups. Onset of clinical signs occurred from 14 to 29 days post inoculation. The time to the onset of first clinical signs was negatively correlated with the log of the dose (r^2 = 0.13, P=0.047). Three animals developed clinical signs during the end phase of the trial. Notable clinical signs included anorexia, skin lesions at the inoculation site and often in the inguinal area (**Figure 4.1**), increased basking

behaviour, and oedema of the neck and legs (**Table 4.1**). Changes in rollover reflex and activity in an open field were also observed inconsistently before and during the trial. For every ten-fold increase in viral dose the odds of developing any clinical signs increased by 3.39 ($P < 0.01$, 95% CI 1.81-6.36). The mean starting weight of infected individuals that did not develop clinical signs, developed clinical signs, and those that went on to develop severe clinical signs was 9.78g (8.6-11.5), 9.93g (8.5-12.5), and 9.87g (8.6-12.2) respectively. Starting weight did not have a significant effect on the odds of developing clinical signs.

Table 4.1 Clinical signs summary statistics stratified into dose groups. The number of animals in each group (n). Percentage in group that developed clinical signs (Morbidity). Percentage of animals that died i.e. were euthanased due to severe clinical signs (Mortality). Median time (days) from inoculation to the onset of clinical signs (CS_{50}). Median survival time in days from the start of the trial to death for those with severe clinical signs (ST_{50}). Median time (days) from the onset of clinical signs to severe clinical signs (CS-SCS). The number of animals at the end of the trial with no clinical signs (NCS), clinical signs (CS), and severe clinical signs i.e. euthanased (SCS) that reacted in PCR assay for ranaviral DNA in liver.

Dose ($TCID_{50}$)	n	Morbidity (%)	Mortality (%)	CS_{50}	ST_{50}	CS-SCS	PCR				An	L	E	B	An_{50}	L_{50}	E_{50}	B_{50}	Wt
							NCS	CS	SCS	Total (%)									
$1x10^{5.33}$	10	90	70	15	18	3	1/1	2/2	7/7	100	80	90	80	30	19	15	15	16	9.7 (8.6-12.2)
$1x10^{4.33}$	10	90	50	18	22	4	1/1	4/4	5/5	100	50	70	80	20	21	20	15	17	9.97 (8.8-11.4)
$1x10^{3.33}$	10	80	30	20	24	9	1/2	5/5	3/3	90	20	70	80	30	20	16	19	21	9.92 (8.5-12.50)
$1x10^{2.33}$	10	20	0	28	n/a	n/a	0/8	2/2	0/0	20	20	10	10	10	30	28	17	28	9.85 (8.8-11.5)
$1x10^{1.33}$	10	20	0	24	n/a	n/a	0/8	2/2	0/0	20	0	20	20	0	n/a	26	23	n/a	9.88 (8.6-11.8)
Control	3	0	0	n/a	n/a	n/a	0/3	0/0	0/0	0	0	0	0	0	n/a	n/a	n/a	n/a	11 (10.1-12.5)

Severe clinical signs

Of the 50 inoculated turtle hatchlings, 15 were euthanased due to severe clinical signs (referred to as deaths/mortalities throughout). Percent mortality ranged from 0-70% in the different dose groups. The highest level of mortality was observed in the highest dose group (7/10, 10^5 $TCID_{50}$ BIV). Zero mortality was observed in the control group and two lowest dose groups (10^2 $TCID_{50}$ & 10^1 $TCID_{50}$) (**Table 4.1**). For every ten-fold increase in viral dose the odds of developing severe clinical signs increased by 3.71 ($P < 0.01$, 95% CI 1.76-7.80).

Clinical signs in the hatchlings euthanased due to severe clinical signs developed between 14-20 days post inoculation (median 15 days). The median survival time (ST_{50}) in days, from inoculation to mortality, was 22 and ranged from 16-25 days when all groups were combined. The ST_{50} was 18 (16-24 days), 22 (17-25 days), and 24 (22-24 days) for the three highest dose groups $10^{5.33}$ $TCID_{50}$, $10^{4.33}$ $TCID_{50}$, and $10^{3.33}$ $TCID_{50}$ respectively (**Table 4.1**). The time between the onset of clinical signs and euthanasia due to severe clinical signs for all groups ranged from 2-10 days (median 5 days).

PCR

Ranaviral DNA was detected, via PCR, in the livers of individuals from all dose groups (**Table 4.1**). All of the liver samples from animals in the two highest dose groups reacted in the PCR assay. Ranaviral DNA was detected in the livers of all of the turtle hatchlings with severe clinical signs (15/15). All turtle hatchlings that developed clinical signs but were not euthanased until the end of the trial, reacted in the PCR for ranaviral DNA in the liver (15/15). Two turtles that did not develop clinical signs also reacted in the ranaviral PCR assay.

Gross pathology

Gross lesions noted at necropsy were limited to individuals that developed clinical signs and included oral haemorrhaging and necrotic plaques on the mucosa and nearby keratinised tissues (**Figure 4.2**), oedema of soft tissues, multifocal raised yellow-white areas and pale discoloration or mottling of the liver, discoloration of the spleen, and haemorrhaging of the intestinal epithelium.

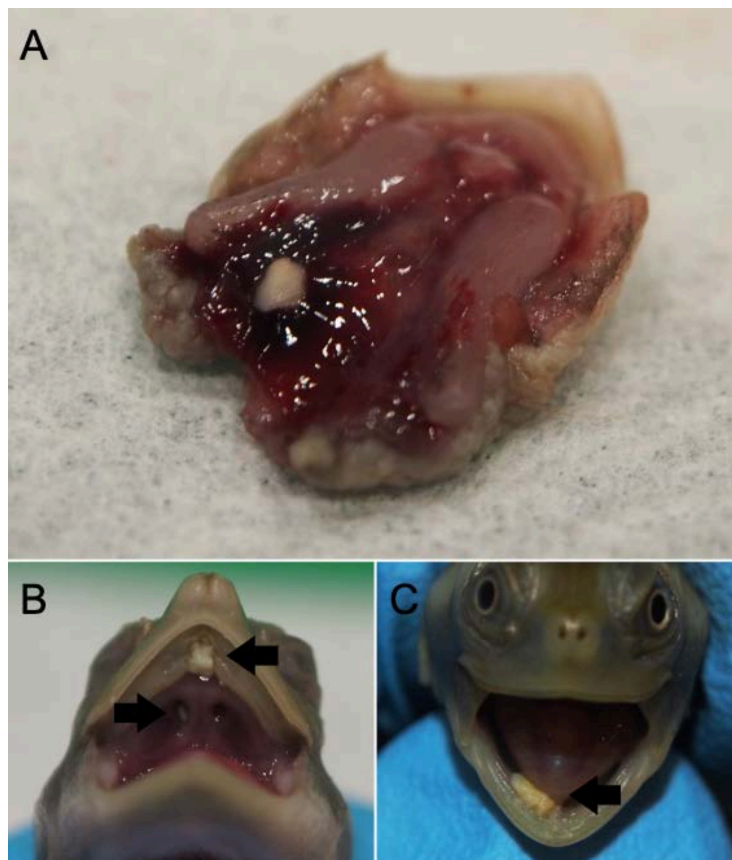

Figure 4.2 Examples of oral lesions observed in turtles during necropsy. A, extensive haemorrhagic lesion anterior to the oesophageal opening, B, necrotic plaques between the maxillary tomial and in the nostril (arrows). C, lesion associated with the mandible (arrow).

Histopathology

Systemic histological changes were noted in all of the individuals that developed severe clinical signs; however, no clear dose related trends were observed (**Table 4.2**). Inflammation (determined by infiltration of immune cells) was most prominent in the skin (14/14) (associated with skin lesions), oral cavity (14/14), lungs (14/14) and liver (14/14) (**Figure 4.3**). Inflammation was also noted in the GIT (9/15) and kidneys (7/13) of about half of the individuals. Necrosis was observed in the skin and smooth muscle (15/15) (associated with skin lesions), spleen (11/13), liver (10/14) and oral cavity (7/14). A few individuals were observed with necrosis in the kidneys (3/13) and GIT (2/15). Necrosis was observed in three incidentally sampled gonads from infected individuals (**Figure 4.4**). However, no gonad tissue was obtained from any of the control animals.

Table 4.2 Histological changes in turtle hatchlings that developed severe clinical signs after intramuscular inoculation with BIV. Gastrointestinal tract (GIT), epidermal necrosis (EN), dermal haemorrhaging (DH), dermal inflammation (DI), muscular necrosis (MN), necrosis of oral cavity (ON), glossitis (G), stomatitis (S), necrosis of the gastrointestinal mucosa (GN), gastrointestinal inflammation (GI), necrosis of pulmonary tissue (LN), pneumonitis (LI), hepatic necrosis (HN), hepatitis (HI), splenic necrosis (SN), necrosis of the renal tubules (KN), nephritis (KI). Histological changes are marked by 'y' if they were observed. Not recorded (NR).

		Skin			**Muscle**	**Oral cavity**			**GIT**		**Lung**		**Liver**		**Spleen**	**Kidney**	
Dose ($TCID_{50}$)	**ID**	**EN**	**DH**	**DI**	**MN**	**ON**	**G**	**S**	**GN**	**GI**	**LN**	**LI**	**HN**	**HI**	**SN**	**KN**	**KI**
	A13	y	y	y	y		y	y		y	NR	NR	y	y	y		
	B9	y	y	y	y		y	y		y	y	y	y	y	y	y	y
	F8	y		y	y		y	y	y	y		y	y	y	y	y	y
$10^{5.33}$	I10	NR		NR	y	y	y	y				y		y	y		
	K2	y		y	y	NR	NR	NR		y		Y	y	y	y		
	I3	y	y	y	y		y	y		y		y	y	y	NR	NR	NR
	J2	y	y	y	y	y	y	y			y	y	y	y	y	NR	y
	H1	y	NR	y	y	y	y	y				y	y	y	y		
	A5	y	y	y	y		NR	y	y	y	y	y	y	y	y	y	y
$10^{4.33}$	B5	y	y	y	y		y	y		y	y	y	y	y	y		y
	D14	y		y	y			y				y		y			
	L11	y	y	y	y	y	y	y		y		y		y	y		y
	A1	y	y	y	y	y		y		y		y	NR	NR	y		NR
$10^{3.33}$	B11	y	y	y	y	y	y	y			y	y	y	y	NR		y
	I2	y	y	y	y	y		y				y		y			
Overall	**(%)**	**100**	**71**	**100**	**100**	**50**	**77**	**100**	**13**	**60**	**36**	**100**	**71**	**100**	**85**	**23**	**54**

Systemic inflammation was observed in the histological sections of individuals that developed less severe clinical signs and survived to the end of the trial. Inflammation of the oral cavity, GIT, lungs and liver was observed, however, necrosis of these organs occurred in only a few individuals.

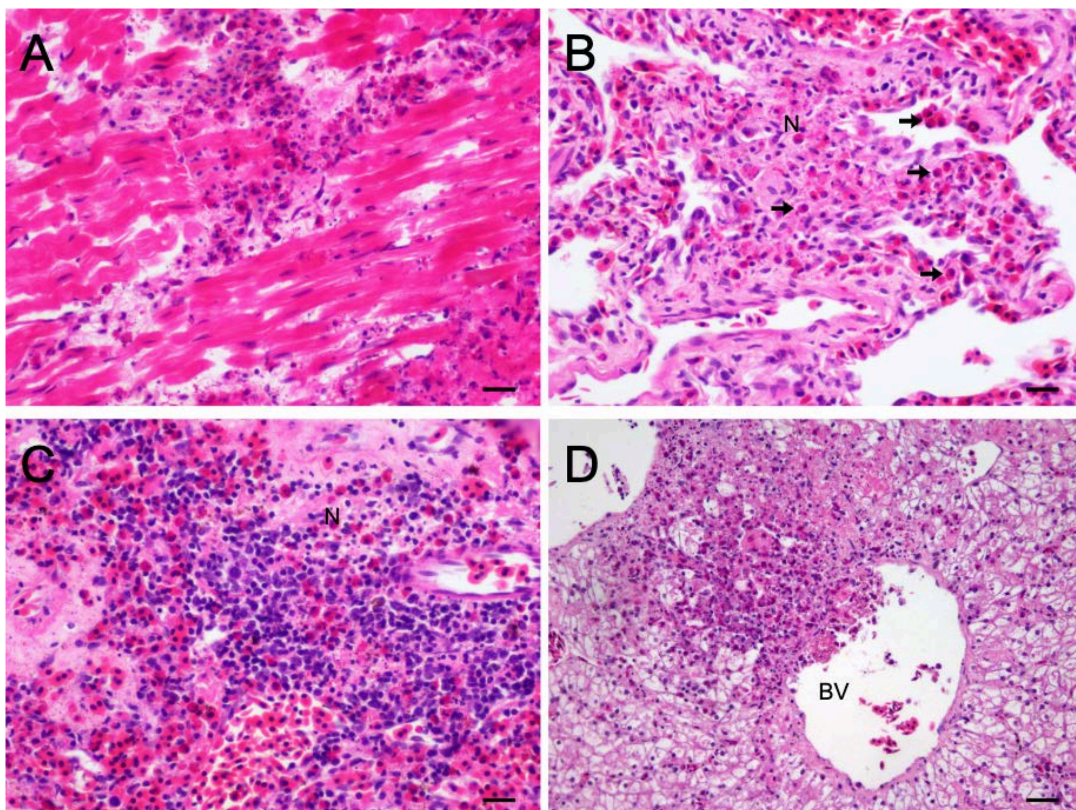

Figure 4.3 Example histological lesions in turtle hatchlings following IM inoculation with BIV. A**,** haemorrhagic necrosis of the smooth muscle near the site of injection. B, pulmonary necrosis (N) with infiltration of eosinophils (arrows). C, hematopoietic necrosis (N) in the spleen. D, large necrotic area adjacent to a blood vessel (BV) in the liver (scale bar 40 µm). The scale bar is 20 µm unless otherwise stated.

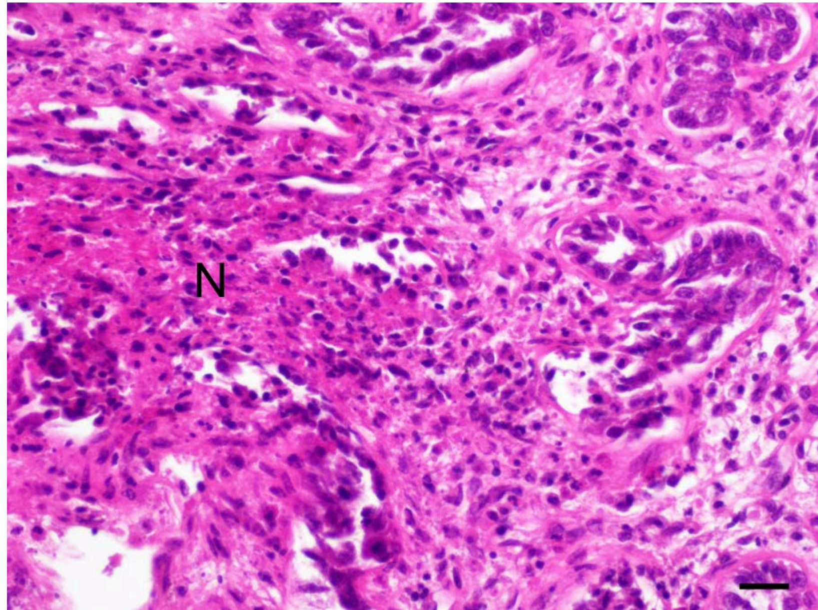

Figure 4.4 Extensive necrosis (N) in the testis of a Krefft's river turtle hatchling inoculated intramuscularly with $1 \times 10^{5.33}$ $TCID_{50}$ BIV. The scale Bar is 20µm.

There was some evidence of gastrointestinal inflammation and infiltration of lymphocytes into the oral cavity amongst individuals that survived to the end of the trial without developing clinical signs. No other histological changes were observed in this group. The livers of the three individuals with subclinical infections (ranaviral DNA in liver at the end of the trial but no clinical signs) had no obvious histological changes.

Discussion

Krefft's river turtle hatchlings are susceptible to low doses and others are capable of surviving high doses of BIV, while remaining PCR reactive for weeks. The LD_{50} of BIV was $10^{4.43}$ $TCID_{50}$ mL^{-1} (95% CI: $10^{3.81} - 10^{5.19}$) for hatchlings inoculated via the intramuscular route. Among the

groups of turtles exposed to doses of $10^{2.33}$ and $10^{3.33}$ $TCID_{50}$ mL^{-1} there was a large increase in mortalities (0-30%) and morbidity (20-80%). This range encompasses the ID_{50}, i.e. the dose at which 50% of individuals react in the PCR assay or develop clinical signs. The ID_{50} was calculated as $10^{2.52}$ $TCID_{50}$ mL^{-1} (95% CI: $10^{1.98} – 10^{2.93}$) and is about one hundred times less than the LD_{50} ($10^{4.43}$). The ID_{50} is a useful metric for turtles as they have been identified as possible carriers of ranaviral disease. Asymptomatic yet infected individuals, may transmit the virus to more susceptible amphibian species sharing their environment (Brenes et al., 2014). However, the rate of viral shedding was not determined in this study, although it is quantified in the experimental infection presented in Chapter 6 of this thesis.

The lower limit of infection (the dose that produces no clinical signs or PCR positive animals) and upper limit of disease (the dose that causes severe clinical signs in all animals) were both outside the range tested in this study. It is possible that given enough time more turtles would have gone on to develop severe clinical signs as we saw turtles continuing to develop disease until the end of the study (e.g. day 31). There was a large amount of variation within the individuals in this study; some developed ranaviral disease in the lowest dose group (2/10, $10^{1.33}$ $TCID_{50}$ mL^{-1}) while an individual showed no clinical signs in the highest dose group (1/10, $10^{5.33}$ $TCID_{50}$ mL^{-1}). We know ranaviruses (based on detection of antibodies to ranaviruses) are present in the environment where these animals are found (Ariel et al., 2017a). Therefore, it is possible that this variability is the result of selective pressure from wild ranaviral infections, i.e. we are seeing the results of evolutionary responses to the pathogen. Although we know the maternal line of the hatchlings used in this study (oviposition was induced), the study was not designed to determine heritability

of resistance. Future studies using detailed host genetics may be able to explain the variability observed here.

Based on the results of Chapter 3, the intramuscular inoculation route was selected for this chapter as the inoculum could be easily administered, the route could effectively produce clinical signs, and it has been used in previous ranaviral challenge studies in turtles (Johnson, Pessier & Jacobson, 2007; Allender et al., 2013b, 2018). There is some evidence that vector transmission may occur in turtle populations and thus intramuscular inoculation could represent a natural infection pathway (Kimble et al., 2014). In addition, the sporadic nature of ranaviral epizootics and the low density and solitary lifestyles of many turtles suggests that indirect routes of transmission may be more likely (Brunner et al., 2015).

The majority of clinical signs and gross pathological changes in this study (inappetence, oedema, oral lesions, multifocal liver discolouration, haemorrhages) were previously reported for ranaviral infection in turtles (Wirth et al., 2018). Basking, a possible form of behavioural fever, was observed more frequently in higher dose groups, however, the thermal potential was not assessed, and turtles may have just been escaping the water and not thermoregulating (Rakus, Ronsmans & Vanderplasschen, 2017). Skin lesions reported in our study have not been reported in other turtle challenge studies, however, external skin lesions are often reported in lizards with ranaviral infections (Wirth et al., 2018) and ulceration has been reported in a ranaviral infection of eastern box turtles (De Voe et al., 2004). Skin lesions were found only in the intramuscularly inoculated individuals and were associated with the injection site. Skin lesions were not observed when twelve

hatchlings of the same species were inoculated via intracelomic with BIV (Ariel et al., 2015), therefore, these skin lesions are likely inoculation route dependent.

Systemic inflammation, consistent with ranaviral infection in other turtle species, was observed in all the turtles that developed severe clinical signs. Necrosis occurred in several organs, most commonly in the skin and muscle (at the injection site), the spleen and liver. Although not originally a target of the histological analysis, three infected hatchlings had gonad tissue associated with their kidney samples (this is not unexpected due to the anatomic proximity of these tissues). When examined, some necrosis was observed in the gonads (2 ovaries and 1 testis) of these individuals. Sexual transmission has not previously been implicated in ranaviral infection in reptiles and should be investigated in future studies.

The results presented here were comparable to the timing of disease in Krefft's river turtle hatchlings inoculated with BIV (1×10^{3} $TCID_{50}$ mL^{-1}) via the intracoelomic route (Ariel et al., 2015). The IC inoculated hatchlings died between 16-29 (ST_{50} 24) days post inoculation, and mortalities of hatchlings in our study, with a comparable dose ($1\times10^{3.33}$ $TCID_{50}$ mL^{-1}), occurred between 22-24 (ST_{50} 24) days post inoculation.

The median survival time for four juvenile cryptodiran turtle species inoculated via the intramuscular route with FV3 (5×10^{5} $TCID_{50}$) was 8 (at 22 ˚C) and 11 (at 27 ˚C) days (Allender et al., 2018). The median survival time for Krefft's river turtles from our study at a comparable dose ($1\times10^{5.33}$ $TCID_{50}$ mL^{-1}) and temperature (28 ˚C) was 7 days longer (ST_{50} 18 days). This suggests that cryptodiran species are more susceptible to ranaviral infection (succumb quicker) than Krefft's

river turtle hatchlings. The LD_{50} in these cryptodiran species is likely to be lower based on this comparison. Due to the longer disease time frame in Krefft's river turtles they may be more effective spreaders than other species with shorter durations of infection, e.g. red-eared sliders. It is also possible that these differences in susceptibility may represent differences in ranaviral isolates as well as host. Although studies have not compared the virulence of FV3 and BIV in reptiles, there is some evidence that FV3 is more pathogenic than BIV in amphibians (Bayley, Hill & Feist, 2013).

The turtles in the study present in this chapter took longer to develop disease than adult wood frogs (*Rana sylvatica*) in a similar dose experiment with orally inoculated FV3 (Forzán et al., 2015). Both initial clinical signs and mortalities took 8 days longer on average in Krefft's river turtles when the results from all doses in this study were combined and compared with wood frogs. The interval between the onset of clinical signs and death was also longer in turtles (2-10 days) compared with the wood frogs (0-4.5 days). Similarly, IM inoculated eastern water dragons (*Intellagama lesueurii*) succumbed to infection quicker than the hatchlings in this study (Maclaine et al., 2018). Eastern water dragons developed clinical signs between 1−5 days and mortalities occurred 5−8 days post inoculation with $10^{5.33}$ $TCID_{50}$ mL^{-1} BIV, whereas the turtles developed clinical signs between 14−30 days and mortalities occurred 16−24 days post inoculation with the same dose.

The PCR assay detected ranaviral DNA in three animals that did not go on to develop clinical signs. These sub-clinically infected animals were from the three highest dose groups (one in each group). It is possible that they were still infectious (i.e. shedding virus) despite not developing

signs and could have acted as carriers, however, viral isolation would have been required to confirm this hypothesis. Other ranaviral infected aquatic turtles are capable of infecting amphibians through water (by virion shedding), while remaining sub-clinically infected themselves (Brenes et al., 2014). Although viral shedding was not examined in this study, infected turtle hatchlings could still spread the infection to susceptible predacious fish, e.g. barramundi, if consumed (Moody & Owens, 1994).

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 and poster. 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 and completed daily husbandry for the turtle hatchlings prior to and during the study;
- I developed the web-based husbandry tracker that was used to keep records of the health, diet, and morphometrics of the animals in this study;
- I performed the 10-fold dilution of viral inoculum;
- I inoculated the turtle hatchlings used in this study;
- I conducted the necropsies of turtle hatchlings and undertook sample collection for, PCR and histology;

- I prepared and analysed the histological slides;
- I extracted the DNA from samples for analysis by PCR;
- I performed the PCR on the samples;
- The results of this chapter were combined with the results of Chapter 3 and accepted for publication in a peer-reviewed journal, I was the lead author on this publication and drafted the manuscript; and
- I prepared the presentation that I presented on this work 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 (found in the appendix of this thesis) and conference presentation:

- Appendix 3 - **Wirth, W**., Schwarzkopf, L., Skerratt, L. F., Tzamouzaki, A., & Ariel, E. (2019). Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with *Ranavirus* isolate (Bohle iridovirus, *Iridoviridae*). Journal of General Virology, jgv001324.
- **Wirth, W.** & Ariel, E. (2017). Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with Bohle iridovirus (Ranavirus sp, Iridoviridae). The 4th International Symposium on Ranaviruses. Budapest, Hungary.

Conclusion

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

1. Determine influence of viral dose on ranaviral disease development in Krefft's river turtle hatchlings including:
    a. The effect of viral dose on the incubation period
    b. The effect of viral dose on clinical presentations
    c. The effect of viral dose on histopathology

Incubation period ranged from 14 to 29 days post-inoculation and was negatively correlated with viral dose. For every 10-fold increase in dose, the odds of developing any clinical signs or severe clinical signs increased by 3.39 [$P<0.01$, 95 % confidence interval (CI): 1.81–6.36] and 3.71 ($P<0.01$, 95 % CI: 1.76–7.80), respectively. Systemic histological changes were noted in all of the individuals that developed severe clinical signs; however, no clear dose-related trends were observed.

2. Determine the viral dose for the mid-point of ranaviral infection and disease i.e.
    a. The dose that causes server clinical signs in 50% of animals (LD_{50})
    b. The dose at which 50% of animals become infected i.e. the 50% infectious dose (ID_{50})

The median lethal and infectious doses of BIV for intramuscularly inoculated hatchlings were $10^{2.52\ (1.98–2.93)}$ and $10^{4.43\ (3.81–5.19)}$ $TCID_{50}$ ml^{-1}, respectively.

In this chapter, I presented the first study of the influence of viral dose on ranaviral infection in turtles. The midpoint values, ID_{50} and $LD_{50,}$ determined in the study are extremely useful for studying ranaviral disease. Now that we know the dose that will cause 50% of animals to become infected or develop severe clinical signs, we can use these values as a baseline for future studies to measure the influence of other factors on infection and disease.

The lower limit of infection (the dose that produces no infections) and upper limit of disease (the dose that causes severe clinical signs in all animals) were both outside the range tested in this study. The fact that these values were outside the range tested is important to the central question of this thesis i.e. why don't we see ranaviral epizootics in Australian freshwater turtle populations? While it appears that Krefft’s river turtles are less susceptible to ranaviral infection when compared to some cryptodiran species, some Krefft’s river turtles (20% in this study) still developed clinical signs of ranaviral disease even at the lowest doses of viral inoculum. There could be some factors that reduce resistance and allow infections to spread even at these low doses.

Environmental temperature is an important factor to consider in the diseases of ectotherms, as it can influence their immune response. Basking, a possible thermoregulatory behaviour, was observed more frequently in higher dose groups of this study, though I didn’t examine the thermal differences of these basking animals. In the following chapter (Chapter 5) I examine how

environmental temperature can influence ranaviral infection rates in Krefft's river turtle hatchlings.

Chapter 5

THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON RANAVIRAL INFECTION IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

Aims

1. Determine the influence of environmental temperature on ranaviral infection rates and viral loads in freshwater turtle hatchlings.
2. Estimate the temperature of maximum ranaviral infection rate.
3. Make recommendations for the use of temperature therapy to limit ranaviral infection in Australian freshwater turtle hatchlings.

Introduction

Reptile physiology is strongly influenced by the temperature of their surrounding environment, by extension, so is the pathogenesis of ranaviral infections. The environmental temperature-dependent physiology includes many aspects of the ectothermic immune system (both adaptive and innate), many immune components have optimal temperatures beyond which the efficacy diminishes (Zimmerman, Vogel & Bowden, 2010). Understanding the effect of temperature on infection has important ramifications for understanding wild disease dynamics. Knowing the range of temperatures that infection can occur at and the temperature at which infection is maximised, it

may be possible to identify at-risk populations or predict temperature driven epizootics. As the climate continues to change it is important to understand how variation in temperature may affect aspects of ranaviral disease. In this chapter, utilising the results of the previous chapter, I determine how environmental temperature influences the infection rate of hatchlings inoculated with an ID_{50} dose of Bohle iridovirus.

Methods

All experiments were carried out under a James Cook University (JCU) Animal Ethics permit (A2344) and a Department of Environment and Science Scientific Research Permit (WISP13270413).

Animals

Hatchlings used in this study were obtained following the methods described in Chapter 2. Briefly, wild gravid turtles were induced to lay eggs with an injection of 10 IU kg^{-1} of syntocinon (synthetic oxytocin 10 IU mL^{-1}, Troy laboratories). After approximately 50 days of incubation at 29 °C the eggs hatched. Newly emerged hatchlings were kept at the JCU Turtle Health Research Facility, where they were housed in tanks with water between 24 and 28 °C. Access to dry areas for basking were provided. The hatchlings were fed commercial turtle pellets (Exo Terra, aquatic turtle food) *ad libitum* for 10-15 minutes per day. During this husbandry phase (four months) and during the experiment (three weeks) feeding observations and morphometric data were collected from each individual. The average weight of hatchlings used in this study was 9.5g (**Table 5.1**).

Source of virus

The Bohle iridovirus (BIV) isolate (FV3 isolate) used in this study was originally isolated in Townsville, Australia and was produced according to the methods of Maclaine et al. (2018). Briefly, the viral isolate was propagated at 25°C in fathead minnow cells grown in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific), supplemented with 100× antibiotic-antimycotic (Thermo Fisher Scientific) and 10% fetal bovine serum (Bovogen Biologicals). Viral titre ($TCID_{50}$) was determined using the methods described by Reed & Muench (1938). The viral stock was stored at -80 °C until the time of challenge study and then diluted in DMEM to $10^{2.5}$ $TCID_{50}$ mL $^{-1}$.

Experimental design

One week prior to the start of the experiment animals were randomly assigned to control and infection groups (**Table 5.1**) and moved into the infection room. The hatchlings were kept in individual 500mL plastic containers on a slant to create a dry area for basking. The containers were placed, in groups of three or four, in temperature chambers set to 16, 22, 28, or 34 °C with a 12-hour light cycle (Greenspan et al., 2016). The hatchlings were given one week to acclimatise to the temperature chambers before the start of the experiment. Husbandry continued as previously described. During the acclimatisation period and throughout the experiment hatchlings were monitored for clinical signs including: inappetance, skin lesions, increased basking behaviour, and oedema of the neck and legs as described by Wirth et al. (2019) (Chapter 3 and Chapter 4) for ranaviral infection in Krefft's river turtle hatchlings. The dose rate and time to euthanasia in this experiment was based on findings in Wirth et al. (2019) (Chapter 4) to allow for sufficient time for the infection to get established and potentially be influenced by temperature above and below

the temperature used in that study, before the samples were collected. On day zero of the experiment all hatchlings in the exposed groups received an intramuscular injection of $10^{2.5}$ $TCID_{50}$ mL^{-1} BIV stock into the hind leg. Injection volume was determined by multiplying the individual's weight (g) by 2.5 (mean 23.8 µL; range 20.25-30.5 µL). Control animals received an equivalent dose of virus free DMEM as a placebo.

Table 5.1 Experiment design and animal numbers at the start of the experimental infection. During the study one animal for the both the control and exposed 16 ˚C temperature groups were removed (*) the weights of these animals are not included in the mean starting weight.

Group	Temperature (˚C)	Number of hatchlings	Mean Starting Weight (g)
Control	16	2*	10.8
	22	3	9.4
	28	3	9.7
	34	3	9.3
Exposed	16	9*	9.4
	22	10	9.5
	28	10	9.4
	34	10	9.5

Endpoint and sample collection

On the day of euthanasia final morphometric data was collected. Hatchlings were randomly selected for euthanasia 21 days post inoculation and all hatchlings were euthanased over the following 3 days. Hatchlings were euthanased with a MS-222 overdose according to the methods described by Conroy et al. (2009). A necropsy was performed on each animal using a different set

of sterile instruments (disinfected with Virkon and washed with detergent). Gloves were changed between animals and surfaces were decontaminated with bleach (4% by volume). A section of liver was collected for qPCR analysis and frozen at -80 °C.

Real-time quantitative PCR (qPCR)

DNA was extracted from the thawed liver samples using an ISOLATE II Genomic DNA Kit (Bioline) following the manufacturer's protocol for DNA extraction from tissues. Viral loads were determined using the Ranavirus major capsid protein gene (MCP) gene assay described by Leung et al. (2017). A single copy conserved vertebrate gene, EBF3N, was used to standardise the number of viral copies. A section of the BIV MCP gene and EBF3N gene were cloned into pGEM-easy vectors following the manufacturer's protocol (Promega). Plasmids were linearised using a PstI restriction digest (New England Biolabs) and quantified using a QuantiFluor dye system (QuantiFluor, Promega). Absolute standard curves were constructed in triplicate from 10^8-10^1 copies μL^{-1} and 10^7-10^1 copies μL^{-1} for the MCP and EBF3N plasmids respectively. The qPCR was performed on a MIC real-time thermocycler (Applied Biosystems) with the conditions described by Leung et al. (2017). The assays were run as a duplex with the MCP probe on the green channel (FAM probe) and ENF3N on the red channel (Cy5 probe). It was assumed that there is one EBF3N gene per *E. m. krefftii* genome. Viral loads were determined by dividing the number of MCP copies μL^{-1} multiplied by two by the number of EBF3N copies μL^{-1} and are referred to as copies per cell throughout this manuscript.

Statistics

All analysis was performed using the Statsmodels module (0.10.2) and Pandas (0.24.3) and SciPy (1.4.1) libraries in Python 3.6 (McKinney, 2010; Seabold & Perktold, 2010). A 2 x 4 chi-squared contingency table was used to compare proportions of infected animals in the temperature groups. The Kruskal-Wallis H-test for independent samples was used to compare variation in viral loads of infected turtles between temperature groups. Conover's test of multiple comparisons was used as post hoc test following a significant Kruskal-Wallis H-test. A normal distribution was fit to the portion of infected animals from each temperature group (using mean temperature, **Table 5.2**) to estimate the temperature of maximal infection rate and its standard deviation for the temperature range tested.

Results

Temperatures

The incubators used in this study were effective at maintaining the average environmental temperature within a degree of the target temperature (**Table 5.2**).

Table 5.2 Descriptive statistics for temperature logger data from the incubators used to house the animals in this study. Q1 and Q3 represent the lower and upper quartiles respectively. All data are expressed as degrees Celsius (˚C).

Group	Mean	Std	Min	Q1	Median	Q3	Max
16	16.24	0.72	15.9	16	16.1	16.3	23.3
22	22.63	1.13	21.6	22	22.3	22.5	27.6
28	27.79	1.12	22.9	27.8	28.1	28.4	29.1
34	34.12	0.35	32.1	33.8	34.1	34.4	35

Deaths

During the study two animals in the 16 °C group were found dead in their enclosures on 3 and 8 days post-inoculation. One animal was from the control group and one from the infection group. Following a necropsy, it was determined that both animals had drowned and not died from a ranaviral infection (they were qPCR negative). The animals were removed from the study and not included in any analysis. No other animals died or developed clinical signs severe enough to warrant euthanasia.

Clinical signs

Only two animals developed clinical signs in the timeframe of this experiment. One animal in the 16°C group developed ocular and nasal lesions 17 days after exposure. One animal in the 22°C group developed an oral lesion 14 days after exposure. No animals developed skin lesions at the site of inoculation as described by Wirth et al. (2019) (Chapter 4). Diet consumption appeared to be strongly linked to environmental temperature and there was no clear evidence of inappetence

caused by ranaviral infection (control and infected groups were affected similarly across all temperatures). Both exposed and control hatchlings in the 16 °C group consistently ate little to none of their food and appeared lethargic. The animals in the 34 °C group consistently ate most of the food offered and were active throughout the experiment.

qPCR

Of the 39 (one was removed) infected turtles, 20 of them reacted in the qPCR assay for ranaviral DNA in the liver 21 days after exposure. However, the distribution of reactors was not even (**Figure 5.1**). Significantly more turtles (9/10 liver samples reacted in the qPCR assay) were infected in the 22 °C group ($\chi^2 = 13.3$; p-value < 0.01). The lowest infection rate was in the turtles kept at 34 °C, where only 1/10 liver samples reacted in the qPCR assay (10%). The mean and standard deviation of the normal distribution fit to the infection rates was 23.2 °C and 4.5 °C respectively.

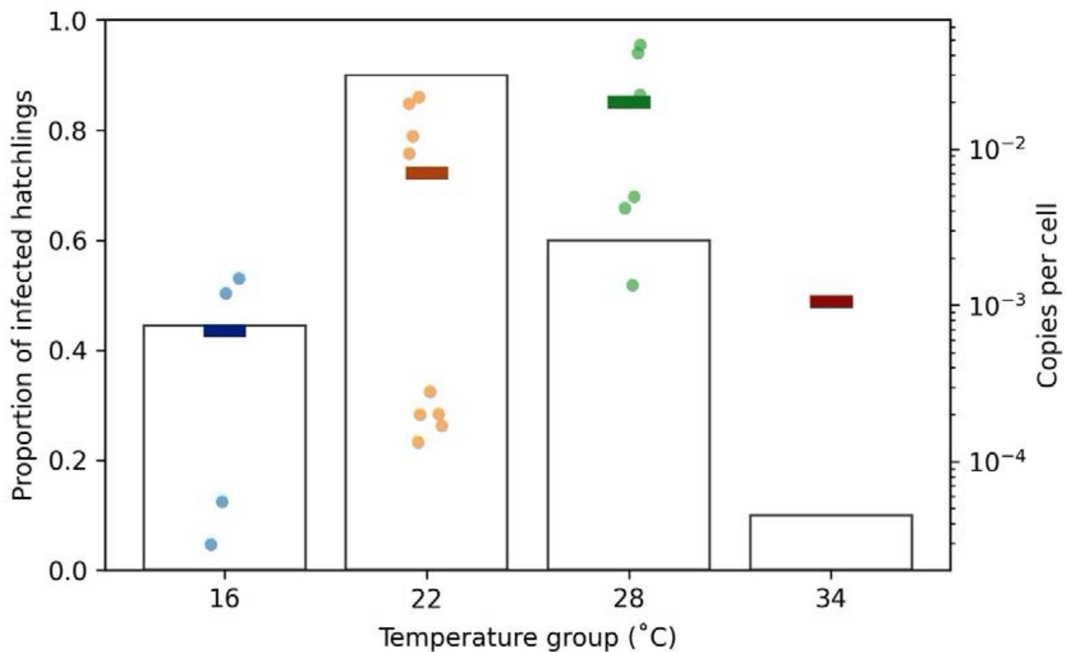

Figure 5.1 Ranaviral infection rate and intensity 21 days post exposure for Krefft's river turtle hatchlings held at different temperatures. The boxes are the proportion of animals from each group that reacted in the qPCR assay for ranaviral DNA in their liver. The dots are the copies per cell (log scale) of ranaviral DNA in each infected animal's liver. The solid line is the mean copies per cell for all infected animals in that temperature group. All groups had a total of ten exposed animals except the 16℃ group that had nine.

There was large variation in ranaviral MCP copy numbers within the different temperature groups (**Figure 5.1**). As there was only one reactor in the 34 °C temperature group it was removed from the statistical analysis of viral loads. The Kruskal-Wallis test found a significant difference (0.05 alpha) between ranaviral MCP copy number between the 16, 22, and 28 °C temperature groups (H statistic = 6.57; p-value = 0.037). Post hoc analysis with the Conover's test of multiple comparisons revealed that this difference was between the 16 and 28 °C temperature groups (p-value = 0.027).

Discussion

Based on the distribution of infection rates and temperature logger data, the predicted temperature for maximal ranaviral infection (i.e. the maximum number of infected animals after 21 days) in Krefft's river turtle hatchlings is 23.2 °C. Assuming that uninfected turtles would not later develop a detectable infection (i.e. turtles that did not react in the qPCR assay have cleared the infection), we can say that temperature has a significant effect on the rate and length of infection. This will have impacts on the transmission of the virus. The more animals that are infected and the longer an animal is infected for, the greater the chance of viral transmission.

Clinical signs observed in this study were similar to those that were reported for ranaviral infection in other turtles, although not as extensive as previous reports (Wirth et al., 2019). Given more time these animals may have either recovered or developed more clinical signs, however, terminating the experiment at 21 days allowed us to prevent unnecessary suffering of the animals (they did not have time to develop clinical signs) while still being able to study the effect of temperature on infection.

There was a large variation in the viral loads observed within temperature groups. Interestingly, the copies per cell of ranaviral DNA within each temperature group appears to divide equally into 'high' and 'low' reactors with at least a 10-fold difference between the mean high and low copy numbers. Such a difference in individuals that received the same treatment may be explained by some dichotomous biological trait, such as those associated with sex, that increases or decreases viral replication. Unfortunately, the sex of these hatchlings cannot be easily identified via physical

exam and was not recorded at necropsy. Krefft's river turtles do not have temperature dependent sex determination and so the sex distributions within the temperature groups is assumed to be 50/50 which would explain the equal numbers of high and low viral loads in the temperature groups if this is the result of the sex of the animals. Future work should investigate the possible effects of sex (or other dichotomous variables) on ranaviral infection in turtles.

Post hoc analysis revealed that there was a significant difference in viral loads of infected animals only between the 16 °C and 28 °C groups (p-value = 0.027). At lower temperatures viral replication is likely reduced thus resulting in reduced viral load. Adult red ear sliders (*Trachemys scripta elegans*, suborder Cryptodira) inoculated with a ranavirus and held at either 22 °C or 28 °C had significantly reduced ranaviral loads and halved mortality in the 28 °C group (Allender et al., 2013b). Similarly, we found that the infection rate was higher in the 22 °C group compared to the 28 °C group. However, we observed no significant difference in the viral loads between the 22 and 28 °C groups.

This pattern of temperature dependent mortality did not hold for a study on four cryptodiran species of juvenile turtles (including red eared sliders). These turtles did not have reduced mortality in the higher temperature group, both groups (22 °C and 27 °C) had 100 % mortality, however, median survival time was reduced in the 27 °C group for two (red eared sliders and Mississippi map turtles) of the four species (Allender et al., 2018). Similar patterns of reduced time until death but lower mortality rates with increasing temperature have been seen with other environmental temperature-dependent host-pathogen systems such as amphibians with chytridiomycosis (Berger et al., 2004). Krefft's river turtles exhibit age dependent ranaviral pathogenesis, with hatchlings

being more susceptible than adult turtles (Ariel et al., 2015). The same may be true for red eared sliders; the dose given to the juvenile turtles may have overwhelmed the turtles and thus negated the effect of temperature, resulting in 100% mortality in both temperature groups (Allender et al., 2013b, 2018). Because adult red eared sliders they are less susceptible, the effect of temperature on viral replication/the immune system may still be prominent, thus reducing mortality in the higher temperature group (Ariel et al., 2015; Allender et al., 2018; Wirth et al., 2018).

In our study we chose to use a median infectious dose (ID_{50}), as determined by Wirth et al. (2019) (Chapter 4) for Krefft's river turtle hatchlings at 28 °C. As expected, the infection rate in the 28 °C group in this study was close to 50 % (60 % in this study). Using a median dose allows for easy detection of change in the dependent variable (infection, death, etc.) as a result of changing different factors (e.g. environmental temperature). We chose the ID_{50} over the median lethal dose (LD_{50}; $10^{4.43}$ $TCID_{50}$ mL^{-1}) for this study for welfare reasons. The results of an infectious dose study are still valuable, although more applicable to a disease transmission than pathogenesis. We are not sure how these temperatures will affect Krefft's river turtles exposed to a higher dose (such as an LD_{50}). It may be that when Krefft's river turtle hatchlings are exposed to higher doses the amount of virus will overwhelm any prophylactic effects of temperature, as seen with red eared sliders. In any case, we still do not understand the natural transmissions dynamics and so it is difficult to determine what dose would be useful to accurately reflect wild disease.

Temperature therapy at greater than 34 °C may also be useful for treating acute ranaviral disease, although further study will be required to determine the effects of temperature on pathogenesis. While 34 °C appears effective at reducing infection rates, BIV itself is not inactivated at 34 °C (La

Fauce et al., 2012) and thus once an animal returns to cooler temperature the infection may re-establish if the virus is not completely cleared. To reduce the chances of ranaviral infection in captive animals we would recommend that turtles are provided with a basking area of at least 34 °C, thus allowing the animals to perform behavioural fever at temperatures high enough to reduce infection. Lower temperatures (e.g. 16 °C or less) may also be effective at reducing infection rates, however, these temperatures are not optimal for turtle health and result in reduced appetite and activity.

The optimal temperature for ranaviral isolate propagation in a range of cell lines is 24 °C (10, 15, 20, 24, and 28 °C tested; Ariel et al., 2009). The average annual temperature in Townsville (where this study was conducted) is 24.1 °C. Both the optimal temperature for ranaviral propagation and the average annual temperature in Townsville are close to the temperature of maximum infection rate (23.2 °C) estimated in this study. Australian freshwater turtles (like Krefft's river turtles) are semi-aquatic and spend the majority of their time in the water. When in the water, the turtles' body temperatures are in thermal equilibrium with water temperature (Manning & Grigg, 1997). Ranaviral infection rates would be expected to be highest in months when water temperature is around 23 °C i.e. May-June and September-October. However, the temperature most effective at reducing infection rate used in this study (34 °C) is not uncommon in Northern Australia where these turtles are native. Thus, ranaviral infected Krefft's river turtles could have the opportunity to use behavioural fever to reduce infection rates, although finer scale studies of thermal and basking site availability are required to quantify this opportunity.

In this chapter I used reaction in a qPCR assay of the liver as a proxy for infection. While the liver is a major target of ranaviral replication, all conclusions reported in this chapter should be interpreted in light of the fact that reaction in a qPCR assay does not equal infection.

Publication and outputs

This chapter includes results from a peer-reviewed paper. I was the first author of this 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 and completed daily husbandry for the turtle hatchlings prior to and during the study;
- I developed the web-based husbandry tracker that was used to keep records of the health, diet, and morphometrics of the animals in this study;
- I inoculated the turtle hatchlings used in this study;
- I conducted the necropsies of turtle hatchlings and undertook sample collection for qPCR;
- I extracted the DNA from samples for analysis by qPCR;
- I performed the qPCR on the samples;
- I performed the statistical analysis presented in this chapter; and
- The results of this chapter were accepted for publication in a peer-reviewed journal, I was the lead author on this publication and drafted the manuscript.

Results from this study are included in the following peer-reviewed publication (found in the appendix of this thesis):

- Appendix 4 - **Wirth, W**. & Ariel, E. (2020). Temperature-dependent infection of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with a ranavirus isolate (Bohle iridovirus, *Iridoviridae*). FACETS.

Conclusions

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

1. Determine the influence of environmental temperature on ranaviral infection rates and viral loads in freshwater turtle hatchlings.

The rate of infection differed across the temperature treatment groups. Infection rates were 44 %, 90 %, 60 %, and 10 % for the 16 °C, 22 °C, 28 °C, and 34 °C temperature groups respectively. Highest viral load was observed in the 28 °C temperature group and there was a statistically significant difference in viral load between the 16 °C and 28 °C temperature groups (p-value = 0.027).

2. Estimate the temperature of maximum ranaviral infection rate.

The temperature of maximum infection rate for ranaviral infection in Krefft's river turtle hatchlings is estimated to be 23.2 °C (SD = 4.5).

3. Make recommendations for the use of temperature therapy to treat ranaviral infection in Australian freshwater turtle hatchlings.

The animals in the 34 °C group had the lowest infection rate out of those tested. I would, therefore, recommend an environmental temperature of 34˚C for treating acute ranaviral infection in Krefft's river turtle hatchlings.

Environmental temperature is integral to ranaviral disease, a disease of ectotherms. The results of this chapter have shown that Krefft's river turtles (suborder Pleurodira) exhibit temperature dependent infection. Using the results of this study I have provided recommendations for temperature therapy of acute ranaviral infection, optimal temperature for behavioural fever in captive turtle enclosures, and estimates of times of the year when infections rates are expected to be high in natural settings. Understanding how temperature influences infectious diseases, like ranaviruses, is important in our changing climate. Results from studies like this one can provide data to climate-disease models and help form a foundation for understanding impact on reptile populations.

We need methods to identify ranaviral disease in Australian freshwater turtles to know when to apply the temperature therapy treatment described in the chapter. Other diseases may have different temperature responses and could be exacerbated by the temperature recommendations

provided here. So far in this thesis ranaviral infectious status has been determined based on PCR of the liver. While the liver is a common sample type for ranaviral assays, it is not very practical for determining ranaviral infection status in living animals. Because of the varied and non-specific clinical signs of ranaviral infection, diagnosis cannot be made from clinical presentation alone. Reliable antemortem sampling methods are required so that ranaviral disease can be identified as early as possible.

From the work present in this and the previous two chapters, we now have an understanding of how inoculation route, viral dose, and temperature influence ranaviral infection in Krefft's river turtle hatchlings. All these inferences have been made at a single time point, late in infection, however, ranaviral disease is not a static process. We have a description of how the clinical signs develop during infection, but we don't know when the underlying histopathological changes occur. To give some depth to our understanding of ranaviral disease in Australian freshwater turtles, we need to determine how this disease develops over time. In the next chapter I determine the influence of infection duration on ranaviral disease in Krefft's river turtle hatchlings, thus allowing us to determine the pathogenesis and optimal sampling methods for ranaviral infection.

Chapter 6

THE INFLUENCE OF INFECTION DURATION ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

Aims

1. Describe the progression of histopathological changes of ranaviral infection in Krefft's river turtle hatchlings
2. Determine the tissue tropisms of ranaviruses in Krefft's river turtle hatchlings
3. Compare the effectiveness of different methods for detecting ranaviral infection in Krefft's river turtle hatchlings

Introduction

Finding regarding route, viral dose, and temperature in respect to ranaviral infection of Krefft's river turtle hatchlings were described in Chapter 3, Chapter 4, and Chapter 5, however, we still don't have a detailed understanding of ranaviral disease development in Australian freshwater turtles. All published studies of ranaviral pathology in turtles (including those presented in the previous three chapters) have looked at a single timepoint of infection i.e. it is not known how length of ranaviral infection influences the underlying histopathological changes. Understanding the time-dependent pathogenesis of ranaviral infection in turtles will help us to develop better

conceptual models of ranaviral infection and the disease process, thus allowing us to make recommendations about diagnostic protocols (e.g. when and where to sample). Here, I present the results of an experimental infection with time-staggered sampling designed to investigate the pathogenesis of ranaviral infection in Australian Krefft's river turtle hatchlings.

Methods

All experiments were carried out under a JCU Animal Ethics permit (A2344) and a Department of Environment and Science Scientific Research Permit (WISP13270413).

Source of virus

The Bohle iridovirus (BIV) isolate used in this study was kindly provided by Alicia Maclaine and Narges Mashkour following the methods described in Maclaine et al. (2018). This isolate was the original 1992 isolate obtained from ornate burrowing frogs (*Platyplectrum ornatum*) and has been sequenced (Speare & Smith, 1992; Hick et al., 2016). The viral isolate was propagated at 25 ˚C in fathead minnow 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 % foetal bovine serum. The inoculum was titrated to an approximate LD_{50} dose for Krefft's river turtle hatchlings, $10^{4.33}$ $TCID_{50}$ mL^{-1} (Chapter 4; Wirth et al., 2019).

Source of host

The hatchlings used in this study were obtained following the methods described in Chapter 2 and husbandry follow the methods of Chapter 3.

Experimental design

Two weeks prior to commencing the experiment, 36 three-month old hatchlings were randomly selected and assigned to the infection (n=27) or control (n=9) groups. Animals were randomly assigned in groups of 4 (3 infected, 1 control) to a predetermined endpoint for euthanasia: 0.33, 1, 2, 4, 8, 12, 16, 20, and 24 days post-inoculation (dpi). Infected and control hatchlings were kept in separate rooms to prevent contamination. Hatchlings were housed in slanted 1L plastic containers with a dry area for basking and 500mL of water that was changed daily after feeding. The hatchlings were fed *ad libitum* with commercial turtle pellets (Exo Terra, aquatic turtle food) for 10-15 minutes per day. Room temperature was maintained at 28 ˚C via air conditioning, water temperature was an average of 25.5 ˚C (SD = 0.57) throughout the experiment. On day zero of the experiment, hatchlings were inoculated by intramuscular injection with a $1x10^{4.33}$ $TCID_{50}$ dose of BIV, or a placebo control (phosphate buffered saline). Morphometric measurements (mass, straight carapace length, and straight carapace width) were recorded at the start of the trial and dietary intake was monitored throughout.

Clinical signs

Prior to, and during, the infection trial, animals were monitored for the development of clinical signs of ranaviral infection, as described by Wirth et al. (2019) (Chapter 4) for turtles with the same dose ($1x10^{4.33}$ $TCID_{50}$ mL^{-1}) and route of infection (intramuscular). These clinical signs included: inappetence, lethargy, skin lesions, increased basking behaviour, and oedema of the neck and limbs.

Endpoint

Turtles were euthanased by MS222 overdose at their predetermined endpoint or when they developed severe clinical signs according to Wirth et al. (2019) (Chapter 4). Each turtle received an intracoelomic injection of 200 µL of 5 % neutral buffered MS222. Following loss of response to stimulus, a 300 µL 50 % overdose of MS222 was given (Conroy et al., 2009).

Gross pathology and sample collection

At euthanasia, final morphometric data was recorded, and a 1 mL water sample was taken from turtle holding containers (after feeding, before water change). A separate tray and instrumentation were used for each animal. Instruments were sterilised in a 4 % hypochlorite solution for at least 5 minutes before each use and washed with surgical soap after each use. Gloves were changed between animals and surfaces were decontaminated with a hypochlorite solution (4%). Prior to euthanasia, photos were taken of any external clinical signs (lesions or swellings). Oral swabs were collected by rolling a cotton swab (Medical Wire, tubed sterile dryswab MW100) back and forth on the tongue 10 times and stored in 500 µL of DMEM. A 27½-gauge needle was used to collect between 100-200 µL of blood from the external jugular vein. The blood sample was left to clot overnight at 4 ˚C and then centrifuge-separated. Following euthanasia, a necropsy was performed, and gross pathological changes were noted. Kidney, liver, and lung samples from all animals in a group, and spleen from one animal per group, were collected and stored at -80 ˚C until DNA extraction and qPCR analysis. The remaining viscera and hind legs were fixed in 10 % neutral buffered formalin for histological examination.

Real-time quantitative PCR (qPCR)

DNA was extracted from the thawed samples (blood clot, swab, water, kidney, liver, lung, spleen) using an ISOLATE II Genomic DNA Kit (Bioline) following the manufacturer's protocol for DNA extraction from the appropriate sample type. The quantitative PCR assays were produced and run according to the methods described by Leung et al. (2017). In short, a section of the BIV major capsid protein gene (MCP) and EBF3N (a single copy gene conserved in vertebrates) were cloned into pGEM-easy vectors following the manufacturer's protocol (Promega). Plasmids were linearized using a PstI restriction digest (New England Biolabs) and quantified using a QuantiFluor dye system (QuantiFluor, Promega). Absolute standard curves were constructed in triplicate from 10^8-10^1 copies μL^{-1} and 10^7-10^1 copies μL^{-1} for the MCP and EBF3N plasmids respectively. The qPCR was performed on a MIC real-time thermocycler (Applied Biosystems) with the conditions described by Leung et al. (2017). The assays were run as a duplex with the MCP probe on the green channel (FAM probe) and ENF3N on the red channel (Cy5 probe). Viral loads were determined by dividing the number of MCP copies μL^{-1} multiplied by two by the number EBF3N copies μL^{-1} and are referred to as copies per cell throughout this manuscript.

Histology

Formalin-fixed tissues were processed for histological examination as per routine standard operating procedures (Bancroft & Gamble, 2008). Samples selected for histological processing included: head (right half), tongue, leg (injection site), liver (right lobe), heart (top), lung (right inferior lobe), intestine (including yolk sac remnant), stomach (pylorus), spleen, a kidney, and a gonad. Sections that included bone were decalcified in Gooding and Stewart's decalcifying fluid before embedding. Immunohistochemistry (IHC) and in situ hybridisation (ISH) were used to

visualise ranaviral protein and ranaviral RNA in infected tissue respectively. The methods for IHC and ISH followed those described by Maclaine et al. (2019).

Results

Animals

The median starting mass of hatchlings used in this study was 7.2 g (range, 5.4–8.9 g). Two hatchlings were euthanased ahead of their scheduled endpoint due to the development of severe clinical signs. A13 was euthanased six days ahead of schedule on day 18, and E3 was euthanased four days ahead of schedule, on day 16. For analysis, A13 was grouped with those euthanased on day 20, and E3 was grouped with those euthanased on day 16.

Clinical Signs

Clinical signs observed in this study, in order of first appearance, included: slight swelling at the inoculation site (leg) (4 dpi), increased basking behaviour (7 dpi), development of raised skin lesions at or near the site of injection (8 dpi), swelling around the neck (14 dpi), and inappetence (15 dpi).

qPCR

To determine the ranaviral tissue tropisms in addition to viral spread in the Krefft's river turtle hatchlings, viral loads were determined using qPCR at selected time points after infection. Ranaviral DNA was detectable in samples from individuals at every time point, but there was large variation in the replicates. The highest viral loads were detected in the two animals that were

euthanased early due to severe clinical signs. Viral loads in the kidney, lung, and liver tissues increased exponentially i.e. linear increase on a log scale. Viral loads in the liver and lung increased at each time point from 2 until 20 dpi, while kidney increased from 4 dpi and began to plateau at 16 dpi. There was an approximate 1000-fold increase in mean viral load in most samples from the first time point (0.33 dpi) to the peak viral load (20 dpi) (**Figure 6.1**).

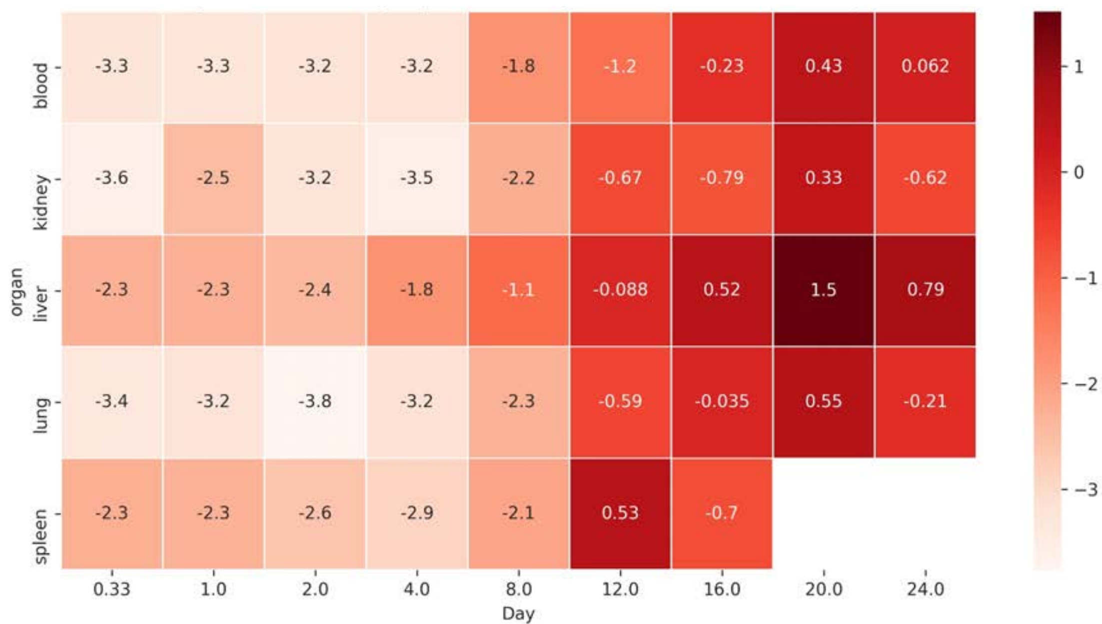

Figure 6.1 Heatmap of mean copies per cell in different organ samples throughout infection. Data are expressed as log_{10} of viral copies per cell. Darker colours indicate higher viral load.

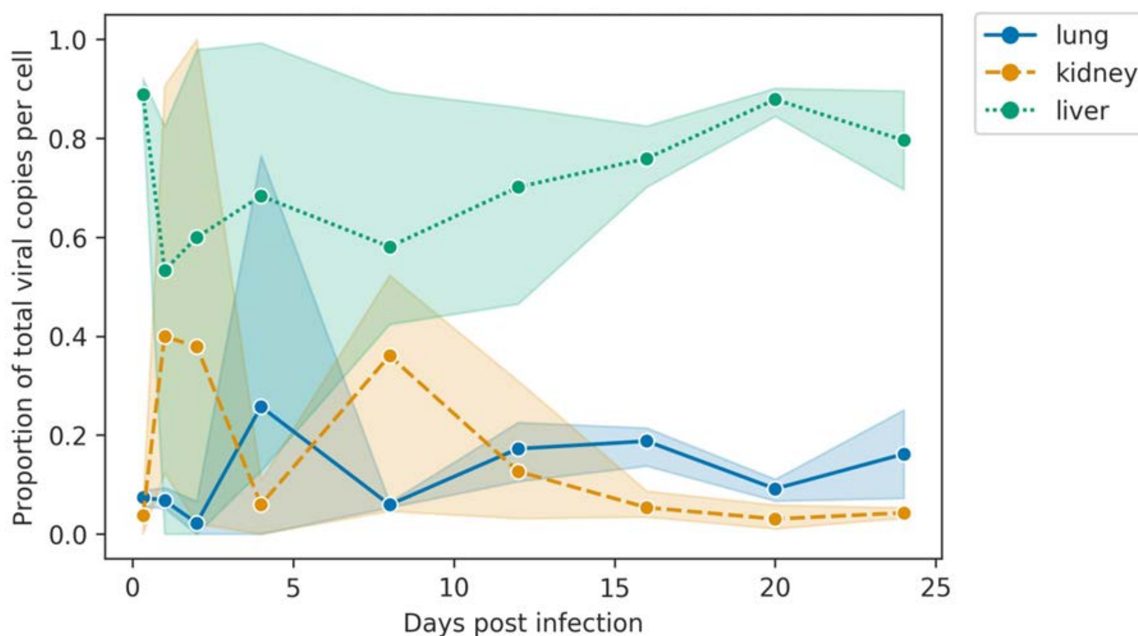

Figure 6.2 Tropisms of Bohle iridovirus for lung, kidney, and liver post infection of Krefft's river turtle hatchlings after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle iridovirus. Plot shows the mean proportion of total viral load for each sample type (lung, kidney, and liver) at each time point. The shaded area represents the 95 % confidence interval. Day 16 and 20 include data from the animals euthanased early due to severe clinical signs.

The mean proportion of total viral load was calculated to determine tissue tropisms of this viral infection, i.e. which tissues had the highest viral load throughout the course of infection. The highest mean proportion of total viral load was recorded in the liver, which was consistently high and ranged from 50-90 % of total viral load (**Figure 6.2**). There was large variation in the proportions of total viral load until 12 dpi, after which the liver consistently had the highest load. The spleen had the highest proportion of total viral loads 0.33 dpi and decreased until 8 dpi and then peaked again in the individual sampled at 12 dpi (**Table 6.1**).

Table 6.1 Tissue tropisms of Bohle iridovirus in hatchlings that had their spleens assayed by qPCR after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$). Each day represents an individual hatchling e.g. 1 dpi is hatchling B7. Viral loads (top) expressed as log_{10} copies per cell for each organ. The percentage represents the proportion of total viral load in each hatchling's lung, kidney, liver, and spleen samples (e.g. at 1 dpi 47.7% of the sum of the copies per cell were in the liver of B9). Cells are coloured by the proportion of total viral load, darker is a higher proportion.

	dpi						
Organ	**0.33 (C7)**	**1 (B9)**	**2 (A4)**	**4 (A10)**	**8 (E8)**	**12 (B6)**	**16 (C5)**
Lung	-3.55	-3.33	-3.29	-3.53	-1.84	-0.47	-0.80
	(3.1%)	(3.5%)	(5.0%)	(0.7%)	(5.8%)	(6.4%)	(16.7%)
Kidney		-2.85	-3.05		-1.95	-1.15	-1.12
	(0.0%)	(10.4%)	(8.7%)	(0.0%)	(4.5%)	(1.3%)	(8.1%)
Liver	-2.49	-2.19	-2.19	-1.39	-0.66	0.15	-0.29
	(35.7%)	(47.7%)	(62.2%)	(96.2%)	(86.5%)	(27.0%)	(54.0%)
Spleen	-2.26	-2.28	-2.60	-2.87	-2.08	0.53	-0.70
	(61.2%)	(38.4%)	(24.1%)	(3.1%)	(3.3%)	(65.2%)	(21.1%)

To compare the viability of different sample types during ranaviral infection the viral loads of environmental water, blood clots, and oral swabs were also quantified. Viral DNA was detected in the water from infected hatchling environments 0.33 dpi and decreased until 8 dpi but was not detected in the water from 2 dpi (**Figure 6.3**). No swabs reacted in the ranaviral qPCR assay until

16 dpi. The mean viral copies per cell increased from 16 dpi until the last endpoint (24 dpi), however, only one individual was positive at each time point. Oral swabs from both the individuals that were euthanased early due to severe clinical signs (A13 on day 18 and E1 on day 16) reacted in the qPCR assay. Viral DNA was detected in blood samples from individual turtles from every endpoint, but blood samples from all three infected animals from an endpoint were not positive until 16 dpi and one individual was still negative at 20 dpi (**Figure 6.3**).

Gross Pathology

Gross lesions, only observed in individuals after 12 dpi, included raised oral plaques surrounded by erythema and petechial haemorrhaging, soft tissue oedema, splenomegaly, darkened spleen sometimes with greyish multifocal to extensive discolorations, air in the digestive tract (tympanism), congested intestinal blood vessels, and multifocal discoloration or mottling of the liver suggestive of hepatic necrosis.

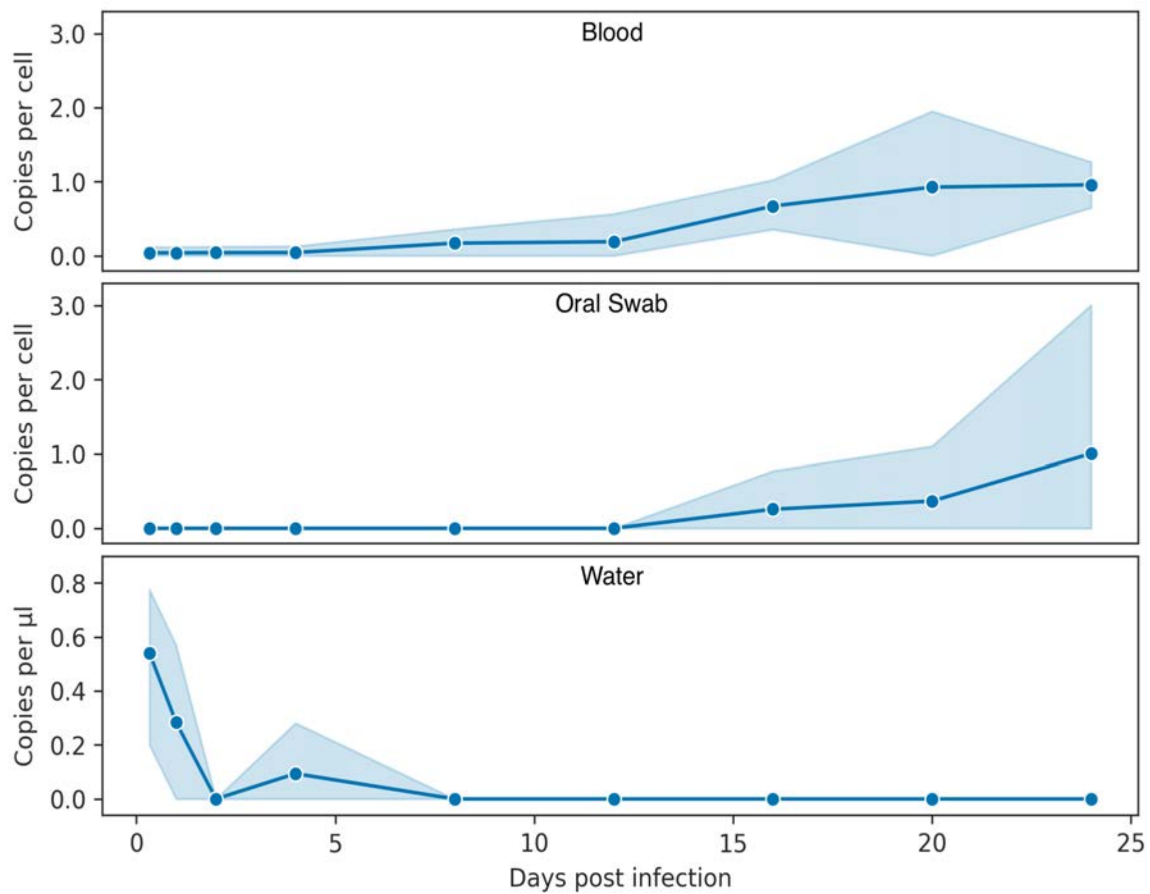

Figure 6.3 Viral copies in different ranaviral sample types over time. All data have been cube-root transformed to reduce right skew and improve visualisation. The data can be converted back to standard units by cubing (y^3) each value. Viral load (copies per cell) in blood clots (top). Viral load (copies per cell) in oral swabs (middle). Number of viral copies per µL of water from the environment of infected turtles (bottom). All points represent means at that time point and the shaded area is the 95% confidence interval. Day 16 and 20 include data from the animals euthanased early due to severe clinical signs.

Table 6.2 Observed histological changes in Krefft's river turtle hatchlings euthanased at different days post infection (dpi) after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle Iridovirus. Data are expressed as the fraction of all animals examined at that time point with the histological change. Includes a turtle hatchling (E3) that was euthanased early due to severe clinical signs (†). Includes a turtle hatchling (A13) that was euthanased early due to severe clinical signs (+).

		dpi								
Organ	**Lesion**	**0.3**	**1**	**2**	**4**	**8**	**12**	**$16^{\dagger}$**	**20^{+}**	**24**
skin	skin haemorrhage							1/4	2/3	1/2
skin	skin necrosis							1/4	1/3	
skin	epi/dermal inflammation	1/3			1/3	2/3	3/3	3/4	3/3	1/2
bone	bone marrow necrosis									1/2
bone	bone marrow IC bodies									1/2
oral mucosa	epithelial necrosis				1/3	2/3				
vascular	vascular necrosis (w/ inflammation*)					2/3	3/3	3/4	1/3	
liver	parenchymal necrosis								2/3	
tracheal/bronchial	inflammation								1/3	1/2
tracheal/bronchial	necrosis								1/3	1/2
kidney	interstitial inflammation/necrosis			1/3						1/2
spleen	hematopoietic necrosis							1/3	2/3	1/2
gonad	necrosis							1/4	1/3	

Histopathology

Systemic histological lesions were observed in infected individuals only (**Table 6.2**). Histopathological lesions included dermal inflammation and vascular necrosis near the inoculation site from 8 dpi, necrosis in the spleen (**Figure 6.4**) and gonads (**Figure 6.5**) from 16 dpi onwards and necrosis in the liver after 20 dpi (**Figure 6.6**). Inflammation and necrosis of the trachea and bronchi was observed 20 dpi and in samples collected at the final end point.

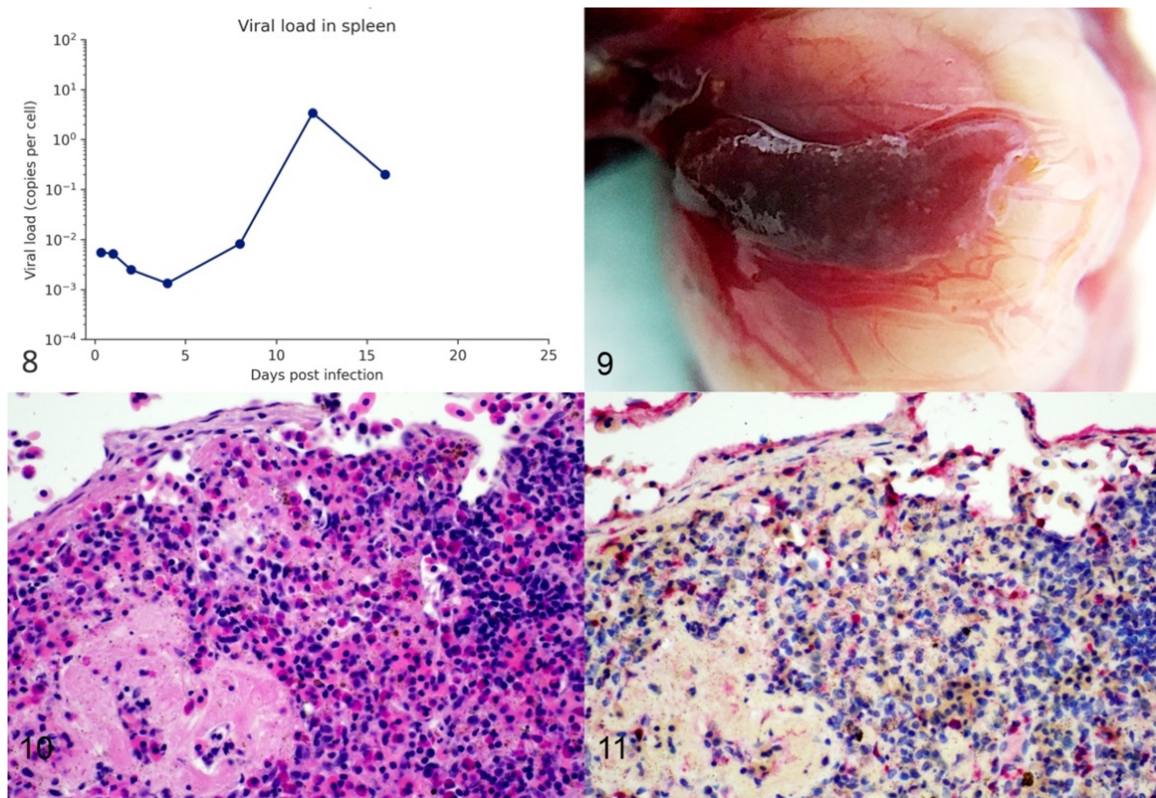

Figure 6.4 Images relating to the spleen from a ranaviral infected Krefft's river turtle hatchling euthanased at 18 days post infection after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle iridovirus. Viral load in liver at each time point during infection, the data are expressed as $\log_{10}$ of the viral load for the animals at that time point, only one spleen was sampled for qPCR analysis at each timepoint (8). Enlarge and discoloured spleen with congestion of surrounding blood vessels (9). Necrosis of the spleen (H&E, 10) with associated IHC staining (11).

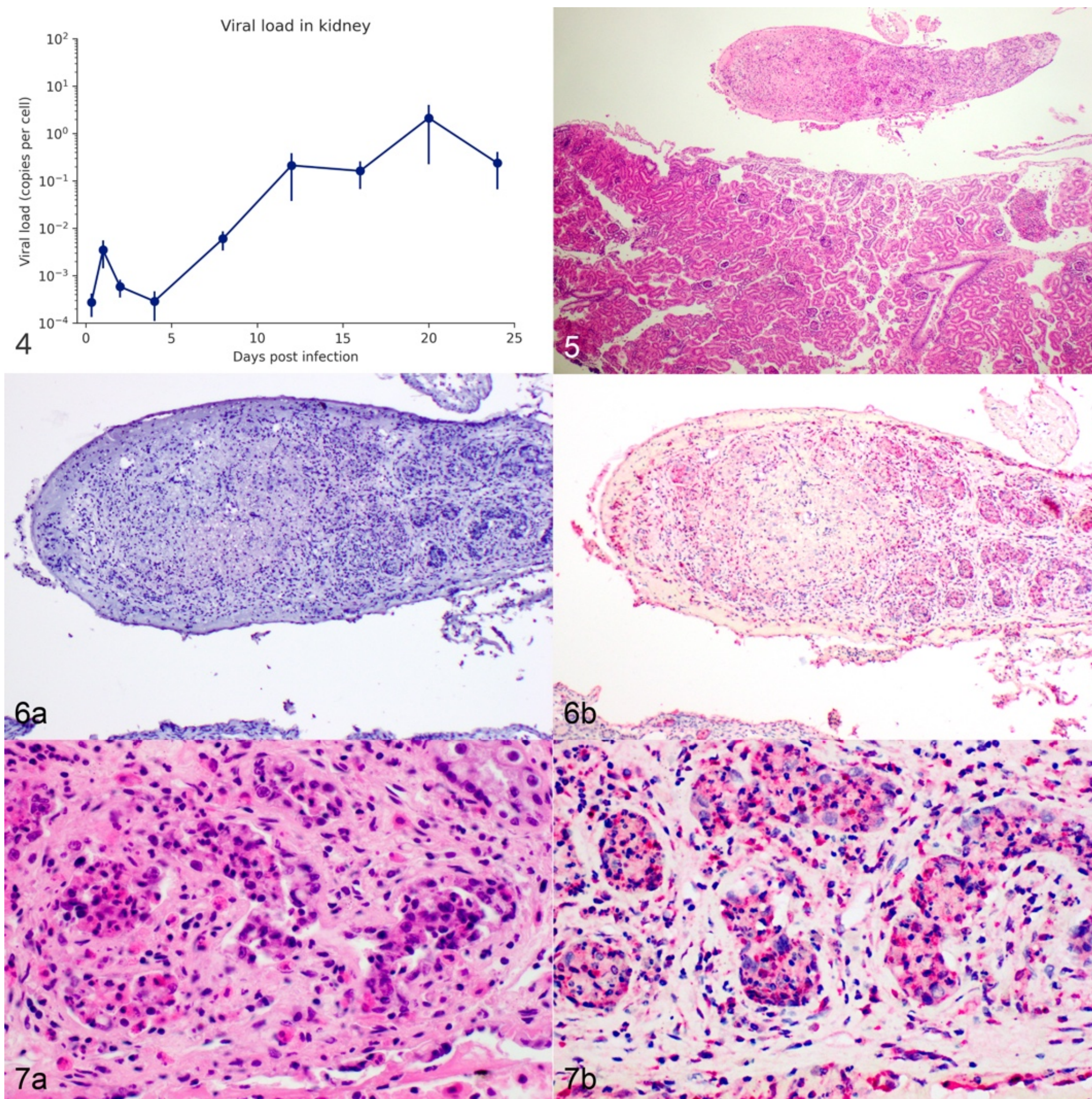

Figure 6.5 Images relating to the gonads and kidney from a ranaviral infected Krefft's river turtle hatchling euthanased at 18 days post infection after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle iridovirus. Viral load in liver at each time point during infection, the data are expressed as log_{10} of the mean viral load +/- standard error of the mean (4). Gonad and kidney tissues proximity at low magnifications, H&E (5). Large necrotic area in the gonad associated with IHC (6b) but not ISH (6a) labelling. Staining of gonads with H&E (7a) and IHC (7b).

No immunolabelling (IHC, **Table 6.3**) was present in any tissue prior to 4 dpi, and probe RNA detection (ISH, **Table 6.4**) was not evident until 16 dpi. Ranaviral RNA and protein staining by ISH and IHC, respectively, were associated with histopathological changes, however, it was difficult to differentiate background staining in the early stages of infection. There was some weak IHC staining in the bone marrow haematopoietic cells near injection site 4 dpi. Skin was positive for IHC stain on day 12dpi. More generalised IHC staining was observed at 16 dpi at which point virus in splenic, testicular, and oral mucosal tissues could be distinguished visually (**Table 6.3**).

Table 6.3 Immunohistochemical labelling of tissues from Krefft's river turtle hatchlings euthanased at different days post infection (dpi) after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle Iridovirus. Data are expressed as the fraction of all animals examined at that time point with positive IHC stain for ranaviral major capsid protein. Includes a turtle hatchling (E3) that was euthanased early due to severe clinical signs (†). Includes a turtle hatchling (A13) that was euthanased early due to severe clinical signs (+).

	dpi								
Organ	**0.33**	**1**	**2**	**4**	**8**	**12**	**16†**	**20+**	**24**
Tongue									
Brain									
Skin						3/3	2/4	1/3	
Bone marrow				1/3			1/4		
Oral/nasal/lingual mucosa							1/4		
Heart									
Trachea/bronchi								1/3	1/2
Lung									
Kidney									
Ureter or oviduct									
Liver								1/4	
Oesophagus								1/4	
Stomach									
Small intestine									
Testes							1/4	1/4	
Ovary									
Spleen							2/3	2/3	2/2

Table 6.4 In situ hybridisation labelling of a subset of tissues from one to three Krefft's river turtle hatchlings euthanased at different days post infection (dpi) after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle Iridovirus. Data are expressed as the fraction of all animals examined at that time point with positive ISH signal for ranaviral major capsid protein. Empty cells indicate no signal was present in that organ or tissue. turtle with positive liver signal euthanased early due to severe clinical signs 16 dpi, tissues from the two other turtles euthanased 16 dpi had no signal (*). Turtle with positive signals in various tissues was euthanased early due to severe clinical signs 18 dpi (#). [np=not present on slide (not examined); e=signals mostly in endothelial cells rather than organ tissue]

	dpi							
Organ	**0.33(1)**	**1(1)**	**2(1)**	**4(1)**	**8(1)**	**12(1)**	**16(3)**	**18#(1)**
Skin								1e/1
Oral/nasal/lingual mucosa								1e/1
Liver							1*/3	1/1
Spleen			np		np			1/1
Heart								1e/1
Blood vessels, various								1e/1
Lung								1/1
Kidney								1/1
Gonad		np		np	np	np	np	

Splenic tissues had consistent immunolabelling (two or more individuals) from 16 to 24 dpi (**Table 6.3**). Only one liver sample (18 dpi) was clearly positive for IHC staining (**Table 6.3**), this sample was from an animal that was euthanased due to clinical signs (A13). Positively stained intracytoplasmic inclusion bodies were observed in the liver of A13 at 18 dpi (**Figure 6.6**). Trachea/bronchi and oesophageal staining was observed at 18 dpi and the trachea and bronchi were positive for one animal at 24 dpi. Clear ranaviral RNA staining was not observed in the liver until after 16 dpi. Wide-spread labelling of ranaviral RNA was observed in the individuals that were euthanased early (day 16 and 18) because they developed severe clinical signs (**Table 6.4**). This widespread staining was predominantly observed in endothelial cells rather than organ tissue, although clear staining was observed in the kidney, lung, spleen, and liver.

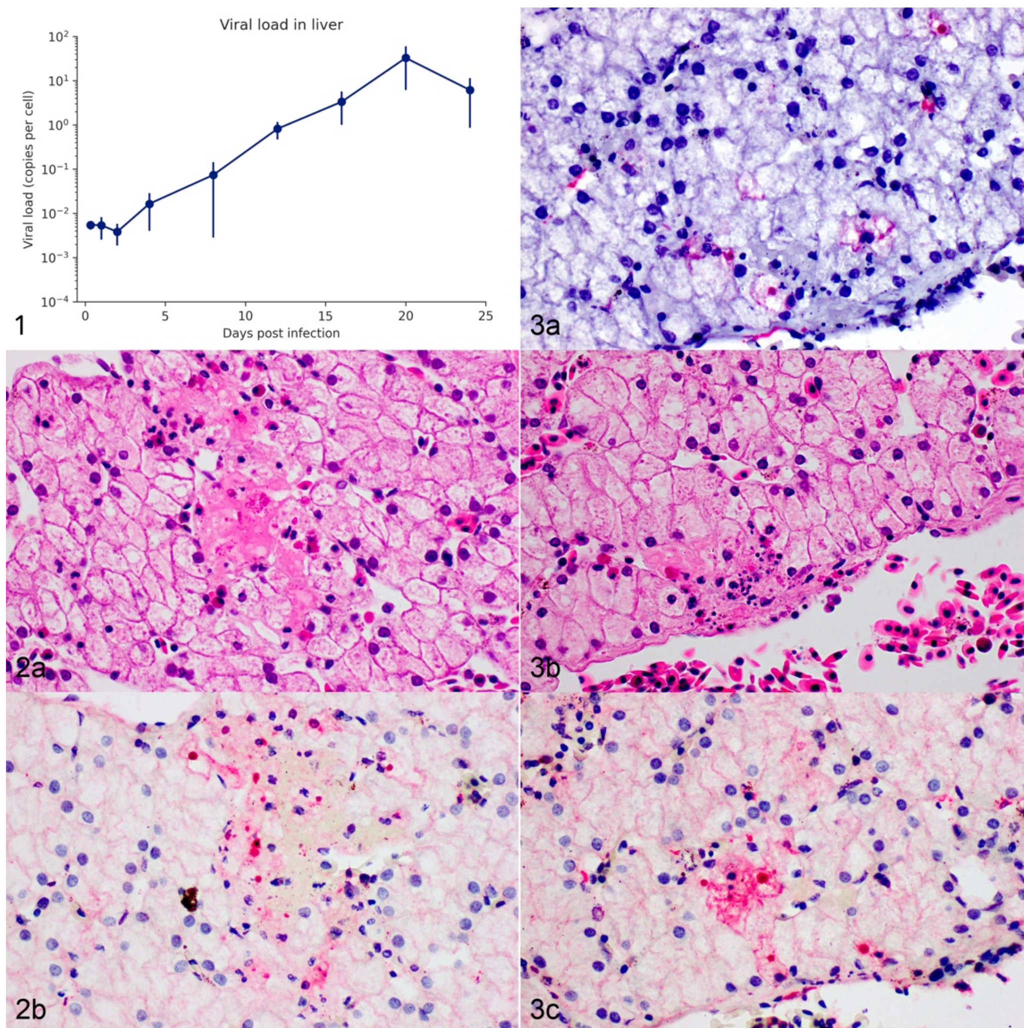

Figure 6.6 Images relating to the liver from a ranaviral infected Krefft's river turtle hatchling euthanased at 18 days post infection after intramuscular inoculation with an LD_{50} dose ($10^{4.33}$ $TCID_{50}$) of Bohle iridovirus. Viral load in liver at each time point during infection, the data are expressed as $\log_{10}$ of the mean viral load +/- standard error of the mean (1). Infiltration of heterophilic monocytes, focal and blood vessel associated hepatocyte necrosis, and basophilic intracytoplasmic inclusion bodies, H&E (2a & 3b). Some hepatocytes and sinusoidal endothelial cells are positive for ranavirus major capsid protein gene RNA via in situ RNA hybridisation (3a). Strong immunolabeling for ranavirus major capsid protein via immunohistochemistry is associated with the areas of necrosis and intracytoplasmic inclusion bodies are clearly stained (3b & 3c).

Discussion

In this chapter I presented a time-dependent pathogenesis study of a ranaviral infection in Australian freshwater turtles using Krefft's river turtles as a representative model. Ranaviral infection in Krefft's river turtles is a slow process and here I have shown how the duration of infection influences disease with implications for diagnosis and sampling protocols.

The quantitative PCR assay used in this study detected ranaviral DNA in all sample types at some point during infection. The most effective (non-destructive) method for detecting ranaviral DNA in these turtles was conducting qPCR on blood, where at least one turtle was positive at every time point. Allender et al. (2013b) found that oral swabs and whole blood had comparable sensitivities in turtles that were challenged with a ranaviral isolate via intramuscular injection. In this study blood samples were consistently more sensitive for ranaviral detection compared with oral swabs from intramuscularly inoculated individuals. Oral swabs reacted in the ranaviral assay only on the last 3 time points (16 dpi (1/4), 20 dpi (1/4), 24 dpi (1/2)) and two of these reactors were samples from the animals that were euthanased due to severe clinical signs. Therefore, swabs may only be effective at identifying late-stage infections in Australian freshwater turtles. Similarly, oral swabs are also only effective in the late stages of ranaviral infection in wood frogs (Forzán et al., 2017). Studies of wild turtles have also demonstrated inconsistency in detection rate between sample types from the same turtles (Allender et al., 2013a; Goodman, Miller & Ararso, 2013; Kimble et al., 2017). In a study of ranaviral reinfection in eastern box turtles (Cryptodira), Hausmann et al., (2015) found that while ranaviral loads in blood samples peak first, oral swabs contain viral DNA

for a longer period of time. It is possible that as infection continues (i.e. past the 24 days of this study) more oral swabs from the Krefft's river turtles will become positive. It is also unclear what the influence of reinfection will be on optimal sample choice. Further studies will be required to elucidate these complexities.

The natural ranaviral infection route for turtles is still not known, and we know that clinical signs can change with inoculation route which may have implications for best sampling methods (Chapter 3; Wirth et al., 2019). In a resource-constrained setting, collecting blood samples from Australian freshwater turtles with potential ranaviral infection should be prioritised over other non-destructive sample types.

Environmental DNA (eDNA) from water has been used to detect ranaviruses in amphibian environments (Hall et al., 2016, 2018; Miaud et al., 2019; Vilaça et al., 2020). Although ranaviral infection in Krefft's river turtles has a long incubation period, they do not appear to shed virus for the majority of this time. Ranaviral DNA was only detected in the water from the environment of infected hatchlings at the start of infection. It is possible that this early detection (0.33, 1, and 4 dpi) is the result of leakage from the infection site and not viral replication. These results suggest that eDNA may not be an effective method for ranaviral detection in Krefft's river turtles under the current experimental setting. This study was scheduled for termination at 24 dpi and although acute clinical signs appeared well within this time period, potential survivors and carriers may initiate shedding of virus at a later stage. Krefft's river turtles may not be effective viral spreaders (in a waterborne epidemic); however, more extensive studies will be required to investigate the full extent of viral shedding in this species. Although viral shedding was not observed in this study,

as suggested in Chapter 4, infected turtle hatchlings could still spread the infection to susceptible predacious species, e.g. barramundi, if consumed (Moody & Owens, 1994).

Ranaviral transmission via vectors has been suggested, and ranaviral DNA has been detected in mosquitoes associated with a ranaviral epizootics in turtles (Kimble et al., 2014). Freshwater leeches, a common parasite of aquatic turtles (and often found on Krefft's river turtles), could also serve as possible ranaviral vectors (McKenna et al., 2005; Wirth et al., 2018). The work presented here adds to the evidence for possible vector borne transmission. We show that ranaviral DNA was detectable in the blood of infected turtles throughout infection. Although DNA presence is not evidence of infectious virus, it is plausible that a blood feeding vector (mosquito, leech, etc) could ingest the virus when consuming the blood meal. Ranaviruses share a common ancestor with iridoviruses that infect invertebrate (Jancovich, Steckler & Waltzek, 2015), so it is also conceivable that these invertebrate vectors still play a role in their biology, although transmission studies will be required to elucidate the potential of vector transmission in ranaviral disease.

Bone marrow has been identified as an effective sample type for ranaviral detection in turtles (suborder Cryptodira), especially in cases where other samples have degraded (Butkus et al., 2017). While bone marrow was not tested with qPCR, IHC staining was visible at 4 and 16 dpi. This suggests that bone marrow samples may also be suitable for Krefft's river turtles, although a direct study would be useful to determine the suitability of this sample type in Australian turtles.

Allender et al. (2013b) found that kidney (after tissues at inoculation site) had the highest viral load in post-mortem samples from ranaviral infected red eared sliders (suborder Cryptodira). In

this study liver had a constantly higher mean viral load when compared with lung and kidney samples. If samples are collected at post-mortem examination from turtles of the Pleurodira ranaviral screening, it is advisable to collect liver or spleen samples. Both sample types were positive throughout infection, although only a few spleens were obtained for qPCR. These samples also had the highest viral loads during infection, which will improve detection rates when using a less sensitive assay.

Ranaviral infection in Krefft's river turtle hatchlings was a slow process, resulting in a long incubation period. Infected yet asymptomatic animals may go unnoticed, especially in areas where populations are not routinely monitored. Clinical signs observed in this study were consistent with ranaviral infection, however the onset of clinical signs occurred sooner than in previous reports (Chapter 4; Ariel et al., 2015; Wirth et al., 2019). Clinical signs included: oedema of the neck and legs, increased basking behaviour, skin lesions at or near the site of injection and inappetence. The lack of specificity of clinical signs further complicates detection of ranaviral infection outside experimental settings.

Histological labelling methods like IHC and ISH can be used to show the association of ranaviral histopathology with viral protein and nucleic acid. Using a combination of these labelling methods it was possible to determine viral replication was occurring via multiple lines of evidence. However, these methods were only useful in the late stage of infection. The spleen had the most severe histopathological changes (common for ranaviral infection). Surprisingly, although ISH is generally considered a more sensitive detection method, IHC was more effective in detecting viral presence at earlier stages of infection. Budgetary constraints resulted in only a small subset of

animals tested by ISH, so it is possible that our lack of detection had to do with individual variability. Alternatively, the difference may have to do with the techniques themselves. The ISH method used in this study is based on detection of viral RNA, whereas the IHC antibodies detect capsid proteins. Perhaps a paucity of cells in active ranaviral RNA transcription at the time of sampling could explain the low detection sensitivity of the ISH.

Histopathological changes followed from the site of inoculation to the surrounding tissues, to the spleen and gonads and finally the liver and upper respiratory tract. This is similar to the progression of disease in orally infected Eastern water dragons (*Intellagama lesueurii lesueurii*), in which splenic necrosis precedes hepatic necrosis (Maclaine et al., 2019). Immunological staining and qPCR results also suggest that the spleen is a primary site of replication in Krefft's river turtles. Immunological staining was consistently observed in the spleen from 16 dpi. In orally infected wood frogs (*Rana sylvatica*) splenic and hepatic necrosis occurred concurrently (Forzán et al., 2017). Difference in pathogeneses between these studies may be a result of different study designs (e.g. viral dose, inoculation route, etc.) or it may be a reflection of taxonomic differences between hosts.

Ranaviral damage to the host is believed to be driven by the inflammatory response (Grayfer et al., 2015). Inhibition of leukotriene (lipid mediators of inflammation and immune regulation) synthesis can dramatically reduce hepatic damage in rodent models exposed to ranaviruses (Hagmann et al., 1987). While necrosis is observed late in infection the extent of ranaviral RNA and protein labelling (via ISH and IHC) is limited. Two possible explanations for this immunolabelling pattern include: the necrosis is the direct result of viral replication, but the virus

has since dissipated (thus no staining); or the pattern of immunolabelling is a result of inflammation-driven pathogenesis due to an overactive immune response as seen in other animals. Indeed, other studies of ranaviral infection in Krefft's river turtles have also reported an influx of immune cells at the sites of necrosis, although, it is difficult to unravel the order of events (Chapter 4; Ariel et al., 2015; Wirth et al., 2019).

Chapter 4 includes the first report necrosis in the gonads of a turtle with a ranaviral infection (Wirth et al., 2019). In this chapter I have shown that there is necrosis in the gonads after 16 dpi and that viral antigens were associated with this necrosis. Tropisms for reproductive organs have not been identified in other ranaviral reports and may have implications for conservation of species if this necrosis causes a loss of reproductive potential or enables sexual or vertical transmission.

Descriptions of ranaviral infection in wild turtles often include lesions in the respiratory track (Wirth et al., 2018). Our study indicates that histopathological changes in the respiratory tract do not occur until late in infection. It is possible that turtles presenting with respiratory clinical signs are already in the later stages of infection. There are several reports of failed treatment for ranaviral infection in turtles (De Voe et al., 2004; Johnson et al., 2008), it is possible that once these animals appear sick enough (i.e. have respiratory distress) to receive veterinary intervention they are already too far into infection to benefit from such treatment (Wirth et al., 2018). A similar time-dependent study to the one presented here, assessing the effectiveness of treatments (antiviral drugs, fluids, temperature therapy) at different stages of infection (during the incubation, prodromal, and ranavirosis phases) would be useful for veterinarians trying to decide if continued treatment is worth the cost and potential prolonged suffering of the affected animal. Oral-

respiratory disease is also not pathognomonic for ranaviral infection and is very common among turtles (Doneley et al., 2017). Ranaviral infection should not be ruled out if there are no respiratory signs, as the animal might be in the prodromal stage of infection, before ranavirosis has established.

Publication and outputs

This chapter includes results presented as a poster at two scientific conferences. 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 and completed daily husbandry for the turtle hatchlings prior to and during the study;
- I developed the web-based husbandry tracker that was used to keep records of the health, diet, and morphometrics of the animals in this study;
- I prepared the inoculum used to infect the animals in this study;
- I inoculated the turtle hatchlings used in this study;
- I, with the help of a volunteer, conducted the necropsies of turtle hatchlings and undertook sample collection for histological and qPCR analysis;
- I, with the help of Maria Forzán, interpreted the histological findings;
- I extracted the DNA from samples for analysis by qPCR;
- I performed the qPCR on the samples; and
- I performed the statistical analysis and produced most of the figures found in this chapter;

Results from this chapter are included in a poster that was presented at two scientific conferences (found in Appendix 7 of this thesis):

- **Wirth, W**., Forzán, M., & Ariel, E. Pathogenesis of Bohle Iridovirus (Genus *Ranavirus*) in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*). The 5th international Symposium on Ranaviruses. 2019. Townsville, Australia.
- **Wirth, W**., Forzán, M., & Ariel, E. Pathogenesis of Bohle Iridovirus (Genus *Ranavirus*) in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*). Wildlife Disease Association Australasian Section Conference. 2019. Hobart, Australia.

Conclusions

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

1. Describe the progression of histopathological changes of ranaviral infection in Krefft's river turtle hatchlings

Histopathological changes were first detected at the site of inoculation, followed by the surrounding tissues, then the spleen and gonads, and finally the liver and upper respiratory tract.

2. Determine the tissue tropisms of ranaviruses in Krefft's river turtle hatchlings

The liver and the spleen are the major targets of viral replication and have the highest viral loads throughout infection.

3. Compare the effectiveness of different methods for detecting ranaviral infection in Krefft's river turtle hatchlings

The most effective (non-destructive) method for detecting ranaviral infection in Krefft's river turtle hatchlings was qPCR on blood.

This research describes the time-dependent histopathological changes in turtles with a ranaviral infection and has implications for diagnosis and sampling. Studies of pathogenesis, like the work presented here, provide researchers with a baseline level of knowledge that allows them to assess the factors influencing wild disease, design better experiments, and begin to untangle the complexity of this infection. The results of this study were used to inform the sampling methods used in the molecular survey of wild turtles presented in the following chapter.

Chapter 7

A MOLECULAR SURVEY FOR RANAVIRUSES IN WILD AUSTRALIAN FRESHWATER TURTLES

Aims

1. Describe the distribution and prevalence of ranaviral infection in North Queensland populations of turtles

Introduction

Turtles are iconic on a global scale and are of great cultural significance to many traditional owner groups. Long-lived animals, like turtles, are sensitive to environmental changes. Turtles are an excellent indicator species for ecosystem health, making them highly valuable to researchers and environmental managers. Australia is home to over 20 species of freshwater turtles and is a priority area for turtle conservation (Buhlmann et al., 2009).

Ranaviruses are global pathogens that can cause disease in turtles (Wirth et al., 2018), and Australia is no exception (Duffus et al., 2015). The distribution of ranaviruses and freshwater turtles overlaps in Australia (Speare & Smith, 1992; Ariel et al., 2015). Based on the findings from the previous chapters of the thesis, it seems likely that ranaviral infections could be detected in Northern Australian turtles Australian turtles (i.e. Krefft's river turtles) are susceptible to multiple routes of infection (Chapter 3), low doses of viral inoculum can result in infection (Chapter 4), and

the temperature at which infection rates are maximised is close to the average temperature in parts of North Queensland (Chapter 5). Turtles can remain infected for weeks; throughout infection, their blood contains detectible levels of ranaviral DNA (Chapter 6).

Wild Australian freshwater turtles with a high seroprevalence of anti-ranaviral antibodies have been documented in Australia, indicating natural ranaviral exposures do occur (Ariel et al., 2017a). However, no Australian freshwater turtles have been identified with active ranaviral infections. This lack of baseline infection data is likely because there have been no molecular surveys for ranaviruses in wild Australian turtles. To get an idea of the baseline levels of ranaviral infection in Australia, a molecular survey for ranaviral DNA in the blood of various freshwater turtle species was performed.

Methods

Study sites and sampling frequency

The Ross River (-19.309223, 146.765670), Alligator Creek (-19.429079, 146.943646), Johnstone River (-17.348406, 145.591781), Elizabeth Creek (-18.142562, 144.327572), and Archer River (-13.430315, 142.503514) were the study sites included in this study (**Figure 7.1**). These study sites were selected to provide a wide range of freshwater turtle habitat conditions and geographical spread. The Ross River runs through the centre of Townsville and is considered urbanised. Alligator Creek runs through the Bowling Green Bay National Park area, and the primary sampling site was immediately outside this national park. The Johnstone River, at the points of sampling (i.e. Malanda), is associated with farmland, primarily dairy cattle. Elizabeth Creek is part of an

inland waterway that is surrounded by beef cattle pastureland. The Archer River is a river located on the Cape York Peninsula that remains in a natural state with very little development. One-off sampling occurred at the Johnstone River (23/11/2016 - 25/11/2016), Elizabeth Creek (14/8/18 - 15/8/18), and Archer River (25/05/2017 - 02/06/2017). Jason Schaffer collected the samples from the Archer River. Multiple sampling events occurred at the Ross River and Alligator Creek from 2014-2019.

Figure 7.1 Map of study sites included in the molecular survey for ranaviral infection in freshwater turtles in North Queensland. It is approximately 1000 km from Alligator Creek to Archer River.

Study animals and sample collection

Capture techniques included either passive sampling (using baited cathedral traps, see Chapter 2) or active sampling (using snorkelling/hand capture). These two methods minimised biases arising due to using only one of these capture techniques. After capture, a physical exam was performed and morphometrics were collected (**Figure 7.2**). The physical exam included: an ocular-oral-nasal examination, an external assessment for soft and hard tissue damage (e.g. swelling, bruising, shell cracks), a general activity level assessment, freshwater leeches parasitising the animals were counted, and adult female turtles were palpated for eggs. Morphometrics collected included: weight, the curved carapace length, the straight carapace length and width, the straight plastron length and width, tail lengths from the tip of the tail to the plastron, from the tip of the tail to the carapace, and from the tip of the tail to the cloacal opening.

Figure 7.2 Gudjuda Rangers collect morphometrics from an adult Krefft's river turtle (*Emydura macquarii krefftii*).

Blood samples were collected from either the femoral vein or external jugular vein using a 27G gauge needle and a 1 mL syringe (Becton Dickinson, USA). Blood samples were deposited in a 1.5 mL microfuge tube and kept cool until they were transported back to the lab. Once in the lab, blood samples were allowed to clot overnight at 4 ˚C. Clotted samples were separated by centrifugation (500 g for 5 min) and serum was removed using a micropipette. The blood clots were stored at -20 ˚C until DNA extraction.

Sample preparation and analysis

To reduce the cost of sample analysis, samples were combined in a multiplexed DNA extraction, i.e., material from three blood clots were included in each extraction. Due to the doubling nature of PCR, batching a small number of samples has a minimal effect on the sensitivity of the assay. Multiplexed samples that react in the downstream assays can be re-extracted, and re-analysed to determine individual reactors. This protocol is useful when prevalence is expected to be low, or resources are constrained. DNA was extracted from the multiplexed samples following the protocol of the previous chapter. The same quantitative PCR assay (Leung et al., 2017) used in the previous chapter was used in this study to assay the extracted samples for ranaviral DNA.

Statistical analysis

All analysis was performed using the Statsmodels module and Pandas library in Python 3.7 (McKinney, 2010; Seabold & Perktold, 2010). Prevalence was calculated using the Wilson score interval with a 95% confidence interval, assuming all turtle species in the same location in the same month were assumed to be part of the same population.

Results

A total of 379 turtles were collected from the five study sites (**Table 7.1**). The majority of samples were collected from turtles in the long-term monitoring sites (Alligator Creek and Ross River). The other sites (Archer River, Elizabeth Creek, and Johnstone River) were only sampled once. Five different turtle species were included in this study (**Table 7.1**). The majority of samples were collected from saw-shelled turtles (*Myuchelys latisternum*) followed by Krefft's river turtles (*Emydura macquarii krefftii*). All the turtles in this study are short-necked turtles apart from the Cann's turtle (*Chelodina canni*) which is an Australian snake-necked turtle species.

Table 7.1 Number of and location of species tested for ranaviral infection during survey.

Site	Species	Count
Alligator Creek	*Emydura macquarii krefftii*	117
	Myuchelys latisternum	148
Archer River	*Chelodina canni*	14
	Emydura subglobosa worrelli	4
	Myuchelys latisternum	7
Elizabeth Creek	*Myuchelys latisternum*	16
Johnstone River	*Elseya irwini*	24
Ross River	*Chelodina canni*	3
	Emydura macquarii krefftii	39
	Myuchelys latisternum	7
	Total	**379**

None of the 379 samples reacted in the qPCR assay for ranaviral DNA in the turtle blood. Hence, ranaviral infection was either absent or generally at very low prevalence (0-1% confidence limits) across all samples. However, confidence limits for prevalence estimates for monthly ranaviral infection, based on the number of animals testing negative, ranged from 0 up to 6-80 % (95% confidence limits) depending on the location of sampling. Therefore, it is possible that ranaviruses are still present within turtles in North Queensland but were missed by this survey (**Table 7.2**).

Table 7.2 Prevalence estimates for ranaviral infection in five North Queensland waterways on different sampling dates. Upper and lower CI represent the range of the 95% confidence intervals for the proportion of infected animals (all species combined).

Site	Year-month	Total # tested	Total # positive	Lower CI (%)	Upper CI (%)
Alligator Creek	2014-10	31	0	0	11.03
	2015-02	20	0	0	16.11
	2016-06	7	0	0	35.43
	2016-07	22	0	0	14.87
	2016-10	59	0	0	6.11
	2016-11	24	0	0	13.8
	2017-05	18	0	0	17.59
	2017-07	21	0	0	15.46
	2018-06	25	0	0	13.32
	2018-07	17	0	0	18.43
	2018-10	5	0	0	43.45
	2019-03	3	0	0	56.15
	2019-10	13	0	0	22.81
Ross River	2017-03	5	0	0	43.45
	2017-04	1	0	0	79.35
	2017-05	1	0	0	79.35
	2017-06	1	0	0	79.35
	2017-09	9	0	0	29.91
	2018-04	19	0	0	16.82
	2018-07	5	0	0	43.45
	2018-12	4	0	0	48.99
	2019-03	4	0	0	48.99
Archer River	2017-02	25	0	0	13.32
Elizabeth Creek	2018-08	16	0	0	19.36
Johnstone River	2016-11	24	0	0	13.8

Discussion

To my knowledge, this study is the first molecular survey for ranaviral infection not only in Australian turtles, but any species from the suborder Pleurodira. While no ranaviral infections were detected, the results of this survey are still valuable as they help to define the distribution of ranaviral infection and its prevalence in wild Australian freshwater turtles. This survey was also valuable to define the general health of wild Australian freshwater turtles. Because we were monitoring turtles at Alligator Creek, for example, we noticed an outbreak of cutaneous lesions in the turtle population that would probably otherwise have gone unnoticed. A report on the cutaneous lesions was recently published, and is included as Appendix 5 of this thesis (Wirth et al., 2020).

The majority of ranaviral infection reports in free-living turtles have been associated with mortality events (Allender et al., 2006; Johnson et al., 2008; Belzer & Seibert, 2011; Farnsworth & Seigel, 2013; Perpiñán et al., 2016; Agha et al., 2017; Adamovicz et al., 2018). It is possible that ranaviral epizootics in Australian turtles follow a sporadic pattern, similar to that seen in other turtles, thus making it difficult to detect ranaviral infections outside an active epizootic. It is not clear what controls epizootics in wild turtles, or if similar influencing factors may apply to Australian turtle species. For an epizootic to occur, stressors, environmental conditions, distribution, susceptible coexisting species and coinfections may all have to align. Indeed, from work presented in this thesis, it seems that different inoculation routes, doses, temperatures, and infection durations can influence outcomes of ranaviral infection in Australian turtles.

Outside of epizootic events, there have been several reports of low to zero baseline prevalence of ranaviral DNA in wild cryptodiran turtle populations (Hanlon et al., 2016; Archer et al., 2017; Kolesnik, Obiegala & Marschang, 2017; Winzeler et al., 2018). It is possible that Australian pleurodiran turtles in North Queensland are either generally not infected with ranaviruses (with occasional epizootics) or similar to international cryptodiran turtles have low levels of baseline infection prevalence.

Low baseline levels of ranaviral prevalence in cryptodiran turtles may be due to the acute and rapidly lethal nature of ranaviral infection in these animals, such that infected animals die before they can be tested (Brunner et al., 2015). This possibility is consistent with low observed seroprevalence in cryptodiran turtles, suggesting that exposed animals either die or fail to seroconvert (Johnson et al., 2010). In Australian turtles, however, experimental ranaviral infection and disease is a long process (Chapter 6) and in the wild seroprevalence is high (greater than 50%) at some of the same locations surveyed in this study (Ariel et al., 2017a), so exposed animals are not dying and if they do they are unlikely to die quickly (compared to ranaviral infection in other species). It is possible that the high level of seroprevalence in wild turtles is the result of cross reactivity and not ranaviral infection. However, ranaviruses (e.g. BIV) are present in the environment of wild turtles and so exposure would be expected, although, experimentally inoculated animals failed to seroconvert (Speare & Smith, 1992; Ariel et al., 2015).

Ranaviral seropositivity correlates with body size in wild Australian freshwater crocodiles and turtles (Ariel et al., 2017a). This correlation suggests that larger turtles may be more likely to seroconvert, or produce a more robust humoral response, or that the probability of an exposure

event accumulates throughout life. Ranaviral infection in Australian turtles could be primarily a disease of younger turtles. Older turtles may produce a robust, long-lasting, neutralising antibody response that prevents reinfection (resulting in low overall prevalence). Younger turtles that survive their first ranaviral encounter may become resistant to the viral infection, thus explaining the higher seroprevalence in larger turtles as survivorship bias. Repeated exposure studies would be required to test this hypothesis in Australian turtles.

It is possible that the design of this study has led to low levels of ranaviral DNA detection. In this study I chose to use blood samples. Blood samples have been used successfully in previous ranaviral surveys and results from Chapter 6 show that turtles are viremic throughout infection (Wirth et al., 2018). However, other studies on ranaviral infection in reptiles have shown inconsistencies in detection rates from different sample types (Wirth et al., 2018). A recent study in gopher tortoises (*Gopherus Polyphemus*, Cryptodira) found high ranaviral prevalence using oral–cloacal swabs, but none of the blood samples tested reacted (Cozad et al., 2020). Future studies should examine ranaviral prevalence in Australian freshwater turtles using alternative sampling techniques e.g. oral–cloacal swabs.

It was difficult to obtain consistently high numbers of animals, and so the monthly confidence intervals on prevalence estimates were large. However, in cases where a large sample size was obtained, we still detected no ranaviral DNA. Possibly, the sampling technique was biased against capturing infected animals. For example, food-baited traps might not be the most effective method for capturing turtles with a disease that causes inappetence (Chapter 4). However, blood samples from infected turtles will likely react in a ranaviral PCR assay before they develop clinical signs

like inappetence (Chapter 6). This study also used an active capture method (snorkelling) that may be biased towards the capture of slow-moving or lethargic turtles (another sign associated with ranaviral infection, see Chapter 4). While we cannot totally dismiss the possibility that there was some sampling bias, reducing our probability of capturing diseased turtles, it seems unlikely that it was related to the capture method.

On the other hand, our study was size-biased. Turtles captured in this survey ranged in straight carapace length between 69 and 325 mm. If infections occur primarily in young turtles, then this survey missed the target population. Indeed, hatchlings are susceptible to ranaviral infection under experimental conditions (Chapters 3-6), and adult turtles do not necessarily become infected when similarly inoculated with the same ranaviral dose that causes disease in hatchlings (Ariel et al., 2015). In Australian freshwater turtles there is likely some threshold of turtle age associated with susceptibility to ranaviral infection. The smallest turtle captured in this survey (69 mm) was significantly larger than the average size of hatchlings (~40 mm) used to model ranaviral disease in the previous chapters of this thesis. The growth rate of the hatchlings used in the thesis was ~1 mm per month. If this growth rate is constant; it will take several years for hatchlings to reach the size of the smallest turtles captured in the survey. It is quite possible that by the time turtles are large enough to be captured by the methods used in this study, the optimal age at which it is possible to detect a ranaviral infection may have already passed.

The best way to reliably obtain turtle hatchlings is to incubate their eggs in the laboratory (see Chapter 2). Although captive raised hatchlings are useful for experimental work (Chapters 3-6), this method does not help us understand this cryptic life stage in situ. The difficulty of finding wild

turtles in the first phase of their life is a well-known problem in sea turtle research, where this life history stage is referred to as the 'lost years'. It seems that even less is known about this period in a freshwater turtle's life. When turtles are at their most vulnerable, they disappear. If we are going to help turtles, we need to understand them, and to understand them, we first must find them. In freshwater turtles, the hatchling life-stage seems like an opportune target for future scientific research and conservation efforts.

Contributions

My overall contribution to this study 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, with the help of many volunteers, captured the turtles used in this study;
- I built the freshwater turtle health web application for maintaining and distributing data generated in this project;
- I collected blood from the majority of animals in this study;
- I extracted the DNA from samples for analysis by qPCR;
- I performed the qPCR on the samples; and
- I performed the statistical analysis and generated the figures presented in this chapter.

Conclusions

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

1. Describe the distribution and prevalence of ranaviral infection in North Queensland populations of turtles.

Based on the results of this study, it is likely that the distribution of ranaviral infection in North Queensland is limited and prevalence of active ranaviral infections in the populations surveyed is low. While no active ranaviral infections were detected in the turtle populations surveyed, this study has still helped to refine our understanding of ranaviral infection in Australian freshwater turtles.

Chapter 8

GENERAL DISCUSSION

Many environmental and host factors can influence ranaviral disease development and severity, and the degree of influence has implications for the epidemiology and pathology of infection (Gray, Miller & Hoverman, 2009; Miller, Gray & Storfer, 2011; Brunner et al., 2015). The ultimate outcome of understanding disease is the ability to prevent it; however, preventing disease requires not only understanding factors that influence disease in the laboratory, but also the extent of infection in the wild. This thesis examined the influence of inoculation route, viral inoculation dose, environmental temperature, and infection duration on critical aspects of ranaviral disease, and examined the extent of ranaviral infection in wild turtle populations in North Queensland, Australia.

The first overall aim of this thesis, i.e. *to determine factors that influence ranaviral disease in Australian freshwater turtles*, was addressed in four separate chapters and formed the bulk of the thesis:

CHAPTER 3 - THE INFLUENCE OF INOCULATION ROUTE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

An experimental ranaviral infection was performed with Krefft's river turtle (*Emydura macquarii krefftii*) hatchlings. Hatchlings were inoculated with a single viral dose ($10^{5.33}$ $TCID_{50}$) via one of three different inculcation routes (intracoelomic, intramuscular, and oral) at a constant

environmental temperature (28 ˚C). The influence of inoculation route on ranaviral disease was compared among the control and inoculation-route groups.

CHAPTER 4 - THE INFLUENCE OF VIRAL DOSE ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

During an experimental infection, hatchlings were inoculated with one of five different 10-fold viral dilutions ($10^{1.33}$, $10^{2.33}$, $10^{3.33}$, $10^{4.33}$ and $10^{5.33}$ $TCID_{50}$) via the intramuscular route at a constant environmental temperature (28 ˚C). The influence of viral dose on ranaviral disease was compared among dose groups and controls.

CHAPTER 5 - THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON RANAVIRAL INFECTION IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

During an experimental infection, hatchlings were inoculated with an infectious ranaviral dose ($10^{2.33}$ $TCID_{50}$) via the intramuscular route, at one of four different environmental temperatures (16, 22, 28, and 32 ˚C). The influence of environmental temperature on ranaviral infection was compared among temperature groups and controls.

CHAPTER 6 - THE INFLUENCE OF INFECTION DURATION ON RANAVIRAL DISEASE IN AUSTRALIAN FRESHWATER TURTLE HATCHLINGS

During an experimental infection, hatchlings were sampled at one of nine different time points after inoculation with a ranaviral dose ($10^{4.33}$ $TCID_{50}$) via the intramuscular route at a constant environmental temperature (28 ˚C). The influence of infection duration on ranaviral disease profile and severity was compared among time point groups.

The second overall aim of this thesis, i.e. *to describe the distribution of ranaviral infection in wild Australian freshwater turtle populations*, was addressed in the seventh chapter of this thesis:

CHAPTER 7 - A MOLECULAR SURVEY FOR RANAVIRUSES IN WILD AUSTRALIAN FRESHWATER TURTLES

A molecular survey was performed on wild freshwater turtles in North Queensland, Australia. Multiple species of free-living pleurodiran turtles were surveyed over several years at multiple locations using sampling methods validated in other chapters of this thesis. Prevalence of ranaviral infection was estimated across sampling times and locations.

Human activities are driving the emergence of infectious diseases around the globe, and these diseases are contributing to species declines (Wilson, 1995; Wake & Vredenburg, 2009; Butler, 2012; Ceballos et al., 2015; Zohdy, Schwartz & Oaks, 2019). Ranaviruses are pathogens of ectothermic vertebrates, and humans are furthering their spread (Duffus et al., 2015). Ranaviruses can infect fish, amphibians, and reptiles and have caused population declines in some species (Price et al., 2014, 2017). The majority of ranaviral epizootics in reptiles are reported in populations of turtles, however, research on this topic has focused on only one of the turtle suborders, the Cryptodira (Wirth et al., 2018). There have been no reports of wild ranaviral infection in the other suborder of turtles, the Pleurodira, despite susceptible species and serological evidence of exposure in wild pleurodiran turtles in Australia (Ariel et al., 2015, 2017a). The two suborders of turtles split more than 150 MYA, and so it seems possible that the perceived difference in ranaviral disease between the groups is real and not just a product of a difference in

research effort, although a lack of research effort on the Pleurodira is likely also contributing (Wirth et al., 2020). Possible causes contributing to real differences in ranaviral disease prevalence include: turtle species in the suborder Pleurodira may be less susceptible to ranaviral infection than those of the Cryptodira; there may be some factors that influence ranaviral disease in the Pleurodira causing fewer ranaviral epizootics; ranaviruses themselves may be less abundant or virulent in areas inhabited by turtles of the suborder Pleurodira. Hence, this thesis set out to test how specific factors influence ranaviral susceptibility and disease development in an Australian pleurodiran turtle species, and to determine the extent of baseline ranaviral infection in free-living pleurodiran turtle populations in North Queensland, Australia.

Before we can begin to test how specific factors influence ranaviral susceptibility and disease development in turtles, we need to develop a reliable model system in which to test these factors. This led to the second chapter in the thesis:

CHAPTER 2 - METHODS FOR THE SAFE COLLECTION AND EFFECTIVE INCUBATION OF EGGS FROM THE KREFFT'S RIVER TURTLE (EMYDURA MACQUARII KREFFTII)

Bohle iridovirus infection in Krefft's river turtle hatchlings was chosen as a model of ranaviral infection in turtles of the suborder Pleurodira (which includes most Australian turtles). The methods for obtaining Australian freshwater turtle hatchlings presented in Chapter 2 enabled the establishment of the model of ranaviral infection in Australian freshwater turtles. This model formed the basis of the four experimental chapters of this thesis that examined the influence of

different factors (inoculation route, viral dose, environmental temperature, and infection duration) on ranaviral infection in Australian freshwater turtles.

Some of the basic requirements of an animal model are reproducibility and applicability (Davidson, Lindsey & Davis, 1987). While using a mouse model of ranaviral disease in turtles may be reproducible (there is little variation in mice from a single strain), the information obtained from mice will be less applicable to ranaviral disease in wild freshwater turtles than an animal model that uses turtles as subjects, i.e. close homologs are generally better analogs (Davidson, Lindsey & Davis, 1987). Using wild (i.e., outbred) animals as a model includes a source of genetic variation; however, this variation means that the model more accurately reflects wild-type populations (Tuttle et al., 2018). Producing inbred colonies of turtles was outside the scope (and timeline) of this thesis, although having such colonies would likely improve aspects of turtle research and reduce animal numbers required for experimental studies. One method proposed to improve recruitment in Australia turtle populations is 'head starting', i.e. releasing captive-raised turtle hatchlings (Spencer, Van Dyke & Thompson, 2017). The methods and data presented in Chapter 2 may also prove useful for those interested in attempting something similar in Krefft's river turtles or related species.

Having obtained hatchlings using the methods described in Chapter 2, it was now possible to begin testing factors that influence disease. The first factor examined was inoculation route, i.e., how changes in the entry pathway of the virus into the host changes the disease.

The influence of inoculation route on ranaviral disease in Australian freshwater turtle hatchlings

Inoculation route was the first factor examined for its influence on ranaviral infection using the Krefft's river turtle hatchling model. Krefft's river turtle hatchlings were susceptible to ranaviral infection via all inoculation routes tested (oral, intracoelomic, and intramuscular). This information is useful as it indicates that more plausible routes of natural transmission may be possible, i.e., ingestion (oral), or wounds or vectors (intramuscular). Other turtles from the Cryptodira have shown varying susceptibility to oral challenge (Wirth et al., 2018). Changes in the inoculation route affect disease presentation and will likely have implications for wild infection dynamics (Gray, Miller & Hoverman, 2009). The natural route of infection remains unknown, but the findings in this thesis have provided a list of possibilities.

Many of the ranaviral challenge studies in species of the suborder Cryptodira have used intramuscular injection as an inoculation route. Because of this, and the practicality of this method, an intramuscular injection was selected for use in the other experimental challenge studies included in this thesis (Wirth et al., 2018). This experimental infection study, like all others on ranaviral disease in reptiles, used a single high dose of virus to examine susceptibility. However, during natural infection viral dose is likely variable, and this is bound to also influence disease severity (Brunner, Richards & Collins, 2005). Having identified a reliable route of infection for producing ranaviral disease in the Krefft's river turtle hatchlings model, the next step was to determine how viral dose influenced the disease development.

The influence of viral dose on ranaviral disease in Australian freshwater turtle hatchlings

Chapter 4 contains the most valuable results of this thesis; it is the first study in reptiles to examine the influence of dose on ranaviral disease. Critically, this chapter produced estimates of the ID_{50} and LD_{50} ranaviral dose in Krefft's river turtle hatchlings. By identifying the viral doses that cause infection or death (severe clinical signs) in 50% of Krefft's river turtle hatchlings, it is now possible to conduct repeatable experiments of infection and disease.

The knowledge of the effect of viral dose on disease outcome gave us a foundation to test other factors that might influence ranaviral disease in turtles. For example, there are no known treatments for ranaviral infection in turtles; however, this may be the result of the design of studies used to assess potential treatments. Acyclovir is used to treat herpes virus infections, inhibiting the viral DNA polymerase after activation by a viral thymidine kinase (Beutner, 1995). Some ranaviruses share this kinase, so it could be a potential treatment option (Allender, 2012; Ferguson et al., 2014). Previous studies on the efficacy of acyclovir at treating ranaviral infection in cryptodiran turtles have been variable (De Voe et al., 2004; Johnson, 2006; Allender, 2012; Ferguson et al., 2014). On several occasions acyclovir use has failed to stop ranaviral disease progression in cryptodiran turtle species; however, this is clinical evidence that is complicated by multiple factors of wild infection and treatment regimes (De Voe et al., 2004; Johnson et al., 2008). Because we now know the LD_{50} for ranaviral infection in Krefft's river turtles, we could design an experiment that examines the effect of acyclovir or other treatment on the percentage of animals that develop clinical signs when given an LD_{50} dose. This experiment would be more sensitive and realistic to determine the efficacy of a drug than a typical experimental infection using an unquantified viral dose or a dose where the expected disease outcome is not known.

Therefore, if we are going to evaluate treatments and factors that critically influence ranaviral disease, we need reliable indicators of success. The ID_{50} and LD_{50} values estimated in this thesis are the foundation of future work on ranaviral disease in Australian turtles as this allows us to more accurately measure the effects of interventions and environmental factors.

The influence of environmental temperature on ranaviral infection in Australian freshwater turtle hatchlings

An important factor that has been identified for disease severity in turtles is environmental temperature. The widespread impacts of climate change will likely be the defining feature of this century (Parmesan & Yohe, 2003). As environmental temperatures change so do the distributions of diseases (Altizer et al., 2013). Climate change is especially critical for ectotherms, in which both infectious pathogens and the host immune responses are dependent on environmental temperature (Tait, 1969; Brunner et al., 2015).

Temperature can influence ranaviral infection rates in Australian turtles: the results of the experiment in chapter 5 showed that a change in temperature of only 8˚C (22-34 ˚C) was enough to reduce the number of infected individuals from 90 to 10%. The majority of this effect is possibly because Bohle iridovirus has limited replication in cell culture at temperatures above 32˚C (Speare & Smith, 1992). Also, several studies have shown decreased ranaviral disease at higher temperatures (Brunner et al., 2015) This has implications for the spread of ranaviral disease as local environmental temperature may influence the number of animals that are infected. Ranaviruses have primarily been isolated in more temperate climates (Brunner et al., 2015). While

the current perceived distribution of ranaviruses may be the result of the research effort in areas like Europe, China, and the USA (Duffus et al., 2015; Wirth, Lesbarrères & Ariel, 2020), it may also represent the influence of temperature on the viral distribution. Tropical places like South and Central America, Africa, and northern Australia have susceptible species and ranaviruses, but they also have higher average temperatures which may reduce overall ranaviral dispersal (Brunner et al., 2015; Duffus et al., 2015). There is evidence that ranaviral disease is correlated with season and latitude (Brunner et al., 2015). In Australia, there appears to be a north-south divide between the two ranaviruses found here: BIV and EHNV. It is unclear if this geological divide is driven by temperature, or the distribution of susceptible host species. EHNV has caused epizootics in redfin perch, a species found predominantly in the south of Australia (Whittington et al., 1996). BIV was originally isolated in ornate burrowing frogs, a species restricted to north-eastern Australia (Speare & Smith, 1992). The Krefft's river turtle and saw-shelled turtle species are susceptible to BIV infection during experimental challenge, and are also only found in the north-eastern part of the Australian continent (Ariel et al., 2015). Ranavirus distributions may be limited by environmental factors such as temperature, but to determine if these factors are influencing the dispersal of ranaviruses in the wild, we need reliable testing methods and an understanding of the disease development process.

The influence of infection duration on ranaviral disease in Australian freshwater turtle hatchlings

Ranaviral disease is not an instant process, this fact is supported by the results of the pathogenesis study in Chapter 6. Histopathological changes were similar to other ectothermic species with ranaviral infections, i.e. changes first occurred in the spleen and liver, then spread to other tissues

(Miller et al., 2015). While blood samples were positive at all sampling points throughout infection, and therefore potentially a good sample for detecting ranaviral DNA at any time during the infection, this may be artificial and a result of the inoculation route (i.e., intramuscular injection) used in the study. Intramuscular injection is an effective method for producing ranaviral disease in turtles; however, we still do not know the natural infection route for turtles, and we know clinical signs can change with inoculation route which may have implications for best sampling methods (Chapter 3; Wirth et al., 2019). While oral swabs were not a sensitive diagnostic sampling method until late in infection, they may be more effective in studies of wild animals if ranaviral infection occurs orally. Future studies should examine this possibility.

To summarise the findings from the experiments addressing the first aim of the thesis, i.e. *to determine factors that influence ranaviral disease in Australian freshwater turtles*:

- Krefft's river turtles were susceptible to ranaviral infection via multiple routes of inoculation, and some individual animals were infected at low doses.
- The environmental temperature associated with the highest number of infections (23.2 °C) is common in North Queensland.
- Turtles can remain infected for weeks; throughout infection, their blood contains detectible levels of ranaviral DNA

Given these findings, it seems likely that ranaviruses should be detectible in wild Australian turtles. Although susceptible turtle species co-occur with BIV in north-eastern Australia, a targeted

molecular survey of freshwater turtles, to find actively infected turtles, had not previously been done.

A molecular survey for ranaviruses in wild Australian freshwater turtles

Using methods validated through experimental infection trials in this thesis, i.e. qPCR on blood clots, a molecular survey was conducted to look for evidence of ranaviral infection in wild pleurodiran turtle populations in North Queensland, Australia. This targeted survey did not detect any active ranaviral infection (i.e., ranaviral DNA) in wild Australian freshwater turtles. Similar to other turtle species (Wirth et al., 2018), baseline levels of ranaviral infection in the Australian freshwater turtles surveyed appear to be either absent or low, despite the reported high levels of antibody prevalence in the 1990s (Ariel et al., 2017a). However, it is clear that we are still missing critical data on infection prevalence in turtle hatchling and young juveniles. These cryptic life stages are difficult to capture. The surveyed population was primarily made up of sub-adult and adult turtles, which did not develop ranaviral disease in a previous ranaviral challenge study, and therefore may not be the best subjects for a molecular survey (Ariel et al., 2015). To understand the full picture of ranaviral ecology in freshwater turtles, we need better information about wild infections in young turtles.

Epizootics form the basis of most publications concerning disease in wild freshwater turtle populations in Australia (Tucker et al., 2002; Flint et al., 2011; Ariel et al., 2017b; Wirth et al., 2020). This reactionary practice means there is a near-complete lack of baseline epidemiological data for free-living Australian freshwater turtle species, despite freshwater turtle conservation being a priority in coastal Australia (Buhlmann et al., 2009). About half of freshwater turtle species

in Australia have a conservation status of threatened or worse, and many populations are declining (Chessman, 2011; Van Dyke, Ferronato & Spencer, 2018; Van Dyke et al., 2019). Studies have found that poor recruitment is likely to or is causing declines in populations of Australian freshwater turtles (Spencer, Van Dyke & Thompson, 2017; Van Dyke et al., 2019). Nest destruction by invasive species, such as foxes, has been identified as a significant threat to Australian freshwater turtles (Spencer & Thompson, 2005; Spencer, Van Dyke & Thompson, 2017). We know that nest destruction is a problem because we have a reliable assay for it (e.g., visually noting a pillaged nest). However, it is unlikely that threats disappear once hatchlings leave the nest. We do not have reliable methods to examine the cause of death of wild hatchlings and young juvenile freshwater turtles. It is possible that despite the low level of wild infection reported in Chapter 7, infections are still occurring in hatchlings, and these infections may be contributing to the observed poor recruitment. Future studies should investigate new methods that enable the sampling of young turtles for ranaviral infection.

Conclusions and future directions

This thesis began with the question: why don't we see ranaviral infection in wild Australian turtles? Australian pleurodiran turtles do appear, overall, less susceptible to ranaviral infection than species of the suborder Cryptodira (Chapter 4; Wirth et al., 2019). However, Australian pleurodiran turtles are susceptible via multiple inoculation routes (Chapter 3; Wirth et al., 2019), the temperature associated with the highest infection levels is typical in the northern Australian environment (Chapter 5; Wirth and Ariel, 2020), and infected turtles remain PCR-positive for weeks (Chapter 6). These results suggest that despite lower susceptibility, it should be possible to detect ranaviral infection in wild turtles if it is occurring. However, if ranaviral infection is predominantly

occurring in the hatchlings of Australian freshwater turtles, then these infection events are probably missed with the current sampling methods (Chapter 7).

When starting this thesis, we had no understanding of how different environmental factors influence ranaviral disease in Australian freshwater turtles. Most Australian turtles belong to the suborder Pleurodira, but the suborder Cryptodira is the focus of the majority of turtle research (Wirth et al., 2018). Turtles in the suborder Cryptodira diverged from Australian turtles over 150 million years ago. Thus, it is inadvisable to blindly extend the results from studies on cryptodiran turtles to Australian pleurodiran turtles. By establishing Krefft's river turtles as a model for ranaviral infection, we now have a much better understanding of ranaviral infection in Australian turtles and a model with which to conduct future studies. Australia is home to a diverse set of freshwater turtles from many genera (Van Dyke, Ferronato & Spencer, 2018). Likely, ranaviral infection in Krefft's river turtles will not precisely reflect this disease in other Australian species. However, Bohle iridovirus infection in Krefft's river turtles is a more applicable model of ranaviral infection in Australian pleurodiran turtles than previous work in cryptodiran turtles (Davidson, Lindsey & Davis, 1987). Results from this Krefft's river turtle model give us a solid starting point for future research.

Wild infection is more complicated than the simple and highly controlled single factor experiments typically used to study disease; a range of varying factors interact and combine to produce diseases like ranavirosis in wild turtles. To examine all the possible combinations of the different factors examined in this thesis (i.e., three infection routes, five doses, four temperatures, and nine time-points) it would require 540 individual experiments, and this would not be practical. We cannot

capture the complexities of wild infection in simple animal models; however, we can begin to understand the influence of each factor, one at a time, by testing them independently under controlled and isolated settings.

While we have drastically increased our understanding of ranaviral infection, it is clear that our understanding of disease in wild Australian freshwater turtles (and turtles of the suborder Pleurodira as a whole) is still deficient. There are many directions in which research on ranaviral infection in Australian turtles could go from here. It is a good idea to look to what has gone before when deciding where to go next. Amphibian research has dominated the study of ranaviruses since their discovery in the 1960s (Price et al., 2017). Amphibian research progression may serve as a useful guide for future ranaviral research in turtles.

Immunology has received a significant amount of attention in the amphibian ranaviral research community (Grayfer et al., 2015). The immunology chapter from a 2015 book on ranaviruses is extensive, yet it does not mention reptiles (Grayfer et al., 2015). An immunological understanding of ranaviral infection in reptiles would help answer many of the questions generated by this thesis. We do not know what was controlling variations in infection and disease severity observed in the temperature and dose-kinetics studies. Applying the tools of immunology to this question will likely yield beneficial results. An immunological perspective will also help in other areas, for example; in understanding the role of antibodies in Australian freshwater turtles' immune response to a ranaviral infection, and the ontogeny of antibodies developed in freshwater turtle hatchlings in response to ranaviral infection.

In additional to immunology, other areas would benefit from further research. The natural transmission route for ranaviral infection in turtles remains unknown, and few transmission studies have been conducted (Brenes et al., 2014). Further research on the possible involvement of vectors (such as leeches) in the transmission of ranaviruses would be useful for understanding their role in wild infection dynamics (Kimble et al., 2014). No studies have looked at the genetic correlates of ranaviral disease in turtles. Australian turtles may be an ideal system in which to examine the evolution of disease resistance, e.g., through comparison to more susceptible species in the suborder Cryptodira. It is not clear if the patterns of resistance (e.g. ID_{50} and LD_{50}) will be the same for other Australian pleurodiran turtle species, it may be useful to conduct similar experiments to those presented in this thesis in some other Australian turtle species (e.g. *Chelodina canni*). Temperatures play an essential role in the disease of ectotherms, and ranaviral disease is no exception; it would be interesting to look into the thermoregulatory behaviours of wild Australian freshwater turtles to determine if they use behavioural fever to reduce ranaviral infection rates, as seen in amphibians (Sauer et al., 2019). Studies on stressors (such as pesticides) and ranaviral infection in turtles have been conducted in the USA and may not be relevant in the Australian environment (Wirth et al., 2018). However, the results of this thesis (e.g. the LD_{50} and ID_{50} estimates) would help in conducting such stressor studies in Australian turtles. Mesocosm experiments might be useful for studying some of these complexities in turtles and have been useful in amphibian ranaviral research (Brunner et al., 2015).

Gene association studies may also be a good starting point for a mechanistic understanding of ranaviral disease. For example, immune gene transcription levels may influence viral loads (like the differences seen in Chapter 5). Using a high throughput method such as RNA-Seq it may be

possible to find immune genes that correlate with viral loads or infection rates. Simpler models (i.e., cell culture) may be required to get a mechanistic understanding of the observed difference (Davidson, Lindsey & Davis, 1987). At the time of writing there are no complete genomes or transcriptomes for Australian turtles published on the NCBI databases (https://www.ncbi.nlm.nih.gov). High-quality, annotated genetic data for Australian turtles would be beneficial for studies on the genetic drivers of ranaviral (or any) disease in Australian turtles. They would help us to understand some of the perceived differences between turtles of the Pleurodira and Cryptodira. Future researchers should endeavour to establish genomic tools for Australian turtles.

Viral diseases present a real threat to Australian freshwater turtles, many of which inhabit a single river system. The extirpation of these turtle populations, through viral epizootics, can result in a significant loss to the species as a whole (Spencer et al., 2018). We need to know more about viral infections in turtle species to prevent further loss of essential and unique biodiversity. Effective animal models of ranaviral infection will enable researchers and environmental resource managers to mathematically model this disease more accurately in wild populations and quantify and manage the susceptibility of at-risk populations. The research presented in this thesis represents the first steps in developing an animal model for studying ranaviral disease in Australian freshwater turtle species. The Krefft's river turtle is now on track to be the standard model for studying Australian pleurodiran turtle host-pathogen interactions.

As custodians of the Earth, we humans have a duty of care to minimise our impact on the planet. Australia is home to a wide range of unique wildlife, many of them under threat of extinction. It is hoped that a better understanding of the role of diseases such as ranaviral infection in freshwater turtles, will ultimately help to manage and preserve the biota of Australia and the planet.

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Appendices

Appendix 1 - Ten years of ranavirology (2010-2019): a scientometric study

1 # Ten years of ranavirus research (2010-
2 2019): An analysis of global research
3 trends

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5

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12 Keywords: ranavirus, scientometrics, bibliometrics, collaboration

Draft

13 ## Abstract

14 Ranaviruses are large nucleocytoplasmic DNA viruses that infect ectothermic vertebrates.
15 Here we report the results of a scientometric analysis of the field of ranavirology for the last
16 10 years. Using bibliometric tools we analyse trends, identify top publications and journals,
17 and visualise the ranavirus collaboration landscape. The Web of Science core collection
18 contains 545 ranavirus related publications from 2010 to 2019, with more publications
19 produced every year and a total of 6830 citations. Research output is primarily driven by the
20 USA and People's Republic of China, who together account for more than 60% of ranavirus
21 publications. We also observed a positive correlation between the average number of co-
22 authors on ranavirus publications and the year of publication, indicating that overall
23 collaboration is increasing. A keyword analysis of ranavirus publications from 2010 to 2019
24 reveals several main areas of research interest including; ecology, immunology,
25 virology/molecular biology, genetics, ichthyology, and herpetology. While ranavirus research

26 is conducted globally, relatively few publications have co-authors from both European and
27 Asian countries, possibly because closer countries (by geographical distance) are more
28 likely to share co-authors. To this end, efforts should be made to foster collaborations across
29 geopolitical and cultural boundaries, especially between countries with shared research
30 interests as ultimately, understanding global pathogens, like ranaviruses, will require global
31 collaboration.

32 Introduction

33 Ranaviruses are a group of large double stranded DNA viruses in the genus *Ranavirus*
34 (family *Iridoviridae*), which infect ectothermic vertebrates including fish, amphibians and
35 reptiles (Gray and Chinchar, 2015). The first of these viruses was isolated in 1965, however,
36 it was not until the late 1980s that they were recognized as pathogens of ecological and
37 economic importance (Gray and Chinchar, 2015). Since their initial discovery, ranaviruses
38 have been isolated from hosts in both temperate and tropical habitats from around the globe
39 (Duffus et al. 2015).
40
41 The wide distribution of ranaviruses has led to global research interest, resulting in the
42 establishment of the Global Ranavirus Consortium (GRC). The GRC, formed in 2011, aims
43 to foster collaboration and knowledge sharing between ranavirus researchers and
44 appropriate stakeholders (Lesbarrères et al. 2011). Since its formation, the GRC has
45 organised five international symposiums on ranaviruses to bring the global ranavirus
46 research community together. Work presented at these symposiums has emphasised the
47 breadth and diversity of the ranavirus research field (Lesbarrères et al. 2011). In 2019, the
48 ISR was held in Australia and covered a range of topics including ecology, pathology,
49 immunology, and diagnostics. Although an important part of fostering research collaboration,
50 and in identifying trends and knowledge gaps, conference presentations are only a small

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51 part of the ranavirus research field and most studies are published in the peer reviewed
52 scientific literature.
53
54 Bibliometrics are a set of tools used to measure and study texts and information associated
55 with interrelated bodies of documents. Commonly, these tools are applied to the scientific
56 literature (scientometrics) for the comparative analysis of research institutes, journals of
57 publications, and individual researchers. Scientometrics can also be used for gaining an
58 understanding of the intra/interdisciplinary collaboration, trends in research topics, and
59 overall structure of a field of scientific research via the generation of bibliometrics maps.
60 Collaboration has been linked to increased impact, research output and citation rates
61 (Larivière et al. 2014; Lee and Bozeman 2005; Figg et al. 2006) and in this study, we report
62 the results of a scientometric analysis of the field of ranavirology for the past 10 years, and in
63 particular, how collaborations have moved this field forward.

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64 Methods

65 The bibliographic data for the publications used in this study were downloaded from the Web
66 of Science (WOS) core collection database using the search term ‘ranavirus’. Only
67 publications from 2010 to 2019 and reported as ‘article’ or ‘review’ were included in the
68 dataset. The filtered dataset contains 545 publications and is referred to as the WOS
69 ranavirus dataset throughout this manuscript. General statistics and figures were
70 generated/produced using Python 3 (McKinney, 2010; Seabold et al., 2010). All correlation
71 analyses were performed using a Pearson’s product-moment correlation.
72
73 Top core ranavirus publications were determined by counting the number of citations from
74 ranavirus related publications in the WOS ranavirus dataset (internal citations). Top
75 auxiliary ranavirus publications were determined by subtracting the number of internal
76 citations from the total number of citations to determine the number of citations each

77 publication received from publications outside of the WOS ranavirus dataset. To assess
78 what are the main ranavirus publication outlets and if these journals were likely to be read by
79 a large audience, we calculated the H-Index of all journals in the WOS ranavirus dataset.
80
81 To visualise research topic groups in the ranavirus field, a co-occurrence map was created
82 for all keywords, with at least five occurrences using the software package VOSviewer (Van
83 Eck and Waltman 2010). The median year of occurrence of each author keyword was also
84 determined to get an idea of research topic trends within the field, and a frequency count of
85 author keywords per year was created to determine how research interests have changed
86 overtime.
87
88 The VOSviewer software package was also used to construct collaboration maps for the
89 ranavirus field (Van Eck and Waltman 2010). Two bibliographic maps were created to
90 visualise the ranavirus field at the country level. First, co-authorship coupling was used to
91 determine which countries are collaborating. While co-authorship is not the only form of
92 collaboration, it remains a reliable and measurable indicator (Larivière et al. 2014). Second,
93 bibliographic coupling was used to determine which countries are producing publications that
94 cite the same publications in their reference lists. Shared references are assumed to
95 represent a similar research interest. Countries with less than five ranavirus related
96 publications were excluded from the visualizations (21 of the 48 ranavirus publishing
97 countries passed this threshold). Fractional counting was used to minimise bias of
98 publications with many co-authors (Perianes-Rodriguez et al. 2016). All other VOSviewer
99 parameters were left as default. To determine the effect of geographic location on co-
100 authorship, distance matrices were created for country nodes in the co-authorship map and
101 for the actual geographical coordinates of the countries; a Mantel test was used to compute
102 the Pearson's product-moment correlation between the two distance matrices.

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103 # Results

104 ## Publications and citations

105 There are 545 publications in the WOS ranavirus dataset published between 2010 and 2019
106 (average per year = 58.2, SD =15.2), with a significantly increasing number of publications
107 per year from 41 in 2010 to 86 in 2019 ($R = 0.87$, $p < 0 .001$; Figure 1). The greatest number
108 of ranaviruses publications between 2010 and 2019 came from the USA and People's
109 Republic of China (PRC) who had 199 and 153 total publications respectively, together
110 accounting for more than 60% of ranavirus related publications in that time period (Table 1).
111 Out of all the countries with more than five publications in the WOS ranavirus dataset, the
112 USA and PRC are the only countries with a strong increase in publication output (Figure 2).
113 Publications in the WOS ranaviurs dataset between 2010 and 2019 were cited a total of
114 6830 times with publications from 2010 having the highest collective citation total (1330)
115 compared to any other year (Figure 3). On average, over the last 10 years, each ranavirus
116 related publication has been cited 12.5 times (SD = 18.4, Median = 7).

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117 ## Top publications and journals

118 The H-index across all publications in the WOS ranavirus dataset was 37. Ranavirus related
119 publications in the WOS ranavirus dataset can be divided into top core (Table 2) and
120 auxiliary (Table 3) publications. The top core ranavirus publications are highly cited by other
121 ranavirus related publications in the WOS dataset. The ranavirus publication with the highest
122 number of internal citations (i.e. citations from other publications in the WOS ranavirus
123 dataset) is Miller et al. (2011, Table 2). The top ten core publication list contains several
124 reviews (e.g. Chinchar et al. 2011, Miller et al. 2011 and Whittington et al. 2010) which, due
125 to their impact, may be a useful starting resource for those interested in ranvirology. The top
126 auxiliary publications are cited by publications from outside the WOS ranavirus dataset. The

127 top auxiliary publication was Hayes et al. (2010) with a total of 176 citations in the WOS core
128 collection.
129
130 Interestingly, Fish & Shellfish Immunology was the top journal publishing ranavirus related
131 research from 2010 to 2019, with over 50 ranavirus publications in the WOS ranavirus
132 dataset (Table 4). Diseases of Aquatic Organisms (DAO) had the highest number of citations
133 (572) while the Journal of Virology had the highest average number of citations per ranavirus
134 related publication (30.0) within the top ten journals. Plos One and DAO both had a H-index
135 of 14 (i.e. 14 ranavirus publications with at least 14 citations).

136 Keywords and research topics

137 There are a large number of common keywords (n=265) used in publications of the WOS
138 ranavirus dataset (Figure 4). These keywords cluster into 5 groups that relate to core
139 concepts in the field between 2010 and 2019 (Figure 4): ecology/emerging
140 disease/amphibians (Group 1), immunology/evolution (group 2), virology/molecular biology
141 (group 3), genetics/ichthyology (group 4) and reptiles (group 5).
142
143 It is not surprising that 'ranavirus' is the most common key word used by authors in the
144 publications in the WOS ranavirus dataset. However, this is not to say the research interest
145 in this field is static (Figure 5). Areas of research (author keywords) with high median year of
146 publication include biosecurity, antiviral immunity, reptiles, etc. (Figure 5). Interests in
147 various infectious agents are changing as new species or strains of viruses get discovered.
148 For instance, the median year of publication of publications from 2010-2019 that use
149 *Epizootic hematopoietic necrosis virus* (EHNV, discovered in the 1980's) as an author
150 keyword was 2011, while the median year of publication for *Andrias davidianus ranavirus*
151 (ADRV, discovered in 2011) was mid 2018. From 2010-2019 over 50 articles have used
152 keywords relating to *Singapore grouper iridovirus* (SGIV) indicating that SGIV is an important

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153 and stable research topic. Interestingly, *Batrachochytrium dendrobatidis* (Bd), another
154 important pathogen of amphibians has appeared less frequently as an author keywords in
155 the ranavirus literature in recent years (Figure 5).

156 Collaborations and landscapes

157 Ranavirus research from 2010 to 2019 has been a global effort with authors in the WOS
158 ranavirus dataset hailing from 48 different countries and an increasing number of co-authors
159 every year (average = 5.8; $R = 0.90$, $p < 0.001$; Figure 6). Using co-author analysis, it was
160 possible to visualise the collaborative landscape of the ranavirus field (Figure 7). Central
161 nodes like the USA and England have more international collaborators, while peripheral
162 nodes (e.g. Hungary, Thailand, Finland) have a less diverse set of co-authors (Figure 7A).
163 Interestingly, there are relatively few co-authorships shared between European (green) and
164 Asian countries (red; Figure 7A). There is a positive correlation between the distance
165 between countries on the co-author map and the actual geographical distance between the
166 countries ($R = 0.338$, $p = 0.005$). For the most part, bibliographic coupling (Figure 7B) is
167 similar to the co-authorship map, suggesting that researchers with similar interests are
168 actively collaborating and refer to the same articles. However, there are a few cases where
169 research interest and co-authorship are less aligned. For example, Germany clusters with
170 the USA, PRC, Australia, Canada, etc. (red group) in the bibliographic coupling map (Figure
171 7B), but does not cluster with the same group in the co-authorship map (Figure 7A). This
172 may indicate that researchers from Germany are citing similar publications to those in the
173 red group, and thus working on similar topics, but are not necessarily working with the
174 researchers behind the publications they cite.

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175 # Discussion

176 Ranaviruses are important global pathogens of ectothermic vertebrates. The importance of
177 and interest in ranavirus research is highlighted by the steady increase in ranavirus related
178 publications over the past 10 years. We ranked the publications in the WOS ranavirus
179 dataset based on citations from other publications in the dataset to determine the core set of
180 ranavirus publications. Inversely, the broader citations in the ranavirus auxiliary publications
181 may be attributed to the fact that these publications tend to be focused on more general
182 concepts such as amphibian decline (e.g. Blaustein et al. 2012; Hayes et al. 2010). More
183 importantly, collaboration is an important component of continued productive ranavirus
184 research output and our study reveals that the average number of co-authors on publications
185 in the field has increased over the 2010-2019 period, possibly resulting in the increase in
186 research output observed (Lee and Bozeman 2005).

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187 ## Journals

188 Fish & Shellfish Immunology has 51 publications from 2010 to 2019, equating to 9.3% of the
189 total publications in the WOS ranavirus dataset. This journal is a specialised immunology
190 journal and its place among the top ranavirus journals indicates the central role of
191 immunology in this field. However, Disease of Aquatic Organisms (DAO) received the most
192 citations (572) during the same period but only represented 8.3% of the total citations for the
193 dataset. DAO was originally established in 1985 and focuses on all forms of life - animals,
194 plants and microorganisms - in marine, limnetic and brackish habitats making it an excellent
195 fit for ranavirus research that often involves aquatic species. PLOS One has the same H-
196 index as DAO (14) for publications in the WOS ranavirus dataset, and a total of 26
197 publications from 2010 to 2019. Although DAO has more publications (44), PLOS One has
198 published higher impact papers (in terms of average citations). PLOS One is an
199 interdisciplinary, open access journal that was founded in 2015 and despite its relatively

200 recent creation, it is still one of the top journals for ranavirus research. The presence of
201 these three top journals demonstrate the breadth of ranavirus research undertaken. Overall,
202 ranavirus research is published in a range journals, with different specialisations and
203 impacts. There is no clear 'ranavirus journal' regardless of the metric used i.e. most citations,
204 most publications, or H-index. The variety of journals publishing ranavirus papers may be a
205 reflection of the variety of research that occurs within the field.

206 Keywords

207 The field of ranavirology is diverse, and there is active research in many areas. Core to
208 ranavirology is the investigation of amphibian population declines and emerging infectious
209 disease; however, other areas of research (molecular biology, genetics, herpetology,
210 ichthyology, etc.) are also well represented. We also observed a cluster of keywords related
211 to reptiles in the keyword map, indicating an increasing interest in viral infections associated
212 with this clade (Wirth et al. 2018). In fact, 3 of the 10 publications in this special collection of
213 FACETS are related to ranaviral infection in reptiles.

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214

215 Between 2010 and 2019, topics of interest in the ranavirus field have been evolving. For
216 instance, changes in the interest for particular viral isolates may be an indication of
217 ecological importance of these viruses (i.e. those causing current epizootics), or simply a
218 reflection of what is new. Interest in SGIV appears to be stable and this is likely the result of
219 increased research output of researchers from Asia in the past 10 years, where SGIV is
220 used as a model system for studying iridoviruses and remains a continuing problem for
221 aquaculture in that part of the world (Qin et al. 2001, Ma et al. 2016). Interestingly, Bd has
222 been used less frequently as a keyword in the WOS ranavirus dataset in recent years. While
223 often combined in early investigations of amphibian population declines, research on these
224 pathogens is now established and ranaviruses and Bd are recognised as separate issues in
225 their own rights.

226 ## Countries and collaborations

227 Bibliographic maps are useful for understanding a scientific landscape. The USA, People's
228 Republic of China (PRC), England, Canada, and Australia were the top countries in terms of
229 total number publications between 2010-2019. The USA has been the centre of ranavirology
230 since the field originated there in 1965 with the discovery of FV3 (Granoff et al. 1965) and is
231 thus central in the bibliographic maps for collaboration and shared citations, indicating that
232 researchers from the USA are working on and collaborating on a diverse set of ranaviral
233 related questions. The USA has also hosted 3 of the 5 International Research Symposia
234 (IRS) since 2011. Due to their central location, researchers from this country should thus
235 play a major role in fostering collaborations between some of the more disparate ranavirus
236 researchers. By contrast, the PRC, despite being the second highest country in terms of
237 research output, has not had the opportunity to host a ISR. The PRC is also less central in
238 terms of the collaboration map when compared to the USA and may thus benefit from an
239 international symposium in terms of increased collaboration. We also noted a positive
240 correlation between collaborating countries and their geographical distance. Reduced
241 physical distance between collaborators would certainly lower the effort required for
242 collaboration and it is also possible that researchers are working on geographically defined
243 issues (where international collaboration is not required). However, there are cases where
244 geographically close countries are not collaborating as much as they could (i.e. Australia and
245 Asian countries). Since Ranaviruses are global pathogens, we recommend that some topics
246 (e.g. phylogenetics, biosecurity, epidemiology, etc.) can or should ultimately be addressed
247 on a global scale, requiring global collaboration.

248 ## Limitations

249 This scientometric analysis was based solely on the core collection of the Web of Science
250 database. While this feature rich database is a standard for bibliometrics analysis, it is not

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251 exhaustive and some ranavirus related references have been excluded (non-peer reviewed
252 material for example). In addition, only the term ‘ranavirus’ was used as a search term to
253 generate the dataset used in this study. We recognise that results may vary when using
254 other databases (e.g. Scopus and Google Scholar) or alternative search terms. However, we
255 chose to limit our search terms to reduce the number of false positives in the dataset, and
256 using a single database provided us with a consistent dataset. We use citations as a proxy
257 for impact and while this is a useful metric, we acknowledge that there are biases when
258 using citation metrics and newer publications may not be favoured (Seglen 1997). Thus, all
259 the conclusions presented here should be interpreted within the context of the limitations
260 defined above.

261 Conclusions

262 In the past 10 years, ranaviruses have remained an active topic of research, with more
263 publications every year. This bibliometric analysis of the ranavirus field has given valuable
264 insight into the structure of the field and how collaborations and publications have evolved
265 over the 2009-2019 period. The GRC was formed to facilitate communication and
266 collaboration among scientists, veterinarians, and others interested in ranaviruses. Our
267 study, and the collaborative nature of the articles published in this Special Collection reveal
268 the success of this endeavour but while the number of collaborators on ranavirus
269 publications have increased, there are still geographical divides in the field. Thus, efforts
270 should be made to foster collaborations across geopolitical and cultural boundaries,
271 especially between countries with shared research interests. Ultimately, maintaining and
272 promoting global research is central to understanding important global pathogens such as
273 ranaviruses.

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Table 1. Publication output from countries with more than 5 publications between 2010 and 2019 in the WOS ranavirus dataset. Total number of publications (Total). Proportion of the total global ranavirus research output (Proportion). Number of publications per year (Average per year). Country of research origin is determined by the corresponding author's address.

Country	Total	Proportion	Average per year
USA	199	37%	19.9
PRC	153	28%	15.3
Canada	32	6%	3.2
Australia	27	5%	2.7
England	22	4%	2.2
Taiwan	16	3%	1.6
Germany	14	3%	1.4
Spain	12	2%	1.2
Netherlands	8	1%	0.8
Singapore	8	1%	0.8
Belgium	6	1%	0.6

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Table 2. Top 10 core publications from 2010 to 2019 in the WOS ranavirus dataset. Publications are ranked by most citations from other publications in the WOS ranavirus dataset (Internal Citations).

	Authors	Title	Year	Journal	Citations	Internal Citations	DOI
1	Miller, D; Gray, M; Storfer, A	Ecopathology of Ranaviruses Infecting Amphibians	2011	VIRUSES	102	91	10.3390/v3112351
2	Whittington, RJ; Becker, JA; Dennis, MM	Iridovirus infections in finfish - critical review with emphasis on ranaviruses	2010	JOURNAL OF FISH DISEASES	123	74	10.1111/j.1365-2761.2009.01110.x
3	Jancovich, JK; Bremont, M; Touchman, JW; Jacobs, BL	Evidence for Multiple Recent Host Species Shifts among the Ranaviruses (Family Iridoviridae)	2010	JOURNAL OF VIROLOGY	87	68	10.1128/JVI.01991-09
4	Hoverman, JT; Gray, MJ; Haislip, NA; Miller, DL	Phylogeny, Life History, and Ecology Contribute to Differences in Amphibian Susceptibility to Ranaviruses	2011	ECOHEALTH	73	64	10.1007/s10393-011-0717-7
5	Teacher, AGF; Cunningham, AA; Garner, TWJ	Assessing the long-term impact of Ranavirus infection in wild common frog populations	2010	ANIMAL CONSERVATION	81	57	10.1111/j.1469-1795.2010.00373.x

6	Price, SJ; Garner, TWJ; Nichols, RA; Balloux, F; Ayres, C; de Alba, AMC; Bosch, J	Collapse of Amphibian Communities Due to an Introduced Ranavirus	2014	CURRENT BIOLOGY	81	54	10.1016/j.cub.2014.09.028
7	Morales, HD; Abramowitz, L; Gertz, J; Sowa, J; Vogel, A; Robert, J	Innate Immune Responses and Permissiveness to Ranavirus Infection of Peritoneal Leukocytes in the Frog Xenopus laevis	2010	JOURNAL OF VIROLOGY	67	53	10.1128/JVI.02486-09
8	Hoverman, JT; Gray, MJ; Miller, DL	Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate	2010	DISEASES OF AQUATIC ORGANISMS	58	51	10.3354/dao02200
9	Geng, Y; Wang, KY; Zhou, ZY; Li, CW; Wang, J; He, M; Yin, ZQ; Lai, WM	First Report of a Ranavirus Associated with Morbidity and Mortality in Farmed Chinese Giant Salamanders (Andrias davidianus)	2011	JOURNAL OF COMPARATIVE PATHOLOGY	69	49	10.1016/j.jcpa.2010.11.012

10	Chinchar, VG; Yu, KH; Jancovich, JK	The Molecular Biology of Frog Virus 3 and other Iridoviruses Infecting Cold-Blooded Vertebrates	2011	VIRUSES-BASEL	64	38	10.3390/v3101959

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Table 3. Top 5 auxiliary publications from 2010 to 2019 in the WOS ranavirus dataset. Publications are ranked by most citations from publications outside the WOS ranavirus dataset.

	Authors	Title	Year	Journal	Citations	Internal Citations	DOI
1	Hayes, TB; Falso, P; Gallipeau, S; Stice, M	The cause of global amphibian declines: a developmental endocrinologist's perspective	2010	JOURNAL OF EXPERIMENTAL BIOLOGY	176	5	10.1242/jeb.040865
2	Rosen, GE; Smith, KF	Summarizing the Evidence on the International Trade in Illegal Wildlife	2010	ECOHEALTH	142	1	10.1007/s10393-010-0317-y
3	Conlon, JM	The contribution of skin antimicrobial peptides to the system of innate immunity in anurans	2011	CELL AND TISSUE RESEARCH	99	2	10.1007/s00441-010-1014-4
4	Liu, CH; Chiu, CH; Wang, SW; Cheng, WT	Dietary administration of the probiotic, Bacillus subtilis E20, enhances the growth, innate immune responses, and disease resistance of the grouper, Epinephelus coioides	2012	FISH & SHELLFISH IMMUNOLOGY	88	2	10.1016/j.fsi.2012.06.012

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5	Blaustein, AR; Gervasi, SS; Johnson, PTJ; Hoverman, JT; Belden, LK; Bradley, PW; Xie, GY	Ecophysiology meets conservation: understanding the role of disease in amphibian population declines	2012	PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	84	4	10.1098/rstb.2012.0011

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Table 4. Top 10 journals from 2010 to 2019 ranked by H index of publications in the WOS ranavirus dataset.

	Journal Title	Total Publications	Total Citations	Average Citations	H Index
1	DISEASES OF AQUATIC ORGANISMS	44	572	13	14
2	PLOS ONE	26	475	18.27	14
3	FISH & SHELLFISH IMMUNOLOGY	51	554	10.86	13
4	DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY	21	276	13.14	11
5	ECOHEALTH	23	479	20.83	11
6	JOURNAL OF VIROLOGY	16	480	30	11
7	VIROLOGY	15	219	14.6	10
8	JOURNAL OF GENERAL VIROLOGY	12	162	13.5	9
9	AQUACULTURE	11	103	9.36	7
10	JOURNAL OF WILDLIFE DISEASES	19	145	7.63	7

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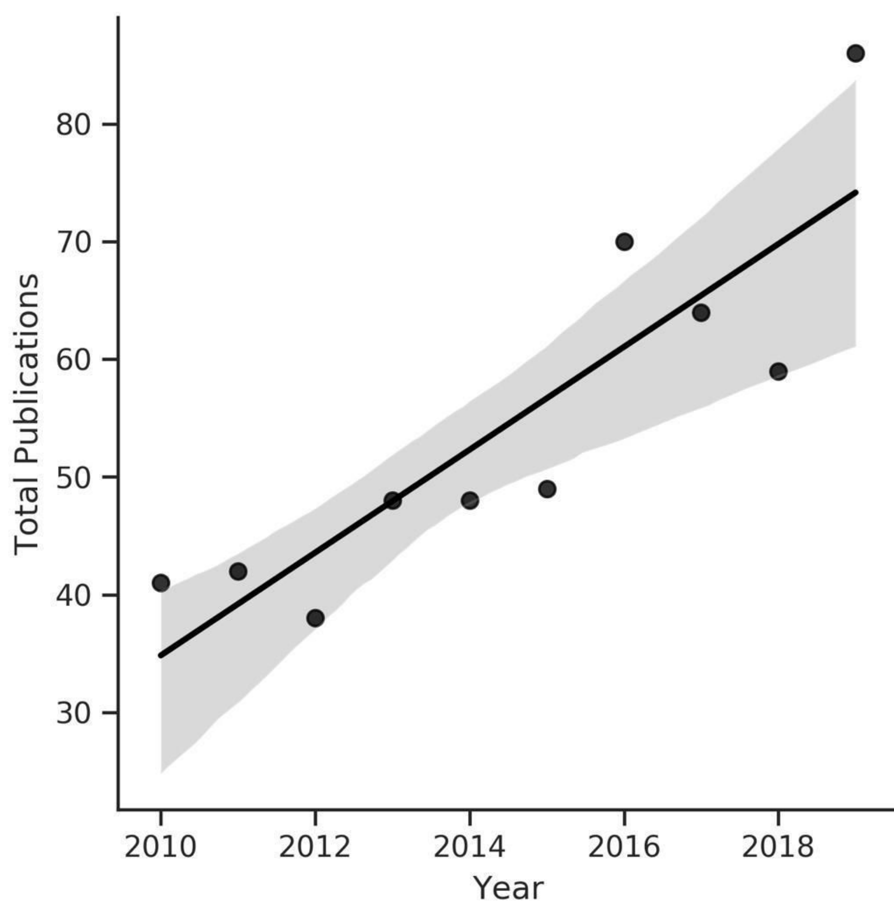

Figure 1. Total number of publications per year in the WOS ranavirus dataset.

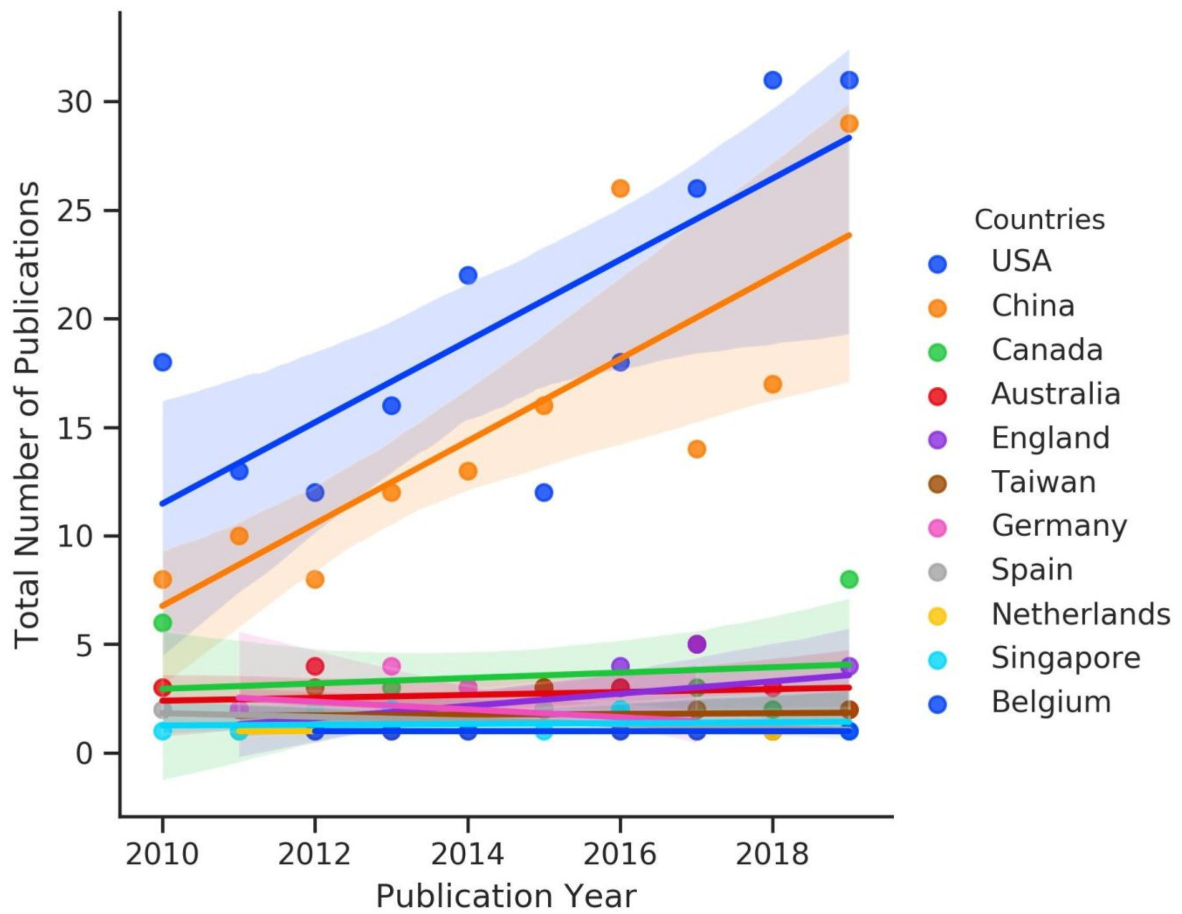

Figure 2. Total number of publications per year per country in the WOS ranavirus dataset. Only countries with greater than 5 ranavirus related publications between 2009 and 2019 are included.

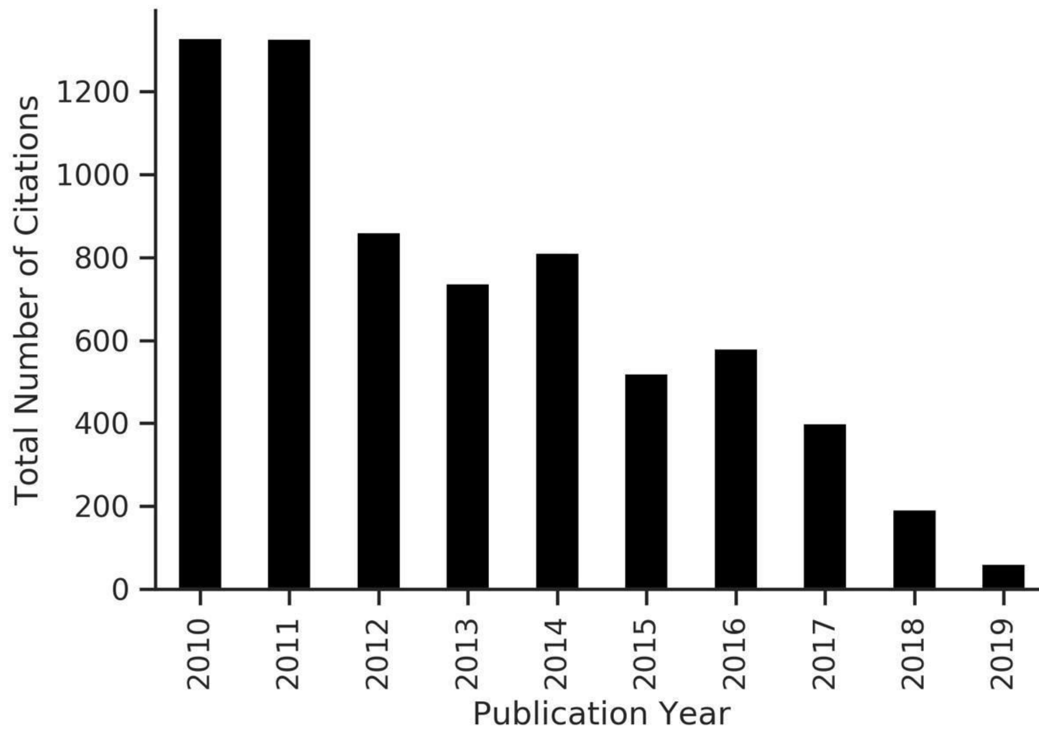

Figure 3. Total number of citations for publications from 2010 to 2019 in the WOS ranavirus dataset.

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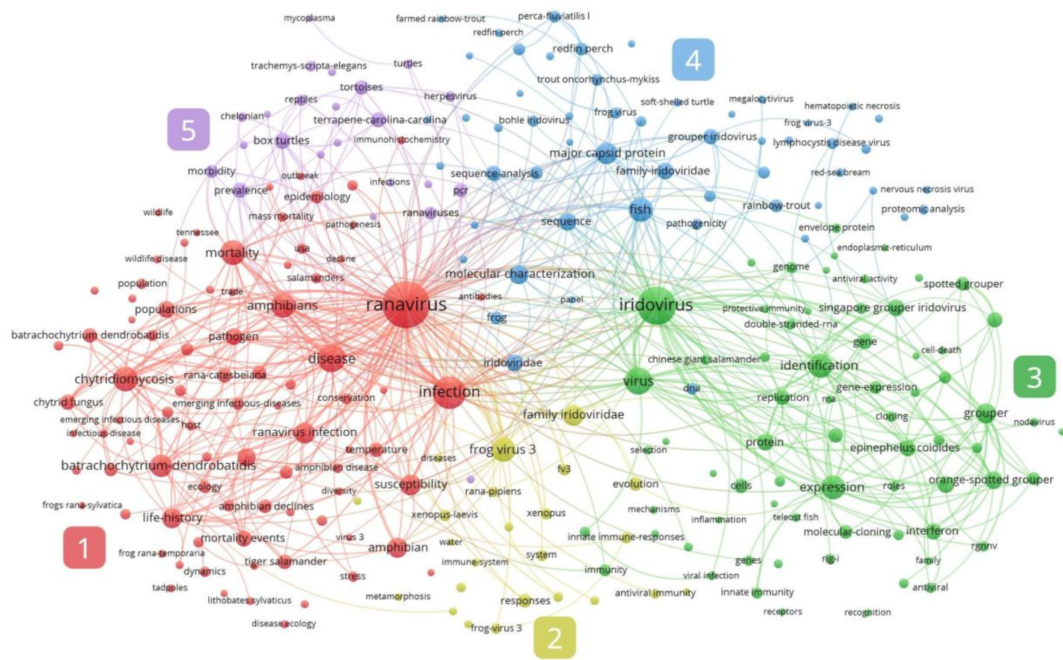

Figure 4. Co-occurrence map of all keywords from 2009 to 2019. There are 256 keywords with at least 5 occurrences each. Size of the nodes indicates the number of occurrences of that word. The lines indicate the strength of association i.e. how likely words are to occur together in the same article. The map is clustered into five related groups. Group 1: ecology/emerging disease/amphibians (red), group 2: immunology/evolution (yellow), group 3: virology/molecular biology (green), group 4: genetics/ichthyology (blue), group 5: reptiles (purple).

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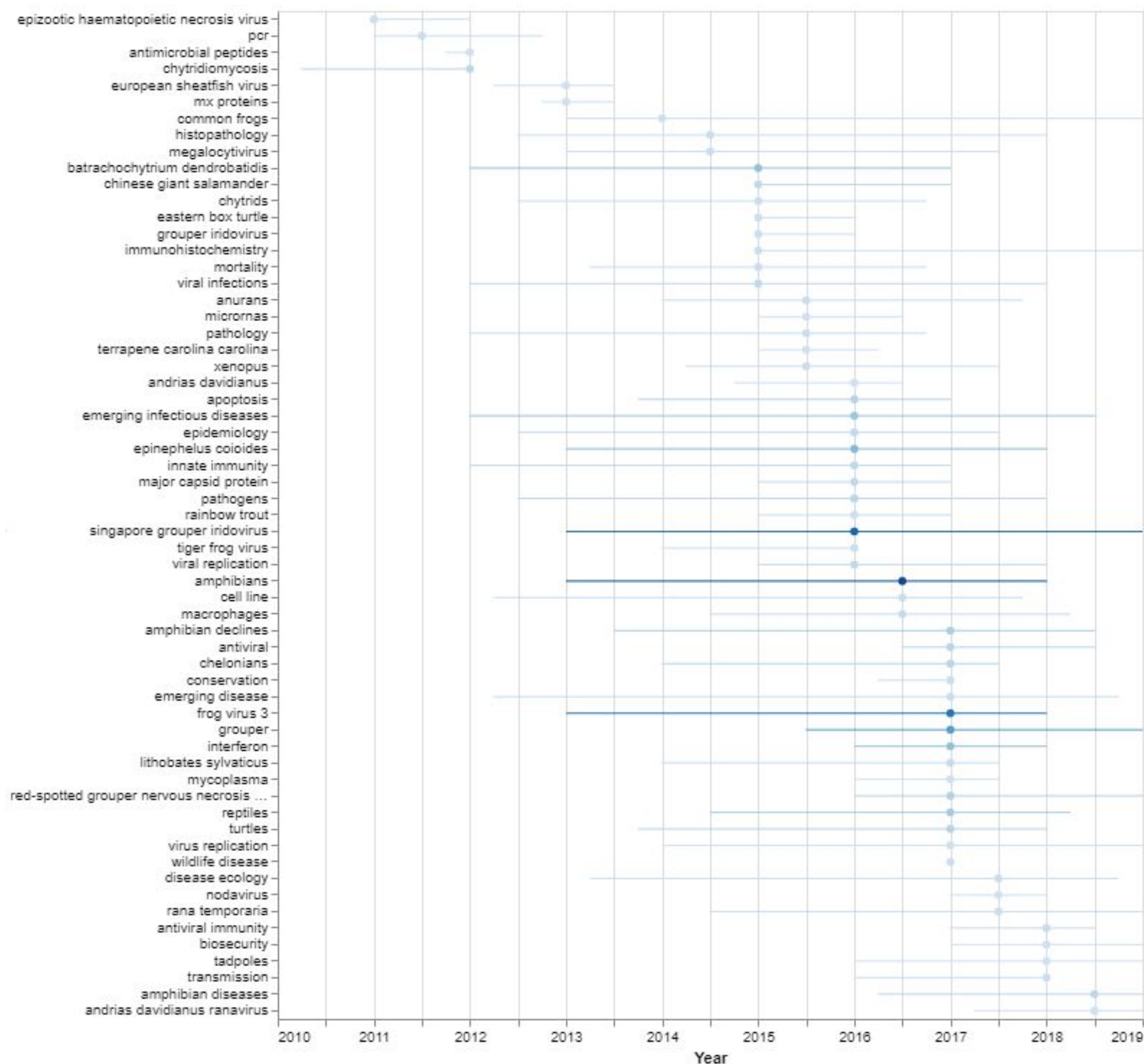


Figure 5. Trends in author keywords from 2010 to 2019. The dots indicate the median year of publication for publications using that keyword and the error bars are the interquartile range. Darker colours indicate more occurrences of that keyword. Only keywords with more than 5 occurrences have been included. The keywords 'ranavirus', 'ranaviruses', 'iridoviridae', 'iridovirus', and 'disease' were removed to improve the visualization.

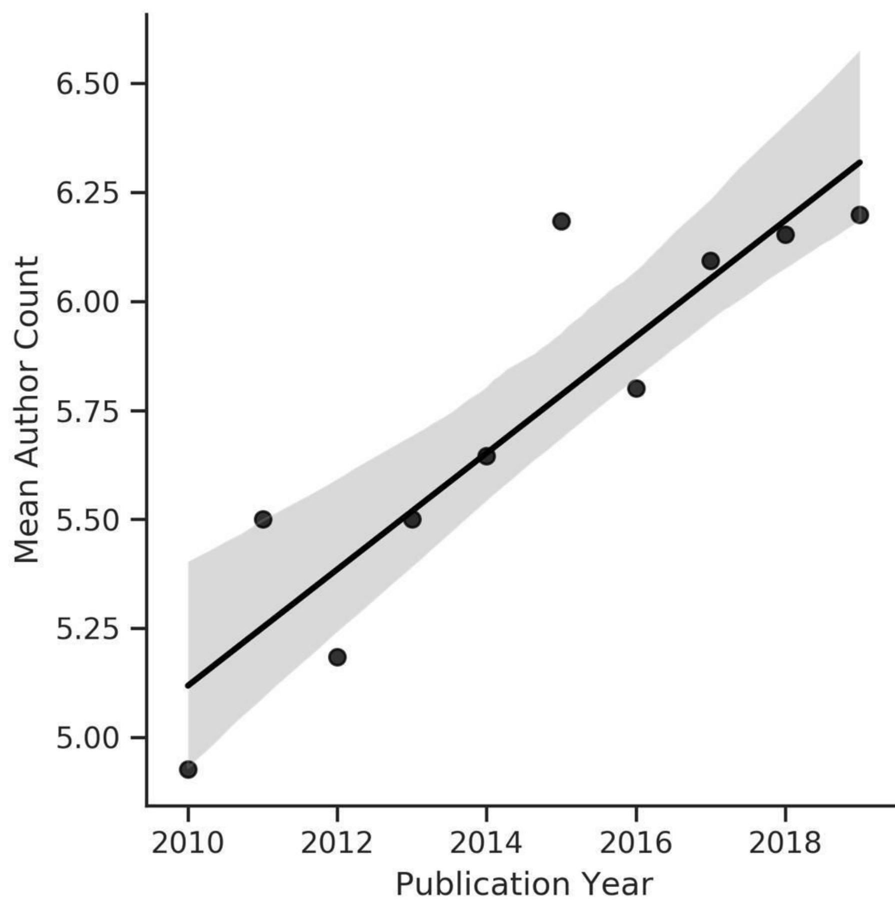
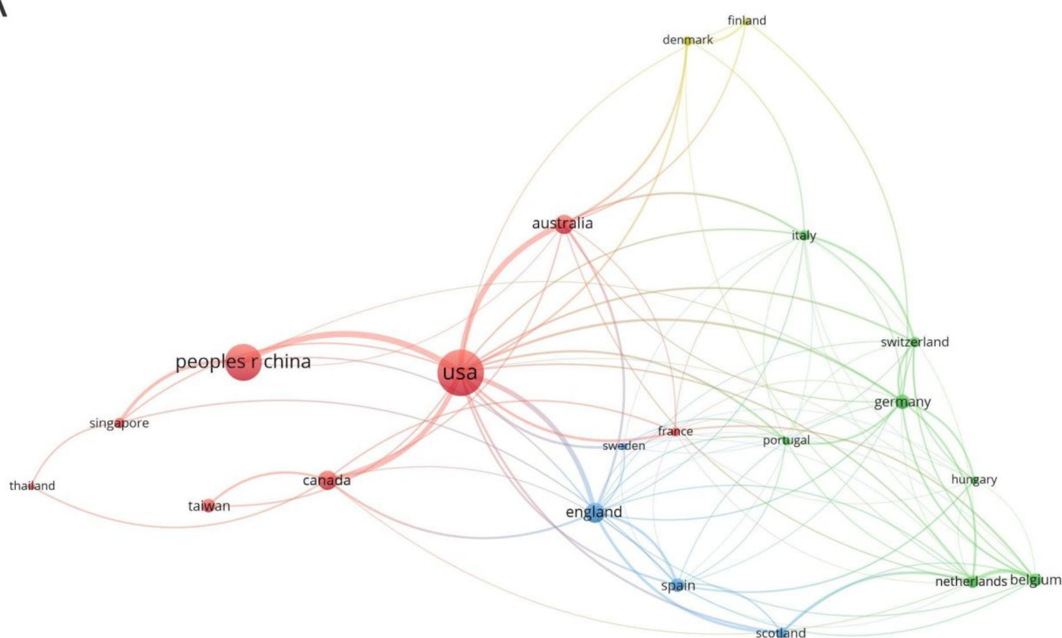


Figure 6. Average number of authors per year on publications in the WOS ranavirus dataset.



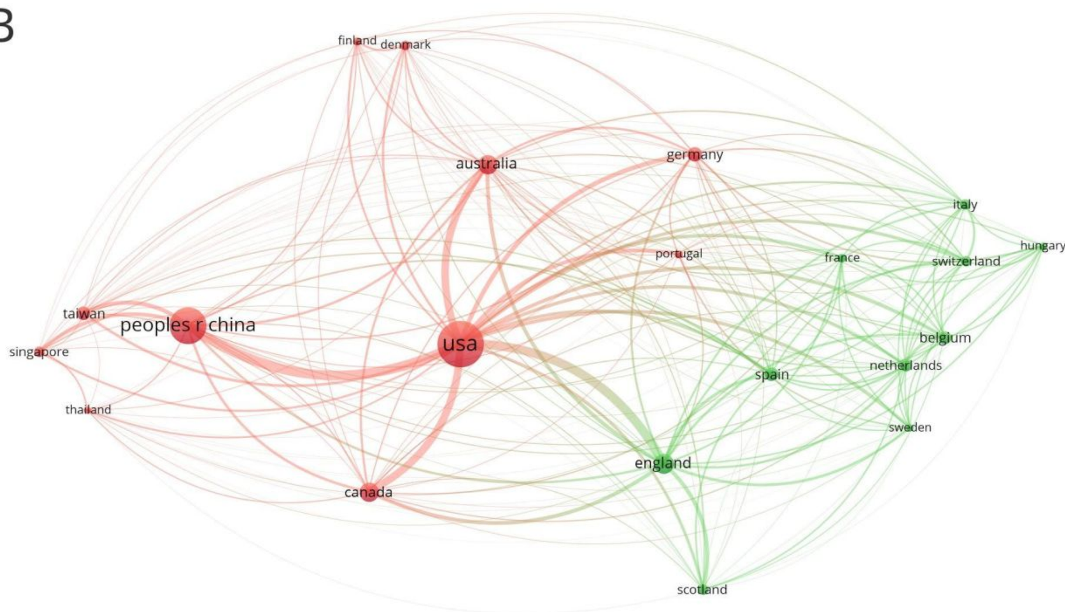


Figure 7. Relationships between countries with highest research output from 2010 to 2019. The countries are denoted by the circular node with different colours indicating clusters. The size of the nodes denotes the number of publications from the country. The countries are connected by lines with different widths, indicating the strength of the association between two nodes. (A) Collaboration coupling, the degree of relatedness is determined by the number of shared co-authors. (B) Bibliographic coupling, the degree of relatedness is determined by the number of shared references.

Appendix 2 - Ranaviruses and reptiles

PeerJ

Ranaviruses and reptiles

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ABSTRACT

Ranaviruses can infect many vertebrate classes including fish, amphibians and reptiles, but for the most part, research has been focused on non-reptilian hosts, amphibians in particular. More recently, reports of ranaviral infections of reptiles are increasing with over 12 families of reptiles currently susceptible to ranaviral infection. Reptiles are infected by ranaviruses that are genetically similar to, or the same as, the viruses that infect amphibians and fish; however, physiological and ecological differences result in differences in study designs. Although ranaviral disease in reptiles is often influenced by host species, viral strain and environmental differences, general trends in pathogenesis are emerging. More experimental studies using a variety of reptile species, life stages and routes of transmission are required to unravel the complexity of wild ranavirus transmission. Further, our understanding of the reptilian immune response to ranaviral infection is still lacking, although the considerable amount of work conducted in amphibians will serve as a useful guide for future studies in reptiles.

Subjects Veterinary Medicine, Virology
Keywords Ranavirus, Reptiles, Taxonomy, Bibliometrics, Diagnostics, Pathology, Transmission, Reservoirs, Susceptibility, Immunology

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Additional Information and Declarations can be found on page 16

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INTRODUCTION

Ranaviruses (family *Iridoviridae*) are emerging lethal pathogens of ectothermic vertebrates. First discovered in 1965 (*Granoff, Came & Rafferty, 1965*), ranaviruses were initially studied for their interesting molecular biology but rose to reportable pathogen status as more epizootics were discovered (*Schloegel et al., 2010*; *Gray & Chinchar, 2015*). The vast majority of research on the genus *Ranavirus* has been conducted in amphibians (*Rana* is Latin for frog), but despite their name, ranaviruses do not occur only in amphibians (*Chinchar & Waltzek, 2014*). This group of viruses infects over 175 species of ectothermic vertebrates; including reptile species from at least 12 different families (*Duffus et al., 2015*). Temperature appears to be the major factor preventing ranaviral infection outside of ectothermic vertebrates; these viruses can replicate in mammalian cell lines, but only when the temperature is below 32 °C (*Gray & Chinchar, 2015*).

Many advances in the field of ranavirology have been made since the discovery of ranaviruses; however, for the most part, this research is specific to amphibians. Reptiles and amphibians are very different physiologically and although they sometimes share habitats, their ecology is different. Some results from one host group can translate to the

other; however, there is no substitute for host-specific research. As ranavirus research continues, it is important to focus efforts on all Classes of hosts, including reptiles.

Since the initial report of ranaviruses in reptiles in the early 1980s (*Heldstab & Bestetti, 1982*), infections have been reported in wild and captive reptiles, and the number of reports continues to grow, representing an increasing problem for reptiles (*Marschang, Stöhr & Allender, 2016*). In this review, we summarise findings in all areas of reptilian ranavirus research. We identify major gaps in this field of knowledge and include recommendations for future research directions.

SURVEY METHODOLOGY

To ensure this review included as many publications focusing on ranaviruses and reptiles as possible, an extensive search of multiple databases using broad search queries was conducted. Databases used in the search strategy included: Web of Science, PubMed, and Google Scholar. The search strategy included keywords such as 'ranavirus' and 'reptiles' and their conjugations as well as more specific terms such as 'turtle', 'lizard', and 'snake'. To broaden the search further, references for articles found in the initial database search were then assessed for content relating to ranaviruses and reptiles. As a baseline for general ranavirus literature, relevant references were extracted from the 2015 *Ranavirus* book (*Gray & Chinchar, 2015*).

TAXONOMY

Ranaviruses are large (~150 nm), nucleocytoplasmic viruses with icosahedral virions and double-stranded DNA genomes that contain approximately 100 genes (*Jancovich et al., 2015*). *Ranavirus* is a genus in the family *Iridoviridae*: a group of five related viral genera. Of the five *Iridoviridae* genera, only ranaviruses cause significant disease in wild reptiles.

The taxonomy of the genus *Ranavirus* is changing; as more viruses are isolated and sequenced a clearer picture of the phylogenetic distribution of this group is developing. The international committee on taxonomy of viruses (ICTV) currently recognises eight species in the genus *Ranavirus* (*Lefkowitz et al., 2018*), none of which were originally isolated from reptiles. The official ICTV process of species recognition takes time and coordination within the scientific community. Many isolates, including isolates from reptiles, remain unclassified (*Chinchar et al., 2017*).

The current phylogeny of the genus *Ranavirus* can be subdivided into five major lineages based on comparison of conserved genes (*Claytor et al., 2017*; *Jancovich et al., 2015*; *Stöhr et al., 2015*; *Price et al., 2017*). No *Ranavirus* lineage exclusively infects reptiles, and the majority of reptile infections appear to originate from putative amphibian specialist viruses (*Price et al., 2017*). The factors that control the host specificity of these viruses remain unknown. Phylogenetic analyses of sequences from different reptilian and amphibian viruses have revealed that viruses found in reptiles are often more closely related to amphibian ranaviruses from the same geographical region than to each other (*Stöhr et al., 2015*). This provides support for the hypothesis that the jump into reptile hosts is relatively recent and has occurred multiple times (*Jancovich et al., 2010*, *Stöhr et al., 2015*).

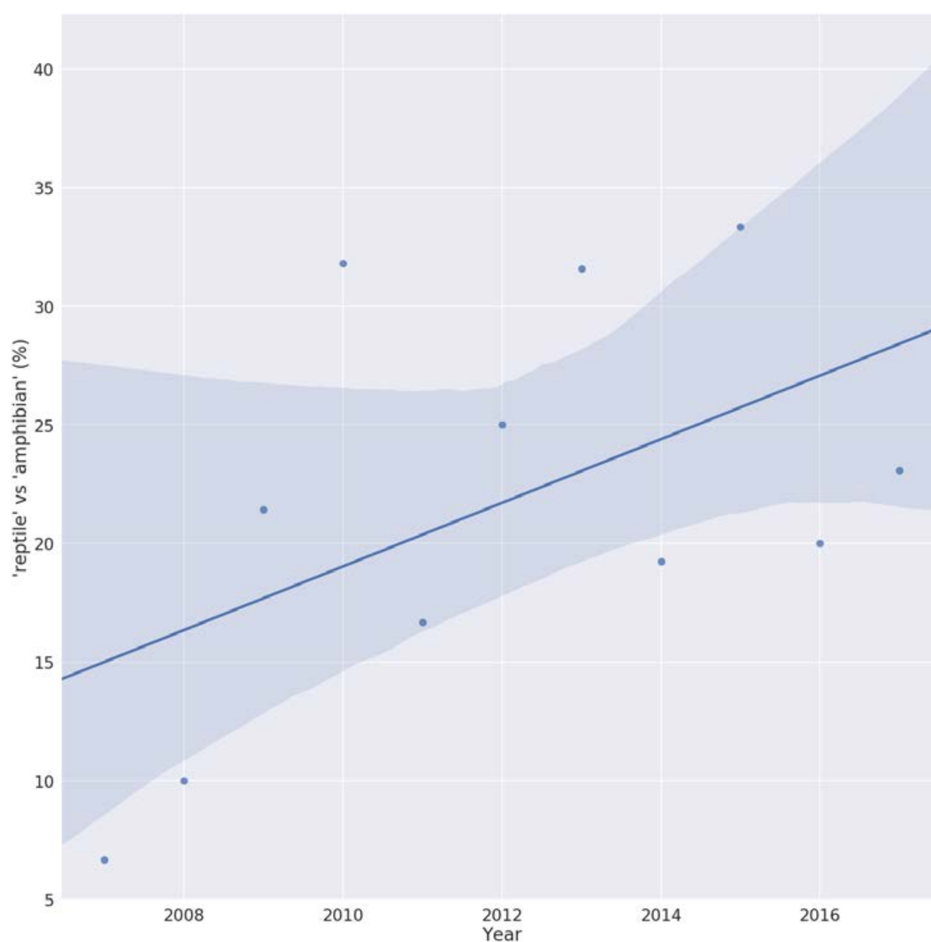
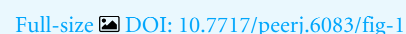


Figure 1 Trend in the number of ranavirus papers referring to reptiles. Ratio of ranavirus papers (n = 449) using the terms 'reptile' vs 'amphibian' in their title or abstract, showing the increase in the relative percent of publications referring to reptiles. A value of 100% would indicate the same number of Ranavirus papers use the term 'reptile' as 'amphibian'. Solid line is the linear trend line fitted with 95% confidence interval (shaded area).


Full-size DOI: 10.7717/peerj.6083/fig-1

BIBLIOMETRICS

Despite a lack of host specificity, the vast majority of ranaviral literature is on amphibians. As of February 2018, 449 references were returned when the Web of Science ™ database was queried for the topic 'ranavirus'. Of these, over 200 used the term 'amphibian' in their title or abstract while fewer than 60 used the term 'reptile'. However, plotting the usage of these terms over the last 10 years shows a steady increase in the ratio of 'reptile' to 'amphibian' terms, possibly reflecting an increase in reptilian ranavirus research or an increased awareness of the role of reptiles in this disease (Fig. 1).

DIAGNOSTICS AND SURVEILLANCE

The World Organization for Animal Health (OIE) provides guidelines for diagnostic methods in their Diagnostic Manual for Aquatic Animal Health (*OIE, 2012*), and *Miller et al. (2015)* summarised the diagnostic techniques used in ranaviral research. The most commonly used methods to confirm the presence of a ranavirus in host samples have included electron microscopy, enzyme-linked immunosorbent assays (ELISAs), viral isolation, immunohistochemistry (IHC), DNA amplification using polymerase chain reaction (PCR), and more recently, next generation sequencing. All of these techniques have been used at some stage in the study of ranaviruses from reptiles; however, the selection of diagnostic technique is highly dependent on the resident expertise in the laboratory, the data required, and the type of study (*Miller et al., 2015*). Before their application in new hosts or against new pathogens, all diagnostic techniques should be thoroughly tested and optimised with appropriate controls (e.g., different species of reptiles or ranaviruses, or both) (*Wobeser, 2007*).

Most ranaviruses can be grown using commercially available fish cell lines (*Miller et al., 2015*). Reptile cell lines such as Russell's viper heart cells, gecko lung cells, turtle heart, and iguana heart cells have also been used successfully to isolate ranaviruses from reptiles (*Hyatt et al., 2002*; *Johnson et al., 2008*; *Alves de Matos et al., 2011*).

Serology

Serological surveys, employing various ELISAs, have been used to assay reptiles for anti-ranaviral antibodies (*Johnson et al., 2010*; *Ariel et al., 2017*). Although these ELISA-based surveys have successfully detected anti-ranaviral antibodies in wild and captive chelonians and experimentally infected reptiles, the utility of such surveys is not clear due to the inconsistency of sero-conversion after ranaviral infection in reptiles. A captive group of chelonians, with a history of iridovirus outbreak, had a low proportion of seropositive individuals, and wild populations of North American chelonians were shown to have low sero-prevalence (*Johnson et al., 2010*). Experimentally-infected Australian reptiles do not show consistent patterns of sero-conversion, although wild populations can have high levels of antibodies (*Ariel et al., 2017*). Sero-surveys of large aquatic reptiles, such as freshwater turtles, could be useful as an indication of ranavirus occurring in freshwater environments but they would likely underestimate the true prevalence of exposure unless the accuracy of the test is determined (*Ariel et al., 2017*; *Johnson et al., 2010*). Exposed animals may fail to sero-convert or die before they are surveyed. Reptile antibody titres vary seasonally (more antibodies are produced in the warmer months), which must be taken into account when determining sensitivity and specificity cut-off values for diagnostic tests (*Wobeser, 2007*; *Zimmerman, Vogel & Bowden, 2010*; *Meddings, 2011*). Seasonal variation of anti-ranaviral antibodies has not been assessed. Using total IgY levels as an internal control may minimise diagnostic errors resulting from seasonal variations in antibody levels.

Polymerase chain reaction

PCR-based assays have been used conventionally and in quantitative real-time assays to detect reptilian ranaviral DNA in a number of sample types including blood, oral and cloacal

swabs, and fresh and fixed tissues (*Pallister Gould et al., 2007*; *Allender, Bunick & Mitchell, 2013*; *Allender et al., 2013a*; *Goodman, Miller & Ararso, 2013*; *Butkus et al., 2017*; *Leung et al., 2017*; *Maclaine et al., 2018*). Molecular surveys of turtle populations for ranavirus have revealed that swabs and blood samples are not equally valid targets for ranavirus detection (*Allender et al., 2013a*). *Goodman, Miller & Ararso (2013)* also found that oral cloacal swabs were not as effective for ranavirus detection when compared with tail clip tissue samples. Given possible differences in sample type sensitivity, it would seem advisable to collect multiple samples (e.g., both blood and swabs) when conducting a molecular survey for reptilian ranaviruses (a method employed in many studies). It is also possible to use bone marrow as a source of DNA for ranavirus detection from reptile carcases in which other viable tissue samples may have decayed (*Butkus et al., 2017*).

The preferred target of ranaviral PCR assays is the major capsid protein (MCP) gene as it is highly conserved throughout the ranaviral lineage (*Miller et al., 2015*). Because of the multispecies host range of most reptile ranaviruses, reptile-specific PCR assays are not required. Many different PCR assays have been used in reptile studies; generally the assay of choice depends on the question asked. PCR assays with broad targets such as those from *Mao, Hedrick & Chinchar (1997)* have been used in surveys (*Goodman, Miller & Ararso, 2013*) and more specific, probe-based PCR assays have also been used in surveys of reptiles (*Allender et al., 2013a*). Probe-based assays generally sacrifice broad detection range for increased sensitivity (*Allender, Bunick & Mitchell, 2013*). Sensitive probe-based assays have also been used in experimental infections with a known viral target to determine levels of infection (*Allender et al., 2013b*). A new qPCR assay developed by *Leung et al. (2017)* should provide more accurate viral load determination by using an internal host control DNA target conserved in reptiles. Large product PCR assays have been used in phylogenetic studies of reptilian ranaviruses (*Stöhr et al., 2015*). As the cost of sequencing decreases, it is becoming increasingly popular to use high throughput sequencing methods to more accurately identify and characterise viral isolates (*Hick et al., 2016*; *Subramaniam et al., 2016*).

Environmental DNA (eDNA) -based detection may be an effective method for assessing the presence of ranaviral DNA in populations of aquatic reptiles. Ranavirus outbreaks in aquatic frog populations have been detected using eDNA PCR methods (*Hall et al., 2016*). Aquatic reptiles (Testudines) with ranaviral infections can shed virions into their surroundings, indicating that eDNA detection may be possible, although no publication has yet confirmed this *in situ* (*Brenes et al., 2014a*). Mosquitoes may be useful targets for detecting ranaviruses in reptile populations. *Kimble et al. (2014)* found ranaviral DNA in mosquitoes associated with a box turtle epizootic. Ranaviral PCR testing of mosquitoes (xenosurveillance) could be combined with DNA barcoding to determine the origin of the mosquito blood meal as well as the presence of ranavirus (*Bitome-Essono et al., 2017*).

Antigen assays

Immunohistochemistry (IHC) has been successfully used in reptilian ranaviral studies to visualise the location of the viral protein in tissue samples (*Hyatt et al., 1991*; *Ariel et al., 2015*). Unlike PCR, IHC targets proteins in histological sections of tissues. Ranaviral IHC

assay results combined with histopathology can be used to correlate pathogenesis with the location of viral antigens (*Becker et al., 2013*; *Ariel et al., 2015*; *De Jesús Forzán et al., 2015*). *Ariel et al. (2015)* used cross-reactive polyclonal anti-EHNV antibodies to detect BIV antigens in infected turtle tissues. They found IHC staining associated with vascular endothelial cells, possibly indicating that viraemia preceded the systemic infection observed in these animals.

Another antigen assay has used anti-ranaviral monoclonal antibodies in a double antibody sandwich ELISA to detect viral particles in soft-shelled turtles (*Zhang et al., 2010*). In this case, the virion was detected with 98% specificity when compared with conventional PCR as the gold standard.

Because of the variability in ranaviral disease signs and severity within and among reptile species (see pathology section), suspected cases of ranaviral disease must be confirmed with laboratory diagnostic techniques. Epidemiological surveys must be adequately designed and powered to ensure ranavirus prevalence is accurately reported (*Gray & Chinchar, 2015*). There have been several studies reporting the negative results of epidemiological surveys (*Hanlon et al., 2016*; *Kolesnik, Obiegala & Marschang, 2017*; *Winzeler et al., 2018*). These results are extremely valuable as they also help describe the distribution and emergence patterns of reptilian ranaviruses; however, it is important to consider the sampling protocols and diagnostic choice when evaluating and comparing epidemiological studies (*Gray & Chinchar, 2015*).

DISTRIBUTION, HOST RANGE AND IMPACT

Ranaviruses capable of infecting reptiles have been found on all continents, except Antarctica (*Duffus et al., 2015*). Ranaviruses have been detected in over 12 families of the orders Testudines (turtles, tortoises and terrapins) and Squamata (lizards and snakes). It is important to note that given reptile populations often share habitat with susceptible fish and amphibian species, it may be possible to infer reptile ranavirus distributions based on amphibian ranaviral prevalence patterns and *vice versa* (*Duffus et al., 2015*). It is not clear if reptilian ecology influences patterns of ranavirus host range or susceptibility. Aquatic reptiles may be more likely to be exposed to ranaviruses; however, ranaviruses are still detected in terrestrial reptiles (*Duffus et al., 2015*). There is some evidence aquatic turtles are less susceptible to ranaviral disease; however, this is far from settled and should be investigated further (*Brunner et al., 2015*). Recently, *Adamovicz et al. (2018)* reported that the use of moist microhabitats is correlated with ranavirus detection in eastern box turtles.

Testudines (turtles, tortoises and terrapins)

Koch's postulates have been confirmed in Testudines with infection and disease demonstrated in red-eared sliders (*Trachemys scripta elegans*) and box turtles (*Terrapene ornata ornata*) infected with a Burmese star tortoise *Ranavirus* isolate (*Johnson, Pessier & Jacobson, 2007*). The first reported cases of ranaviral infections in Testudines were identified microscopically during the 1980s in Hermann's tortoises (*Testudo hermanni*) in Switzerland (*Heldstab & Bestetti, 1982*). Following this, ranaviruses were predominantly

isolated from box turtles (*Terrapene carolina*) and were identified as the aetiological agent of 'red neck disease' in the soft-shelled turtle (*Pelodiscus sinensis*) (*Chen, Zheng & Jiang, 1999*). In the last decade, several new reports of ranaviral infections in Testudines have been published (*Johnson et al., 2008*; *Johnson et al., 2010*; *Belzer & Seibert, 2011*; *Allender, 2012*; *Stöhr et al., 2015*; *Perpiñán et al., 2016*; *Butkus et al., 2017*; *Agha et al., 2017*; *Archer et al., 2017*; *Adamovicz et al., 2018*). Despite the increasing number of reports of infections in the Testudines, ranaviral disease in these reptiles is still likely to be underreported due to a lack of awareness, an incomplete understanding of the pathology caused by the disease, few long-term studies, and minimal population monitoring (*Duffus et al., 2015*). Sea turtles are a group of reptiles that have received little attention from ranavirus researchers, despite the existence of ranavirus infections in marine fish (*Whittington, Becker & Dennis, 2010*).

Squamata (lizards and snakes)

The first reports of ranaviral infection in squamates came after several green tree pythons were seized during an attempt to illegally import them into Australia from Indonesia. *Hyatt et al. (2002)* reported that these snakes were infected with an FV3-like ranavirus isolate. In 2005, *Marschang, Braun & Becher (2005)* reported the first ranaviral infection in lizards. The reports of ranavirus infections in squamates have been, for the most part, restricted to groups of captive lizards, providing little evidence of the role of ranavirus infection in wild squamate populations (*Stohr et al., 2013*; *Behncke et al., 2013*; *Stöhr et al., 2015*; *Tamukai et al., 2016*). Although no epizootics have been reported, ranaviral DNA and seropositive animals have been detected in wild squamate populations (*Alves de Matos et al., 2011*; *Ariel et al., 2017*; *Goodman, Hargadon & Carter, 2018*).

Rhynchocephalia (tuatara), Archosaurs (crocodiles, birds)

There have been no documented cases of ranavirus infections in animals from the other groups of the class Reptilia, namely the Rhynchocephalia (tuatara) and the archosaurs (crocodilians and birds). The tuatara only inhabit parts of New Zealand, and although ranaviruses are believed to be present (i.e., short-finned eel ranavirus, *Bovo et al., 1993*), no studies have been published on the presence of ranavirus in tuatara. While yearling Australian freshwater crocodiles (*Crocodylus johnstoni*) were exposed to ranavirus (BIV) under laboratory conditions, this challenge did not cause any adverse effects in the yearlings and the virus could not be re-isolated (*Ariel et al., 2015*). A serosurvey of wild freshwater crocodiles did show evidence of anti-ranaviral antibodies, indicating that wild populations are likely exposed (*Ariel et al., 2017*). It is important to continue to study apparently resistant species, like crocodiles, as they may give insights into the determinants of immunity. Birds and reptiles are closely related; crocodiles are genetically more closely related to birds than they are to lizards. There are no reports of birds infected by ranaviruses (this is probably related to endothermy); despite this, birds may still play a role in ranaviral transmission. It has been hypothesised that migratory birds, acting as mechanical vectors, are responsible for some of the geographic transmission of ranaviruses (*Whittington et al., 1996*).

PATHOLOGY

The clinical signs and pathogenesis of natural ranaviral infection in reptiles can be extremely variable. Mortality during an epizootic can range from 0–100% and the effect on a host can vary from quite mild to extremely severe, requiring immediate veterinary attention or euthanasia (*Miller et al., 2015*). There is evidence that reptiles can also be asymptomatic carriers of ranaviruses (*Stohr et al., 2013*; *Goodman, Hargadon & Carter, 2018*). Quiescent viral reactivation in amphibians that have recovered from infection is possible; however, the same is not known for reptiles (*Robert et al., 2014*). The complex presentation and inconsistency in the pathogenesis of ranaviral infection in reptiles may occur because of the influence of host physiology and life history, and varying degrees of viral virulence, stressors, and temperatures acting on the course and outcome of infection (see Susceptibility section).

Descriptions of pathogenesis in reptiles infected with a variety of ranaviral strains in several host species under experimental, wild, and captive conditions are presented in Table 1. Despite differences in descriptions of pathogenesis and the fact the reports are often confounded with co-infections (*Sim et al., 2016*; *Adamovicz et al., 2018*), some common patterns of ranaviral pathogenesis have emerged.

General lethargy and inappetence are associated with many cases of ranaviral infection in reptiles; however, such clinical signs are common to many diseases and are not pathognomonic for ranaviral infection. Turtles often present with respiratory signs, including nasal and oral discharge (*Johnson, Pessier & Jacobson, 2007*; *Johnson et al., 2008*; *Allender et al., 2013b*; *Kimble et al., 2017*). Oedema, especially of the eyes or neck, is also commonly associated with this infection in the order Testudines (*Chen, Zheng & Jiang, 1999*; *Johnson, Pessier & Jacobson, 2007*; *Johnson et al., 2008*; *Allender et al., 2013b*). The clinical signs of ranaviral infection in Squamates are scarcely described. This is partially due to the lack of experimental infection trials in this group, which would help describe pathogenesis markers. *Maclaine et al. (2018)* recently demonstrated the susceptibility of an Australian lizard species (*Intellagama lesueurii lesueurii*) to ranaviral infection, documenting that clinical signs and histopathological changes varied with inoculation route. With increasing descriptions of ranaviral infected lizards over the last decade, an emerging trend suggests that skin lesions may be a common occurrence (*Behncke et al., 2013*; *Stohr et al., 2013*; *Tamukai et al., 2016*).

Ranaviral infections are systemic, and there is often extensive damage to multiple organs during infection, especially the liver and spleen in reptiles. Liver lesions are also very common in the pathogenesis of ranaviruses in amphibian and fish species (*Miller et al., 2015*). Histopathology is frequently characterised by inflammation and multifocal necrosis in multiple organs, and is often associated with hematopoietic tissue (*Ariel et al., 2015*). Reptilian hosts of ranaviruses experience a range of histological changes including necrosis and inflammation of the respiratory tract, pneumonia, conjunctivitis, stomatitis, esophagitis, tracheitis, necrosis of endothelial cells and the submucosa of the gastrointestinal tract, glomerulonephritis, multifocal hepatic necrosis, splenitis, intracytoplasmic inclusion bodies in many tissues, and necrotizing myositis (see Table 1). Evidence from epizootics in reptiles indicates that ranaviral infection can be accompanied by secondary pathogens

Table 1 Representative reptilian ranaviral pathogenesis. This table includes only cases of moribund reptiles where sufficient clinical description was given.

Reference	Order	Family	Genus	Species	Population	Clinical signs						Pathogenesis									
						Behaviour	Oral	Nasal	Ocular	Skin	Other signs	Skin	Oral cavity	Gastrointestinal tract	Upper respiratory tract	Lower respiratory tract	Liver	Spleen	Kidney	Pancreas	Muscle
Behncke et al. (2013)	Squamata	Agamidae	*Japalura*	*splendida*	Captive/Wild	CN, DA				L							H, N		N		
Stöhr et al. (2013)	Squamata	Agamidae	*Pogona*	*vitticeps*	Captive					L		I									I
Tamukai et al. (2016)	Squamata	Agamidae	*Pogona*	*vitticeps*	Captive	DA				L, U		I, N, U									
Maclaine et al. (2018)	Squamata	Agamidae	*Intellagama*	*lesueurii lesueurii*	Experimental	An, CN, DA				L, U	Abdomen swelling	N			H, I		N	H, I, N	N		
Stöhr et al. (2013)	Squamata	Anguidae	*Ophiosaurus*	*gracilis*	Captive					L		I, U									
Stöhr et al. (2013)	Squamata	Dactyloidae	*Anolis*	*sagrei*	Captive	DA				L		I, N									
Stöhr et al. (2013)	Squamata	Dactyloidae	*Anolis*	*carolinensis*	Captive					I, U		I, U									
Marschang, Braun & Becher (2005)	Squamata	Gekkonidae	*Uroplatus*	*fimbriatus*	Captive	An							I, N, U				N	I			
Stöhr et al. (2013)	Squamata	Iguanidae	*Iguana*	*iguana*	Captive					L											
Hyatt et al. (2002)	Squamata	Pythonidae	*Morelia*	*viridis*	Captive/Wild	An, DA	U						N	I	I, N	I	N	N	N		N
Duffus et al. (2015)	Squamata	Pythonidae	*Python*	*brongersmai*	Captive								I				I				
Ariel et al. (2015)	Testudines	Chelidae	*Emydura*	*macquarii krefftii*	Experimental	An, DA								I, N			I, N	I, N	I, N	I, N	I, N
Johnson et al. (2008)	Testudines	Emydidae	*Terrapene*	*carolina bauri*	Wild			D	D, E				I					I			
Johnson et al. (2008)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Captive/Wild		D		D, E				I	I		I		I			
Johnson et al. (2008)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Wild				D, E		Aural ab-scesses		I			I		I	I		
Johnson et al. (2008)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Wild				D				N			I		I			
Johnson et al. (2008)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Captive													I			

(*continued on next page*)

Table 1 (*continued*)

Reference	Order	Family	Genus	Species	Population	Clinical signs: Behaviour	Oral	Nasal	Ocular	Skin	Other signs	Pathogenesis: Skin	Oral cavity	Gastrointestinal tract	Upper respiratory tract	Lower respiratory tract	Liver	Spleen	Kidney	Pancreas	Muscle
DeVoe et al. (2004)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Captive/Wild	An, DA			C	A, U	Respiratory distress	I	N	I		I	I	I	I	I	I
Allender et al. (2006)	Testudines	Emydidae	*Terrapene*	*carolina carolina*	Wild	An, DA	L	D	C, D		Weight loss		I, N	I, N		I,N	N		I	I, N	
Johnson, Pessier & Jacobson (2007)	Testudines	Emydidae	*Terrapene*	*ornata ornata*	Experimental	An, DA			D									L			
Johnson, Pessier & Jacobson (2007)	Testudines	Emydidae	*Trachemys*	*scripta elegans*	Experimental	An, DA		D	C, D		Increased basking, Exoph- thalmus, hyphema		I	H, I		T	N, T	L	T		
Allender et al. (2013b)	Testudines	Emydidae	*Trachemys*	*scripta elegans*	Experimental	DA	L	D	D	A	Leg swelling		N, U			I, T	L, T	I, N			I, N
Benetka et al. (2007)	Testudines	Testudinidae	*Stigmochelys (Geochelone)*	*pardalis*	Captive	An, DA	D	I					I, N			I					
Marschang et al. (1999)	Testudines	Testudinidae	*Testudo*	*hermanni*	Captive									I, N			I, N				
Blahak & Uhlenbrok (2010)	Testudines	Testudinidae	*Testudo*	*hermanni*	Captive						Emaciation		I								
Blahak & Uhlenbrok (2010)	Testudines	Testudinidae	*Testudo*	*kleinmanni*	Captive								I		I		I				
Blahak & Uhlenbrok (2010)	Testudines	Testudinidae	*Testudo*	*marginata*	Captive								I			N					
Heldstab & Bestetti (1982)	Testudines	Testudinidae	*Testudo*	*hermanni*	Captive									I, N			I, N	I, N			
Johnson et al. (2008)	Testudines	Testudinidae	*Geochelone*	*platynota*	Captive			D	C		Neck swelling		I	I, N				I	I		
Johnson et al. (2008)	Testudines	Testudinidae	*Gopherus*	*polyphemus*	Wild			D	C, D, E				I					I	I		
Westhouse et al. (1996)	Testudines	Testudinidae	*Gopherus*	*polyphemus*	Wild	DA		D	D		Respiratory disease			I		I, N, U					
Chen, Zheng & Jiang (1999)	Testudines	Trionychidae	*Pelodiscus*	*sinensis*	Captive/ Experimental						Red neck, neck swelling	H					H				

Notes.
A, abscess; An, anorexia; C, conjunctivitis; CN, central nervous disorders; DA, decreased-activity/depression/lethargy; D, discharge; E, oedema; H, haemorrhage; I, inflammation; L, lesion; N, necrosis; T, thrombi; U, ulceration.

that may exacerbate the disease and mask clinical signs of ranaviral infection (*Stohr et al., 2013*; *Sim et al., 2016*; *Archer et al., 2017*).

TRANSMISSION

The natural route of transmission of ranaviruses in wild populations of reptiles is still debated, although experimental data suggest multiple transmission routes are possible (*Brunner et al., 2015*). During an experimental challenge of adult red-eared sliders (*Trachemys scripta elegans*), *Johnson, Pessier & Jacobson (2007)* found that the orally exposed animals were refractory to infection while animals challenged with the same dose via intramuscular injection developed severe disease. In another study, exposure to ranavirus in water *via* cohabitation resulted in subclinical infection in some red-eared slider hatchlings (*T. scripta elegans*), although the route of infection was not determined, and the concentration of virus in the water was not quantified (*Brenes et al., 2014a*). *Ariel et al. (2015)* found that adult freshwater turtles (*Emydura krefftii* and *Elseya latisternum*), freshwater crocodiles (*C. johnstoni*), and several species of snakes were refractory to infection irrespective of the route of exposure. The hatchlings of both species of freshwater turtles were susceptible to infection *via* intra-coelomic exposure although oral inculcation was not attempted. Juvenile Australian eastern water dragons (*Intellagama lesueurii lesueurii*) developed ranaviral disease from all exposure routes tested (oral, intramuscular, and cohabitation) (*Maclaine et al., 2018*).

Differences in susceptibility *via* different routes of exposure may reflect real differences in natural transmission routes between reptiles and other Classes. More experimental studies using a variety of species, life stages, and routes of transmission are needed to resolve this.

Amphibians are highly susceptible to ranaviral infection *via* all tested forms of inoculation (water bath, skin contact, oral inoculation or injection) (*Miller et al., 2015*). Fish are also susceptible *via* multiple inoculation routes, although it appears to be species-dependent (*Bang Jensen, Ersbøll & Ariel, 2009*; *Gobbo et al., 2010*; *Jensen et al., 2011*). Differences in viable transmission routes result in different epidemiologies, and thus research from other host classes with different viable transmission routes may not accurately reflect risks and susceptibility of reptilian populations. It is therefore important to account for variation in transmission routes among reptile species when developing statistical models for reptilian disease.

VECTORS

Humans are contributing to the global spread of ranaviruses, primarily though global animal trade (*Kolby et al., 2014*; *Duffus et al., 2015*; *Stöhr et al., 2015*). Although there are reports of ranaviral infection in traded reptiles (*Hyatt et al., 2002*; *Stohr et al., 2013*), no systematic survey of ranaviral infection in traded reptiles has been conducted. There have been some ranaviral disease outbreaks in private reptile collections and zoos (*Marschang, Braun & Becher, 2005*; *Sim et al., 2016*), but the full extent of disease prevalence is hard

to assess, both because of inapparent infections, and lack of reporting of dead animals amongst reptile breeders and collectors.

Ranaviral DNA sequences have been identified in mosquitoes associated with a ranavirus outbreak in box turtles, providing evidence for vector transmission (*Kimble et al., 2014*). Ranaviral DNA and antigens have been detected in blood and blood-associated tissues of reptiles (*Allender et al., 2013a*; *Ariel et al., 2015*; *Miller et al., 2015*). Leeches are common ectoparasites of aquatic reptile species and can act as vectors for blood-borne diseases (*Siddall & Desser, 1992*; *Watermolen, 1996*; *Readel, Phillips & Wetzel, 2008*). There has been at least one report of a ranavirus-positive leech (PCR for MCP) associated with an infected amphibian host, although there are no reports for leeches of reptiles (*Hardman et al., 2013*). Some low density reptile populations that experience ranaviral epizootics do not appear to be capable of propagating ranaviral disease through physical contact alone (*Brunner et al., 2015*). Despite these indicators of the possible involvement of vectors in ranavirus transmission, no experimental studies have been published that support or refute this hypothesis in reptiles.

RESERVOIRS

Ranaviral virions are extremely stable in controlled settings, they are capable of withstanding high and low pH and temperatures and are resistant to desiccation, remaining viable for days to years (*Granoff, Came & Rafferty, 1965*; *Langdon et al., 1986*; *Langdon, 1989*; *Munro et al., 2016*; *Nazir, Spengler & Marschang, 2012*). These qualities of stability may not to carry to ecological settings as interactions with the aquatic biotic communities can reduce the longevity of infectious ranaviral particles (*Brunner et al., 2015*). *Reinauer, Bohm & Marschang (2005)*, found that tortoise ranaviruses remain infectious in lake water samples and in soil for many days; however, biotic communities were not quantified. It also appears that moisture is important for persistence in soil environments (*Brunner et al., 2015*; *Nazir, Spengler & Marschang, 2012*). Animals, both live and dead, are also probably reservoirs for reptilian ranavirus infections (*Gray, Miller & Hoverman, 2009*). Reptiles are known to consume frogs, fish, and even other reptiles as a part of their natural diet (*Kischinovsky, Raftery & Sawmy, 2017*). Dead and decaying animals continue to release virions and might be consumed by susceptible reptiles (*Brunner et al., 2015*; *Gray & Chinchar, 2015*). Asymptomatic amphibians are sometimes reservoirs; they can spread virus to other susceptible species, and possibly reptiles, in multispecies ranavirus epizootics (*Brenes et al., 2014a*; *Brenes et al., 2014b*; *Brunner et al., 2015*).

CORRELATES OF SUSCEPTIBILITY

Reptiles are ectotherms and so their physiology is strongly influenced by the temperature of their surrounding environment. By extension, the innate and adaptive immune response of reptiles is also linked to available environmental temperatures (*Zimmerman, Vogel & Bowden, 2010*). Ranavirus-infected reptiles, such as turtles, exhibit temperature-dependent pathogenesis (*Allender et al., 2013b*; *Allender et al., 2018*) similar to that observed in fish and amphibians (*Brunner et al., 2015*; *Brand et al., 2016*); however, the replication efficacy of

the virus is also linked to temperature (*Ariel et al., 2009*). Thus, it is difficult to determine the degree to which temperature-dependent pathogenesis is a result of the effect of temperature on the replication of the virus or on the immune system of the turtles. Several studies have quantified the temperature-dependent activity of the innate immune system of reptiles (*Merchant et al., 2006*; *Ferronato et al., 2009*; *Merchant et al., 2012*). In experimental infections of ranaviruses, temperature is often uncontrolled (reported as 'room temperature').

Allender et al. (2013b) suggested that an environmental temperature increase of 6 °C is enough to significantly reduce ranavirus loads and halve morbidity in infected adult turtles. However, in a follow up study, in juvenile turtles, it was found that increased temperature reduced median survival time of all four species tested (*Allender et al., 2018*). Similar patterns of reduced time until death but lower mortality rates with increasing temperature have been seen with other environmental temperature-dependent host-pathogen systems such as amphibians with chytridiomycosis (*Berger et al., 2004*). This pattern of temperature-related susceptibility in reptiles is important for future studies to quantify.

The effects of stressors on reptilian ranaviral disease are poorly understood (*Polakiewicz & Goodman, 2013*). Several studies in amphibians have examined the effects of stressors on disease in experimental infections (*Echaubard et al., 2010*; *Forson & Storfer, 2006*; *Haislip et al., 2012*; *Kerby, Hart & Storfer, 2011*; *Reeve et al., 2013*), and epidemiological studies have looked for correlations between environmental stressors and ranaviral prevalence (*St-Amour et al., 2008*; *Brunner et al., 2015*). The immunosuppressive effects of some anthropogenic stressors (e.g., pesticides, herbicides, and heavy metals) on the reptile immune system suggest a possible mechanism of environmental influence on susceptibility. Future epidemiological studies should consider these factors (*Keller et al., 2006*; *Soltanian, 2016*).

IMMUNOLOGY

Studies of ranaviral host immunity and immune evasion in amphibians are extensive, while similar work in reptiles is limited (*Grayfer et al., 2015*). The immunology section in the 2015 *Ranavirus* book, although comprehensive on amphibians, only mentions reptiles in passing (*Grayfer et al., 2015*). Immunology is an area in which a great number of unknowns remain for ranaviruses and reptiles.

Innate

Antimicrobial peptides (AMPs) are likely involved in amphibian ranaviral defence. Amphibian antimicrobials such as E2P and R2P are cable of inactivating ranaviral virions through direct interaction at all temperatures tested (0–26 °C) (*Chinchar et al., 2001*). Reptile species also possess a range of antimicrobial peptides, primarily cathelicidins and β-defensins (*Preecharram et al., 2010*; *Van Hoek, 2014*; *Ageitos et al., 2017*). Homologs of the anti-ranaviral peptides in amphibians (class-four AMPs) have not been found in reptiles, although defensin-like peptides from the albumin of marine turtles possessed

antiviral activity against enveloped rhabdoviruses (*Chattopadhyay et al., 2006*). No reptilian AMPs have, however, been specifically assayed for anti-ranaviral activity.

Few studies have looked at the role of cytokines against ranaviruses in reptile immunity and these should be investigated in future studies. One study found that IFN-γ appears to have some antiviral activity in ranavirus infected soft-shelled turtle cells, although the mechanisms are unclear (*Fu et al., 2014*).

The reptile serum complement system also deserves further consideration, as it is capable of inhibiting viral replication (*Merchant et al., 2005*). Serum from American alligators (*Alligator mississippiensis*) exhibits antiviral activity against human immunodeficiency virus type-1, which has been attributed to action of the complement system (*Merchant et al., 2005*). The effect of the reptilian complement system on ranaviral replication efficiency has not been investigated.

Extensive work has attempted to elucidate the complex role of amphibian macrophages in ranaviral infection, although work in reptile hosts is limited (*Grayfer et al., 2015*). It has been hypothesized that ranaviral infection is partly dependent on the phagocytic and endocytic activity of macrophages. Ranaviruses overcome the antiviral defences of macrophages and use the cells for persistence and dissemination throughout the host. Ectothermic vertebrates, including reptiles, possess a unique type of phagocytic B cell capable of ingesting foreign particles (*Zimmerman et al., 2010*). It is conceivable that these phagocytic B cells may also be involved in ranavirus dissemination.

Adaptive

Much less is known about the reptilian adaptive response than the innate response system (*Rios & Zimmerman, 2015*). Studies of the role of the adaptive immune system in clearing ranaviral infection have been almost exclusively restricted to amphibians and fish (*Chen & Robert, 2011*; *Grayfer et al., 2015*). The only studies of ranaviruses and the adaptive arm of the reptilian immune system have been through epidemiological studies. Anti-ranaviral IgY is produced as a long-lasting and specific adaptive response to infection and is the preferred target of reptilian serological assays (*Johnson et al., 2010*; *Zimmerman, Vogel & Bowden, 2010*; *Ariel et al., 2017*). The virus neutralising ability of anti-ranaviral antibodies detected in reptile populations has not been determined. Studies of T cell proliferation in response to ranaviral infection have not been conducted in reptiles and it is not clear if reptiles develop long-lasting immunological memory against ranaviral infection. Amphibian researchers have made a start on these questions, providing useful guidance for future studies in reptiles (*Grayfer et al., 2015*).

TREATMENT

For treatment of acute ranaviral infection, several antivirals have been considered and tested (*Allender, 2012*; *Li et al., 2015*; *Sim et al., 2016*). However, there are few examples of their successful use to treat clinical cases (*Johnson et al., 2010*; *Allender, 2012*; *Miller et al., 2015*). Many *in vitro* antiviral studies that show promising results do not carry to *in vivo* models or have not been thoroughly tested *in vivo*.

Acyclovir, the most extensively studied antiviral in reptiles, does not appear to be an effective anti-ranaviral agent. Viral thymidine kinase (present in some herpesviruses and ranaviruses) is required for activation of acyclovir, which then blocks viral DNA replication through competitive inhibition of the viral DNA polymerase (Beutner, 1995). *In vitro* results have been mixed; Johnson (2006) found that acyclovir provided a dose-dependent partial inhibition of a FV3-like ranavirus, and Ferguson et al. (2014) found no statistically significant effect of acyclovir on FV3 replication. Plasma concentrations of orally dosed acyclovir do not reach levels in turtles that have been suggested as sufficient for ranavirus inhibition (Allender, 2012; Gaio et al., 2007). It is difficult to interpret the results of the use of this drug in uncontrolled clinical settings; however, it is clear that in several cases acyclovir has not stopped the progression of reptilian ranaviral disease (DeVoe et al., 2004; Johnson et al., 2008).

Pharmacological studies of the effectiveness of different antivirals at different severities and durations of ranaviral infection in reptiles have not been conducted, but would be extremely useful for guiding the treatment of acute ranaviral infection in reptiles.

Iridoviral vaccine development has been limited to the aquaculture industry (Miller et al., 2015). Frogs can produce long-lasting FV3-specific neutralising antibodies on second exposure (Maniero et al., 2006), suggesting it would be possible to develop vaccines for them. Reptiles can produce anti-ranaviral antibodies during infection (Ariel et al., 2017; Johnson et al., 2010), and vaccines have been developed for other reptilian pathogens with varying success (Horner, 1988; Jacobson et al., 1991; Mohan et al., 1997; Marschang, Milde & Bellavista, 2001; Yang, Pan & Sun, 2007). Vaccine research and development are extremely costly, and more epidemiological research is required to determine if the development of a ranaviral vaccine would be efficacious for wild reptilian populations. However, there are several instances where a vaccine could be useful for small scale use. For example: in zoo collections, for valuable broodstock, and for endangered or at risk populations. Epidemiological studies may feasibly identify and prophylactically treat animals most at risk.

Environmental temperature can have a substantial effect on the humoral immune system (e.g., antibody production) of ectotherms (Tait, 1969), which opens up the possibility of influencing the outcome of an infection *via* control of environmental temperatures (see Susceptibility section). Increased ambient temperature has been suggested as a treatment method for ranavirus infection in reptiles (Hyndman & Marschang, 2017). However, Allender et al. (2018) recently reported that increased temperature (22 °C to 27 °C) resulted in reduced median survival time of ranaviral infected Testudines. It is likely that there is a threshold temperature, which dramatically improves survival as occurs with chytridiomycosis in amphibians (Berger et al., 2009). Further investigation is required to determine the optimal temperature for increasing survival of ranaviral infected reptiles, which may also be viral and host species dependent.

FUTURE RESEARCH AND CONCLUSIONS

The field of ranavirus research is dominated by studies on fish and amphibians, these studies can serve as a guide for the tremendous number of directions ranaviral research in reptiles

could take. An increase in the number of epidemiological studies and surveys of ranaviruses in reptile populations is required to understand the distribution of these viruses in the class Reptilia, and to identify at-risk populations. Pathogenesis and transmission of ranaviruses in reptiles are still poorly understood and will require elucidation before this disease can be correctly modelled and appropriately managed in reptile populations. Reptile ranaviral host immunity and immune evasion strategies of the virus are also under-represented in the literature. From predator to pollinator to prey, reptiles play vital roles in the ecosystems they inhabit, but like amphibians, reptiles are experiencing global declines (*Gibbons et al., 2000*). It is, therefore, imperative that research continues to expand our understanding of reptiles and ranaviruses to help protect this valuable part of biodiversity.

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Author Contributions

- Wytamma Wirth conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Lin Schwarzkopf, Lee F. Skerratt and Ellen Ariel conceived and designed the experiments, authored or reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

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Appendix 3 - Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with *Ranavirus* isolate (Bohle iridovirus, *Iridoviridae*)

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MICROBIOLOGY SOCIETY

Dose-dependent morbidity of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with *Ranavirus* isolate (Bohle iridovirus, *Iridoviridae*)

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Abstract

Ranaviral infections cause mass die-offs in wild and captive turtle populations. Two experimental studies were performed to first determine the susceptibility of an Australian turtle species (*Emydura macquarii krefftii*) to different routes of infection and second examine the effect of viral titre on the morbidity in hatchlings. All inoculation routes (intracoelomic, intramuscular and oral) produced disease, but the clinical signs, histopathology and time to onset of disease varied with the route. The median infectious and lethal doses for intramuscularly inoculated hatchlings were $10^{2.52\ (1.98-2.93)}$ and $10^{4.43\ (3.81-5.19)}$ $TCID_{50}$ ml^{-1}, respectively. Clinical signs began 14 to 29 days post-inoculation and the median survival time was 22 days (16–25) across all dose groups. For every 10-fold increase in dose, the odds of developing any clinical signs or severe clinical signs increased by 3.39 [$P<0.01$, 95% confidence interval (CI): 1.81–6.36] and 3.71 ($P<0.01$, 95% CI: 1.76–7.80), respectively. Skin lesions, previously only reported in ranaviral infection in lizards, were observed in the majority of intramuscularly inoculated hatchlings that developed ranaviral disease. The histological changes were consistent with those in previous reports for reptiles and consisted of necrosis at or near the site of injection, in the spleen, liver and oral cavity. Systemic inflammation was also observed, predominantly affecting necrotic organs. The estimates reported here can be used to model ranaviral disease and quantify and manage at-risk populations.

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Keywords: ranavirus; animal model; freshwater turtle; experimental infection; clinical signs; median lethal dose.
Abbreviations: BIV, Bohle iridovirus; CI, confidence interval; CS, clinical signs; FHM, fathead minnow; FV3, Frog virus 3; GIT, gastrointestinal tract; i.c., intracoelomic; ID50, median probability of infection; i.m., intramuscular; LD50, median probability of death; o.r., orally; SCL, straight carapace length; SCW, straight carapace width; ST50, median survival time; TCID50, median tissue culture infectious dose.
Supplementary material is available with the online version of this article.

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Appendix 4 - Temperature-dependent infection of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with a *Ranavirus* isolate (Bohle iridovirus, *Iridoviridae*)

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RESEARCH ARTICLE

Temperature-dependent infection of freshwater turtle hatchlings, *Emydura macquarii krefftii*, inoculated with a ranavirus isolate (Bohle iridovirus, *Iridoviridae*)

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Abstract

Fish, amphibians, and reptiles exhibit temperature-dependent ranaviral disease. We performed an experimental infection at four different environmental temperatures (16, 22, 28, and 34 °C) to investigate the effect of temperature on ranaviral infection in Krefft's turtle (*Emydura macquarii krefftii*). Infection rates and viral loads were determined by quantitative polymerase chain reaction to detect ranaviral DNA in liver samples at 21 d postexposure. The rate of infection differed across the temperature treatment groups. Infection rates were 44%, 90%, 60%, and 10% for the 16, 22, 28, and 34 °C temperature groups, respectively. Highest viral load was observed in the 28 °C temperature group, and there was a statistically significant difference in viral load between the 16 and 28 °C temperature groups ($p = 0.027$). Based on the results of this study, the temperature of maximal infection rate for ranaviral infection in Krefft's river turtles is estimated to be 23.2 °C (SD = 4.5). The findings of this study can inform management decisions in terms of disease control and treatment and form a platform for modelling disease outbreaks.

Key words: ranavirus, reptiles, turtles, temperature, infection

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Introduction

Ranaviruses are pathogens of ectothermic vertebrates like reptiles. Because reptile physiology is strongly influenced by the temperature of their surrounding environment, so is the pathogenesis of ranaviral infections. The environmental temperature-dependent physiology includes many aspects of the ectothermic immune system (both adaptive and innate) and, while immune function is often severely reduced at lower temperatures, many immune components have optimal temperatures beyond which the efficacy diminishes (Zimmerman et al. 2010).

As with the ectothermic immune response, the replication rate of ranaviruses is also linked to temperature (Ariel et al. 2009). It has been concluded that the decrease in mortality at higher temperatures (>25 °C) is associated with ranaviruses' inability to replicate effectively above 32 °C in cell culture (Chinchar et al. 2009; Allender et al. 2013). However, it is difficult to differentiate the degree to which temperature-dependent pathogenesis is a result of the effect of temperature on the

replication of the virus or on the immune system or other physiological components of the ectothermic host (Brunner et al. 2015).

Given the effects of temperature on the ectothermic immune system and viral replication, ranaviral disease is, in part, determined by the environmental temperature at which infection occurs. Wild outbreaks of ranaviruses are often seasonal and theoretically driven by temperature (Hall et al. 2018). Challenge studies have shown that changes in environmental temperature can affect pathogenesis in exposed animals. Ranavirus-infected turtles exhibit temperature-dependent disease (Allender et al. 2013, 2018). Similarly, fish and amphibians exhibit temperature-dependent ranaviral disease (Whittington and Reddacliff 1995; Rojas et al. 2005; Ariel and Jensen 2009; Jensen et al. 2011; Brunner et al. 2015; Brand et al. 2016).

As temperature can influence ranaviral disease, temperature therapy (exposing animals to environmental temperatures that are associated with reduced disease) may be a cheap and effective method for treating acute ranaviral disease in captive animals if the optimal temperature for survival is known. High environmental temperatures may effectively force the animals into behavioural fever, increasing their immune response and reducing viral replication, which subsequently results in reduced mortality. Behavioural fever is the process by which a diseased ectotherm (e.g., reptile) actively brings about a febrile state through the behavioural selection of higher environmental temperatures (Monagas and Gatten 1983; do Amaral et al. 2002; Merchant et al. 2007). Infected or antigen-challenged ectotherms will generally select an environmental temperature a few degrees higher than control groups, and it is thought that this temperature is correlated with the optimal immune response of the animal (Spellerberg 1972; Monagas and Gatten 1983; Merchant et al. 2007). There is evidence that toads perform behavioural fever to fight ranaviral infections (Sauer et al. 2019). Thus, knowing the optimal temperature of infection, it will be possible to make recommendations of the optimal temperature for temperature therapy and the best temperatures for basking sites in enclosures to ensure behavioural fever is possible.

Understanding the effect of temperature on infection also has important ramifications for understating wild disease dynamics. Knowing the rage of temperatures that infection can occur at and the optimal temperature for infection, it may be possible to identify at-risk populations or predict temperature-driven epizootics. As the climate continues to change it is important to understand how variation in temperature may affect aspects of ranaviral disease.

Australia is home to ranaviruses, *frog virus 3* (FV3) and *epizootic hematopoietic necrosis virus*, and susceptible ectothermic vertebrates such as the Krefft's river turtle (*Emydura macquarii krefftii*) (Ariel et al. 2015; Chinchar et al. 2018). Krefft's river turtles belong to the Pleurodira suborder of turtles, which are distinct from the native turtles of Asia, Europe, and North America and may be a useful model for other Pleurodirid turtles (of Africa, Australasia, and South America). The effect of ranaviral dose at a single temperature has been reported for Krefft's hatchlings (Wirth et al. 2019). This study investigates how infection rate with a local ranaviral isolate (Bohle iridovirus; FV3) varies within a range of environmental temperatures representative of topical Australia (16–34 °C).

Methods

All experiments were carried out under a James Cook University (JCU) Animal Ethics permit (A2344) and a Department of Environment and Science Scientific Research Permit (WISP13270413).

Animals

Hatchlings used in this study were obtained following methods described by Wirth et al. (2019). Briefly, wild gravid turtles were induced to lay eggs with an injection of 1 IU 100 g^{-1} of syntocinon

Table 1. Experiment design and animal numbers at the start of the experimental infection.

Group	Temperature (°C)	Number of hatchlings	Mean starting weight (g)
Control	16	2*	10.8
	22	3	9.4
	28	3	9.7
	34	3	9.3
Exposed	16	9*	9.4
	22	10	9.5
	28	10	9.4
	34	10	9.5

Note: During the study one animal from both the control and exposed 16 °C temperature groups were removed.
*The weights of the removed animals are not included in the mean starting weight.

(synthetic oxytocin 10 IU mL^{-1}, Troy laboratories). After approximately 50 d of incubation at 29 °C the eggs hatched, and newly emerged hatchlings were kept at the JCU Turtle Health Research Facility. The hatchlings were housed in tanks with water between 24 and 28 °C and given access to dry areas for basking. The hatchlings were fed commercial turtle pellets (Exo Terra, aquatic turtle food) ad libitum for 10–15 min per day. During and after this husbandry phase, feeding observations and morphometric data were collected from each individual. The average weight of hatchlings used in this study was 9.5 g (**Table 1**).

Source of virus

The Bohle iridovirus (BIV) isolate (FV3 strain) used in this study was originally isolated in Townsville, Australia and was produced according to the methods of Maclaine et al. (2018). Briefly, the viral isolate was propagated at 25 °C in fathead minnow cells grown in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific), supplemented with 100× antibiotic-antimycotic (Thermo Fisher Scientific) and 10% fetal bovine serum (Bovogen Biologicals). Viral titre ($TCID_{50}$) was determined using the methods described by Reed and Muench (1938). The viral stock was stored at −80 °C until the time of challenge study and then diluted in DMEM to $10^{2.5}$ $TCID_{50}$ mL^{-1}.

Experimental design

One week prior to the start of the experiment animals were randomly assigned to control and exposed groups (**Table 1**) and moved into the infection room. The hatchlings were kept in individual 500 mL plastic container on a slant to create a dry area for basking. The containers were placed, in groups of three or four, in temperature chambers set to 16, 22, 28, or 34 °C with a 12-h light cycle (Greenspan et al. 2016). The hatchlings were given one week to acclimatise to the temperature chambers before the start of the experiment. Husbandry continued as previously described. During the acclimatisation period and throughout the experiment hatchlings were monitored for clinical signs including inappetence, skin lesions, increased basking behaviour, and oedema of the neck and legs as described by Wirth et al. (2019) for ranaviral infection in Krefft's river turtle hatchlings. The dose rate and time to euthanasia in this experiment was based on findings in Wirth et al. (2019) to allow for sufficient time for the infection to get established and potentially be influenced by temperatures above and below the temperature used in that study, before the samples were collected. On day zero of the experiment all hatchlings in the exposed groups received an intramuscular injection of

$10^{2.5}$ $TCID_{50}$ mL^{-1} BIV stock into the hind leg. Injection volume was determined by multiplying the individual's weight (g) by 2.5 (mean 23.8 µL; range 20.25–30.5 µL). Control animals received an equivalent dose of virus-free DMEM as a placebo.

Endpoint and sample collection

On the day of euthanasia final morphometric data were collected. Hatchlings were randomly selected for euthanisa 21 d post exposure and all hatchlings were euthanized over the following 3 d. Hatchlings were euthanised with a MS-222 overdose according to the methods described by Conroy et al. (2009). A necropsy was performed on each animal using a different set of sterile tools (disinfected with Virkon and washed with detergent). Gloves were changed between animals and surfaces were decontaminated with bleach (4% by volume). A section of liver was collected for quantitative polymerase chain reaction (qPCR) analysis and frozen at −80 °C.

qPCR

DNA was extracted from the thawed liver samples using an ISOLATE II Genomic DNA Kit (Bioline) following the manufacturer's protocol for DNA extraction from tissues. Viral loads were determined using the ranavirus major capsid protein gene (MCP) gene assay described by Leung et al. (2017). A single copy conserved vertebrate gene, EBF3N, was used to standardise the number of viral copies. A section of the BIV MCP and EBF3N gene were cloned into pGEM-easy vectors following the manufacturer's protocol (Promega). Plasmids were linearised using a PstI restriction digest (New England Biolabs) and quantified using a QuantiFluor dye system (QuantiFluor, Promega). Absolute standard curves were constructed in triplicate from 10^8 to 10^1 copies μL^{-1} and 10^7–10^1 copies μL^{-1} for the MCP and EBF3N plasmids, respectively. The qPCR was performed on a magnetic induction cycler real-time thermocycler (Applied Biosystems) with the conditions described by Leung et al. (2017). The assays were run as a duplex with the MCP probe on the green channel (FAM probe) and ENF3N on the red channel (Cy5 probe). It was assumed that there is one EBF3N gene per *E. m. krefftii* genome. Viral loads were determined by dividing the number of MCP copies μL^{-1} multiplied by two by the number of EBF3N copies μL^{-1} and are referred to as copies per cell throughout this manuscript.

Statistics

All analysis was performed using the Statsmodels module (0.10.2) and Pandas (0.24.3) and SciPy (1.4.1) libraries in Python 3.6 (McKinney 2010; Seabold and Perktold 2010). A 2×4 χ^2 contingency table was used to compare proportions of infected animals in the temperature groups. The Kruskal–Wallis H-test for independent samples was used to compare variation in viral loads of infected turtles between temperature groups. Conover's test of multiple comparisons was used as post hoc test following a significant Kruskal–Wallis H-test. A normal distribution was fit to the portion of infected animals from each temperature group (using mean temperature, **Table 2**) to estimate the temperature of maximal infection rate and its standard deviation for the temperature range tested.

Results

Temperatures

The incubators used in this study were effective at maintaining the average environmental temperature within a degree of the target temperature (**Table 2**).

Deaths

During the study two animals in the 16 °C group were found dead in their enclosures on day 3 and 8 postinoculation. One animal was from the control group and one from the infection group. Following

Table 2. Descriptive statistics for temperature logger data from the incubators used to house the animals in this study.

Group	Mean	Std	Minimum	Q1	Median	Q3	Maximum
16	16.24	0.72	15.9	16	16.1	16.3	23.3
22	22.63	1.13	21.6	22	22.3	22.5	27.6
28	27.79	1.12	22.9	27.8	28.1	28.4	29.1
34	34.12	0.35	32.1	33.8	34.1	34.4	35

Note: Q1 and Q3 represent the lower and upper quartiles respectively. All data are expressed as degrees Celsius (°C).

a postmortem, it was determined that both animals had drowned and not died from a ranaviral infection (they were qPCR negative). The animals were removed from the study and not included in any analysis. No other animals died or developed clinical signs severe enough to warrant euthanasia.

Clinical signs

Only two animals developed clinical signs in the timeframe of this experiment. One animal in the 16 °C group developed ocular and nasal lesions 17 d after exposure. One animal in the 22 °C group developed an oral lesion 14 d after exposure. No animals developed skin lesions at the site of inoculation as described by Wirth et al. (2019). Diet consumption appeared to be strongly linked to environmental temperature and there was no clear evidence of in-appetence caused by ranaviral infection (control and infected groups were affected similarly across all temperatures). Both exposed and control hatchlings in the 16 °C group consistently ate little to none of their food and appeared lethargic. The animals in the 34 °C group consistently ate most of the food offered and were active throughout the experiment.

qPCR

Of the 40 turtles infected across all temperatures 50% of them reacted in the qPCR assay for ranaviral DNA in the liver 21 d after exposure. However, the distribution of reactors was not even (Fig. 1). Significantly more turtles (9/10 liver samples reacted in the qPCR assay) were infected in the 22 °C group ($\chi^2 = 13.3$; $p < 0.01$). The lowest infection rate was in the turtles kept at 34 °C, where only 1/10 liver samples reacted in the qPCR assay (10%). The mean and standard deviation of the normal distribution fit to the infection rates was 23.2 and 4.5 °C, respectively.

There was large variation in ranaviral MCP copy numbers within the different temperature groups (Fig. 1). As there was only one reactor in the 34 °C temperature group it was removed from the statistical analysis of viral loads. The Kruskal–Wallis test found a significant difference (0.05 α) between ranaviral MCP copy number between the 16, 22, and 28 °C temperature groups (H statistic = 6.57; $p = 0.037$). Post hoc analysis with the Conover's test of multiple comparisons revealed that this difference was between the 16 and 28 °C temperature groups ($p = 0.027$).

Discussion

Based on the distribution of infection rates (Fig. 1) and temperature logger data (Table 2), the predicted optimal temperature for ranaviral infection (i.e., the maximum number of infected) in Krefft's river turtles is 23.2 °C. Assuming that uninfected turtles would not later develop a detectable infection (i.e., turtles that did not react in the qPCR assay have cleared the infection), we can say that

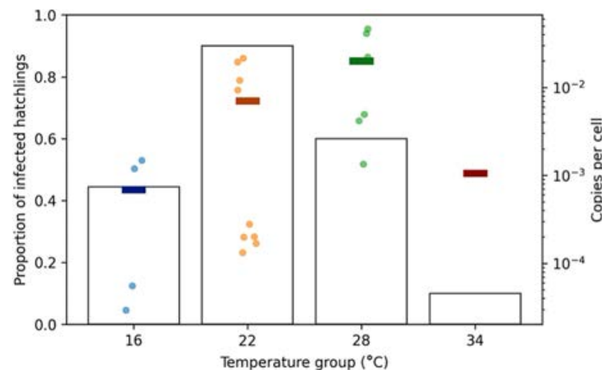

Fig. 1. Ranaviral infection rate and intensity 21 d post exposure for Krefft's river turtle hatchlings held at different temperatures. The boxes are the proportion of animals from each group that reacted in the qPCR assay for ranaviral DNA in their liver. The dots are the copies per cell (log scale) of ranaviral DNA in each infected animal's liver. The solid line is the mean copies per cell for all infected animals in that temperature group. All groups had a total of 10 exposed animals except the 16 °C group that had nine.

temperature has a significant effect on the rate and length of infection. This will have impacts on the transmission of the virus. The more animals infected and the longer an animal is infected, the greater the chance of viral transmission.

Clinical signs observed in this study were similar to those that were reported for ranaviral infection in other turtles, although not as extensive as previous reports (Wirth et al. 2019). Given more time these animals may have either recovered or developed more clinical signs; however, terminating the experiment at 21 d allowed us to prevent suffering of the animals (they did not have time to develop clinical signs) while still being able to study the effect of temperature on infection.

There was a large variation in the viral loads observed within temperature groups. Interestingly, the copies per cell of ranaviral DNA within each temperature group appears to divide equally into "high" and "low" reactors with at least a 10-fold difference between the mean high and low copy numbers. Such a difference in individuals that received the same treatment may be explained by some dichotomous biological trait, such as those associated with sex, that increases or decreases viral replication. Unfortunately, the sex of these hatchlings cannot be easily determined via physical exam and was not recorded at necropsy. Krefft's river turtles do not have temperature-dependent sex determination, so the sex distributions within the temperature groups are assumed to be 50/50, which would explain the equal numbers of high and low viral loads in the temperature groups if this is the result of the sex of the animals. Future work should investigate the possible effects of sex (or other dichotomous variables) on ranaviral infection in turtles.

Post hoc analysis revealed that there was a significant difference in viral loads only between the 16 and 28 °C groups ($p = 0.027$). At lower temperatures, viral replication is likely reduced, thus resulting in reduced viral load. Adult red-eared sliders (*Trachemys scripta elegans*, suborder Cryptodira) exposed to a ranavirus and held at either 22 or 28 °C had significantly reduced ranaviral loads and halved morbidity in the 28 °C group (Allender et al. 2013). Similarly, we found that the infection rate was higher in the 22 °C group compared with the 28 °C group. However, we observed no significant difference in the viral loads between the 22 and 28 °C groups.

In a similar experiment, four Cryptodirid species of juvenile turtles (including red-eared sliders) did not have reduced mortality in the higher temperature group, both groups (22 and 27 °C) had 100%

mortality (Allender et al. 2018). Krefft's river turtles exhibit age-dependent ranaviral pathogenesis, with hatchlings being more susceptible than adult turtles (Ariel et al. 2015). The same may be true for red-eared sliders; the dose given to the juvenile turtles may have overwhelmed the turtles and thus negated the effect of temperature (Allender et al. 2013, 2018). Because adult red-eared sliders are less susceptible, the effect of temperature on viral replication/the immune system is still prominent (Ariel et al. 2015; Allender et al. 2018; Wirth et al. 2018).

In our study, we chose to use a median infectious dose (ID_{50}), as determined by Wirth et al. (2019) for Krefft's turtle hatchlings at 28 °C. As expected, the infection rate in the 28 °C group in this study was close to 50% (60%; **Fig. 1**). Using a median dose allows for easy detection of a change in the dependent variable (infection, death, etc.) as a result of changing different factors (e.g., environmental temperature). We chose the ID_{50} over the median lethal dose (LD_{50}; $10^{4.43}$ $TCID_{50}$ mL^{-1}) for this study for welfare reasons. The results of an infectious dose study are still valuable, although more applicable to a disease transmission than pathogenesis. We are not sure how these temperatures will affect Krefft's river turtles exposed to a higher dose (such as an LD_{50}). It may be that when Krefft's turtle hatchlings are exposed to higher doses, the amount of virus will overwhelm any prophylactic effects of temperature, as seen with red eared-sliders. In any case, we still do not understand the natural transmissions dynamics, so it is difficult to determine what dose would be useful to accurately reflect wild disease.

Temperature therapy >34 °C may also be useful for treating acute ranaviral disease, although further study will be required to determine the effects of temperature on pathogenesis. While 34 °C appears effective at reducing infection rates, BIV itself is not inactivated at 34 °C (La Fauce et al. 2012); thus, once an animal returns to cooler temperature the infection may re-establish if the virus is not completely cleared. To reduce the chances of ranaviral infection in captive animals, we would recommend that turtles are provided with a basking area of at least 34 °C, thus allowing the animals to perform behavioural fever at temperatures high enough to reduce infection. Lower temperatures (e.g., ≤16 °C) may also be effective at reducing infection rates; however, these temperatures are not optimal for turtle health and result in reduced appetite and activity.

The optimal temperature for ranavirus isolate propagation in a range of cell lines is 24 °C (10, 15, 20, 24, and 28 °C tested; Ariel et al. 2009). The average annual temperature in Townsville (where this study was conducted) is 24.1 °C. Both the optimal temperature for ranaviral propagation and the average annual temperature in Townsville are close to the temperature of maximum infection rate (23.2 °C) estimated in this study. Australian freshwater turtles (like Krefft's turtles) are semi-aquatic and spend the majority of their time in the water. When in the water, the turtles' body temperatures are in thermal equilibrium with water temperature (Manning and Grigg 1997). Ranaviral infection rates would be expected to be highest in months when water temperature is around 23 °C, i.e., May–June and September–October. However, the temperature most effective at reducing infection rate used in this study (34 °C) is not uncommon in Northern Australia where these turtles are native. Thus, ranaviral-infected Krefft's river turtles could have the opportunity to use behavioural fever to reduce infection rates, although finer-scale studies of thermal and basking site availability are required to quantify this opportunity.

In this study we, used reaction in a qPCR assay of the liver as a proxy for infection. While the liver is a major target of ranaviral replication, all conclusions reported in this study should be interpreted in light of the fact that reaction in a qPCR assay does not equal infectious virus.

Conclusions

Temperature is integral to ranaviral disease. We have shown that Krefft's river turtles (suborder Pleurodira) exhibit temperature-dependent infection. Using the results of this study we have provided

recommendations for temperature therapy of acute ranaviral infection, optimal temperature for behavioural fever in captive turtle enclosures and estimates of times of the year when infections rates are expected to be high in natural settings. Understanding how temperature influences emerging infectious diseases, like ranaviruses, is important in our changing climate. Results from studies like this one can provide data to climate change models and help form a foundation for understanding the impact on reptile populations.

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Author contributions

WW and EA conceived and designed the study. WW performed the experiments/collected the data. WW analyzed and interpreted the data. WW and EA contributed resources. WW and EA drafted or revised the manuscript.

Competing interests

Ellen Ariel is a guest editor.

Data availability statement

All relevant data are within the paper.

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Appendix 5 - Cutaneous Lesions in Freshwater Turtles (*Emydura macquarii krefftii* and *Myuchelys latisternum*) in a Rainforest Creek in North Queensland, Australia

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Cutaneous Lesions in Freshwater Turtles (*Emydura macquarii krefftii* and *Myuchelys latisternum*) in a Rainforest Creek in North Queensland, Australia

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Freshwater turtles inhabit most rivers and creeks on the east coast of Australia, but some species are only found in specific catchments, which makes them vulnerable to extinction. During annual fieldtrips to Alligator Creek, North Queensland, the resident population of *Myuchelys latisternum* and *Emydura macquarii krefftii* in a natural pond, just outside Bowling Green National Park, have been surveyed for a number of years and demographic data recorded against tagged turtles. Rounded, cutaneous lesions on individual animals were first noted in August 2016, three years after the first survey of the population. Turtles living in the upstream sections of the creek were not affected. An initial investigation into the cause of the lesions ruled out pollutants and although the bacterial communities appeared to be different on turtles with lesions, a causative agent was not identified. Attempts to isolate virus in culture was not successful and specific PCRs for ranavirus, papillomavirus, adenovirus and herpesvirus did not identify their presence. Blood biochemical parameters, body condition and activity levels were not significantly different between affected turtles and those without lesions. The turtles in this pond were monitored regularly over the following three years with 249 *M. latisternum* and 192 *E. m. krefftii* captured, tagged and released. The prevalence of the lesions fluctuated with season from 0 to 77 and 68% respectively, but did not vary significantly between species or sex in adults. There was a tendency for larger animals to be more likely to have lesions. The position of the lesions on the turtles was mostly on dorsal surfaces, distally on the legs and proximal on the tales of males, indicating that the initial lesion may have been associated with a behaviourally induced trauma. Recaptured animals ($n = 43$) during this period, provided records of lesion progression over time and while some healed up between capture events, others persisted for up to 24 months. Some turtles were repeatedly captured without lesions. Intra-species aggression associated with seasonal behaviours could potentially be the primary cause of skin trauma, followed by a secondary invasion of an unusual pathogen present in the environment.

Keywords: freshwater turtles, cutaneous lesions, disease, wildlife, outbreak

INTRODUCTION

Freshwater turtles are vulnerable to many human and natural factors and a systematic analysis of tortoise and freshwater turtle global distribution identified coastal Australia as one of three areas of priority for freshwater turtle conservation (1). In Australia, there are 25 species of freshwater turtles, 11 of which have a conservation status of vulnerable or worse (2). Most of the Australian freshwater turtles belong to the family Chelidae that retract their neck and head under the shell by folding it to one side and are therefore referred to as side-necks (3). They are a totem animal in some Indigenous Australian cultures and although they are collected and consumed, such traditional harvest is not of conservation concern (4). The major risks to Australia turtles include: invasive species, drought, habitat modification, and disease (2).

Due to their longevity and close association with the aquatic environment, freshwater turtles can be considered indicators of aquatic environmental health. In addition to being sentinel species for long term pollution exposure, they are also at risk of habitat loss or degradation, invasive species and diseases among other threats (5–8). The limited range of many Australian freshwater turtle species and the risk of extirpation from any of the abovementioned threats, means that conservation is a very real and urgent issue. Recently, the vulnerability of the range restricted Bellinger River Snapping Turtle (*Myuchelys georgesi*), became apparent when a novel disease affecting the turtles drove the population close to extinction in <1 month (9, 10) and highlights the need for a focus on factors affecting the health of Australian freshwater turtles.

In August 2016, cutaneous lesions were noted on a proportion of freshwater turtles captured as part of regular monitoring of wild living turtles in Alligator Creek, North Queensland. Here we report on the characteristics of the lesions, the spatial and temporal extent of the epidemic and the impact on the population through a longitudinal study.

MATERIALS AND METHODS

All handling of turtles were carried out under permits from James Cook University (JCU) Animal Ethics Committee (A2309) and Department of Environment and Science (WISP13270413 and WA0012830).

Study Site

For the past six years in July/August the JCU Turtle Health Research Team has monitored a pond on Alligator Creek just outside the Bowling Green Bay National Park for freshwater turtle presence, demographics and general health. Alligator Creek originates in the Mt Elliott complex, inside the national park, where there is no industry, agriculture or human dwellings. The pond of interest, the Craill pond, is a hollow in the bedrock, ∼30 m at the widest part, 90 m at its longest and 5 m at its deepest. Alligator Creek runs into and out of this pond. The Craill pond is inhabited by two species of turtles, *Emydura macquarii krefftii* and *Myuchelys latisternum* and a number of freshwater fish species as well as freshwater crustaceans and crocodiles (*Crocodylus johnstoni*). No unusual natural or anthropogenic event preceded the first observations of lesions in this population of turtles.

Turtles

Approximately 50 turtles are captured annually by a combination of baited cathedral traps (passive sampling) and hand-capture (active sampling) to minimize any bias that might arise due to using only one method. *Emydura macquarii krefftii* tend to frequent the downstream reaches of large river systems or creeks, where they forage in the water and are considered omnivorous (11). *Myuchelys latisternum* are chiefly carnivorous and inhabit mainly the headwaters of rivers and tributaries, but can also be found in lagoons and billabongs (11). Both species can grow to 30 cm curved carapace length (CCL) and have a life-span of 20–30 years (12). In early August 2016, during the annual fieldtrip, cutaneous lesions on the soft tissue were recorded in the neck and tail region and on legs and feet of turtles ($n = 15/50$).

Fieldtrips

Field monitoring of the Craill pond (Site 5) was continued at intervals during the next 2 years. Any turtle above 15 cm CCL was tagged with a small titanium tag (National Wing Tags, Jiffy 893) in the webbing of one of the hind-feet and re-captures could therefore be identified and disease progression recorded if relevant. Morphometrics of captured turtles and records of cutaneous lesions were collected from the turtles captured. The size at which male tails start to elongate varies between species and individuals (12). Turtles less than the median CCL of the 10 smallest identifiable males of each species were classed as juveniles, unless their tail was obviously differentiated (13). Physical examinations were performed on all captured turtles, this process included: freshwater leech counts, eye/oral/nasal examination, soft-tissue exam for swelling or bruising, activity level (high, medium, low), damage to shell or missing claws, and adult female turtles were palpated for eggs to assess breeding status. Disease investigation as well as water quality monitoring were also performed to develop an understanding of the etiology of the lesions.

Spatial Pattern

In order to determine the spatial extent of this disease, turtle populations in four ponds upstream from Site 5 were also investigated during September/October 2016, starting with the pond at the highest elevation above mean sea level that the terrain allowed access to (Site 1). The five sites were at least 1 km apart and between 10 and 25 m difference in elevation (https://www.freemaptools.com/elevation-finder.htm), with Site 1 being at the highest elevation and the downstream sites gradually lower. See **Table 1** for GPS location and elevation of study sites. To avoid spreading an un-identified agent upstream, we disinfected all equipment with 5% bleach after each site and moved in a downstream direction between sites on any given day. Turtle populations downstream from Site 5 were not investigated as it is assumed that they will be exposed via the flow to a given pathogen in the water (14). Additionally, the creek below this

TABLE 1 | GPS locations and elevation in meters (m) above mean sea level for the five sites investigated for lesions on turtles following the outbreak at Site 5 in July 2016.

Site	Latitude	Longitude	Elevation	Description
1	−19.4456	146.9746	95	Pond
2	−19.4417	146.9623	82	Pond
3	−19.4408	146.9541	69	Stream
4	−19.4367	146.9477	44	Pond
5	−19.429	146.9437	34	Pond

pond is frequented by saltwater crocodiles, which makes trapping and underwater hand capture too risky.

Environmental Measurements

Conductivity, pH, and macro invertebrate counts and identity were recorded in the Craill pond when the lesions were first noticed (Site 5), as well as upstream from the outbreak. Air temperature historical data for the nearest weather station at Cape Ferguson was obtained from the Australian Bureau of Meterology (www.bom.gov.au) and mapped against proportion of turtles with lesions on the various fieldtrips.

Data Analysis

All analysis was performed using the Statsmodels module (version 0.10.0) and Pandas library (0.24.2) in Python 3.6 (15, 16). Chi Square tests were used to compare the proportion on turtles (post-outbreak) with lesions in each species, sex and age class. The association of CCL with probability of having lesions (odds-ratio) was calculated by logistic regression. Pearson correlation was used to determine the relationship between mean maximum air temperature and the portion of animals with lesions grouped by month of capture.

Laboratory Analysis

Pathology

With permission from relevant authorities, one male *E. m. krefftii* (14.0 cm CCL; 235 g), one male *M. latisternum* (14.8 cm CCL; 280 g) and one juvenile *M. latisternum* (12.0 cm CCL; 145 g) with lesions, were euthanized with MS222 according to Conroy et al. (17). A full necropsy and pathology investigation was performed, with emphasis on the lesions, but samples from all internal organs were collected from all three turtles. Samples were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with Haematoxylin and Eosin as well as Gomori's Methenamine-Silver (GMS), Ziehl-Neilsen (ZN), and Periodic Acid-Schiff (PAS) stains for examination (18).

Bacterial Culture and Initial Identification

Swab samples were collected in the field from 20 lesions of affected turtles and chins of 20 turtles without lesions. These areas were rinsed well with sterile saline before being sampled using dry swabs. Swab samples were also taken directly from liver, spleen, heart and heart blood during necropsy. All samples were immediately cultured on a range of media including Sheep Blood Agar (non-selective, aerobic and anaerobic) and MacConkey (selective for Gram negative bacteria) using standard culture methods. Bacterial identifications were determined using Biolog system and API20NE according to the manufacturer's instructions. All incubations were performed at 28 and 37°C for 48 h.

Bacterial Identification by Sequencing

Bacterial isolates that could not be identified using traditional methods were extracted to isolate genomic DNA using High Pure PCR Template Preparation Kit Version 20 (Cat. 11796828001 Roche, NSW) and Lysozyme (Cat. 10837059001 Roche, NSW) as per manufacturer's instructions. The concentrations of the resulting purified DNA samples were quantified using a Qubit 2.0 fluorimeter (Invitrogen) prior to storage in a −20°C freezer. The bacterial 16S rRNA gene was amplified with a set of universal primers, 27F and 1391R (5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-GACGGGCGGTGTGTRCA-3′; 1350bp) under standard PCR conditions consisting of an initial denaturation of 1 min at 95°C; followed by 30 cycles of denaturation at 95°C for 30 s; annealing at 55°C for 15 s; extension at 72°C for 15 s; and final elongation step at 72°C for 5 min. The PCR products were run on a 1.5% agarose gel to confirm amplification. Following confirmation, the PCR products were sent to Macrogen Inc., Seoul, South Korea for Sanger sequencing. Later, the nucleotide sequences were processed and aligned in Geneious (Biomatters Ltd.) followed by identification using NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Blood Biochemical Parameters

Blood was collected in the field (1 ml) from the femoral vein of 20 turtles with and 20 without lesions using a 1 ml syringe and 27 gauge needle into a lithium heparin paediatric tube. The samples were transported back to the JCU Pathology Laboratory on ice. The samples were separated as soon as possible and the plasma samples stored frozen (−20°C) until analysed within 24 h on an automated Clinical Biochemistry analyser (Beckman Coulter AU480). The blood biochemical parameters were assessed and analysed using a student *T*-Test to determine significant difference in biochemical parameters associated with presence and absence of skin lesions and severity of infection.

Viral Culture

Samples from select organs (lung, spleen, liver, kidney and heart) from the turtles that underwent necropsy were stored at −80°C for viral isolation. Samples were homogenised with 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 100X Antibiotic-Antimycotic and subjected to three freeze/thaw cycles at −20°C before clarification by centrifugation at 12,000 rpm for 5 min. The swabs from lesions were soaked in the same culture medium and treated similar to the necropsy samples. A total of 500 µl supernatant from each sample was added to 80% confluent monolayers of FHM (fathead minnow) cells in a 24-well tissue culture plate (SARSTEDT®). The plates were incubated at 25°C and checked daily for cytopathic effects. Two blind passages were performed for each sample at weekly

intervals, by transferring 100 μl of cell culture supernatant from inoculated wells to corresponding wells with new, non-infected FHM cell monolayers on a separate plate.

Viral Molecular Investigation

DNA extraction was carried out on swabs from lesions by NucleoMag® VET DNA isolation Kit (Macherey-Nagel) according to manufacturer's protocol. PCR was performed on lesion swabs using primers for various viral infections. The swabs were screened for herpesvirus, adenovirus, papillomavirus and ranavirus genome by PCR following the respective methods described by Vandevanter et al. (19), Wellehan et al. (20), Manire et al. (21), and Ariel et al. (22). Any samples that reacted in the assays were sequenced using BigDye v.3.1 Sanger sequencing (Macrogen, Korea) following gel extraction. Non-specific reactors (determined by sequence) were disregarded.

RESULTS

Spatial Extent of Outbreak

During September / October 2016, 23 *M. latisternum* and four *E. m. krefftii* were captured at the four upstream sites investigated for spatial extent of the outbreak (**Table 2**). None of the 27 turtles caught upstream from the Craill pond had cutaneous lesions. *M. latisternum* was the only species found at the three sites at highest altitude. At the site closest to the Craill pond, both species were present. A gravid female was identified at sites 1, 3, and 4, indicating that the turtles were actively involved in reproduction and nesting during this time.

TABLE 2 | Number, sex and age-class of *M. latisternum* and *E. m. krefftii* captured at upstream sites (1-4), during September/October 2016 fieldtrips to Alligator Creek.

	E.latisternum			*E. m. krefftii*	
Site	**Male**	**Female**	**Juvenile**	**Male**	**Female**
1	3	4*	1		
2	4	6	1		
3		2*	1		
4			1	1	3*

*Indicates that one of the females captured were gravid with hard-shelled eggs.

Water Quality

The pH levels were close to neutral for all sites (mean pH: 6.96, 7.22, 7.46, and 7.1 for sites 1-2, 3, 4, and 5 respectively). The water conductivity values at all sites were low (mean μS/cm: 64, 43, 74, and 63 for sites 1-2, 3, 4, and 5 respectively). The macro invertebrate populations were different at Site 5 (the Craill pond). Site 1, 2, 3, and 4 all had highly sensitive macro invertebrates which indicates undisturbed water conditions, while Site 5 only had medium or lower sensitive invertebrates typically associated with creeks found near urban settings.

Season and Proportion of Turtles With Lesions

The proportion of turtles captured with lesions varied over time in a regular manner that appeared to be seasonal (**Figure 1**). Air temperature was overlaid on the graph showing proportion of turtles with lesions over time. The proportion of turtles with lesions is negatively correlated with the mean maximum air temperature during the sampling month ($P < 0.05$, $r = -0.7$).

Gross Pathology

Lesions are characterized by focal areas of white-tan discoloration varying in size from 1 to 3 cm diameter, with irregular to rounded margins, which are occasionally firm, white and contracted, reflecting fibrosis (**Figures 2A,B**). The discolouration of the skin caused the lesions to look white against the darker skin and these white rounded patches were also noticeable when watching a turtle surface in the water. Position of 58 lesions on the skin of turtles were categorized using available photos from 22 females, 24 males, and 3 juveniles. Ninety three % of these type of lesions were on the dorsal surface of tails, legs and necks, the rest

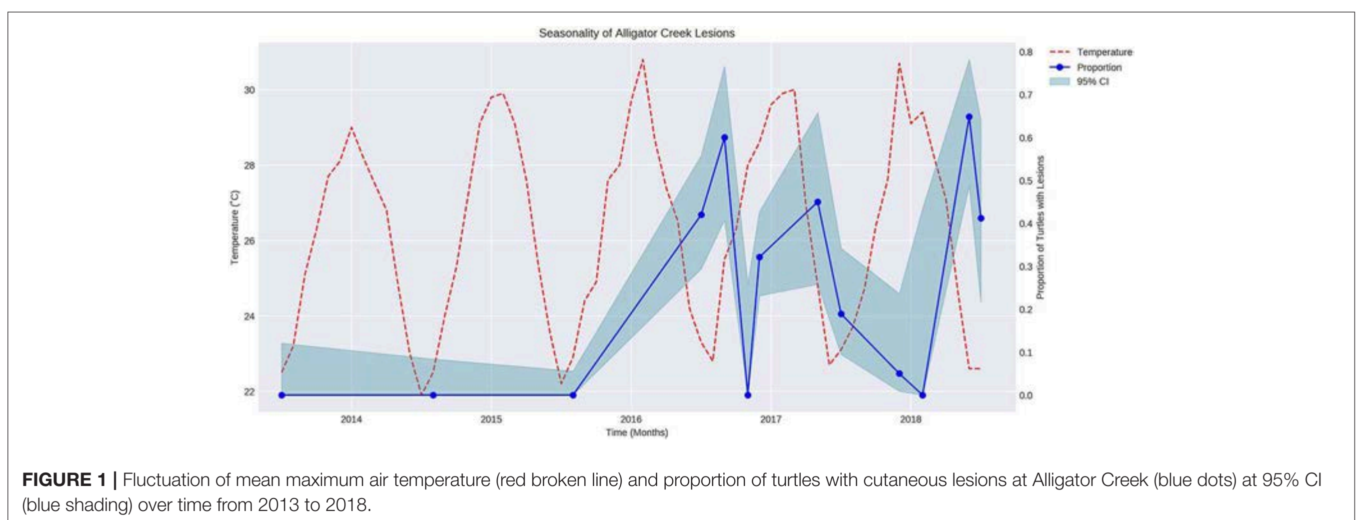

FIGURE 1 | Fluctuation of mean maximum air temperature (red broken line) and proportion of turtles with cutaneous lesions at Alligator Creek (blue dots) at 95% CI (blue shading) over time from 2013 to 2018.

FIGURE 2 | Typical appearance and location of skin lesions on turtles captured at Alligator Creek. **(A)** Skin lesions on top of foot of *E. m. krefftii*. **(B)** Skin lesion on neck of *M. latisternum*.

were lateral, except for lesions near the cloaca on two adult females. The lesions on the tails were mostly proximal (92%), predominantly distal on the legs (93%) and either proximal (43%) or middle of the neck (43%). Lesions were fairly evenly distributed between adult males and females for neck and leg lesions, but the tail lesions were recorded predominantly on males (92%). Turtles with lesions did not otherwise appear affected in terms of body condition and activity levels. The three turtles euthanized for necropsy were in good health with full gastro-intestinal tracts, plenty of body fat and no lesions other than the cutaneous lesions.

Histopathology

Skin samples from all three turtles were similar, with each demonstrating a severe, subacute to chronic, multifocal, necrotizing, suppurative epidermitis and dermatitis. The epidermis was multifocally eroded to ulcerated and overlain by a dense serocellular crust embedded with colonies of coccobacilli and rods (predominantly Gram negative) (**Figure 3**) and filamentous organisms (Gomori's methenamine silver stain (GMS) positive) (**Figure 4**). No organisms were observed with Ziehl-Neelsen stain. In some areas there was also a layering effect of keratin and exudate. At the dermo-epidermal junction, there was a band of inflammation and often, granulation tissue; the former consisted largely of granulocytes and macrophages. In the dermis, there was patchy infiltration of granulocytes and small blood vessels were congested and frequently cuffed by moderate numbers of lymphocytes and plasma cells. Vascular fibrinoid degeneration was occasionally observed, but rare. In the adjacent intact epidermis, there was frequently spongiosis of the basal cell layer. No significant abnormalities were noted in other organs.

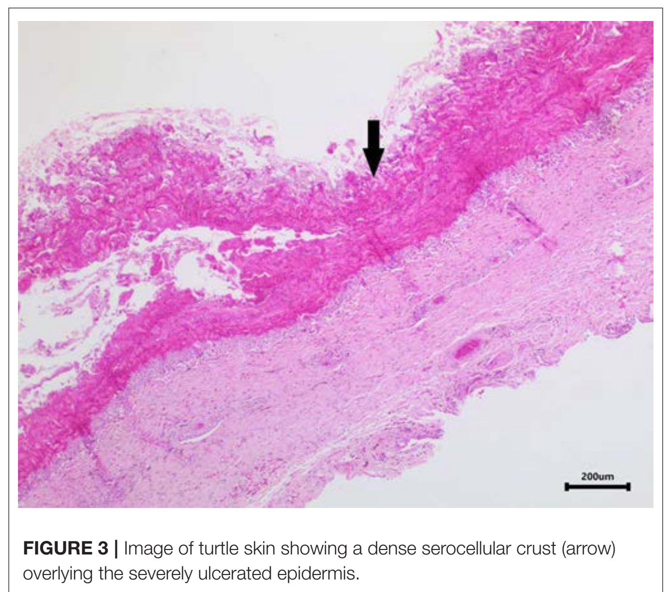

FIGURE 3 | Image of turtle skin showing a dense serocellular crust (arrow) overlying the severely ulcerated epidermis.

Bacteriology

Bacterial culture of swabs from internal organs during necropsy were negative, while a number of bacterial strains were cultured and identified from swaps of normal skin in turtles with and without lesions as well as directly from lesions (**Table 3**).

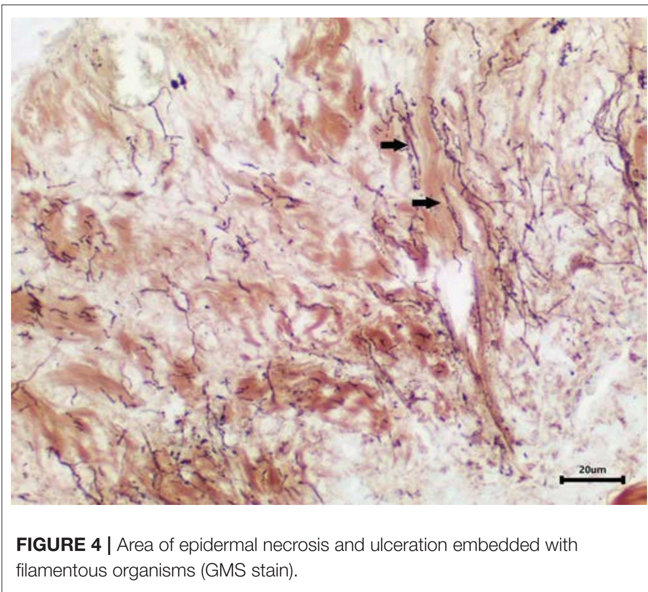

FIGURE 4 | Area of epidermal necrosis and ulceration embedded with filamentous organisms (GMS stain).

TABLE 3 | Number of bacterial strains isolated for 14 species of bacteria from 20 turtles without lesions and normal skin and lesions in 20 diseases freshwater turtles.

	Turtle without lesion	Turtle with lesion	
	Normal skin	Normal skin	Lesion
Serratia marcescens	2	3	9
Staphylococcus capitis	1	2	4
Fictibacillus and *Bacillus*	1	1	4
Aeromonas sobria		2	3
Aeromonas hydrophila		4	1
Chryseobacterium			1
Acinetobacter		3	
Aquitalea		1	
Diaphorobacter and *Acidovorax*		1	
Microbacterium sp.		1	
Pseudomonas fluorescens		1	
Bacillus	1		
Citrobacter youngae	1		
Pseudomonas aeruginosa	1		

Blood Biochemical Parameters

No statistically significant differences were observed in the blood parameters of turtles from different species, age groups, sex, sites, and presence or absence of lesions.

Virology

There was no development of CPE in cell cultures after three blind passages and the PCRs to detect ranavirus, herpesvirus, adenovirus, and papillomavirus genome were also negative for the samples collected.

TABLE 4 | The total number of *M. latisternum* and *E. m. krefftii* captured as well as those presenting with lesions in the Craill pond, during fieldtrips to Alligator Creek at various dates from 2013 to 2018.

Date	*M. latisternum*		*E. m. krefftii*	
	Total	Lesions	Total	Lesions
21/07/2013	15	0	13	0
10/08/2014	25	0	17	0
2/08/2015	44	0	21	0
31/07/2016	26	12	24	9
10/09/2016	13	10	12	5
6/11/2016	10	0	1	0
10/12/2016	45	17	39	10
5/05/2017	13	5	7	4
30/07/2017	21	4	16	3
10/12/2017	9	1	11	0
10/02/2018	1	0	4	0
28/06/2018	18	11	19	13
29/07/2018	9	2	8	5
	249	62	192	49

Turtles Captured

In the period from July 2013 to July 2018, 249 *M. latisternum* and 192 *E. m. krefftii* were captured in Alligator Creek at Site 5 (**Table 4**). All size groups of turtles were continuously captured over the period for both species (**Figure 5**).

The Curved Carapace Length (CCL) of turtles captured ranged in size from 7.8 to 30.0 cm for *M. latisternum* and 8.4 to 28.5 cm for *E. m. krefftii*. For both species, juveniles comprised approximately 1/3 of the captured turtles, while females dominated the *M. latisternum* captures (ratio male: female = 54 : 119) and males the *E. m. krefftii* turtles (male: female = 99 : 39) (**Table 5**).

Presence of Lesions Over Time

Cutaneous lesions were not recorded in this sub-population of freshwater turtles before July 2016, nor in any other freshwater turtles studied in the region, before or since. The number of turtles caught on each fieldtrip varied, as did the proportion of animals with lesions (**Figures 6A,B**). Presence of cutaneous lesions on turtles captured on fieldtrips since July 2016 varied from 0 to 77% for *M. latisternum* and 0 to 68% for *E. m. krefftii.*

Considering data from the first records of the outbreak in July 2016 and onwards from Site 5 only, there was no significant difference in proportion of turtles with cutaneous lesions between the two species (X^2 (1, $N = 306$) 0.262296; $p > 0.05$), nor was there any significant difference between sex in adult *M. latisternum* (X^2 (1, $N = 108$) 0.908654; $p > 0.05$), nor for *E. m. krefftii* (X^2 (1, $N = 102$) 0.728571; $p > 0.05$).

There was however, a significant difference in proportion of turtles with lesions between adults and juveniles (X^2 (1, $N =3$ 06) 16.67154; $p < 0.001$), with juveniles being less likely to have lesions than adults for the two species combined. For a 1 cm

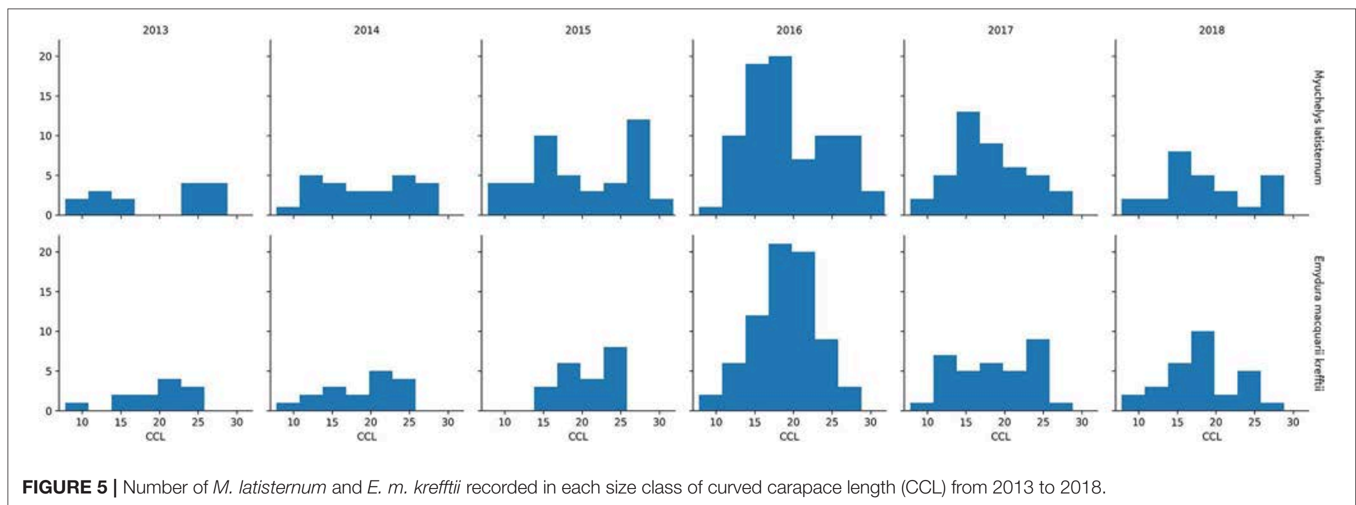

FIGURE 5 | Number of *M. latisternum* and *E. m. krefftii* recorded in each size class of curved carapace length (CCL) from 2013 to 2018.

TABLE 5 | Male, female and juvenile *M. latisternum* and *E. m. krefftii* captured at Alligator creek from 2013 to 2018.

	M. latisternum	*E. m. krefftii*
Males	54	99
Females	119	39
Juveniles	76	54

increase in CCL there is a 15.2% ($p < 0.001$, 95% CI 8.9–21.9) increase in odds of having a lesion.

Lesion Progression Over Time

In order to assess the progression of the disease over time, a subset of the data from 2013 to 2018 was investigated using turtles ($n = 43$) that had been recaptured since the initial recorded outbreak in July 2016. Spanning 11 fieldtrips over 2.5 years, 15 turtles remained without lesions on subsequent fieldtrips spaced from 2 to 30 months apart. Two of these turtles were previously recorded with lesions, but had recovered. Fourteen turtles that were registered with lesions, still had lesions 2–24 months later. One of these turtles (number 52854, **Figure 7**) was initially registered with lesions, which appeared to heal over a 3-month period, but it was recaptured with lesions 22 and 24 months later. Eight of the recaptured turtles were initially registered without lesions, but were recapture with them between 3 and 24 months later. On the other hand, five turtles with lesions were found to have healed on subsequent fieldtrips 2 to 5 months later (see **Figure 7**).

DISCUSSION

Several threats to freshwater turtles have been identified as contributors to their decline, including urban development (23–26) and introduced predators (4, 27), however, the study on the impact of disease on wild animals including populations of freshwater turtles is hampered by their cryptic and often remote habitats (22). Occasionally, spectacular outbreaks in public areas draw the attention of the general public and scientists alike and spur in-depth investigations with a multi-disciplinary approach (9, 10). The appearance of cutaneous lesions on a proportion of freshwater turtles captured as part of regular monitoring of wild living turtles in Alligator Creek, North Queensland was likewise an opportunity to study the course of disease development in a wild population over time.

Spatial and Temporal Extent of the Epidemic

The epidemic was confined to a natural pond on Alligator Creek, the Craill pond (Site 5), and although turtles were found upstream from the pond, these did not have lesions during the initial stages of the outbreak in 2016. Downstream populations were not investigated due to presence of saltwater crocodiles and associated risk, but were considered infected according to general principles guiding zoning for diseases in streams (14). Prior to July 2016, there were no records of such lesions in this area or other streams or rivers in the region despite regular annual sampling, but turtles were continuously caught with lesions in this pond for the 3-year monitoring that followed.

Water quality parameters tested did not indicate an acute contamination event although the Craill pond was considered more disturbed than the upstream sites, based on the macro invertebrates found there. However, it was not possible to completely rule out a pollution, contamination, or other toxic event as water was only sampled at one timepoint (during the initial outbreak).

An inverse relationship between temperature and the proportion of turtles captured with lesions throughout the year was identified. The relationship may not be a direct one, but temperature fluctuate with season and other seasonal factors such as migration and aggression in response to reproduction, basking sites or food availability (28) may also have increased the risk in this pond at certain times of the year.

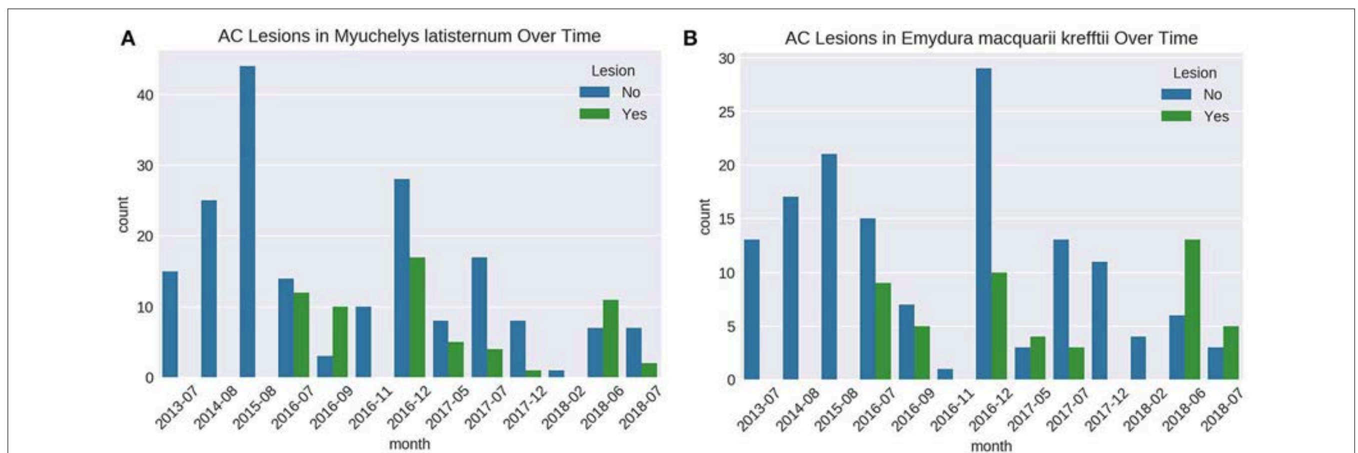

FIGURE 6 | Number of captured *M. latisternum* **(A)** and *E. m. krefftii* **(B)** turtles without cutaneous lesions (blue) and with lesions (green) on fieldtrips to Alligator Creek from 2013 to 2018.

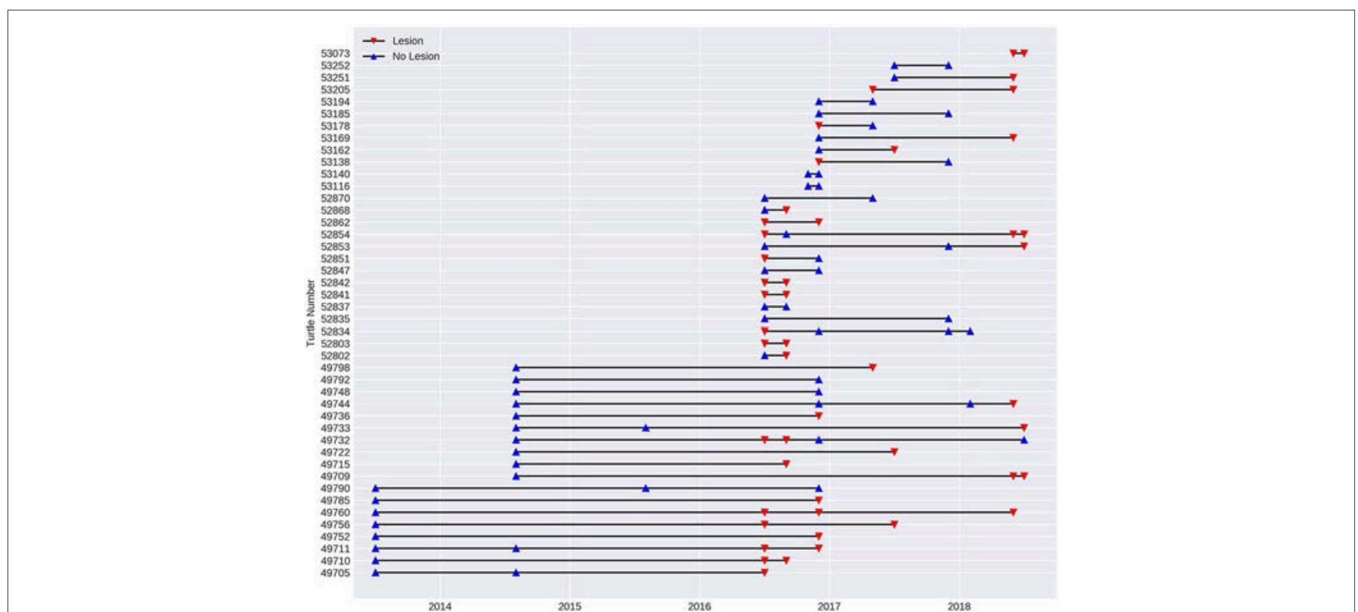

FIGURE 7 | Presence of lesions (red triangle) and no lesions (blue triangle) in the 43 turtles recaptured at Site 5, Alligator Creek between 2013 and 2018. Individual turtles are identified with tag number.

Characteristics of the Lesions

Histological examination revealed that the condition was largely confined to the epidermis, while all other organs in the animals investigated appeared normal. Skin lesions associated with ranaviral infection have been reported in lizards and turtles, but in most cases internal organs were also infected (29–32). While there was no detectable bias toward sex or species of turtles, the odds ratio indicates that the larger the turtle, the higher the risk of a lesion, and juveniles were rarely affected. The position of the lesions on the turtles indicated a non-random distribution on the skin and it is therefore likely that the initial skin lesion may have been caused by trauma and associated with a behavior where certain parts of the body would be at higher risk. The lesions were predominantly dorsal indicating that the trauma may have originated from above the turtle rather than below. The lesions on legs were mostly distal, while neck lesions were both proximal and middle of the neck. Tail lesions were essentially all proximal and on males apart from lesions near the cloaca on two females.

Given that peak lesion prevalence (May-September) precedes the nesting season of in this region, which is October to January for *E. m. krefftii* and September to March for *M. latisternum* according to Cann (12) and as evidenced by hard-shelled eggs detected via palpation on fieldtrips, it is possible that courtship behavior would put adult turtles in a higher risk group, either through intra-species aggression during courtship and mating (28) or because this behavior favors another risk factor such as a

predator. Biting during courtship and mating could also explain the lesions on the male tails as they may be targeted by competing males as seen in other freshwater turtles and sea turtles (28, 33). Alternatively, the males have larger tails than the females and would therefore be an easier target, but lesions on legs were mostly distal and on the feet, while the tail lesions were primarily proximal, which indicates that male tails are somehow a different target from legs. The lesions on the cloaca of two females could be associated with skin trauma during mating.

Courtship and mating is a natural occurrence and by itself would not cause such lesions. The investigation therefore focused on identifying a unique infectious agent, which could have gained entry following skin trauma.

Agent Causing the Lesions

Occasionally there are reports of wild populations of turtles succumbing to unknown pressures: several hundred *E. macquarii*, for instance, were unaccountably found dead at Lake Boga, South Australia, during the first half of 1976, while many others were clearly emaciated (12). Such mass mortalities are possibly caused by intoxication or infectious diseases. Most publications concerning the influence of disease on freshwater turtle populations in Australia are based on mass mortality events or general health problems in a population (7, 34). It is therefore challenging to interpret the presence of microorganisms in sick turtles as pathogenic or commensal due to lack of baseline data.

A number of bacterial strains were cultured and identified from swaps of normal skin in turtles with and without lesions as well as directly from lesions. The only bacteria isolated only from a lesion and not normal skin on any of the turtles was *Chryseobacterium*. This was only isolated in one instance, and is therefore not considered a causative agent. Some of the other bacteria are normal skin flora (*Aeromonas sobria, Aeromonas hydrophila, Serratia marcescens, Staphylococcus capitis, Fictibacillus,* and *Bacillus*), but they can on occasion turn pathogenic (22). In this study they were isolated at a higher rate from lesions and could therefore be secondary pathogens. There seemed to be a different composition of bacteria on skin of some turtles with and without lesions, but again, they do not appear to have any causation effect.

The blood biochemical parameters in turtles with lesions did not differ from turtles without lesions and none of the swab samples reacted in the virological assays for ranavirus, herpesvirus, adenovirus, or papillomavirus, which have been reported in reptiles on previous occasions (35). Ranaviral infections in turtles have been associated with intra-cytoplasmic inclusion bodies (36, 37), but these were not detected. A toxic etiology would also seem unlikely in this case, based on the distribution of the lesions, as it would be expected that the lesions would be random and affect all areas of the animal, rather than just the dorsum.

The Impact on the Population Through a Longitudinal Study

The lesions in the turtles at Alligator Creek, did not appear to have a dramatic impact on their overall health over the 3-year monitoring period following the first appearance of the lesions. There was no obvious decline or change in the demographics of the turtle populations and some turtles recorded with lesions were recaptured the following years, indicating that the affliction was not definitively lethal, but slow progressing. In addition, the post mortem examination following euthanasia of three adult males in clinically good health, but with skin lesions, revealed a good amount of body fat and a gastro intestinal tract full of ingesta, indicating that the animals were thriving, despite the winter temperatures and presumed mating season.

Field monitoring was intensified in the months after the initial reports of lesions so there are more data from late 2016 than other years, and fieldtrips in 2016–2018 were also spaced over more months and seasons. Following the initial outbreak in 2016, we continued to capture turtles of all sizes and both species of turtles, including recapture of 43 tagged turtles. This sub-sample of recaptured turtles was very useful in defining disease progression in the population. It revealed that some turtles stayed free of lesions over several years even if they co-existed with animals with lesions. Some turtles were repeatedly registered with lesions over several fieldtrips, while others had healed between captures. Other turtles were initially registered without lesions, but developed them between subsequent fieldtrips. Future monitoring will be required to determine if the population will fully heal over time.

This pattern of disease progression suggests that the agent is not a lethal one and that healing may occur naturally. It also suggests that exposure to the agent in the environment is not sufficient to cause disease, but there must be a contributing risk factor involved to create a portal of entry in a non-random fashion. These findings fits into the theory of an initial skin trauma associated with certain seasons, followed by entry and establishment of a pathogen present in the local environment. The seasonal aggression associated with courtship and mating could potentially be the cause of the skin trauma, but the identity and drivers for the appearance of a unique pathogen in this population is still under investigation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by James Cook University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

EA and WW contributed conception and design of the study. All authors contributed to the acquisition, analysis and interpretation of data for the work. WW and KD organized the database and photos. WW performed the statistical analysis. EA wrote the first draft of the manuscript. All authors wrote individual sections of the manuscript and revising it

critically for important intellectual content and approved the submitted version.

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Conflict of Interest: EE was employed by the company Ausphage.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix 6 - Poster: Clinical signs and their time to development vary with infection route in Australian freshwater turtle species: *Emydura macquarii krefftii*

Clinical signs and their time to development vary with infection route in Australian freshwater turtle species: *Emydura macquarii krefftii*

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Introduction

Ranaviruses cause disease in wild and captive turtle species around the world, often resulting in high levels of mortality[1-4]. *Ranavirus* pathogenesis has been studied in turtles from the sub-order Cryptodira, however, little research has gone into understanding this disease in species from the other living suborder of turtles, the Pleurodira. Australian side-neck turtles from the suborder Pleurodira (family Chelidae) live in habitats where ranaviruses have previously been isolated and a member of this group of turtles was susceptible to *Ranavirus* infection during experimental challenge[5,6]. Despite the potential for disease in these Australian turtles, little else is known about the pathogenesis of ranaviral disease and factors that influence its development. Inoculation route is an important factor to consider in experimental infections as different inoculation routes may result in different disease presentation, some more accurately reflecting the natural course of infection. To determine an infection route for use in future experimental challenge studies and to describe clinical signs and changes in pathogenesis produced by alternative routes of infection; **an experimental *Ranavirus* infection was performed with *Emydura macquarii krefftii* hatchlings (family Chelidae).**

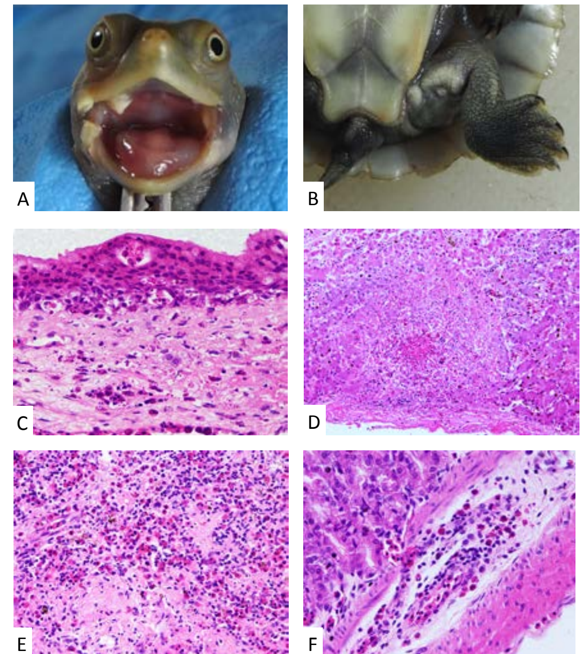
Methods

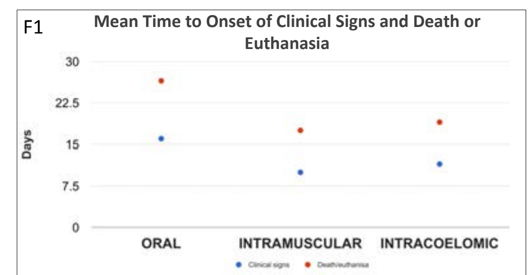
- Two *E. krefftii* hatchlings (two months old) were assigned to different infection route groups (oral, intramuscular, and intracoelomic) and each received an equal dose of *Bohle iridovirus* (BIV; $10^{5.33}$ $TCID_{50}$ ml^{-1})
- For each infection group one control hatchling received, by the same root, a placebo inoculation of phosphate buffered saline
- Throughout the trial animals where monitored for the development of clinical signs
- At the end of the trial (1 month) or at the onset of severe clinical signs the animals were euthanised with tricaine methanesulfonate (MS222)
- Following euthanasia gross pathological changes were recorded and various tissues where collected for analysis by real-time polymerase chain reaction (PCR) and histopathology

Australian 'side-neck' turtle hatchlings (*E. Krefftii*)

Results

	CLINICAL SIGNS	HISTOLOGY	PCR
ORAL	One hatching developed severe clinical signs including necrotic oral plaques (A), inappetence, lethargy, and soft tissues eodema. The other hatchling in this group showed no signs of disease and was euthanised at the end of the trial.	The diseased animal in this group had an influx of granulocytes into the liver and GIT, necrotizing splenitis and necrosuprative stomatitis and glossitis (C). The other hatchling had a slight increase in hepatic granulocytes, however, no other histological changes were evident.	Liver samples tested positive for *Ranavirus* in only the diseased animal.
INTRAMUSCULAR	Both hatchlings in this group developed severe clinical signs more rapidly than any other group (F1), these signs included lethargy, inappetence, oral haemorrhaging, periocular and soft tissues oedema, swelling at the injection site and increased basking behavior. One hatchling developed skin lesions at and around the site of injection (B) and produced a bloody discharge from the cloaca.	These animals had an influx of granulocytes into the liver and multi-focal epidermal necrosis. The hatchling with skin lesions had a more severe dermal and epidermal necrosis and haemorrhaging of the GIT.	Both liver samples tested positive for *Ranavirus*.
INTRACOELOMIC	Both hatchlings developed severe clinical signs including lethargy, inappetence, petechial haemorrhaging of the tongue, soft tissues oedema and increased basking behavior.	These animals had large necrotic areas in the liver (D) and increased granulocytes. One animal had necrotizing splenitis (E) and the other developed necrosis and inflammation of the intestinal submucosa (F).	Both liver samples tested positive for *Ranavirus*.





Conclusions

Emydura macquarii krefftii hatchlings were susceptible to disease via all inculcation routes, however, oral inoculation only produced disease in one of the exposed hatchlings. Time to development of clinical signs depended on inoculation route, however, larger studies will be required to determine statistical significance. Lethargy, inappetence and soft tissue eodema where common to all groups. The disease was generally systemic with multiple tissues affected. Group specific pathogenesis appeared to relate to the site of inoculation i.e. the oral and intramuscular groups developed lesions at the site of inoculation. This study describes the variability of clinical signs that depend on inoculation route. Future studies should consider this variability when designing challenge studies and elucidating transmission routes.

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Appendix 7 - Poster: Pathogenesis of Bohle Iridovirus (Genus *Ranavirus*) in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*)

Pathogenesis of a Ranaviral Infection in an Australian Freshwater Turtle Species (*Emydura macquarii krefftii*)

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INTRODUCTION

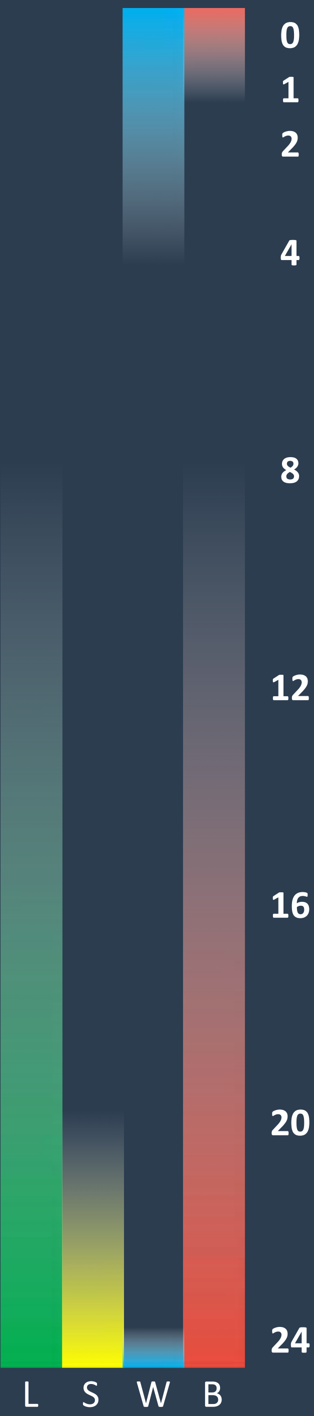
Ranaviruses are responsible for mass die-offs in many populations of ectotherms, including fish, amphibians and reptiles (Gray and Chinchar, 2015). For the most part, ranaviral disease research has focused on fish and amphibians, however, these viruses also cause disease in wild and captive reptile species around the world, often resulting in high levels of mortality (Duffus et al., 2015). Krefft's river turtle (*Emydura macquarii krefftii*) hatchlings are one such reptile species susceptible to ranaviral infection (Ariel et al., 2015). Little is known about the process of development of ranaviral disease in reptiles. Here, we present the results of an experimental infection with time staggered sampling designed to determine pathogenesis.

METHODS

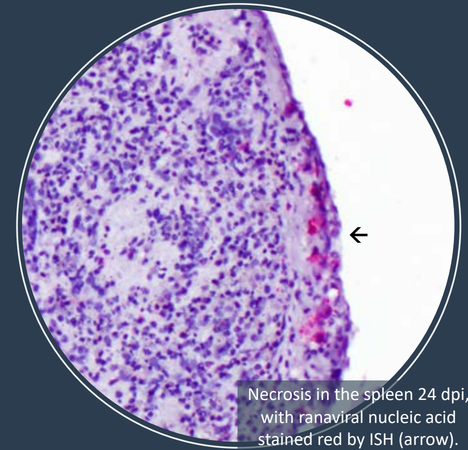
Captive-raised hatchlings (3 months old) were intramuscularly inoculated with a $10^{4.33}$ $TCID_{50}$ dose of Bohle iridovirus (*frog virus 3,* genius *Ranavirus*), (N=27) or placebo control (N=9). During the trial, animals were monitored for the development of clinical signs. Animals were euthanised in groups of 4 (3 infected, 1 control) at predetermined endpoints: 0.33, 1, 2, 4, 8, 12, 16, 20, and 24 days post-inoculation (dpi). At the time of euthanasia, samples (liver, oral swab, water, and blood) were collected for ranaviral polymerase chain reaction (PCR) and the remaining viscera was processed using standard histological methods. Immunohistochemistry (IHC) and *in situ* hybridisation (ISH) was used to associate viral antigens with observed pathogenesis.

RESULTS

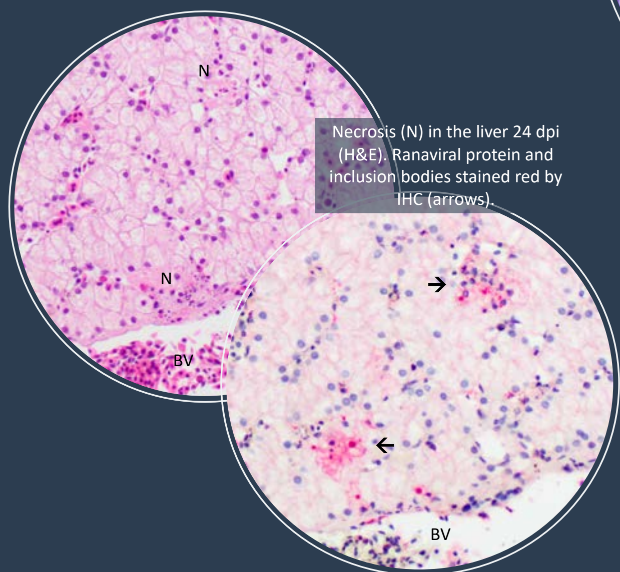
The median starting weight of hatchlings used in this study was 7.2 g (range, 5.4–8.9 g). Mild clinical signs were observed in most individuals after 7 dpi and were consistent with ranaviral infection, though one animal was euthanized 6 days ahead of its scheduled euthanasia (24 dpi) due to severe clinical signs. Clinical signs observed in this study in order of first appearance included: slight swelling of leg observed at inoculation site (2 days dpi), increased basking behavior (7 dpi), development of raised multifocal skin lesions at or near the site of injection (13 dpi), and swelling around the neck (14 dpi). Gross lesions, only observed in individuals after 16 dpi, included oral lesions and hemorrhage, soft tissues edema, splenomegaly, air in the digestive tack, multi-focal discoloration or mottling of the liver, and discoloration of the spleen. Ranaviral DNA was detected, via PCR, in the livers (L) and oral swabs (S) of infected individuals from 8 and 20 dpi respectively. Ranaviral DNA was detected in water (W) from the enclosures for the first four dpi then not again until the last day of the trial (day 24). The PCR on blood (B) showed similar results to the liver samples, and also reacted in the first two endpoints (0.33 and 1 dpi). Systemic histological changes included: dermal inflammation and vascular necrosis near the inoculation site from 8 dpi, hematopoietic necrosis in the spleen from 16 dpi, necrosis in the liver after 20 dpi, and inflammation and necrosis of the trachea and bronchi was observed in the final end point (24 dpi). Ranaviral staining by ISH and IHC was associated with histopathological changes, however, strong staining was not observed until after 20 dpi.



> ***"Pathogenesis of ranaviral infection in Krefft's turtle hatchlings follows from the site of inoculation, to the spleen, then liver and respiratory tract.***



Necrosis in the spleen 24 dpi, with ranaviral nucleic acid stained red by ISH (arrow).



Necrosis (N) in the liver 24 dpi (H&E). Ranaviral protein and inclusion bodies stained red by IHC (arrows).

> ***"Blood is the best non-destructive sample-type for detecting ranaviral infection in Krefft's river turtles by PCR.***

Skin lesions developed at (arrow) and near the inoculation site after 14 days.

CONCLUSIONS

Ranaviral infection in Krefft's turtle hatchlings is a slow process. The most effective (non-destructive) method for testing for ranaviral infection in these turtles appears to be PCR of blood. Histological labelling methods like IHC and ISH can be used to show the association of ranaviral histopathology with viral protein and nucleic acid; however, these methods were only useful in the late stage of infection. Pathogenesis appears to follow from the site of inoculation (i.e. the leg), to the spleen then liver and respiratory tract. Although ranaviral infection in Krefft's turtles has a long incubation period, they do not appear to shed virus during the majority of this time as viral DNA only detected in water 24 dpi (viral DNA in the water at the start of the trial is most likely leakage from injection site). This lack of shedding suggesting that Krefft's turtles may not be effective viral spreaders; however, more extensive studies will be required to see the full extent of viral shedding in this species.

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It is late and I am pipetting in the dark

It wasn't dark when I started

Ultraviolet lamina glow surrounds me

I whistle through the night

A new way home a new discovery

I lie down in bed and grin

And think of my morning coffee