UNIVERSITY OF NEW ENGLAND

THE ECOLOGY AND CONSERVATION OF THE ENDANGERED BELL'S TURTLE (*MYUCHELYS BELLII*)

A Dissertation submitted by
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For the award of
Doctor of Philosophy
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August 2021

ABSTRACT

Worldwide, herpetofauna are in decline and are increasingly of concern for conservation organizations as climate change, habitat loss, emerging infectious diseases, invasive species, and human exploitation all impact populations. Turtles in particular are an imperilled group, with more than half of all living species being considered vulnerable or worse by the International Union for the Conservation of Nature (IUCN). Turtles are thus considered high priority for conservation efforts around the globe. In Australia, many of the >20 species of native turtles are under threat. Several Australian species are endemic to small ranges, with some restricted to single river catchments, adding urgency to the necessity of their protection. Bell's turtle (*Myuchelys bellii*), "yiwaang" in Nganyaywana or "yiwanga" in Dhanggati, is a large freshwater turtle species endemic to some rivers in the New England Tablelands of New South Wales and Queensland. Like many Australian turtle species, the Bell's turtle is under threat, with nest raiding by invasive red foxes (*Vulpes vulpes*) of chief concern due to its negative impacts on recruitment. Other threats include potential competition with the Murray River turtle (*Emydura macquarii*), which is expanding its range into Bell's turtle habitat, and cataract-like eye abnormalities in some Bell's turtle populations. These threats, causing particularly low recruitment and limited species' range, have prompted the IUCN to list the Bell's turtle as endangered.

The aim of this thesis was to develop and test new conservation methods for protecting turtle nests against fox depredation, identify the species of turtle eggshells by eggshell microstructure, and to investigate the potential threats posed by interspecific competition and disease. Two nest protection methods were trialled in an attempt to curtail nest depredation rates: the use of large nesting refuge structures, and ultrasonic animal repellent devices. Results were unfortunately inconclusive. While nesting female turtles showed some apparent interest in the refuge structures, favoured nesting habitat is within the flood zones of streams for this species, and refuge structures were frequently inundated and damaged by flooding. Surprisingly, no foxes were recorded on the sites chosen to test the ultrasonic repellent devices, both before or after activation of the devices, so their utility at repelling foxes specifically remains uncertain. However, other mammal species that can hear in the ultrasonic range did not show any detectable aversion to the repellent devices, inferring that this method would be an ineffective deterrent to foxes. Neither nest protection method were therefore considered to be successful.

Turtle nests are often raided by predators before they hatch, and determining the turtle species that laid the nest can be challenging if there are multiple species nesting in a location. To test whether ootaxonomy (diagnosis of the provenance of eggs) was possible using microstructural features of eggshells, eggshell fragments from four native turtle species (Bell's turtle, Murray River turtle, eastern long-necked turtle (*Chelodina longicollis*), and Bellinger River turtle (*M. georgesi*)) were scanned with a scanning electron microscope (SEM), and microstructural features of the eggshells measured and compared. Central plaque size emerged as the most diagnostic feature, with Eastern long-necked turtle not having any visible pores, Murray River turtle having small pores, and both *Myuchelys* species having larger pores. Further refinement of sampling protocols are required, but these initial results suggest that SEM is a promising tool for ootaxonomy in turtles. A dichotomous key for the three species found in New England is proposed.

The potential for competition between Bell's turtles and Murray River turtles was analysed by comparing the size and condition between Bell's turtles that were sympatric or allopatric with Murray River turtles. Sympatric adult Bell's turtles, particularly females, were on-average significantly smaller and had lower body mass than allopatric turtles. As mature, breeding females are of vital importance to population persistence in turtles, further investigations into the interactions of Bell's turtles and Murray River turtles are recommended.

Finally, a landscape level analysis of the cataract-like eye disease was conducted. Abiotic factors were modelled and the most-predictive models identified through model selection. Mean annual solar radiation emerged as the strongest predictor, albeit with low effect size: turtles captured in higher-radiation areas had a higher probability of developing cataracts than turtles in lower-radiation areas. Further avenues to establish the link between eye abnormalities and radiation are suggested. Most concerning, while females with clinical signs were recaptured in later years with status either unchanged or without abnormalities (implying that females recover from the condition), no males captured with clinical signs were ever re-captured. While a skewed sex ratio in the area where the clinical signs are most prevalent could explain these data, more worryingly sex-biased mortality might also be occurring.

The research outlined in this dissertation provides valuable information for establishing policy and best practices for Bell's turtle conservation. These results were achieved in challenging conditions as the study area went through major drought and bushfire periods, followed by research restrictions related to the global pandemic that impacted the entire study. Nonetheless, many of the lessons learned here are likely applicable to other freshwater turtle species. Conservation programs have limited resources, and the null results of the tested nest protection methods would caution organizations from diverting valuable resources towards ineffective strategies. By contrast, the promising results of SEM ootaxonomy show that the method shows promise worth pursuing. Finally, the large-scale modelling of competition and eye disease presence provides avenues for future research efforts to proceed with clear objectives, namely to better study the interspecific interactions between Bell's turtles and Murray River turtles, and to investigate the link between local radiation levels and eye abnormalities in turtles.

CERTIFICATION OF DISSERTATION

I certify that the ideas, experimental work, results, analyses, software and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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Signature of Candidate Date

ENDORSEMENT

[redacted] 13/08/2021

Signature of Principal Supervisor Date

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Signature of Co-Supervisor Date

ACKNOWLEDGEMENTS

This PhD thesis was made possible with funding support by the NSW Environmental Trust through the Saving Our Species initiative, and by the Northern Tablelands Local Land Service. Additional funding was generously provided by the University of New England and the Holsworth Wildlife Research Endowment.

I must first offer thanks to my supervisors, Paul McDonald and Adrienne Burns. I know that they took a risk in inviting a Canadian to move down under and take up this PhD position, and I can only hope that I have made them proud. The support and direction given by my supervisory team has been exemplary and invaluable. With bushfires followed by flooding, never before in my career have the words "come Hell or high water" been so applicable, but your efforts have seen me through.

To my friends and colleagues in the ABEL, LAZER, and REED labs, thank you also for the support, feedback, and friendship that you have given me over the years. In no particular order, thanks go to Lucy Farrow, Jarrod Mesken, Candice Larkin, Lou Streeting, Deb Bower, Julie Kern, Farzaneh Etezadifar, Ahmad Barati, and Eric Nordberg. Further thanks must go to staff at UNE who have made my project (and life) so much easier these past few years: in particular Sandy Watson, Cindy Burton, Tony McKinnon, and Malcolm Lambert deserve thanks for their help over the years.

Further acknowledgement must go to the Turtles Forever organization; rarely have I had the pleasure of working with such a dedicated, passionate group. Particular thanks go to Marty Dillon, coordinator extraordinaire, and to Phil Spark, Bruce Chessman, Darren Fielder, and once again Lou Streeting for their tireless work on behalf of Bell's turtle, and for providing me with mountains of raw data to play with. The wonderful members of the New England community deserve much praise and thanks as well for their efforts and accommodation of turtle research on their properties: Anne and Winston Doak, Richard Daugherty, Judith Cox, and many others have given me so much help and support.

Of course, I have made many friends around UNE and Armidale that have not yet been mentioned, but absolutely must be. To Miriam Khan, Heath Milne, Jessica de Souza Vilela, Garth Bowen, Sharon McGavin, Tom Heddle, and Alva Curtsdotter have all been such wonderful friends to me over the years, as have many others that must go unmentioned lest this section end up as long as the rest of the thesis. From the bottom of my heart, thank you. It is *hard* to uproot your life and move to a new country, and having such splendid folk in my bubble certainly made it so much easier. Special thanks must also go to my dear

friends, James Baxter-Gilbert and Julia Riley, who helped smooth the transition from Canada to Australia for me when I first arrived. Thanks must also go to the friends whom I left behind in Canada, especially Jory Mullen who is looking after my pets while I've been away.

Lastly, but absolutely not the least, I must thank my family. My parents, Norm and Judy, have been endlessly supportive of me making a career of chasing small animals in the bush. My brother Greg, reliable tech support as ever, even from the other side of the planet. My many loving aunts, uncles, and cousins. Special thanks must go to my late grandmother, Joyce Hughes, whose financial support allowed me to start this journey in the first place. To everyone, I love you all and have missed you dearly these last few years, but I hope that I have made you all proud.

Geoffrey N. Hughes

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CHAPTER 1 - GENERAL INTRODUCTION

Despite their superficial similarity, the taxa referred to as "reptiles" are a far more diverse group than commonly understood by many; a deep divide exists within the living Sauropsida (the term which has largely replaced "Reptilia" in phylogenetics), between the Archosauria and the Lepidosauria (Haeckel, 1866; Cope, 1869). The extant living archosaurs include the crocodilians and birds, which are not traditionally included in the "reptiles" but are surviving maniraptoran dinosaurs (Gauthier, 1986; Benton, 2004). The living lepidosaurs include the lizards, snakes, and the tuatara (Evans and Jones, 2010). One clade of reptiles, the turtles, have been much more difficult to place within the phylogenetic tree. They were once considered part of the now-defunct clade Anapsida due to their lack of temporal fenestrae, but are now known to be diapsids that secondarily developed an anapsid condition (Rieppel, 1999; Zardoya and Meyer, 2001). Still, the exact position of turtles within the Diapsida remains contested. The most recent genetic studies suggest that they are closely allied to the Archosauria (Field *et al*., 2014; Crawford *et al*., 2015).

Turtles (Order Testudines, sometimes Chelonia) are an ancient clade with fossil specimens of potential turtle ancestors dated to 250 million years old, such as *Pappochelys rossinae* and *Eunotosaurus africanus* (Joyce, 2015; Schoch and Sues, 2015). The extant Testudines exhibit a deep phylogenetic divide between the Pleurodira, which fold their necks sideways into their shells for protection, and the Cryptodira, which fold their necks vertically and pull their heads backwards into the body cavity (Fig. 1.1). A related taxon of near-turtles, the Meiolaniformes, survived in Australia until the Pleistocene, and on the islands of the South Pacific until ~3000 years ago, but only true chelonians exist today (White *et al*., 2010; Rhodin *et al*., 2017).

There are three broad ecotypes found within the Testudines: marine turtles (7 species), terrestrial turtles including the tortoises (Testudinidae - 65 species) and box turtles, and freshwater turtles or terrapins (284 species; Rhodin *et al*., 2017). The majority of the extant species and ecotype diversity is found in the Cryptodira, with 263 species of cryptodirans (including all of the marine and fully terrestrial species) to 93 pleurodirans species (Rhodin *et al*., 2017). Extant pleurodirans are all freshwater or semi-terrestrial, though marine forms are known from the fossil record (Ferreira *et al*., 2015).

Turtles are a taxon in trouble. Of the 356 extant chelonian species recognised by the International Union for the Conservation of Nature (IUCN), more than half are considered threatened with extinction (Rhodin *et al*., 2017). Of these living species, 179 (50%) are considered at risk (either Vulnerable, Endangered, or Critically Endangered), and 12 other species have become extinct since 1500 CE (Rhodin *et al*., 2017). Numerous reasons are attributed to the sharp declines of turtle species in recent centuries, but they can generally be attributed to chelonian life history strategies being vulnerable to human exploitation or alteration of their habitats.

Turtles are characterised by a "bet hedging" reproductive strategy. Adults are long-lived and have high reproductive output with little investment in individual offspring (Congdon *et al*., 1994; Heppell, 1998; Tuberville *et al*., 2008). This strategy results in a high attrition rate of juveniles, with only a small percentage reaching adulthood (Gibbons, 1968; Iverson, 1991). While turtle populations are thus robust against the loss of juveniles (within limits), they are highly vulnerable to the loss of adults. Without these long-lived adults sustaining a high level of reproductive output, a population will eventually crash without recruitment (Brooks *et al*., 1991; Gibbs and Shriver, 2002). Human collection of turtles for food is a major cause of adult mortality in turtles (Shiping *et al*., 2008; Mancini and Koch, 2009); collection for the pet trade is also a major source of "mortality" (i.e. loss of breeding individuals from a wild population) in some species (Ernst and Lovich, 2009). Other factors affecting freshwater turtle species include habitat loss, road mortality, and subsidised or invasive predators consuming eggs or juveniles at unsustainable levels (Gibbs and Shriver, 2002; Hamilton *et al*., 2018; Lovich *et al*., 2018; Karson *et al*., 2019). All of these threats affect the turtles of Australia as much as they do turtles in other parts of the world.

1.1 Australia's Turtles

Of Australia's native freshwater turtle species, all but one are side-necked turtles of the family Chelidae (Table 1.1; Cann and Sadlier, 2017; Rhodin *et al*., 2017), with the taxonomy of Australia's chelid turtles often being a controversial subject. Disputes occur amongst authors over whether a particular taxon constitutes a full species or a subspecies, and even the status of some genera are debated (Kehlmaier *et al*., 2019). Consequently, it is difficult to definitively state the number of turtle species living on the continent; most sources list between 20 and 30. There are 7 well-accepted genera of Australian chelid turtles with varying numbers of species depending upon the source consulted (Table 1.1).

Australia's freshwater species are generally found on the margins of the continent, avoiding the dry interior (Cann and Sadlier, 2017); the greatest turtle diversity is found in

Queensland and the Northern Territory (Rhodin *et al*., 2017). Australian freshwater turtle fauna shows high degrees of endemism. Several species are restricted to particular catchments, with examples including the Mary River turtle (*Elusor macrurus*), Fitzroy River turtle (*Rheodytes leucops*), and Johnstone River snapping turtle (*Elseya irwini*). This high degree of endemism is thought to be due to populations of formerly widespread species becoming isolated and diverging as Australia began to dry in the Miocene (Martin, 2006; Byrne *et al*., 2008; Todd *et al*., 2013). Endemism is a strong predictor of extinction risk, due to endemic species typically having small populations and restricted species ranges (Gaston, 1998; Purvis *et al*., 2000; Kamino *et al*., 2012). As freshwater turtles often fill important aquatic grazer or scavenger niches in their ecosystems, the loss of these species can have cascading effects on the local watersheds (Lovich *et al*., 2018).

1.2 Study Species

The Bell's turtle (*Myuchelys bellii*; "yiwaang" in the Nganyaywana language or "yiwanga" in the Dhanggati language) is a highly threatened endemic species, and is the chosen study species for this research. Several concerns for the species' future have prompted this study, of which the Bell's turtle's restricted distribution in the New England region of eastern Australia is paramount. Limited literature exists on the species, and there are significant knowledge gaps on the Bell's turtle's behaviour, reproduction, and ecology, which potentially reduces the efficacy of any amelioration measures proposed to improve the species' conservation status.

The Bell's turtle and its close relatives have variously been included in the genera *Phrynops*, *Hydraspis*, *Elseya*, and *Wollumbinia*, before being placed in *Myuchelys* by Thomson and Georges (2009; Fielder *et al*., 2015a; Kehlmaier *et al*., 2019). The Bell's turtle has 2 or 3 extant congeners, depending on the source consulted. The common saw-shelled turtle (*M. latisternum*) and the critically endangered Bellinger River turtle (*M. georgesi*) are both generally accepted as members of the genus. The endangered Manning River turtle has been included in *Myuchelys* as *M. purvisi*, but has also been elevated to its own genus as *Flaviemys purvisi* by Le *et al*. (2013; Fig. 1.2). This elevation has been contested by other authors, such as Spinks *et al*. (2015; Fig. 1.2). Kehlmaier *et al*. (2019) suggested that the contradictory results of these two sources may be due to an ancient mitochondrial capture by the ancestors of the Manning River turtles, leading to a discrepancy between nuclear and mitochondrial phylogenies. Whether the Manning River turtle is included or not, *Myuchelys* is a largely imperilled taxon, with only *M. latisternum* being widespread and abundant (Cann and Sadlier, 2017; Rhodin *et al.*, 2017).

The Bell's turtle is the largest member of *Myuchelys* (Chessman, 2015; Fielder *et al.*, 2015a), with mature females reaching 30 cm carapace length. Males are smaller than females, known to reach ~23 cm in length (Chessman, 2015; Fielder *et al.*, 2015a). Like other *Myuchelys* species, Bell's turtles have relatively flat, smooth carapaces, darkly coloured and with little patterning in adults, and sharply-serrated rear marginal scutes (Fig. 1.3). The *Myuchelys* species all have fleshy tubercules on their chins and smooth, keratinous 'helmets' on their crania. Bell's turtles also have fleshy tubercules on their necks, features that distinguish them from the smooth-necked *M. georgesi* (Fielder, 2010). They are distinguished from *M. latisternum* by pale yellow stripes on their lower jaw and neck, while *M. latisternum* have no distinctive facial markings (Fielder, 2010). Manning River turtles have bright yellow facial markings that include a light coloured beak, while Bell's turtles have black beaks.

The Bell's turtle is endemic to the New England Tablelands of northern New South Wales (NSW), with a single additional population found in southern Queensland (QLD; Fig. 1.4). Although there are some morphological distinctions between the NSW populations and the disjunct QLD population, genetic analysis shows that they are a single species with no subspecies (Fielder, 2010). The species' range consists of the some of the upper-most catchments of the Murray-Darling Basin: the Macdonald River, Gwydir River, and some streams in the Border Rivers catchment (Fig. 1.4). Bell's turtle habitat consists of upland streams (above 600 m elevation) that are characterised by pools up to 4 m in depth, interspersed with shallower riffle areas (Fielder *et al.*, 2015a). Previous studies suggest that the turtles nocturnally forage in these riffles, and take refuge in the deeper pools during the day (Fielder *et al.*, 2015a).

The Bell's turtle is considered an imperilled species. The IUCN lists the species as 'endangered' (Rhodin *et al.*, 2017); it is also listed as 'endangered' in NSW (Threatened Species Scientific Committee, 2008), and as 'vulnerable' in QLD and by the Commonwealth government (Department of Environment and Heritage Protection, 1992; Department of Environment, Water, Heritage, and the Arts, 2008). There is a species recovery plan in place in NSW due to the Bell's turtle's endangered status (NSW Office of the Environment and Heritage (NSW OEH), 2014), but QLD and the commonwealth governments do not have recovery plans at present.

As discussed, endemism is a strong predictor of extinction risk due to limited ranges and often small populations (Gaston, 1998; Purvis *et al*., 2000; Kamino *et al*., 2012). There is often less "redundancy", in both numbers of breeding individuals and in genetic diversity, within populations of endemics. A catastrophic stochastic event can leave endemics on the brink of extinction, as was demonstrated in 2015 by the Bell's turtle's congener, the Bellinger River turtle (Spencer *et al*., 2018). The Bellinger River turtle was subjected to a highly infectious viral disease that devastated the population; human intervention has preserved the species for now, but the Bellinger River turtle's future is in doubt (Cann and Sadlier, 2017; Spencer *et al*., 2018). The Bell's turtle's situation has similar potential for disaster, though potentially mitigated by the species' segregation into several un-connected sub-populations (Fig. 1.4).

Seemingly peculiar to Bell's turtle is an eye disease that afflicts individuals, leading to the development of abnormalities that resemble cataracts. This disease is most prevalent in the Macdonald River, where as many as 10% of captured turtles have one or both eyes affected (Fielder *et al*., 2015a). Afflicted individuals have also been occasionally captured in the Gwydir River, Severn River, and Deepwater River (Chessman, Fielder, pers. comm. 2020). The cause, vector, and impacts on fitness of this disease are not well understood, but afflicted turtles do not appear to have reduced body condition compared to unafflicted turtles (Chessman, 2015). There are reports of an afflicted adult female being taken into captivity for observation, whereupon the cataracts cleared without medical intervention (Cann and Sadlier, 2017). One individual was captured in 2006 with abnormalities and was recaptured in 2015 with the abnormalities still in place, potentially showing that the turtles can survive long-term with the affliction (Chessman, 2015). While this particular illness appears to have little effect on the health of the turtles, the catastrophic disease that affected the Bellinger River turtle in 2015 does show how vulnerable turtle populations can be to such events, and emerging infectious diseases are a concern for turtle populations worldwide (Herbst *et al*., 1994; Knöbl *et al*., 2011; Ariel *et al*., 2017; Spencer *et al*., 2018). Thus, researchers cannot afford to be cavalier about dismissing the implications of this disease on the future of the Bell's turtle.

Like other chelonians, Bell's turtles are characterised by a bet hedging life history (Fielder *et al*., 2015b). Populations are reliant on long-lived adults to sustain high amounts of reproductive output, to offset high rates of juvenile attrition and thus allow for recruitment (Congdon *et al*., 1994; Heppell, 1998; Tuberville *et al*., 2008). Bell's turtles have particularly long maturation times even for turtles, as females only mature at ~20 years old (Fielder *et al*., 2015b). Introduced red foxes (*Vulpes vulpes*) are a major threat to Australian turtle populations due to nest raiding, with >90% nest mortality in some years (Thompson, 1983; Spencer, 2000; Van Dyke *et al*., 2019). While turtle populations are generally robust to the loss of juveniles, such continuous losses are likely unsustainable (Spencer *et al*., 2017). Red foxes are also known to attack nesting female turtles in Australia (e.g. Spencer, 2000), and turtle populations are much less robust against losses of breeding adults than losses of eggs or juveniles (Heppell, 1998). Furthermore, feral pigs are known predators of turtle nests in other parts of Australia (Fordham *et al*., 2006; Whytlaw *et al*., 2008) and pigs do occur within the Bell's turtle's range, although have not been directly observed raiding Bell's turtle nests.

Competition with other turtle species is also a potential cause for concern. Bell's turtles co-occur with two other native turtle species. The eastern long-necked turtle (*Chelodina longicollis*) is sympatric with the Bell's turtle in all catchments and has very different dietary and habitat requirements (Chessman, 1984), so the two species are unlikely to compete in any significant way. The Murray River turtle (*Emyudra macquarii*) is sympatric with Bell's turtles in only two streams, the Deepwater River and parts of Bald Rock Creek, but is the more abundant species in both areas of overlap (Chessman, 2015). The two species have similar life histories, which has led to concerns that the Murray River turtle, which is widespread throughout Australia, may outcompete the endangered Bell's turtle where they co-occur (Chessman, 2015). While the Deepwater River and Bald Rock Creek may be natural zones of sympatry, there are potential avenues for invasion of the Gwydir River by Murray River turtles (Chessman, 2015), which could be problematic to the local population as it has existed without a high degree of interspecific competitors. It is widely reported that introduced turtle taxa can have profound effects on native turtle species (e.g. Cadi and Joly, 2003; 2004). For example, the highly-invasive North American red-eared slider (*Trachemys scripta elegans*) has established colonies in some Australian urban areas (O'Keeffe, 2009; Robey *et al*., 2011), but has not been recorded within the Bell's turtle's range.

The Bell's turtle is apparently a habitat specialist, only found in upland streams above 600 m elevation (Fielder *et al*., 2015a). Bell's turtles have not been recorded in still waters, such as wetlands or artificial dams, nor in lower-elevation rivers, and have not been recorded travelling long distances over land. It is not known if Bell's turtles could survive or thrive in other aquatic habitats like its congener, the common saw-shelled turtle (*M. latisternum*), which may be more of a generalist and able to use a broader range of habitats (Freeman and Cann, 2014). However, assuming that Bell’s turtles are habitat specialists until demonstrated otherwise, they would be particularly vulnerable to local alteration and destruction of riverine habitats (Sarre *et al.*, 1995; Foufopoulis and Ives 1999). According to the journals of explorer John Oxley, the pre-colonial landscape of the New England region was mainly open woodland, but much of that habitat has been cleared for livestock pasture starting in the 1830s. The river banks and beds of the Bell's turtle's streams are often damaged by cattle trampling and pugging, which causes compaction and erosion of river banks and siltation of adjacent waterways. Further, water contaminated with livestock faeces (pers. obs.) can cause eutrophication in these rivers. These disturbances almost certainly affect the general health of the river (Gregory and Gamett, 2009; Wilson and Everard, 2018), but the direct effects on the Bell's turtles are not known.

1.3 Study Sites

Due to the highly-restricted distribution of the Bell's turtle, this study took place entirely in the New England Tablelands of New South Wales, Australia. The Tablelands are a highland plateau that varies between 600 m and 1000 m above sea level, with some peaks reaching ~1500 m elevation. The region is largely agricultural, particularly cattle and sheep pasture, with the hills and deeper river gorges tending to be un-cleared and more densely forested.

Bell's turtles are restricted to three west-flowing catchments in the upper Murray-Darling basin: the Macdonald River and Gwydir River in NSW, and the Border Rivers catchment in NSW and QLD. The study sites were all in NSW, with most work conducted in the Macdonald River and Gwydir River and their tributaries, with some work done on the Deepwater River and Severn River in the Border Rivers catchment. For Chapters 4 and 6, historical data from these streams and from Bald Rock Creek in QLD were also incorporated. Work was primarily done on private lands with permission from the landholders, but also in travelling stock reserves (TSRs) with the cooperation of Northern Tablelands Local Land Service.

The Macdonald River is the southernmost catchment in the study area. Bell's turtles are known to inhabit its length from the headwaters (near Niangala NSW) to Warrabah National Park (Chessman, 2015), where the Macdonald River becomes the Namoi River (Fig. 1.4). Tributaries of the Macdonald where Bell's turtles were captured during this study are the Cobrabald River, Carlisle Gully, and Watson's Creek. Previous analyses on the water quality of the Macdonald River within the Bell's turtle range show a water quality index of "poor", particularly for nitrogen, phosphorus, and dissolved oxygen (NSW DPIE, 2020a). It should be noted that these water quality parameters may not be reflective of these rivers' quality as Bell's turtle habitat.

The Gwydir River lies between the Macdonald River and Border Rivers (Fig. 1.4). Bell's turtles are known to inhabit the catchment from the headwaters (near Yarrowyck NSW) to Lake Copeton near Inverell NSW. Murray River turtles (*Emydura macquarii*) inhabit Lake Copeton itself, and also the Gwydir River downstream of the lake. Tributaries of the Gwydir River where Bell's turtles were captured during this study are Roumalla Creek, Georges Creek, Rocky River, Laura Creek, Boorolong Creek, Moredun Creek, Limestone Creek, and Cope's Creek. The water quality in the Gwydir River was rated from poor to fair within the Bell's turtle's range, with particularly poor index ratings for nitrogen, phosphorus, and pH (NSW DPIE, 2020b). Laura Creek was analysed in the same study, receiving a "good" index rating (NSW DPIE, 2020b).

The Border Rivers catchment is the most northern catchment within the Bell's turtle range, and several rivers within the catchment contain Bell's turtle populations with no known population connectivity among them (Fig. 1.4). The Severn River, near Glen Innes NSW, is the southernmost of these rivers, along with its tributary, Beardy Waters. The Severn flows into the McIntyre River, but Bell's turtles are not found downriver of Pindari Dam in the Severn. Further north is the Deepwater River, near the towns of Bolivia and Deepwater NSW. The Deepwater River contains both Bell's turtles and Murray River turtles, and flows into the Mole River, which only contains the latter species. Bald Rock Creek, located in Girraween National Park in QLD, is the northernmost known population of Bell's turtles. Murray River turtles are found in the lower reaches of the creek, but not in the upper reaches. Bald Rock Creek flows into Accommodation Creek near Ballandean QLD. Water quality data in the Bell's turtle-inhabited Border Rivers are only available for the Severn River, which was rated as "poor", particularly for nitrogen, phosphorus, and pH (NSW DPIE, 2020c).

Although most of these streams did not receive high ratings for indices of water quality, the relationship of these variables to the individual and population health of Bell's turtles is not clear. In general, the effects of nutrient loads and pH on freshwater turtles has not been studied, although turtle mortalities have been noted in eutrophic lakes with blue-green algae blooms (e.g. Chen *et al.*, 2009). Dissolved oxygen is likely to be highly important, as Bell's turtles spend the winter submerged for long periods of time, absorbing oxygen from the water (Fielder, 2012).

While potential long-term threats such as habitat degradation are important to examine in depth, such studies should not come at the expense of short-term issues. At present, Bell's turtle captures are highly adult-biased, with little evidence of significant recruitment occurring (Chessman, 2015; Fielder *et al.*, 2015b). To ensure that the Bell's turtle persists into the future, more immediate conservation concerns must also be addressed.

1.4 Summary

The Bell's turtle's limiting factors (endemism, habitat specialism, bet hedging lifestyle) and the high number of threats facing the species (invasive predators, habitat alteration, competition, the potential for disease outbreaks) has led to serious concerns for the future of the Bell's turtle. This PhD thesis explored in greater detail some of the current conservation concerns identified for the Bell's turtle.

Chapter 2 tested a method of passively protecting Bell's turtle nests from predation, namely the use of exclusion structures to serve as nesting refuges. This technique could provide a valuable tool for conservation efforts if shown to be effective. These refuge structures were based on a design used successfully in the United States for diamondback terrapins (*Malaclemys terrapin*), and were intended to allow Bell's turtle females to enter and nest, while excluding foxes and other predators from the enclosure (Quinn *et al.*, 2015). The intention was to provide a low-cost, low-effort alternative to locating and protecting individual nests, as has been done successfully for many turtle species (e.g. Riley and Litzgus, 2013). Protecting a larger area of suitable nesting habitat and enticing nesting females inside would be simpler than locating individual nests, and would not require the services of (potentially expensive) detection dogs. This method may also be suitable for other ground-nesting species in need of protection from nest predators, such as other small reptiles or birds, by providing a protected area that can be accessed by the protected species but not by predators.

Chapter 3 tested an active exclusion method, employing motion-triggered ultrasonic repellent devices to repel foxes and other predators from nesting beaches. In turtle species where hearing range has been examined, none could hear into the ultrasonic range (e.g. Heffner and Heffner, 2007), so these devices would hypothetically not disturb a nesting turtle, whilst startling or inflicting discomfort on any foxes or other mammalian predators that moved within range. Such devices are beneficially low cost and not labour-intensive to employ, potentially providing another tool for conservation efforts if they are shown to be effective. This method may also be suitable for other ground-nesting species that cannot hear ultrasonic frequencies, by excluding mammalian predators that can hear those frequencies.

Chapter 4 tested a technique for determining the species of origin of turtle eggshells. All too frequently, researchers discover turtle nests only after they have been raided by a predator. Valuable data may still be gleaned from these raided nests, but it may be difficult to determine which turtle species laid a given nest, so assigning these data to a particular species becomes problematic. Eggshells of known provenance from four Australian turtle species were examined using a scanning electron microscope, to determine if diagnostic features were apparent among these species. If so, a powerful new tool may become available for turtle research and conservation.

Chapter 5 tested the hypothesis that Bell's turtles and Murray River turtles compete with each other in streams where they co-habit. This study made use of recent and historical capture records of Bell's turtles to compare carapace length, mass, and standardised mass index of Bell's turtles captured within and without the zones of sympatry. Bell's turtles were divided into separate life history categories (juveniles, sub-adult females, adult females, and adult males) for these comparisons. These biometric variables were compared between presence and absence of *Emydura*, and to selected abiotic factors of these rivers as a measure of degree of effect presence/absence has on Bell's turtle growth and condition.

Chapter 6 explored the occurrence of cataracts and other eye abnormalities across landscape-level spatial scales, to determine if patterns of the disease could be identified. Using a long term mark-recapture dataset of Bell's turtles, the occurrence of eye disease across catchments was examined and compared to abiotic factors and anthropogenic disturbances within the Bell's turtle streams. Model selection was used to test for likely predictors of cataract occurrence.

Appended to this thesis is a version of a natural history note, published in Herpetological Review as Hughes *et al*. (2020). During collection of stomach contents from Bell's turtles to better understand diet, two samples from a pair of large, hand-captured females were collected. Both samples were completely composed of large quantities of *Daphnia* spp., a smaller free-swimming crustacean. Previous work on the diets of Bell's turtles had been performed (Fielder *et al*., 2015a), but *Daphnia* had not been identified as a food source. This may be the first recorded instance of a large freshwater turtle consuming mass quantities of small prey. Appendix II provides supplementary figures for Chapter 5.

The goal of this PhD was to aid in the preservation of an endangered, endemic species of freshwater turtle. This goal was pursued via two broad objectives: one applied conservation (Chapters 2, 3, and 4) and one investigative ecology (Chapters 5 and 6). The methods trialled and threats explored herein will assist conservation organizations for this and potentially other turtle species, by informing policy and best practices for future conservation efforts, and providing guidance for future research efforts.

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1.6 Figures

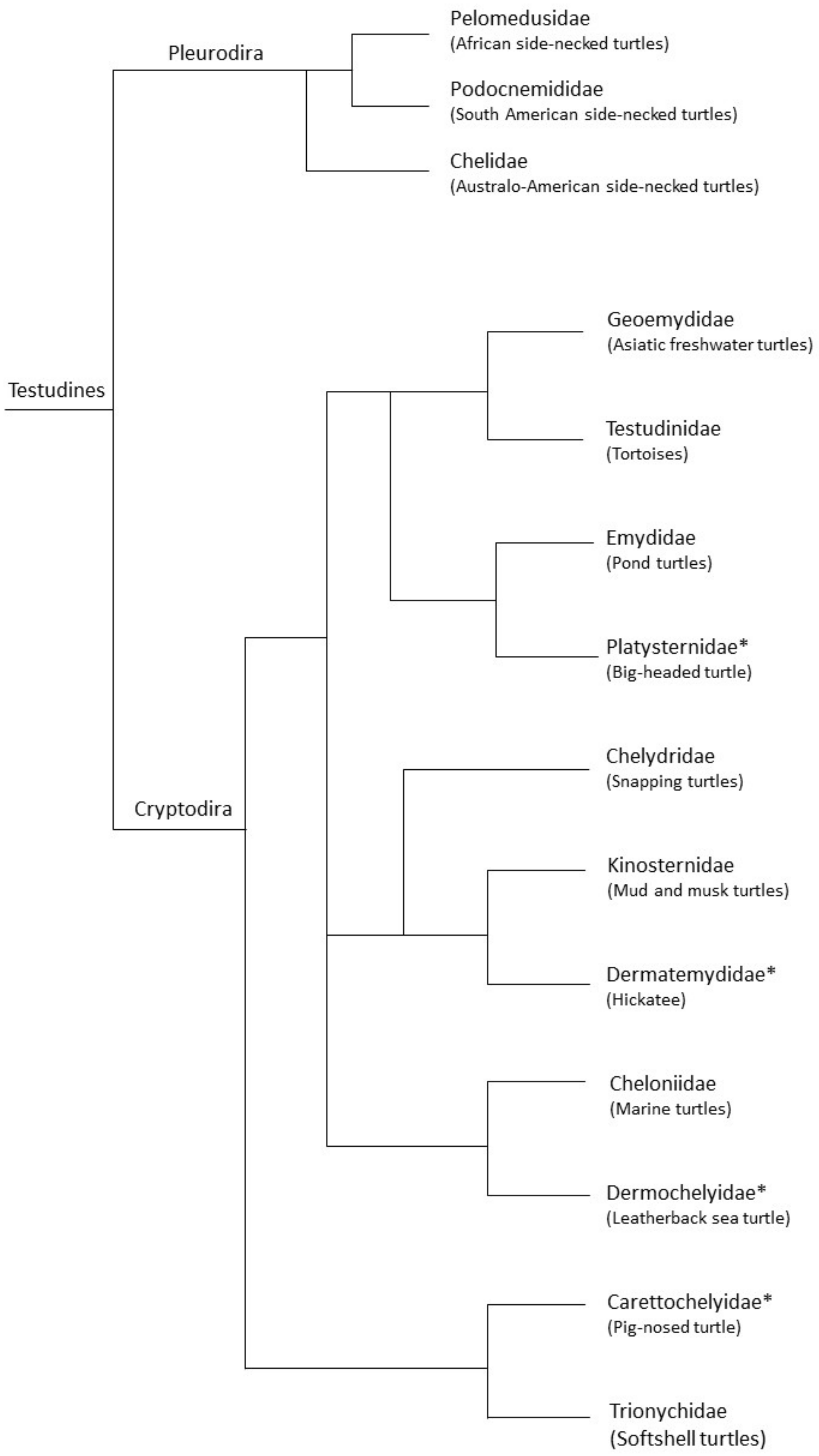

Figure 1.1 - Extant turtle families, derived from Guillon *et al*., 2012. An * denotes a monotypic family.

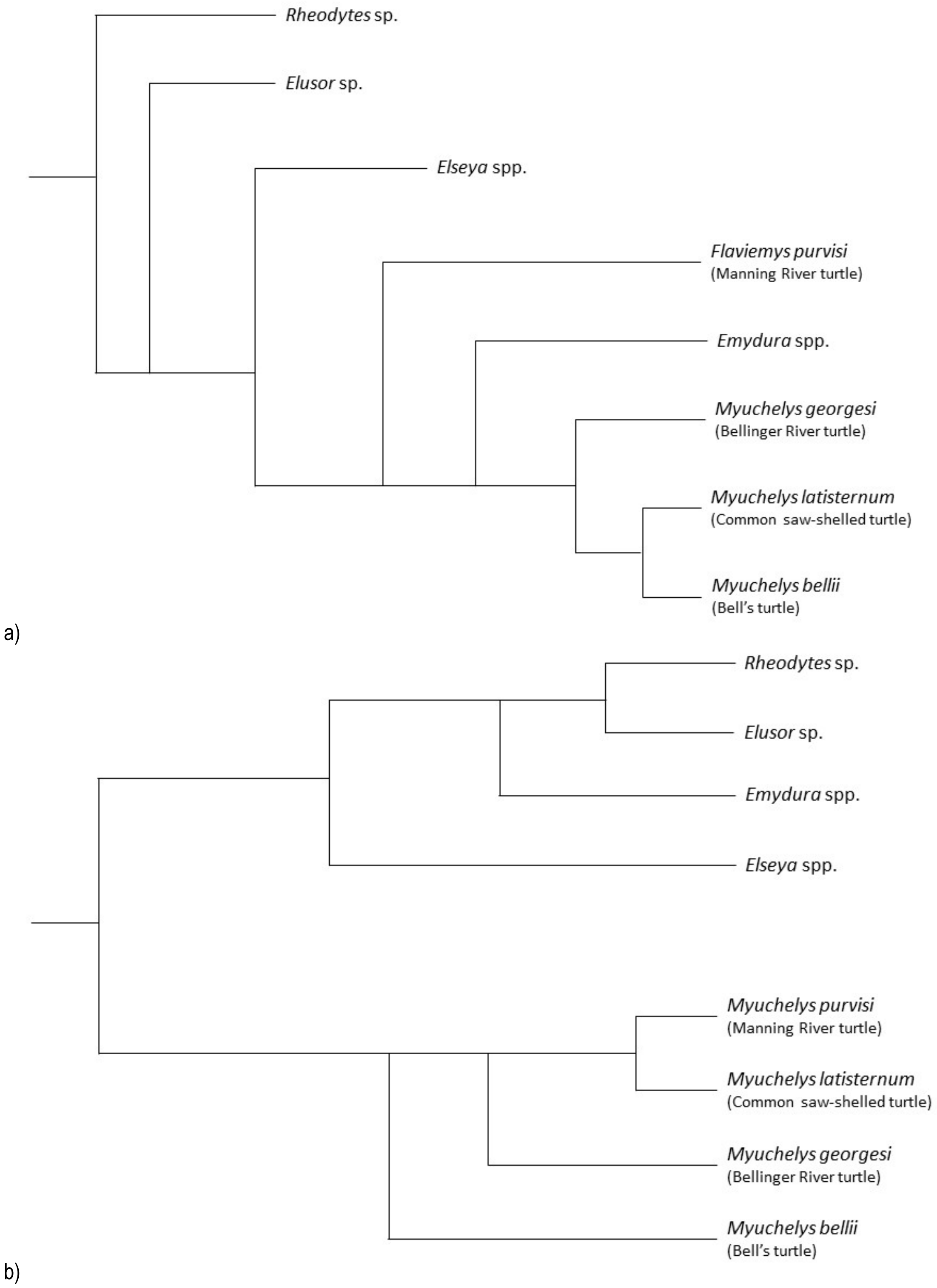

Figure 1.2 - Proposed phylogenies of the Australian short-necked turtles, with particular focus on the genus *Myuchelys*. Cladograms are derived from a) Le *et al*., (2013) and b) Spinks *et al*., (2015).

Figure 1.3 - Members of the Australian freshwater turtle genus *Myuchelys*. a) *M. bellii*, the Bell's turtle, b) *M. latisternum*, the common saw-shelled turtle (photo credit: D. McKnight). c) *M. georgesi*, the Bellinger River turtle (photo credit: R.-J. Spencer). d) *M?. purvisi*, the Manning River turtle (photo credit: P. Spark); placement of this species in the genus is unclear, but included here for completeness. All credited images are used with permission.

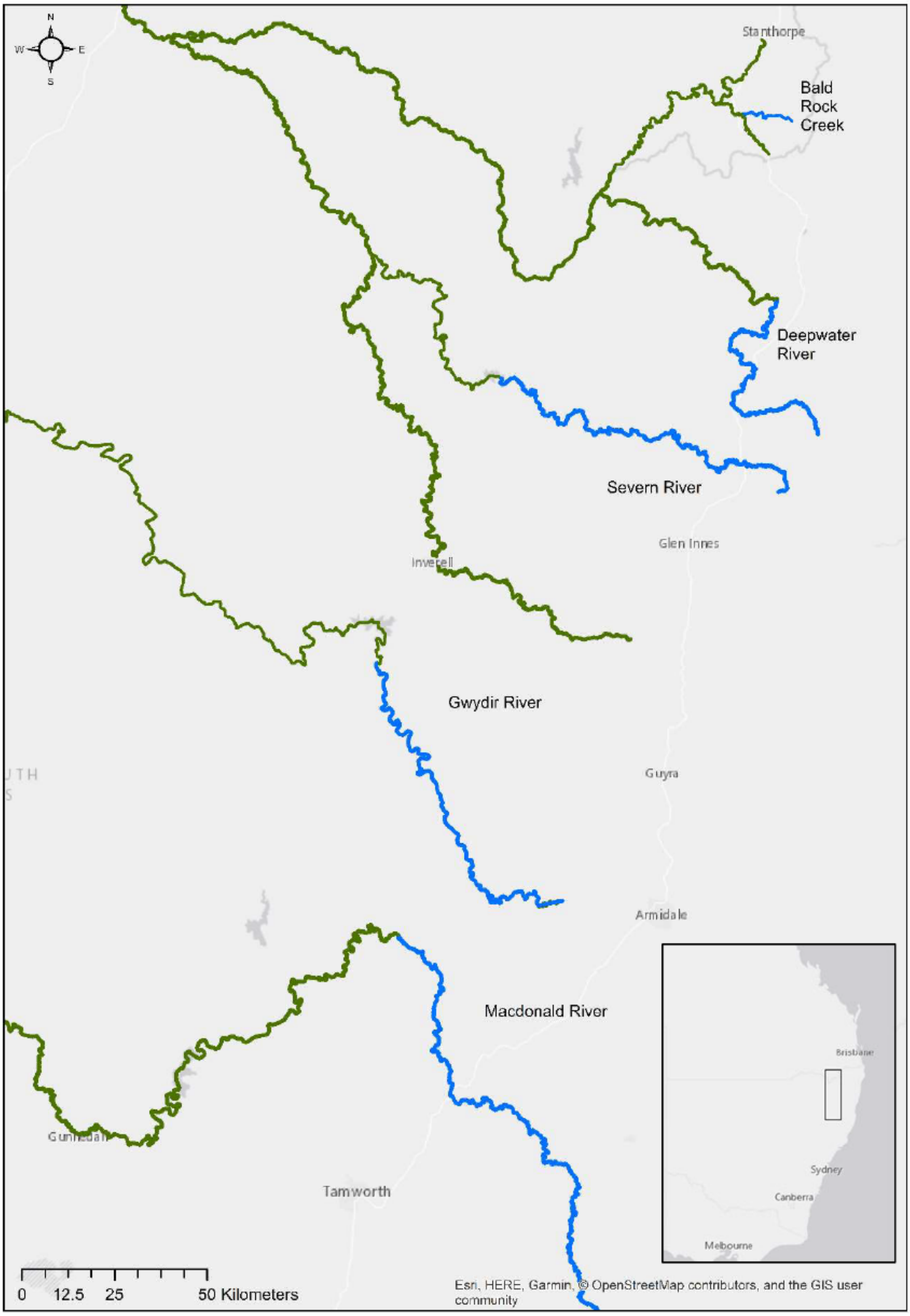

Figure 1.4a - Catchments inhabited by Bell's turtle (*Myuchelys bellii*). Blue lines indicate rivers inhabited by Bell's turtles, green lines indicate connected downstream rivers not inhabited by Bell's turtles. Inset shows the location of the study area in eastern Australia.

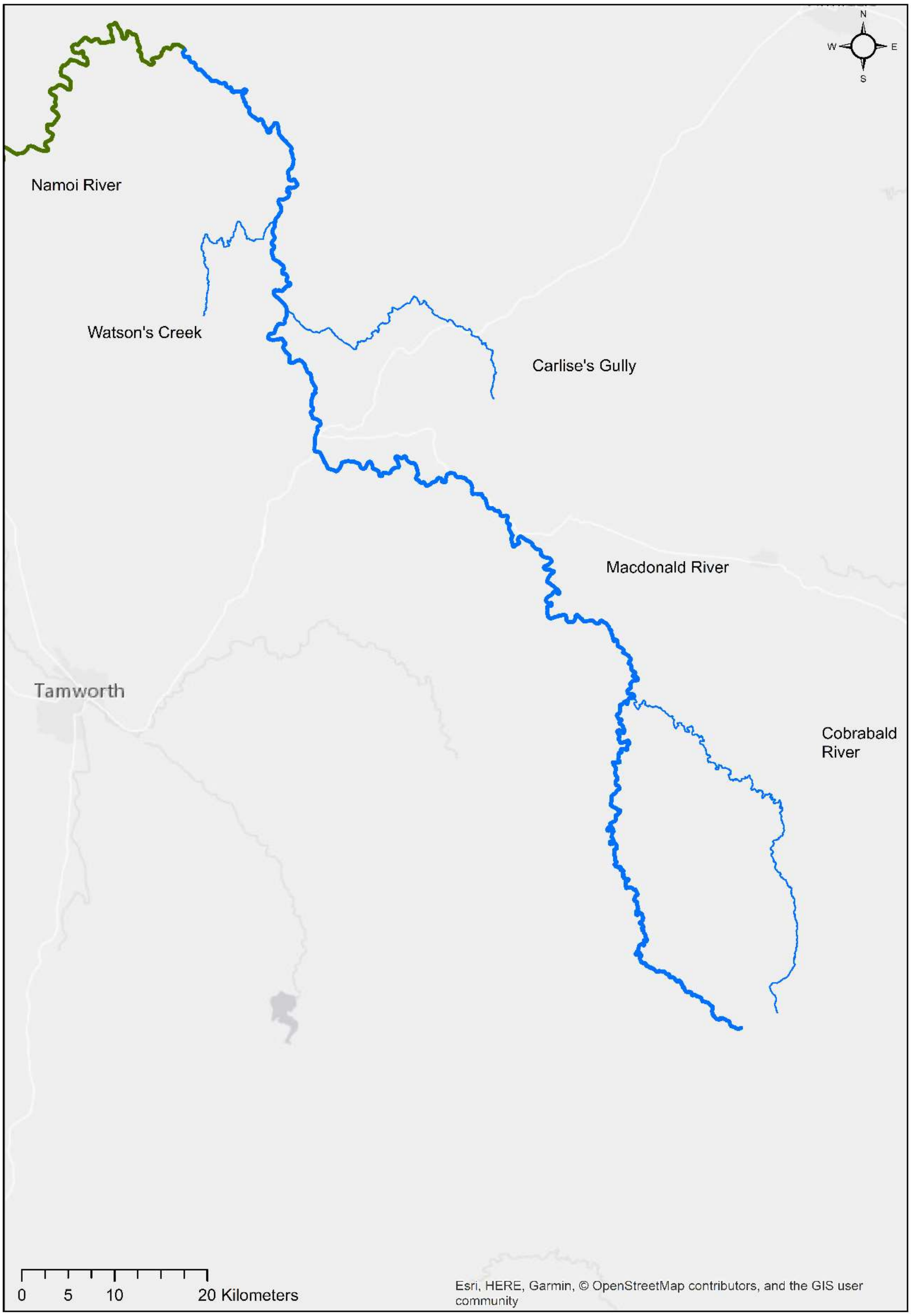

Figure 1.4b - Streams in the Macdonald River catchment inhabited by Bell's turtle (*Myuchelys bellii*). Blue lines indicate rivers inhabited by Bell's turtles, green lines indicate connected downstream rivers not inhabited by Bell's turtles. Thinner blue lines denote a tributary.

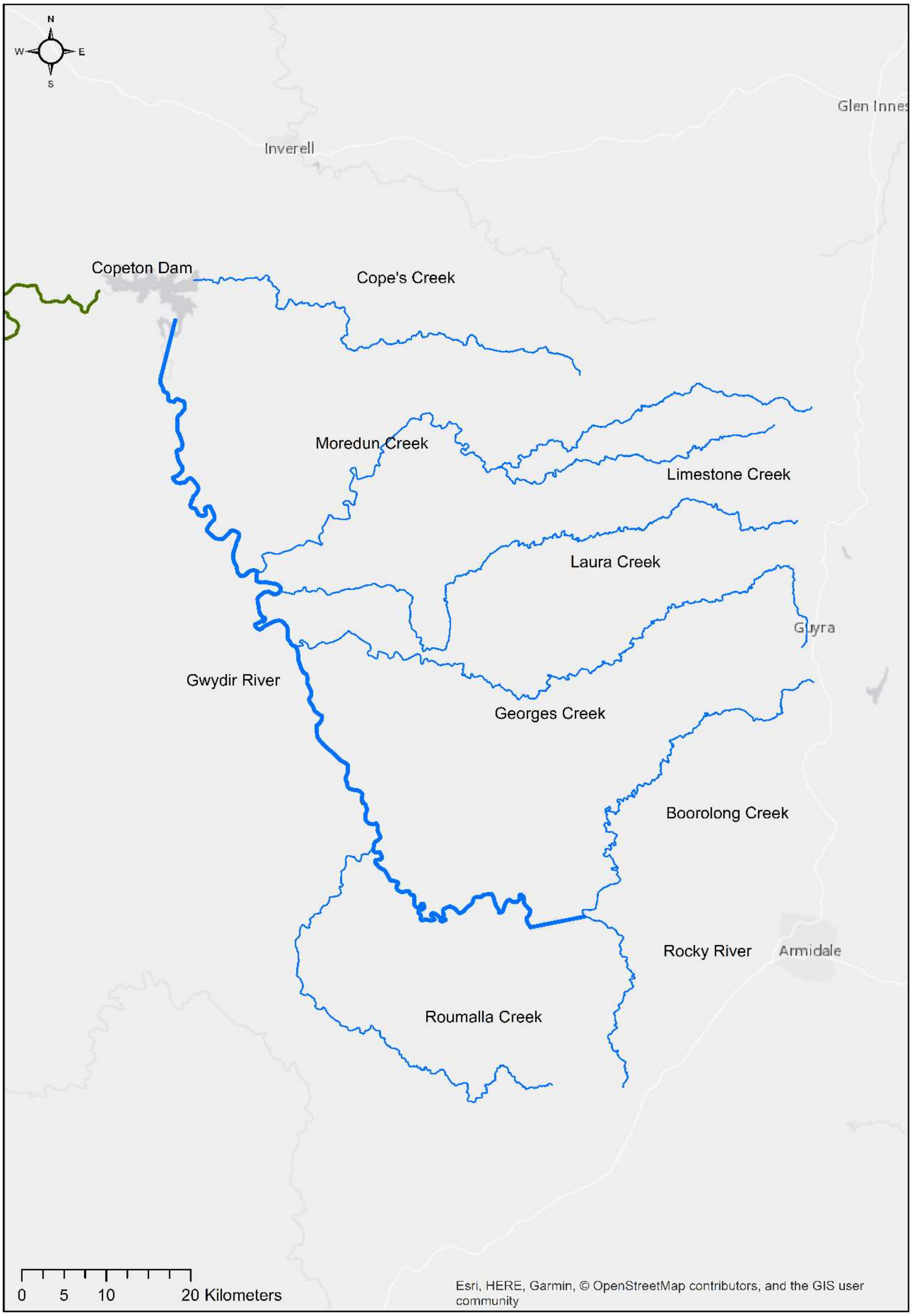

Figure 1.4c - Streams in the Gwydir River catchment inhabited by Bell's turtle (Myuchelys bellii). Blue lines indicate rivers inhabited by Bell's turtles, green lines indicate connected downstream rivers not inhabited by Bell's turtles.Thinner blue lines denote a tributary.

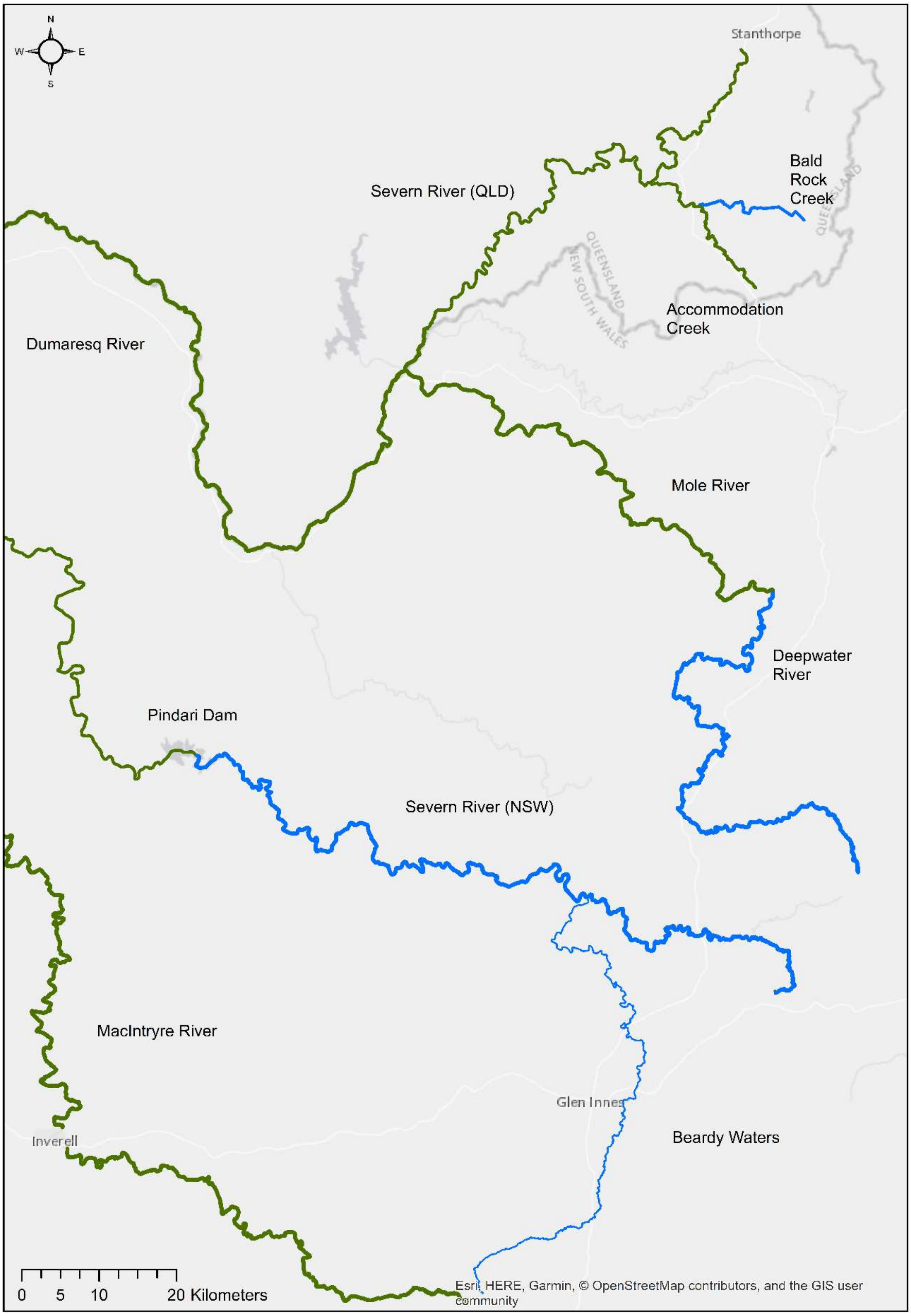

Figure 1.4d - Streams in the Border Rivers catchment inhabited by Bell's turtle (*Myuchelys bellii*). Blue lines indicate rivers inhabited by Bell's turtles, green lines indicate connected downstream rivers not inhabited by Bell's turtles.Thinner blue lines denote a tributary.

1.7 Tables

Table 1.1 - Freshwater turtle genera of Australia. Derived from Cann and Sadlier (2017) and Kehlmaier *et al*., (2019).

Suborder	Family and Subfamily	Genus	Description
Pleurodira (Side-necked turtles)	Chelidae -Chelodininae	*Chelodina*	Commonly called long-necked turtles and snake-necked turtles. Between 7 and 9 species in Australia depending on source, other species found in Papua New Guinea, Timor-Leste, and Indonesia. Australian species conservation status ranges from Least Concern to Near Threatened.
		Emydura	Commonly called short-necked turtles; distinguished from other Australian short-necked genera by the smooth marginals on the read of the carapace. Between 4 and 7 species depending on source, endemic to Australia. Conservation status is generally Least Concern.
		Elseya	Commonly called Australian snapping turtles. Generally five species are accepted for Australia, although the specific names are disputed; other species found in Papua New Guinea and Indonesia. Australian species conservation status is generally Least Concern, except for *E. albagula* which is Endangered.
		Myuchelys	Commonly called saw-shelled turtles for the exaggerated serrations on the rear marginal scutes of the carapace. Between 3 and 4 species are accepted depending on source, all endemic to Australia. One species is Least Concern, others are Endangered or Critically Endangered. Some sources refer to this genus as *Wollumbinia*.
		Rheodytes	Fitzroy River turtle - monotypic and endemic to Queensland. Listed as Vulnerable.
		Elusor	Mary River turtle - monotypic and endemic to Queensland. Listed as Endangered.
		Flaviemys	Manning River turtle - monotypic and endemic to New South Wales. Listed as Data Deficient. A controversial taxon that may be nested within *Myuchelys.*
	Chelidae -Pseudemydurinae	*Pseudemydura*	Western swamp turtle - monotypic and endemic to Western Australia. Listed as Critically Endangered.
Cryptodira (Hidden-necked turtles)	Carettochelyidae	*Carettochelys*	Pig-nosed turtle - monotypic. Found in the Northern Territory and in New Guinea. Listed as Endangered.

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	**% of contribution**
Candidate	Geoffrey N. Hughes	80
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	Adrienne Burns	10

Name of Candidate: Geoffrey N. Hughes

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STATEMENT OF ORIGINALITY

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We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of Work	Page number(s)
All text, tables, and figures	1 to 24

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CHAPTER 2 - NESTING REFUGE STRUCTURES ARE INEFFECTIVE AT PROTECTING BELL'S TURTLE (*MYUCHELYS BELLII*) NESTS

A version of this chapter is in press with Chelonian Conservation and Biology as:

Hughes, G.N., A. Burns, and P.G. McDonald. 2021. Nesting refuge structures are ineffective at protecting Bell's turtle (*Myuchelys bellii*) nests from red fox (*Vulpes vulpes*) depredation. *Chelonian Conservation and Biology*. In Press.

2.1 Abstract

Invasive red foxes (*Vulpes vulpes*) are a serious conservation issue for Australia's freshwater turtle species, including the endangered Bell's turtle (*Myuchelys bellii*). As many as 96% of Australian freshwater turtle nests may be depredated in a season by foxes. Current methods of turtle nest protection are labour intensive and rely on early detection of nesting activity, followed by nest-specific structures to prevent predation. A less labour intensive method to provide protection against fox raiding was tested, which used a nesting refuge structure based on a design successfully implemented in the United States to protect diamondback terrapin (*Malaclemys terrapin*) nests. Six wood and chicken wire structures were placed within typical Bell’s turtle nesting habitat beside large riverine pools on the Macdonald and Gwydir Rivers, north-eastern New South Wales, Australia in the summers of 2019-20 and 2020-21. Prior to placement, the soil was tilled with a rotary hoe, as Bell's turtles had been previously seen to nest in disturbed soils. Although females did approach the structures and in one case entered, no females were recorded nesting inside. Further, no foxes were shown attempting to enter the structures. While the structures effectiveness at protecting nests from predators was inconclusive, severe flooding in both years damaged and/or displaced 4 of the 6 structures, effectively preventing a valid assessment of the technique for either purpose. Rigid nest protection structures such as these were therefore not shown to be an effective conservation method for this species, due to environmental hazards rather than a fault of the design, despite their success in other regions for other species. Null results such as these are important for conservation studies, as they guide conservation efforts away from expending limited resources on ineffective methods and strategies.

2.2 Introduction

In biodiversity terms, Australia is as much a gigantic island as it is a small continent (Simpson, 1961). Prolonged isolation from the rest of the Earth's land masses has resulted in a wide variety of unique Australian lifeforms and communities (Simpson, 1961; Lomolino and Channell, 1995). Like many islands, however, Australia's species and ecosystems are vulnerable to invasion (Mooney, 2005). For example, the arrival of dingos (*Canis familiaris*) circa 5,000 years ago is thought to have contributed to the extirpation of marsupial predators such as the thylacine (*Thylacinus cynocephalus*) and Tasmanian devil (*Sarcophilus harrisii*) from the mainland (Johnson and Wroe, 2003; Savolainen *et al*., 2004; Letnic *et al*., 2012). When European explorers and settlers arrived in Australia, they brought rats (*Rattus* sp.), mice (*Mus musculus*), cats (*Felis catus*), and rabbits (*Oryctolagus cuniculus*) among other animal and plant species, which have all taken a heavy toll on native Australian wildlife communities through depredation, competition, or displacement (Abbott, 2011). The invasive mammalian predators have had a particularly devastating impact on numerous reptile populations through direct predation (Woinarski *et al*., 2018a; 2018b). In the context of Australian freshwater turtle conservation, predation of nests and nesting female predation by the red fox (*Vulpes vulpes*) is of paramount concern (Thompson, 1983; Van Dyke *et al*., 2019). Freshwater turtles are largely protected in the adult life stage, however are vulnerable to these terrestrial predators when returning to land to nest (Spencer, 2002).

Red foxes are generalist meso-predators with a naturally Holarctic distribution, but were introduced to Australia in the 1860s or 70s, probably for sport (Saunders *et al*., 2010; Abbot, 2011). Since then, the species has spread across much of the continent where they generally outcompete native meso-predators like quolls (*Dasyurus* sp.; Glen and Dickman, 2008), and have caused declines in native prey species such as bettongs (*Bettongia* sp.), numbats (*Myrmecobius fasciatus*), and a range of bandicoot species (Family: Peramelidae; Mahon, 2009; Abbott, 2011). The impact of red foxes on Australian freshwater turtles has been highlighted by Van Dyke *et al*. (2019), who argue that there is a pressing need to devise management strategies for freshwater turtles that address fox depredation of turtle nests. The endangered Bell's turtle (*Myuchelys bellii*) is a frequent victim of nest raiding by foxes (NSW Office of the Environment and Heritage (NSW OEH), 2014). Bell's turtles are a species of freshwater turtle found in the west-flowing streams of the New England Tablelands in northern New South Wales and southern Queensland (Fielder *et al*., 2015a). Bell's turtles are highly aquatic; they will leave the water to bask on rocks or logs, but nesting females generally stay close to the water’s edge, nesting on the slopes of the riverbank (NSW OEH, 2014; Fielder *et al*., 2015a; Cann and Sadlier, 2017). Fox depredation of *Emydura macquarii* nests have been reported to exceed 96% (Thompson, 1983; Spencer, 2002) and it is suspected that fox predation of *M. bellii* nests may also be high where fox control is minimal or absent (NSW OEH, 2014; Fielder *et al*., 2015b). Bell's turtle populations are measurably ageing with little apparent recruitment (Chessman, 2015), leading to concerns of an incipient population crash in the future if measures are not taken to increase immediate recruitment.

Worldwide, various methods have been trialled in attempts to protect turtle nests and other vulnerable native species from fox depredation: poison baits, shooting and trapping, and nest caging are a few examples (Fagerstone *et al*., 2004; Gentle *et al*., 2007; Riley and Litzgus, 2013). While these methods show short-term successes (e.g. Spencer, 2000), in the long term they are generally not effective, or are extremely labour-intensive to maintain indefinitely (Harding *et al*., 2001; Gentle *et al*., 2007; Spencer *et al*., 2016; 2017). Additionally, some landholders and community members may be reluctant to allow lethal fox control methods on their properties, for fear of pets and livestock being harmed. As such, it is important to consider additional protection methods, which can be used in lieu of, or in concert with, these existing strategies.

Whilst nest caging is an effective method of nest protection (Riley and Litzgus, 2013), protecting a small area of nesting beach and enticing female turtles to nest within is an alternative that has seen success in previous studies (e.g. Quinn *et al*., 2015), and if successful here could be employed on a broad scale to increase recruitment. In this study, a nesting refuge structure design was tested in the summers of 2019-20 and 2020-21 at sites within the Bell's turtle's range. These structures were intended to allow female turtles to nest within while excluding foxes; the females could thus nest unharmed, and the embryos could develop without the nest being raided. Intensive searches with researchers and dogs for individual nests would not be required, only construction of the refuge structure prior to commencement of the nesting season. This study had sought to test two questions: will female Bell's turtles use the structures as designed, and will the structures successfully exclude foxes? It was hypothesized that these fox-exclusion structures could increase recruitment of Bell's turtles, as the lack of positive stimulus for foxes of obtaining eggs or adult turtles as prey items would not reinforce foraging activity on nesting beaches. It was predicted that these structures would allow female Bell's turtles to enter and nest un-harassed, while excluding red foxes and other potential predators (e.g. corvids, pigs). If successful, these nest refuges could provide a long-term, cost-effective method of protecting nests from fox predation, creating a tool for Bell's turtle conservation efforts to use.

2.3 Methods

Nesting refuge structures. — Six nesting refuge structures were constructed and placed in selected sites across the New England Tablelands where Bell's turtle nesting activity was known or anticipated. Refuge structures were placed at sites where mature Bell's turtle females had previously been recorded (M. Dillon, pers. comm.). These structures were based on designs successfully employed by Quinn *et al.* (2015) for diamondback terrapins (*Malaclemys terrapin*) in the United States. However, several differences in behaviour and habitat use by Bell's turtles and diamondback terrapins necessitated alterations in Quinn *et al.* (2015)'s design. Bell's turtles live in winding upland streams with loam soil and granite outcrops, and females nest close to the water's edge (NSW OEH, 2014; Fielder *et al.*, 2015a; Cann and Sadlier, 2017). Diamondback terrapins live in coastal, brackish wetlands, and may move longer distances from the water to find a suitable nesting site in dune habitats (Burger and Montevecchi, 1975; Seigel, 1980). Because Bell's turtles have not been observed far from the water's edge (Chessman, 2015), road mortality may not be a serious concern as it is in terrapins. As such, the nest refuge structures were deployed individually at different sites to prevent site bias in our experiment, rather than in long arrays as in Quinn *et al.* (2015), which were partially designed to intercept nest-searching terrapins before they could reach a road.

The nest refuge structures consisted of wooden frames (2.4 m long x 1.8 m wide x 0.6 m tall), with chicken wire covering all sides except the entrance and bottom (Fig 2.1). The entrance was 15 cm high with an electric fence wire (100 mm x 12 mm polytape) placed along the top of the entrance (Fig. 2.1); this was intended to allow turtles into the structure while excluding foxes and other predators. The wire was energised with a Thunderbird S18B solar fence charger (Thunderbird Ag, Mudgee NSW, Australia) set to full charge. Drift fencing (4 - 6 m in length depending on the position of the structure relative to the water's edge) was placed at either side of the entrance and ran outward at an angle (dependent on the shape of the beach) to funnel nest-searching females into the entrance. This drift fencing was composed of 30 cm tall rigid plastic garden edging in 2019-20, and was replaced with a 50 cm tall flexible rubber mesh in 2020-21 (Fig. 2.1).

The structures were built and deployed in November 2019 through to January 2020 (during the austral summer). The structures were re-deployed from November 2020 to January 2021 with some modifications to the original design: as mentioned, the drift fencing was replaced with a different material, and additionally the wooden beam at the bottom of the structure entrance was removed and replaced with metal mesh buried 5 cm deep to prevent foxes from burrowing under the electric wire, and half of the structure's top was replaced with a removable frame to allow researchers easier access to the interior. These changes aimed to reduce any aversion to the structures by increasing the size of the entrance.

A rotary hoe was used to churn patches of soil close to the river's edge, over which the structure was set. A mattock was used to dig a trench in the outline of the structure, down to the hard earth beneath the tilled soil to reduce the chances of foxes digging under the structure and bypassing the entrance. The edges of the structure were lowered into this trench with the entrance facing the river, and the edges buried; the soil inside the structure was raked to evenly distribute disturbed soil within the structure. In 2019-20, the soil inside of the structure was also doused with ~10 L of water taken from the river with a garden watering can; enough to saturate the soil. The interior of the structure was doused with each subsequent site visit, to simulate rainfall moistening soil. Disturbed soils have been noted as potential attractors to nesting Bell's turtles (G. Hughes, pers. obs.; P. Spark pers. comm.), and rainfall is commonly associated with the start of nesting season in other turtle species (e.g. Mortimer and Carr, 1987; Burke *et al.*, 1994; Czaja *et al.*, 2018). Conditions changed between field seasons from severe drought in the 2019-20 field season (Filkov *et al.*, 2020), through to much higher rainfall in the 2020-21 season; water dousing was thus discontinued in the second season.

The structure was surrounded with electric fence to prevent livestock from approaching and potentially damaging the structure or being injured, and also to further make digging under the sides of the structure difficult for foxes (Fig. 2.1). Two lines were run around the exterior of the structure on posts, one at ~10 cm above the surface and one ~50 cm above the ground; these were connected to the fence charger. This exterior fencing was removed in 2020-21 out of concern that female turtles were discouraged from approaching the structure by it (Fig. 2.1).

Each refuge structure had two camera traps monitoring the structures, either LTL Acorn camera traps (Little Acorn Australia, VIC, Australia) or Bushnell Trophycam camera traps (Bushnell Corporation, KS, USA). Camera traps were always the same model at each site. In 2019-20, one camera was placed at one side of the entrance, approximately 30 cm from the structure and 10 cm above the ground, facing across the entrance. The second camera was placed beside the structure, facing outward to capture animals moving near the structure. In 2020-21, both cameras were placed 2 m from the entrance on either side, ~ 10 cm above the ground, facing inwards at a 45° angle. These camera traps were intended to detect any turtles entering the structure, or any foxes or other predators interacting with the structure entrance. Sites were visited once per fortnight to exchange memory cards and replace batteries as necessary.

Study sites. — Three sites were located in the Macdonald River catchment near Bendemeer NSW, and three sites were located in the Gwydir River catchment near Bundarra NSW (Fig. 2.2). For landholder anonymity, the property names and exact locations are not published so the sites are hereafter named Macdonald 1, 2, and 3 and Gwydir 1, 2, and 3. Some landholders engaged in active fox control (i.e. shooting or poison baiting) as outlined below, which potentially limited exposure of structures to foxes in this study.

Macdonald 1 was located in an area of restored parkland. Invasive trees and weeds had been removed in the previous year, and the structure was deployed on 22 November 2020, on a slightly inclined patch of open, loamy soil, with the entrance 2.5 m from the water's edge and <1m above the waterline. There was shade to the south and northwest from remaining native trees (primarily Casuarinaceae), but the patch received full sun from directly overhead, and to the east and north. Flooding in January 2020 forced the structure to be relocated to a different location within the park for the 2020/21 nesting season. The structure was modified and redeployed at a nearby site (~100 m downstream) on 30 October 2020 on a higher bank (~1.5 m above the waterline). Surrounding conditions were similar, with primarily Casuarinaceae forest to the south and open ground to the east, north, and west, ensuring sunlight coverage of the interior of the structure. In late December 2020, the area was again flooded and the structure was irreparably damaged. Local landholders did not engage in fox control of any kind during these field seasons.

Macdonald 2 and Macdonald 3 were located on a private cattle and sheep farm on the Macdonald River. The two sites were ~1 km apart and separated by a roadway and several fence lines; both structures were deployed on 22 November 2019. Macdonald 2 was located on a flat, sandy beach <1 m above the waterline, ~2 m from the edge of the water. There were no nearby trees or other sources of shade. Macdonald 3 was located on a high sand-and-gravel riverbank, with some shade from a large willow tree to the north but otherwise no obstacles to sun exposure. The same flooding event that threatened Macdonald 1 in January 2020 completely inundated and irreparably damaged Macdonald 2, and Macdonald 2 was not used in 2020/21. Macdonald 3 was modified and redeployed 11 November 2020 and used for the 2020/21 field season. Landholders engaged in fox control with 1080 baits on their property during these field seasons.

Gwydir 1 was located on a cattle and horse farm with intact riparian forest along the Gwydir River. The structure was placed on 18 November 2019 in a flat area of loamy soil, ~2m from the water's edge and 1 m above the water line. The site was heavily shaded by trees, but was open to the north and west, so was considered to receive enough sun exposure for the purposes of this experiment, and no more-suitable sites were available on the property. In January 2020 a flood event irreparably damaged the structure, and it was not used in the 2020/21 field season. Landholders engaged in limited fox control (shooting only) on their property during these field seasons, reporting 4 foxes removed in per year.

Gwydir 2 was located on a large (~12,000 ha) cattle station, on a tributary of the Gwydir River which will remain confidential to protect the landholder's privacy. The structure was placed on 18 November 2020, in a flat area of loamy soil 2 m from the water's edge and 1 m above the water line. The site was shaded by a single willow tree to the south, but had good sun exposure from all other angles. In 10 January 2020, a flood event slightly damaged the structure. The structure was repaired and modified, and was redeployed on 3 November 2020 for the 2020/21 field season; it was moved ~80 m from the first site, with sandy soil and less shade; the new site was 2 m from the water and <1 m above the waterline. Landholders engaged in intensive fox control (shooting only) on their property during these field seasons, reporting >300 foxes killed over these two years across the whole property.

Gwydir 3 was located on a private cattle and sheep farm along a patch of open riparian woodland. The structure was placed on 27 November 2019, on a high bank (2 m) above the river with some shade from a large eucalypt to the north east but otherwise in a location well exposed to sunlight. The structure was modified and reset on 3 November 2020 for the 2020/21 field season, but was irreparably damaged by flooding in January 2021. Landholders engaged in limited fox control (1080 baiting) on their property during the 2019-20 field season, but did not engage in fox control during 2020-21.

Analysis. — Images from the camera traps were examined for Bell's turtle activity in or near the structures. Particular focus was placed on observing the turtles entering or exiting the structure, or attempting to nest (i.e. manipulating soil with hind legs). Additionally, signs of digging activity in or near the structures were searched for visually during site visits. Any raided turtle nests near the structures were also noted, in particular the distance between these nests and the structure's entrance.

Images taken by the camera traps were examined for fox activity in or near the structures. Attention was focused on observing the foxes investigating the structure entrances, and any interactions with the wires. Activity of other egg-laying reptile species, such as eastern water dragons (*Intellagama lesueurii*) and eastern long-necked turtles (*Chelodina longicollis*) were also noted, as potential indicators of nesting habitat viability. During site visits, any tracks, scat, or apparent attempts to dig under the structure from the sides were noted if found. Animals of any species entering or exiting the structures were recorded.

2.4 Results

Bell's Turtle Activity. — Female Bell's turtles were recorded in front of the refuge structure entrances in both field seasons, and one female entered a structure in 2020/21; however, no nesting activity was directly observed (Fig. 2.3 - 2.6). No Bell's turtle activity was recorded at Macdonald 2 or Gwydir 1 during either year.

One female was recorded in front of the Macdonald 1 structure on 26 November 2019 from 1723 hrs to 1730 hrs; this female began digging with her hind feet directly in front of the structure entrance (Fig. 2.3), but left the hole unburied after ~7 minutes, indicating a possible test dig. Between 2 and 3 females were recorded in front of the Gwydir 3 structure on 2 January 2020 from 1617 hrs to 1652 hrs (Fig. 2.4), including one female that was recorded digging with her hind feet directly in front of the structure entrance from 1620 hrs to 1652 hrs, but left the hole unburied after 32 minutes. At 1634 hrs, two turtles were recorded in front of the structure, including the one that was digging. A small pit was recorded inside the structure at Gwydir 2 on 27 November 2019 (Fig. 2.5), and another directly in front of the structure entrance at Macdonald 3 on 14 December 2019, but the digging activity was not recorded on camera in either instance so could not be assigned to a species (Fig. 2.6).

One female was recorded inside of the Gwydir 2 structure on 13 November 2020 (Fig. 2.5). The turtle was not recorded entering the structure, but was seen fully inside the enclosed space at 1158 hrs with its nose close to the soil, possibly searching for a suitable nesting site. The turtle was next recorded leaving the structure at 1205 hrs, without apparently digging any soil inside. However, the female did have some soil on her back (Fig. 2.5), which may indicate some digging as seen in other turtle species that will flip soil onto their backs during nesting (Harding and Bloomer, 1979). One female approached the Gwydir 3 structure on 21 November 2020 at 1439 hrs (Fig. 2.6), remaining stationary in front of the entrance for 1 minute before it moved away from the structure entrance. One female was recorded directly in front of the Macdonald 3 structure on 3 December 2020 at 2014 hrs (Fig. 2.6); the image was dark, so the turtle's activity was not discernible, however a later site visit showed a pit dug in approximately the location where the turtle was photographed, suggesting a test dig.

A cluster (n=3) of raided turtle nests were located ~5 m from the Gwydir 2 strructure on 4 December 2020, ~50 cm from the water's edge. A cluster (n=5) of raided turtle nests were located ~10 m from the Macdonald 1 structure on 17 December 2020, ~1 m from the water's edge.

Red Fox Activity. — Red foxes were recorded near some structures in 2019/20, but no fox activity was recorded near any structures in 2020/21. No foxes were recorded attempting to gain entry to the interiors of any structures, either through the entrance or by digging under. Gwydir 2 had the highest rates of fox activity, with near-nightly recordings of foxes; a later site visit located a fox den ~20 from the structure. Macdonald 3 had high levels of fox activity, with foxes passing close to the structures frequently during January 2020. Macdonald 1 had two visits in 2019/20, although the foxes did not approach close to the structure and appeared to be passing the structure by. However, foxes only appeared to investigate the structure entrances at Gwydir 3: in the early morning (0333 hrs) and late evening (2154 hrs) of 4 December 2020, and in the late evening (2330 hrs) of 4 January 2020, where the fox appeared to be sniffing at the site where the female Bell's turtle had been digging two days prior. The foxes approached the close to the structure entrance, but it is unclear if they interacted with the electrified wire at all.

Other Species. — A large black animal, speculated to be an Australian raven (*Corvus coronoides*) was recorded inside Gwydir 2 on 27 November 2019, however the image is unclear so species identification is not certain. Eastern water dragons were frequently recorded inside the Gwydir 2 structure, but it is unclear if they were nesting. Australian wood ducks (*Chenonetta jubata*) and eastern water dragons were frequently recorded entering and leaving the structure at Macdonald 1 in 2020/21 with no signs of aversion. There was no indication that the water dragons were nesting. Australian magpies

(*Gymnorhina tibicen*) were recorded entering and exiting the structure at Gwydir 3 in 2020/21 with no signs of aversion.

Flooding. — All six structures were inundated at least once during the two years of the field season. Macdonald 2 and Gwydir 1 were irreparably damaged in 2019/20, and Macdonald 1, and Gwydir 3 were both irreparably damaged in 2020/21.

2.5 Discussion

While a female Bell's turtle did enter 1 structure, and 4 out of 6 structures were directly approached by females, no Bell's turtles were detected nesting inside the structures. Nor were other egg-laying species detected nesting within the structures, including eastern water dragons and eastern long-necked turtles that are sympatric with Bell's turtles. Red foxes were detected near 4 of 6 structures, and foxes appeared to investigate the entrance of 1 structure, but were not detected attempting to enter any structures. Although some female Bell's turtles were detected near the refuge entrances and even digging in front of them, and raided turtle nests were found near 2 of the structures, the persistent risk of unpredictable flooding renders nest refuge structures of this design ineffective as tools for Bell's turtle conservation. Thus, while the effectiveness of the refuge designs at excluding nest predators was inconclusive, the point is rendered moot by the environmental hazards posed to the structures.

Nonetheless, some useful information about Bell's turtle nesting behaviour was exposed during this study. Bell's turtles sometimes nest in soil that has been previously disturbed, for example in patches of torn-up soil where trees have been removed (G. Hughes, pers. obs; P. Spark pers. comm.). The use of a rotary hoe to disturb the soil was thus intended to entice the females to nest inside the structure, and 4 of 6 structures did show at least some interest by female Bell's turtles. This may be further evidence that females prefer soil that has been recently turned-over as nesting substrata. Bell's turtles may be reluctant to make use of the nest refuge structures as designed, however the use of a rotary hoe or similar tools to create enticing nesting habitat may be incorporated into other nest protection methods. Using a rotary hoe to create or enhance nesting substrata prior to the nesting season may serve to entice females to nest inside protected areas. Care should be taken to not over-use this technique, however, as it could increase erosion of riverbanks at these sites. Used sparingly and with due consideration to the long-term integrity of riverbanks, this technique warrants further investigation; turning patches of soil could be part of an effective nest protection strategy for the future that targets key potential nesting habitat, and warrants further investigation.

The differing behaviours and habitats of Bell's turtles and diamondback terrapins necessitated some changes from Quinn *et al.* (2015)'s structure design and placement; primarily, Bell's turtles nest much closer to the water than terrapins do (Burger and Montevecchi, 1975; NSW OEH, 2014; Cann and Sadlier, 2017). Consequently, the structures in this study had to be placed quite close to the river's edge, which placed them at risk of damage or displacement due to flooding. All 6 structures were inundated at least once during the two field seasons, and 4 of the 6 were irreparably damaged by flooding. Evidently, a rigid, immobile structure is not suitable for long-term deployment for protecting Bell's turtle nests, as placing such structures inside the narrow band of riverbank that Bell's turtles nest in will always place them at risk from flooding.

No foxes were shown to gain entry to the structures, however it is inconclusive whether the structures as designed could effectively exclude the foxes. Without turtles or other animals nesting inside the structures, foxes would have had little incentive to gain entry. Foxes were only recorded investigating the structure entrances at Gwydir 3, and no interaction with the electric wire was directly recorded. Quinn *et al.* (2015) reported that raccoons (*Procyon lotor*) were not able to gain entry to their structures if there was an electric wire across the entrance, and it is unlikely that foxes could have entered through the structure entrance without contacting the wire. It is less clear whether they could have burrowed under the sides to gain entry, although none attempted to do so. Other potential egg predators, such as magpies and ravens, were able to enter the structures without difficulty. Ravens have been observed scaring nesting female Bell's turtles back into the river and then consuming the eggs from the exposed nest (L. Streeting, pers. comm.), and Spencer (2002) records magpies as frequent nest predators in the lower Murray-Darling catchment. Apparently, the structures as designed would have afforded the turtles little protection from avian predators. However, while egg predation by birds has been recorded it is unclear how great of a threat it is to the species' persistence, compared to the likely threat posed by foxes (Fielder *et al.*, 2015a; 2015b).

The importance of null results in research is often overlooked but are necessary to consider when testing new methods or techniques, particularly in conservation studies (Axford *et al.*, 2020). Conservation organizations have limited resources, and investing these resources in an ineffective strategy when more effective alternatives may be available is an error to be avoided. Other avenues for protecting Bell's turtle nests should be investigated in the future such as conditioned food aversion, which has been shown to reduce nest predation by foxes on ground nesting birds (Tobajas *et al.*, 2020). Investigation into the proximate and ultimate questions underpinning the choice of Bell's turtles to nest on frequently flooded riverbanks may yield interesting evolutionary adaptations. Such studies may also provide evidence for an ethological trap, similar to that described by Spencer *et al.* (2016) in the context of predation, if flooding has become more intense and less predictable since colonization. Instead of nesting refuge structures, alternative strategies such as conditioned food aversion could be potentially combined with turning over soil to entice Bell's turtles to nest in protected nesting areas, which may prove more effective than the use of a refuge structure while still enabling managers to devote less time to protecting individual nests, and thereby operate more efficiently over a breeding season.

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2.7 Figures

Figure 2.1 - Examples of nesting refuge structures: a) initial structure design used in 2019-20, b) modified structures used in 2020-21. Modifications in the second year were: rubber mesh as drift fencing, a removable frame on the top, and removal of the wooden beam at the bottom of the structure's entrance.

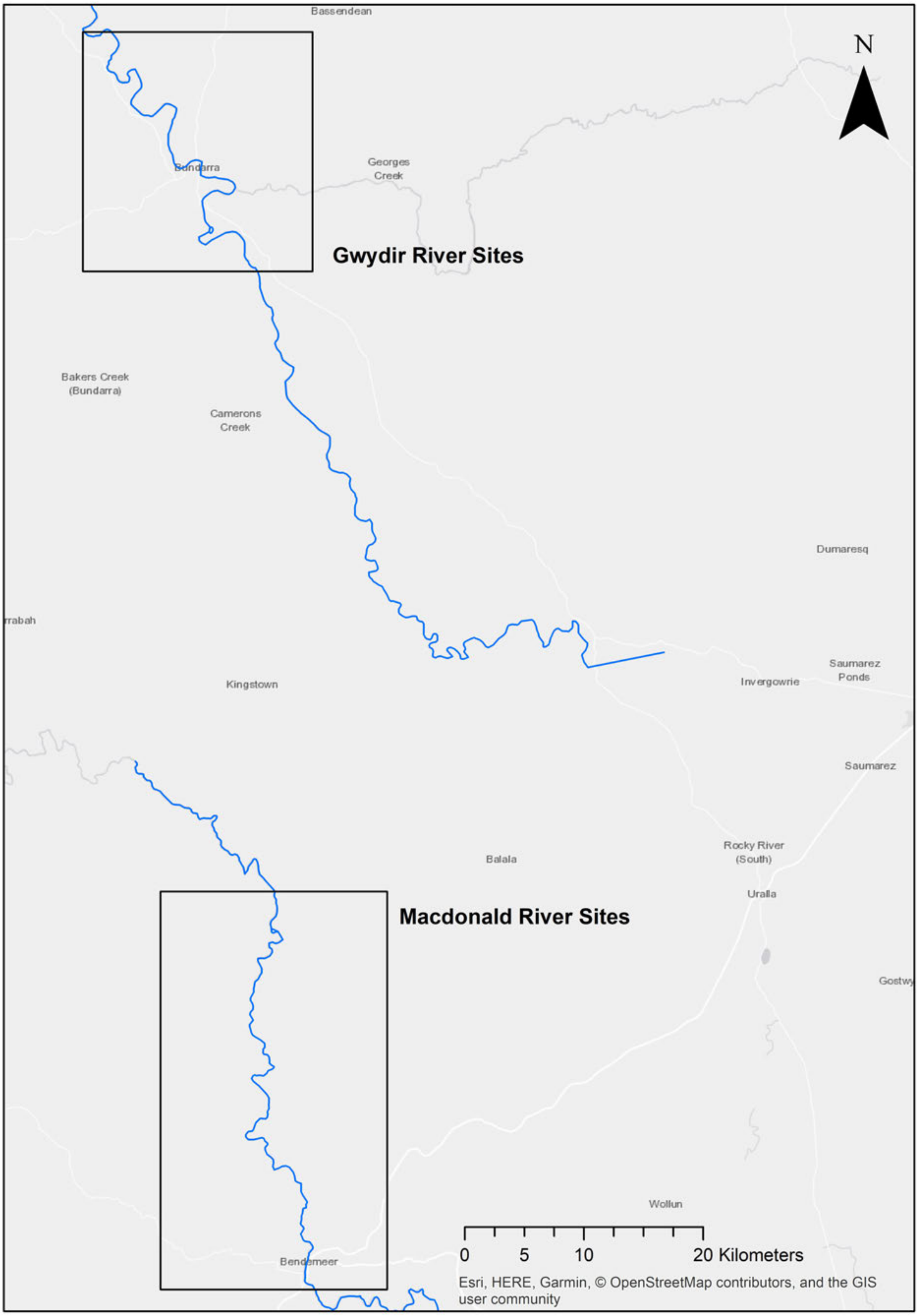

Figure 2.2 - Approximate locations of sites for structure deployment. Exact sites were kept confidential at the landholders' requests.

Figure 2.3 - Bell's turtle (*Myuchelys bellii*) activity at the Macdonald 1 site nest refuge structure. Sequence of photos: a) Female approaching the nesting refuge structure (26 November 2019), b) same female digging in front of the nesting refuge structure, c) female abandoning the dig and retreating from the structure.

Figure 2.5 - Bell's turtle (*Myuchelys bellii*) activity at the Gwydir 2 site nest refuge structure. Sequence of photos: a) Female Bell's turtle inside the nesting refuge structure (13 November 2020), circled for clarity, nose close to the soil which may indicate nest-searching behaviour, b) Bell's turtle (presumed same female) leaving the nest structure.

Figure 2.5 - Bell's turtle (*Myuchelys bellii*) activity at the Gwydir 3 site nest refuge structure. Sequence of photos: a) female Bell's turtle retreating from nesting refuge structure entrance (2 January 2020), b) two females in frame, one digging close to the entrance of the nesting refuge structure and one retreating from the entrance (2January 2020), c) a female approaching the entrance of the structure (21 November 2020).

Figure 2.6 - Bell's turtle (*Myuchelys bellii*) activity at the Macdonald 3 site nest refuge structure. Sequence of photos: a) partially-dug hole in front of the structure entrance, potentially dug by a turtle (discovered 14 December 2019; image taken with a mobile phone), b) Bell's turtle recorded in front of the nesting refuge structure (3 December 2020), circled for clarity.

2.8 Tables

Table 2.1 - Activity of Bell's turtles (*Myuchelys bellii*) and red foxes (*Vulpes vulpes*) near nesting refuge structures in 2019-20 and 2020-21. As individuals could not be distinguished, the table shows the number of days that the structure was approached by at least one animal compared to the number of camera monitoring days for each site.

	Bell's turtles		**Red Foxes**	
Site	**2019-20**	**2020-21**	**2019-20**	**2020-21**
Gwydir 1	0/35	n/a	0/35	n/a
Gwydir 2	0/47	1/45	23/47	0/45
Gwydir 3	1/54	1/57	4/54	0/57
Macdonald 1	1/22	0/50	2/22	0/50
Macdonald 2	0/25	n/a	0/25	n/a
Macdonald 3	0/58	1/49	12/58	0/49

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Higher Degree Research Thesis by Publication
University of New England

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Type of Work	Page number(s)
All text, tables, and figures	27 to 47

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CHAPTER 3 - ULTRASONIC PREDATOR REPELLENCE AS A MEANS OF PROTECTING BELL'S TURTLE (*MYUCHELYS BELLII*) NESTS FROM INVASIVE MAMMALIAN AND AVIAN PREDATORS

3.1 Abstract

Predation of nests is a major conservation concern for Australian turtle species, particularly when this predation is novel and arising from the impact of introduced taxa not normally present in a system. As such, methods to protect turtle nests from predators must be tested for efficacy before deploying them on a large scale, to prevent both a waste of limited conservation resources, and also ensure that effective protection is in place. This study tested the efficacy of ultrasonic animal repellent devices to repel local turtle nest predators from nesting beaches, using a modified before-after impact-control (BACI) experimental design. Ultrasonic animal repellent devices were deployed on suitable nesting beaches of the endangered Bell's turtle (*Myuchelys bellii*), and baited with chicken eggs to attract nest predators. Three beaches had ultrasonic devices deployed on them, while three control beaches did not; all beaches were monitored with 2 camera traps, and all beaches also had 2 additional camera traps facing outwards to monitor nearby game trails. The experiment included a 20-day pre-feeding period when the ultrasonic repellent devices were not active, and a 10-day long post-activation period. The number of daily site visits and total daily site visit duration of several species of interest were monitored with the camera traps at impact and control sites before and after activation of the ultrasonic repellent devices. Key introduced predators of this turtle species, red foxes (*Vulpes vulpes*) and feral pigs (*Sus scrofa*), were detected during initial site visits, but did not consume any baits during the experiment. Ravens (*Corvus* sp.) were the most prevalent nest predator, however raven site visit patterns did not change following the activation of the ultrasonic repellent devices. In addition, mammals that should be able to detect the ultrasonic repellent device signal, such as brushtail possums (*Trichesurus vulpecula*) and several species of large macropods, did not show any aversion to the ultrasonic repellent devices. These results add to a growing body of literature demonstrating a general ineffectiveness of ultrasonic repellent devices at repelling animals from a "protected" area, and suggest that these ultrasonic repellent devices are not a useful method for turtle conservation. Null results in methods studies such as this are useful, as they guide conservation efforts away from wasteful expense of limited resources on ineffective techniques.

3.2 Introduction

Although predation is a natural part of any species' ecology, populations of imperilled species may be unable to cope with high predation rates if populations are small or if natural recruitment is low (Neves and Odom, 1989; Roby *et al.*, 2003). In a natural predator-prey relationship, the predator provides a valuable and oft-times necessary control to prey populations, and balance to the ecosystem as a whole (Sih *et al.*, 1998; Ripple and Beschta, 1998). In human-modified ecosystems, these relationships can become unbalanced, and prey species can begin to decline in the face of heightened predation pressure (Oehler and Litvaitis, 1996). Such predators often target vulnerable juveniles, preventing them from being recruited (Clout and Craig, 1995; Wanless *et al.*, 2007), but may also target adults, removing valuable breeding individuals from a population (Stewart, 1997; Keevil *et al.*, 2018). As such, human intervention via anti-predator measures could potentially assist these imperilled species in population recovery, and in some cases may be necessary for their continued survival (Butchko, 1990). Of particular concern are non-natural predation rates arising from invasive or subsidised predator species, which can lead to population declines as the endangered prey species has not evolved countermeasures against these novel conditions (Strauss *et al.*, 2006; Sih *et al.*, 2010). Such declines have been documented in seabirds (Wanless *et al.*, 2007; Caut *et al.*, 2008), waterfowl (Brzeziński *et al.*, 2020), insects (Rand and Louda, 2006), and in turtles (Thompson, 1983; Butchko, 1990; Boarman, 2003; Esque *et al.*, 2010; Van Dyke *et al.*, 2019).

Turtle populations, with their "bet hedging" reproductive strategy (long-lived adults, high reproductive output, but low recruitment), are generally robust against sustained high losses of juveniles (Congdon *et al.*, 1994; Heppell, 1998; Tuberville *et al.*, 2008). Typically, turtle populations are more strongly threatened by adult mortality than juvenile mortality, as it removes breeding individuals from the population; even slight increases in adult mortality can lead to eventual population crashes (Brooks *et al.*, 1991; Gibbs and Shriver, 2002; Van Dyke *et al.*, 2017). However, there is a limit to the bet hedging strategy when recruitment of juveniles is insufficient to replace adults killed by predators, senescence, or anthropogenic causes like road mortality (Van Dyke *et al.*, 2017; 2019). Much research has been done on predation of turtle nests by a variety of predators, including raccoons (*Procyon lotor*; Quinn *et al.*, 2015; Engeman *et al.*, 2005), striped skunks (*Mephitis mephitis*; Galois, 1999), feral pigs (*Sus scrofa*; Fordham *et al.*, 2006; Whytlaw *et al.*, 2013), nine-banded armadillos (*Dasypus novemcinctus*; Woolard *et al.*, 2004; Engeman *et al.*, 2005), Virginia opossums

(*Didelphis virginiana*; Woolard *et al*., 2004), and red foxes (*Vulpes vulpes*; Spencer, 2000; Spencer *et al*., 2017). Both foxes and pigs have been introduced to Australia, and have become widespread pest animals responsible for declines of native species (Hone, 1990; Mahon, 2009). Numerous freshwater turtle species in Australia suffer near-total nest failure on a yearly basis due to invasive red foxes and feral pigs, alongside losses to native predators such as Australian magpies (*Gymnorhina tibicen*) and native corvids (Spencer, 2002; Dawson *et al*., 2016; Spencer *et al*., 2017; Van Dyke *et al*., 2019). Known victims of these predators include the Murray River turtle (*Emydura macquarii*; Thompson, 1983; Spencer, 2000), the broad-shelled turtle (*Chelodina expansa*; Petrov *et al*., 2018), and the critically-endangered Western swamp tortoise (*Pseudemydura umbrina*; Reavely *et al*., 2009).

The endangered Bell's turtle (*Myuchelys bellii*) may be another victim of nest predation (Fielder *et al*., 2015a; Cann and Sadlier, 2017), and foxes are suspected to be one such nest raider (Mahon, 2009; NSW Office of the Environment and Heritage (NSW OEH), 2014). The Bell's turtle is endemic to the New England Tablelands of New South Wales and Queensland (Fielder *et al*., 2015b), and like many endemic species, is at an especially high risk of extinction due to a limited species' range and small population (Gaston, 1998; Purvis *et al*., 2000; Kamino *et al*., 2012). Trapping studies of the species' sub-populations still have large numbers of adults captured, but a very low number of juveniles (Chessman, 2015; Fielder *et al*., 2015a). This apparently low recruitment is cause for concern of a potential population collapse in the future (Fielder *et al*., 2015b).

As such, protection of Bell's turtle nests from predators is a high priority for the species' conservation. Nest protection strategies tested in the past to protect other turtle nests have included active extermination efforts of predators such as foxes, raccoons, and armadillos in vulnerable areas (e.g. Spencer, 2000; Robley *et al*., 2016; Engeman *et al*., 2005), protective caging or mesh around individual nests (Yerli *et al*., 1997; Riley and Litzgus, 2013), and the construction of nest refuges that exclude predators (e.g. Quinn *et al*., 2019; see Chapter 2 of this dissertation). Another potential method is to discourage predators from entering vulnerable areas, using chemical or technological repellents to exclude these predators without affecting nesting turtles. One such technology that may be of use are ultrasonic repellent devices. These are typically motion-activated, triggering a high decibel sound in the ultrasonic range that is alarming or even painful for animals that can detect it, such as foxes (Isley and Gysel, 1975). To be effective as a nest protection strategy, the ultrasonic repellent device must not impinge on the hearing range of the imperilled species.

The hearing range of the Bell's turtle has not been measured, but red-eared sliders (*Trachemys scripta elegans*; Patterson, 1966; Heffner and Heffner, 2007) and several sea turtle species (Ketten and Bartol, 2005) have been shown to not hear into the ultrasonic range. It is likely that adult Bell's turtles would also be unaffected by such devices.

Ultrasonic repellent devices are readily available commercially, and if shown to be effective at deterring nest predators from turtle nesting beaches, they could provide a cost-effective method of protection against these nest predators across large areas of habitat. This chapter sought to test the efficacy of one commercial model of ultrasonic repellent device in excluding predators from areas in known turtle nesting habitat. It was hypothesized that these ultrasonic repellent devices could reduce predator activity on Bell's turtle nesting beaches where they were deployed. It was predicted that beaches with ultrasonic repellent devices would show lower rates of predator site visits than control sites, after the devices were activated. If successful, these ultrasonic repellent devices could provide a low-cost, low-labour tool for the protection of turtle nests from foxes and other mammalian nest predators.

3.3 Methods

Study Site. — This pilot study was conducted on Congi Station (-30.929°S 151.321°E), near Bendemeer NSW (Fig. 3.1). The site consists of a 900 m long pool in the Macdonald River known to contain Bell's turtles. The pool was bordered by un-grazed cattle pasture on the eastern side that forms part of Congi Station, and on the western side by grazed pasture owned by a neighbouring farm. The riparian edge habitat was a mix of open grassy bank, invasive willows (*Salix* sp.), and native trees including eucalypts (*Eucalyptus* sp.), wattles (*Acacia* sp.), and she-oaks (Casuarinaceae).

Repellent Devices. — The ultrasonic repellent devices used for this experiment were Pestrol Solar Animal Away Elite (Pestrol Pest Control, NSW, Australia). These are motion-triggered devices that are able to generate sonic alarms audible to the human ear, ultrasonic alarms in the 25 - 40 kHz range, and LED strobe effects to repel pest animals. These devices have a reported maximum effective range of 25 m. For this experiment, the devices were set to perform both a focused ultrasonic pulse and a sweeping wide-beam ultrasonic pulse when triggered, along with a bright LED strobe. The audible mode was used prior to deployment to test the motion triggers on the devices, but was not used in the field as the audible alarms could possibly discourage any nesting turtles.

Species of Interest. — Preliminary surveys of the site were conducted using camera traps from 14 October to 20 October 2020, accompanied by visual surveys for tracks and scats, to determine which species of interest were present at Congi Station. These surveys showed the presence of red foxes and feral pigs, both known predators of turtle nests in Australia (Whytlaw *et al*., 2013; Van Dyke *et al*., 2019). Common brushtail possums (*Trichosurus vulgaris*) were also identified on-site, and were considered to be potential nest predators; possums have not been recorded predating on turtle nests but have been recorded consuming birds' nests (Brown *et al*., 1993; Olsen and Trost, 2009) and may raid a turtle nest given the opportunity. Brushtail possums can hear into the ultrasonic range (Osugi *et al*., 2011), so their behaviour in relation to the repellent devices was also of interest, regardless of the likelihood of possums consuming turtle nests. Visits to the study site by large herbivorous mammals, notably several species of macropod including eastern grey kangaroo (*Macropus giganteus*), red-necked wallaby (*M. rufogriseus*), and swamp wallaby (*Wallabia bicolor*), were also recorded and analysed. While not predators, macropods should be able to hear the ultrasonic repellent device alarms (Guppy, 1985), and the patterns of macropod visits to the sites would provide an indicator of the effectiveness of the repellent devices.

Large corvids were also regularly observed on site: most likely Australian ravens (*Corvus coronoides*) or Torresian crows (*C. orru*), although forest ravens (*C. tasmanicus*) were also possibly present in the area. These species are difficult to distinguish from static appearance alone, so were all classed as "ravens" (*Corvus* sp.) for the purposes of this study. Ravens are known predators of turtle eggs in Australia, including Bell's turtle eggs (L. Streeting, pers. comm.). Australian magpies are considered possible nest predators, as they are known to predate on Murray River turtle eggs (Spencer, 2002). Birds are not known to hear in the ultrasonic range (Seamans *et al*., 2013), but may respond to the LED flashes from the ultrasonic repellent device devices.

Experimental Design. — This experiment followed a modified before-after control-impact (BACI) design. Six beaches were selected along the pool in the Macdonald River at Congi Station (Fig. 3.1). Three of these were selected as impact beaches (Impact North, Impact Centre, Impact South), which had repellent device devices on-site, and three were control beaches (Control North, Control Centre, Control South), which lacked ultrasonic repellent devices; further detail on equipment deployment below. Open beaches along the pool with little to no ground vegetation or shade were chosen as the treatment and control sites, as these are the habitats where Bell's turtles are known to nest. The beaches were each ~10 m in length and ≥40 m apart (Fig. 3.1), well beyond the devices' reported effective range (~25 m). Cameras and repellent devices were deployed on 20 November 2020.

Four camera traps (Little Acorn Australia, VIC, Australia) were placed on each beach. Two were placed at either end of the beach, 5 m apart and ~2 m from the water's edge, as Bell's turtles nest on the riverbank close to the water's edge (NSW OEH, 2014; Cann and Sadlier, 2017). These cameras faced inwards toward each other, and were placed ~10 cm above the ground on star pickets, to capture animals interacting with the soil surface (i.e. digging up egg baits). Two other cameras were placed facing away from the water and higher on the bank, to record nearby game trails for animals that may have bypassed or deliberately avoided the sites. The outward-facing cameras were placed 3-5 m from the water's edge, and 6-10 m from each other, depending on the topography of the beaches, and mounted on star pickets approximately 30 cm above the ground, to capture animals passing along nearby game trails. All cameras were set to record still images with 30 second trigger reset intervals.

At the 3 impact beach, a Pestrol device was placed on top of the same post as the inward-facing camera trap (Fig. 3.2), with two devices facing each other. This was intended to provide redundancy if one device failed to trigger when an animal approached the beach, and to potentially magnify the effects if both repellent devices triggered simultaneously. To allow the local wildlife to become habituated to the devices' presence, the devices were placed on site on 20 November 2020, but were not activated until the beginning of the "after" phase of the experiment 20 days later.

Three sentinel nests, each comprised of 3 chicken eggs buried 10 cm below the soil surface, were placed at all beaches to simulate turtle nests. The eggs were buried at the midpoint of each site (~2.5 m from each end) and within view of the camera traps. The sites were checked daily for 10 days, and any missing eggs were replaced and camera trap photos were checked to determine the identity and frequency of animals visiting the sites. After the first period, visits to monitor beaches and refresh baits were reduced to once every 3 days to minimise human scent and disturbance.

The ultrasonic repellent devices were activated on 9 December 2020, after 20 days of pre-feeding to condition the local predators on the availability of food at the beaches. Three fresh sentinel nests were buried at all beaches once the repellent devices were activated. Researchers made a final visit to the site on 19 December 2020, 10 days after activation of the repellent devices, to assess rate of visitor activity. Camera traps were retrieved and images were assessed to determine the identity and frequency of site visits by nest predators or potential nest predators. The 30 days of the experiment were divided into three 10-day phases (referred to as Preliminary 1, Preliminary 2, and Test Phase; Table 3.1).

Analysis. — Analysis was conducted on patterns of animal visits and visit duration for each species of interest at all 6 beaches, both before and after the repellent devices were activated. Animal visits and visit duration to the beaches were also compared to the pattern of researcher visits to each site to determine if activity patterns correlated with the activation state of the repellent devices or to the refreshing of baits and/or the recency of human scent on-site.

Images from the camera traps were analysed for all species of interest, with number and duration of visits to all beaches being recorded by species. The duration of visit was counted in minutes from the time from the first image in the sequence being taken to the last image in the sequence. If a single image only was taken, it was considered a 1-minute long visit. A long sequence of animal photos was considered multiple visits (i.e. the animal leaving and returning later) if the time gap between images was greater than 10 minutes long.

For each day of the experiment, the species of visitor, the number of visits, and total duration of all visits to the beaches were analysed. Visits from each species (or genus in the case of corvids) were analysed separately. Daily visit rates were analysed using general linear mixed models with Poisson distributions, as the models were assessing count data. Daily total visit duration was analysed using linear mixed models. Both visit frequency and visit duration response variables were tested using treatment (control/impact), phase (Preliminary 1, Preliminary 2, and Test), and human visit that day (yes/no) as fixed variables; in all models, beach was used as a random variable. All models were tested for significance using likelihood ratio tests of the full model versus models with dropped terms, including a simplified model with only random terms. Additionally, the number of days per period per beach where depredation of baits was observed (via camera images or during site visits) were compared using ANOVAs. Model fit was assessed via plotting the distribution of residuals for all models, and was assessed as adequate.

All analyses were conducted in R 4.0.3 (R Core Team, 2021), and mixed models were constructed using the 'lme4' package (Bates *et al.*, 2015).

3.4 Results

General Observations. — Although foxes were identified on-site at Congi Station during initial surveys of the site one month prior to the experiment, via direct visual identification, presence of scat near the experimental sites, and images from a 6-day preliminary camera trap survey across the study area, no foxes were captured on camera during the experiment itself. Feral pigs were also identified visually, and through the presence of scats, tracks, and camera trap photos during preliminary site surveys, but were infrequent visitors to only two control sites during the experiment. In terms of adequate number of visits to reach statistical significance, ravens, brushtail possums, and macropods were the only species of interest that were recorded with enough frequency to perform statistical analyses.

Ravens. — Ravens were the most frequent visitors, with 208 visits recorded across all beaches. Ravens were also the only predator species recorded consuming eggs (Fig. 3.3), with 41 recorded visits showing direct evidence of egg consumption. Ravens were recorded consuming eggs on average 7 days (±8.9 SD) after the sites were first baited. All sites except for Control North and Impact North showed raven activity the same day that the ultrasonic repellent devices were activated. Raven site visit frequency (Fig. 3.4) did not significantly differ between treatments (LRT: χ^2_1=0.2, p=0.70), across periods (LRT: χ^2_2=1.6, p=0.45), or due to human activity (LRT: χ^2_1=0.7, p=0.39). Gaps between site visits averaged 3.1 days (±2.98 SD) in Preliminary 1, 2.6 days (±1.84 SD) in Preliminary 2, and 2.4 days (±2.96 SD) in the Test Phase. Raven site visit duration (Fig. 3.5) did not significantly differ between treatments (LRT: χ^2_1<0.1, p=0.92), across periods (LRT: χ^2_2=0.5, p=0.78), or due to human activity (LRT: χ^2_1=0.4, p=0.54). Raven predation activity did not differ among beaches ($F_{(5,12)}$=0.9, p=0.53) or among study periods ($F_{(2,15)}$=1.4, p=0.28).

Brushtail Possums. — Possums were occasional visitors (Fig. 3.2), with 34 separate visits recorded at 4 of the 6 sites. The majority (n=18) of these site visits were at Control

South, while Impact Centre and Control North did not have any possum sightings. Possums were not recorded interacting with eggs, including eggshells that had been exposed on the soil by previous raven activity. Possum site visit frequency did not significantly differ between treatments (LRT: χ^2_1=0.4, p=0.56), across periods (LRT: χ^2_2=3.0, p=0.22), or due to human activity (LRT: χ^2_1=0.2, p=0.66). Gaps between site visits averaged 5.7 days (±4.39 SD) in Preliminary 1, 4.9 days (±3.81 SD) in Preliminary 2, and 5.4 days (±4.09 SD) in the Test Phase. Possum site visit duration did not significantly differ between treatments (LRT: χ^2_1=0.4, p=0.53), across periods (LRT: χ^2_2=1.0, p=0.61), or due to human activity (LRT: χ^2_1=0.8, p=0.39). Possums were recorded on impact sites within 2 days of repellent device activation.

Macropods. — Macropods were occasional visitors (Fig. 3.2), with 30 separate visits recorded across all 6 sites. The majority of macropod site visits were at Control Centre (n=11) and Impact Centre (n=9). Macropod site visit frequency did not significantly differ between treatments (χ^2_1<0.1, p=0.83) or across periods (χ^2_2=1.3, p=0.52), but did differ significantly when compared to the timing of human activity (χ^2_1=4.2, p=0.04); macropods were 18% (±0.08 SE) less likely to visit a site on days when researchers had also visited Congi Station. Gaps between site visits by macropods averaged 7.1 days (±3.36 SD) in Preliminary 1, 4.4 days (±3.26 SD) in Preliminary 2, and 3.8 days (±3.17 SD) in the Test Phase. Macropod site visit duration did not significantly differ between treatments (LRT: χ^2_1=0.9, p=0.34), across periods (χ^2_2=1.2, p=0.54), or due to human activity (χ^2_1=2.0, p=0.16). Macropods were recorded on impact sites the day following the activation of the ultrasonic repellent devices.

Feral Pigs. — Inward-facing camera traps recorded 5 separate visits by pigs to Control Centre during the experiment (Fig. 3.2), and no pigs were recorded at any other sites. Given these limited data, impacts of the ultrasonic repellent devices cannot be quantified. The observed visits at the control site lasted no longer than 2 minutes, indicating that the pig sounder was passing through the beach rather than foraging. A group of 2-3 pigs was recorded by the outward-facing cameras at Control South on 29 November for 4 minutes, and appeared to be foraging higher on the bank.

Australian Magpies. — Magpies were recorded on-site with 3 visits, each at a different site and each visit on different days. This included a visit to Impact South after the repellent devices had been activated. There was no indication that the magpies were consuming eggs.

Other. — Cameras recorded 3 Bell's turtle sightings: 2 sightings at Impact South on 3 and 4 December, and 1 sighting at Impact North on 6 December. All three sightings were of female turtles (identified by the size and shape of the turtle's tail) out of the water, potentially indicating nest-searching behaviour, although no digging was observed. All of these records occurred prior to the activation of the ultrasonic repellent devices.

Three camera trap photos contained unidentifiable animals, either due to the subject being too close to the camera or in motion. Hair was visible in all three cases, indicating a mammal, but exact species could not be identified.

3.5 Discussion

Ultrasonic repellent devices were not shown to be effective at excluding nest predators from turtle nesting beaches. In particular, raven site visits were not affected by the repellent devices, and ravens were the only species of interest recorded consuming egg baits. While a lack of response to ultrasonic frequencies was expected in avian predators (Seamans *et al*., 2013), it should be noted that the additional LED visual flashes were also unable to deter the ravens in this trial. Few other predatory species were recorded at the sites, including the main target species (foxes and pigs), so the effectiveness of ultrasonic devices on deterring them are indeterminate. Based on the available observations of other mammalian species' activity when exposed to the ultrasonic repellent devices, some inferences may by drawn.

Although the results of this experiment were inconclusive regarding fox and pig aversion, some inferences may be drawn from observing the behaviour of other mammals during the experiment. Macropod site visits may have been affected by researcher visits, possibly due to human scent left behind by researchers (Parsons and Blumstein, 2010), but were not affected by treatment; possum visits were not affected by any of the measured factors. This suggests that the mammals visiting the site were not disturbed by the repellent devices, a suggestion that is supported by previous studies on the topic. Bender (2003) showed that Eastern grey kangaroos and red kangaroos (*Osphranter rufus*) did not strongly modify their behaviours when exposed to similar ultrasonic devices. Greaves and Rowe (1969) showed that rodents expressed some mild aversion to ultrasonic signals, but would continue to forage in range of the ultrasonic signals. In general, it is relatively easy for animals to become habituated to sound disturbances; European starlings (*Sternus vulgaris*) were shown to habituate to sounds from jet engines, continuing to forage even when ambient noise surpassed a likely-painful 130 dB (Boudreau, 1968). Rodents have also been observed to be more adverse to mild electric shocks than to pain-inducing sounds (Campbell and Bloom, 1965). While the mammals that were frequently recorded on-site did not re-appear immediately after the repellent devices were activated, the delayed timing of their reappearance does not differ from gaps in visits pre-activation, and cannot be attributed to the ultrasonic signals. Some caution must be taken in drawing conclusions from the models, particularly for brushtail possums and macropods as the data were strongly zero-weighted, which affected model fit. Repetition of the experiment in an area with higher predator presence may yield differing results. However, given the observations from this experiment and from examination of the literature, it is possible to accept with confidence that these devices are not suitable as a conservation tool.

The dearth of foxes and pigs during the experiment was unusual, and was similarly noted in Chapter 2. Both species were recorded at Congi Station during preliminary site visits, and neither Congi Station nor its close neighbours conducted any pest control activities during or immediately prior to the experiment (A. Urun, pers. comm., site manager at Congi Station). Foxes were not recorded at any site during the experiment, and pigs were only occasional visitors to some sites.It is possible that the scent of human researchers visiting the sites would cause aversion in these two species, as some meso-predators like bobcats (*Lynx rufus*) avoid human scents (Heinlein *et al*., 2020). However, Heinlein *et al*. (2020) reported that foxes did not differ in visit frequency to baited camera traps with and without human scent masking. No similar research appears to have been conducted on pigs, though numerous researchers have mentioned the possibility that pigs may be averse to human scent during camera trap studies (e.g. da Cunha Nogueira *et al*., 2007; Holtfreter *et al*., 2008). Human visits to nest refuge structures for Chapter 2 of this dissertation were much less frequent than visits to the test sites for this chapter, and foxes were similarly not recorded during the 2020/21 field season during Chapter 2, so human scent is an unlikely factor preventing the recording of fox or pig activity at Congi Station. It is possible that the severe drought and bushfires from the previous year reduced the population of both foxes and pigs in the New England region (Filkov *et al*., 2020), such that there were not enough present to be recorded in any significant numbers. Due to the small size of the site, the experimental beaches were close together and may have fallen within the territory of a single fox (Towerton *et al*., 2016). The experiment, therefore, may have run the risk of pseudoreplication if any foxes were observed during the experiment. It is possible that the fox observed during preliminary site visits was eliminated by other means (i.e. road mortality, predation, etc.) or was in the process of dispersing. However it is unusual for an empty territory to remain unclaimed for long, even after a fox has been eliminated by human activity (Newsome *et al*., 2014).

While it is unfortunate that results on the effect of ultrasonic devices on foxes and pigs could not be measured directly, the lack of apparent effect on other mammals in the area could suggest that repellent devices may not be effective at excluding these invasive predators, however, further investigation would be required to confirm this. Conservation efforts have limited resources, however, so perhaps should avoid the use of ultrasonic repellent devices until such time as their effectiveness is confirmed experimentally. Conservation efforts would be better served testing other novel methods for nest protection, such as conditioned food aversion (Tobajas *et al*., 2020), or refining existing techniques, such as nest caging (Riley and Litzgus, 2013). Null results such as these are important to conservation efforts, as they can guide conservation programs away from ineffectual strategies (Axford *et al*., 2020). This study showed that ultrasonic repellent devices, far from being a panacea, are apparently ineffective as a nest protection method, and limited conservation resources should be directed elsewhere in similar contexts.

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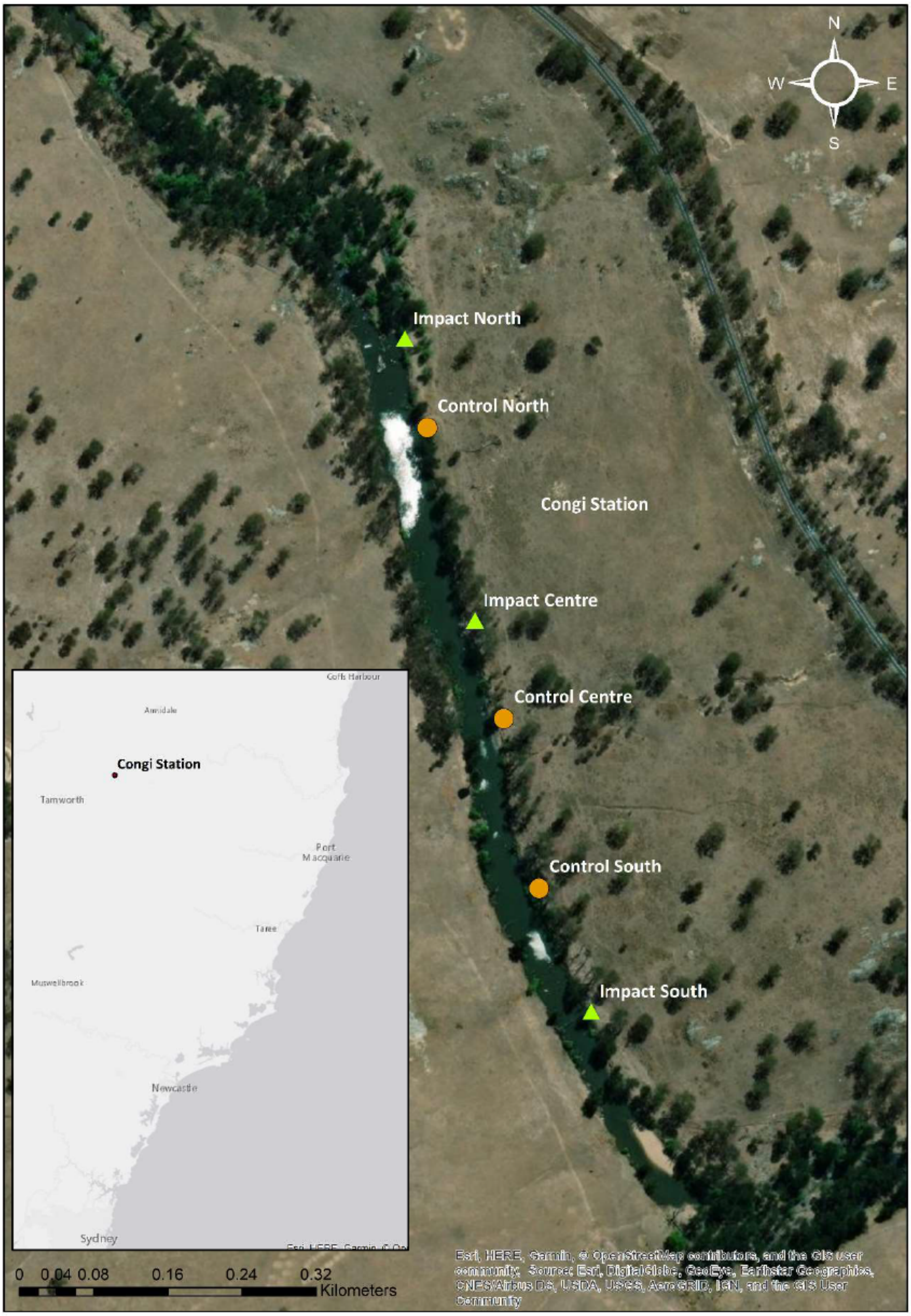
3.7 Figures



Esri, HERE, Garmin, © OpenStreetMap contributors, and the GIS user community. Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 3.1 - Map of the six beaches at Congi Station. Green triangles show impact treatments, orange circles show control treatments. Inset shows the relative location of Congi Station within northern New South Wales.

Figure 3.2 - Example of inward-facing camera trap (below) with ultrasonic repellent device (top of stake) at an impact site (Impact South site). An outward facing camera is visible to the right of the image. Bell's turtle (*Myuchelys bellii*) is visible next to the lower post, possibly a nest-searching female.

Figure 3.3 - Selected images as examples of species of interest visiting sites: a) raven (*Corvus* sp.) at Impact Centre consuming an egg bait, b) common brushtail possums (*Trichurus vulpecula*) at Control Centre, c) feral pigs (*Sus scrofa*) at Control Centre, and d) eastern grey kangaroo (*Macropus giganteus*) at Control Centre.

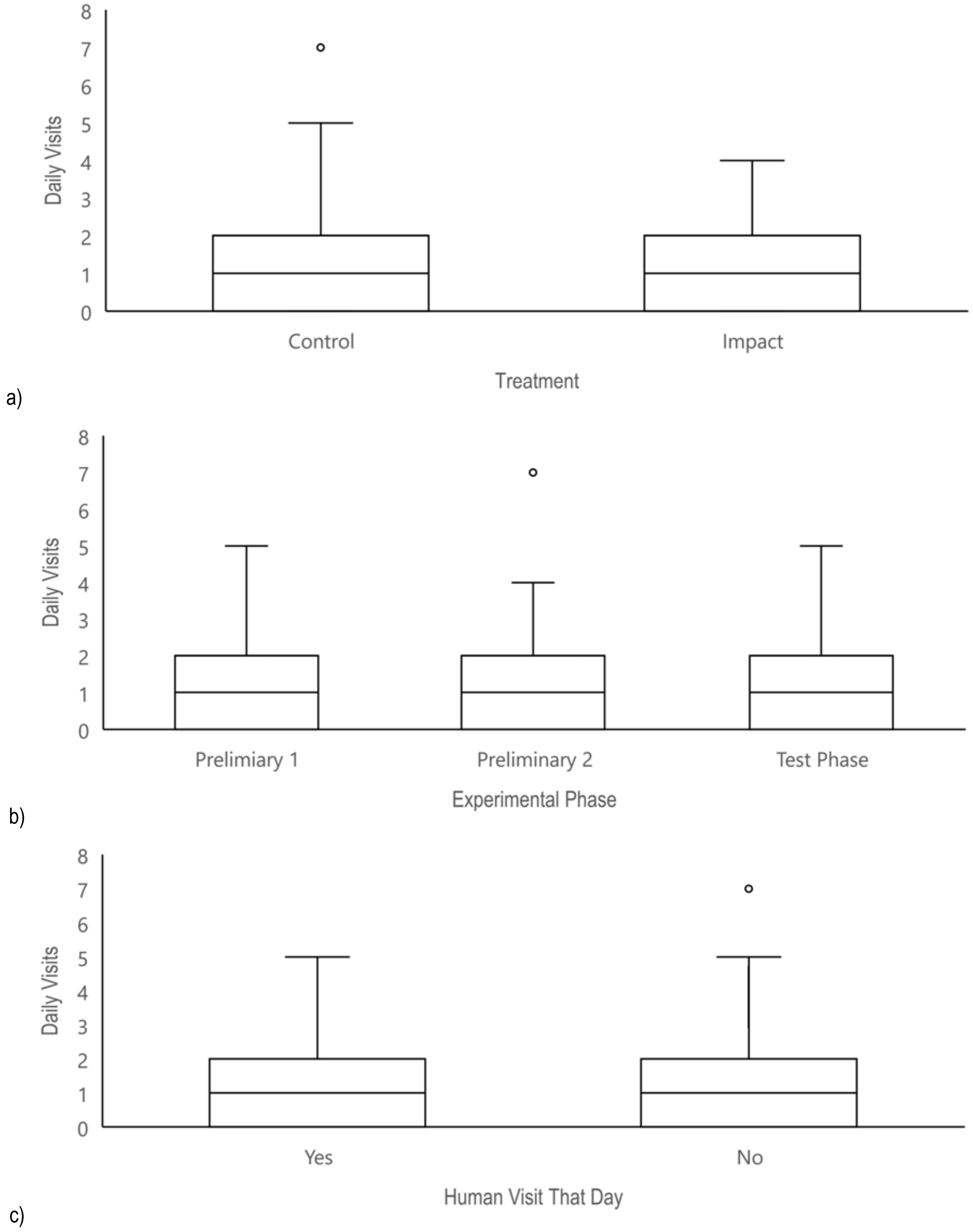


Figure 3.4 - Raven (*Corvus* sp.) daily site visits with a) treatment (LRT: χ^2_1=0.2, p=0.70), b) period (LRT: χ^2_1=1.6, p=0.45), and c) human site visits (LRT: χ^2_1=0.7, p=0.39). Centre line shows the median, boxes show 25% to 75% percentiles, whiskers show 5% to 95% percentiles, and points show outliers.

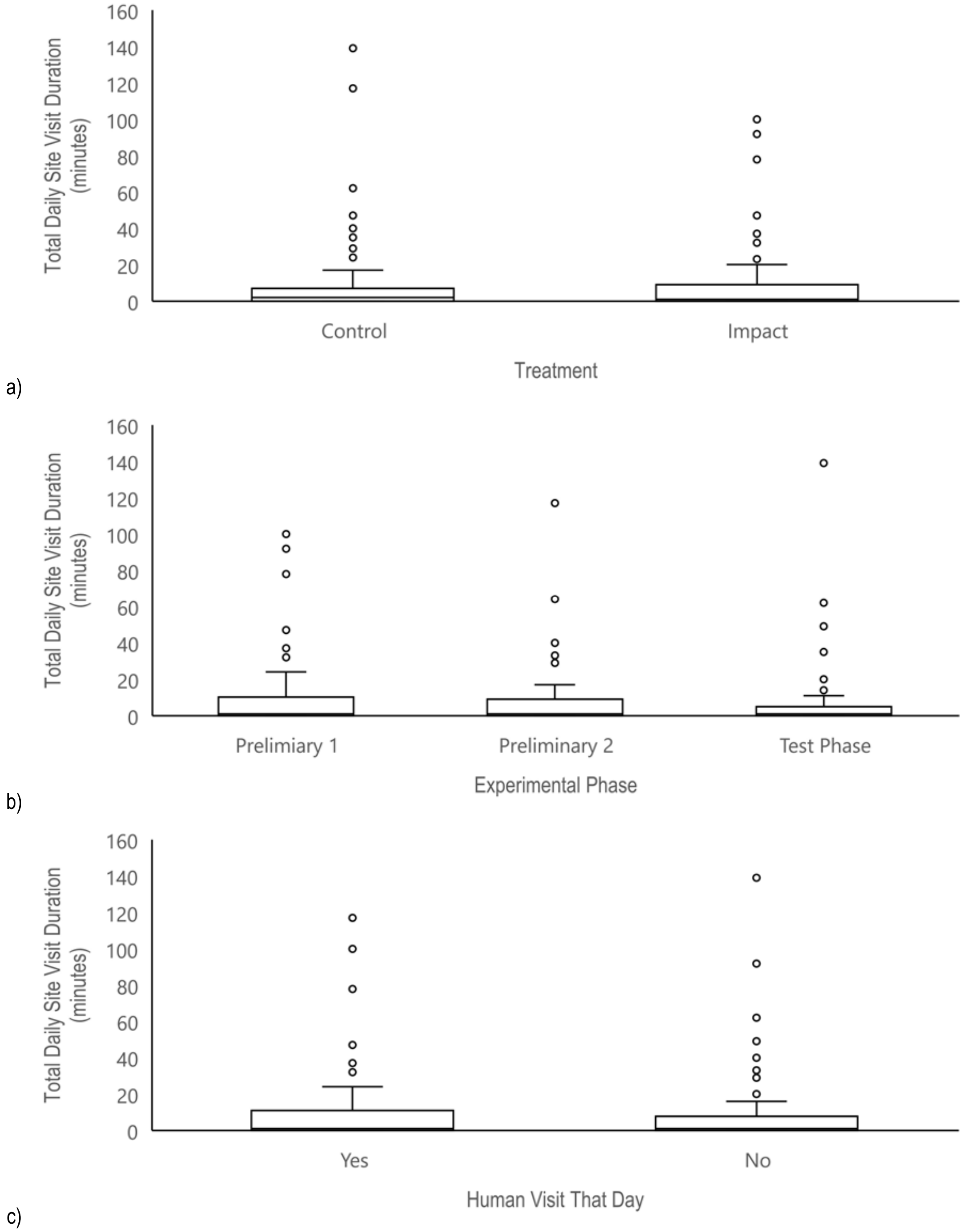


Figure 3.5 - Raven (*Corvus* sp.) total daily site visit duration with a) treatment (LRT $\chi^2_1<0.1$, p=0.92), b) period (LRT $\chi^2_1=0.5$, p=0.78), and c) human site visits (LRT $\chi^2_1=0.5$, p=0.54). Centre line shows the median, boxes show 25% to 75% percentiles, whiskers show 5% to 95% percentiles, and points show outliers.

3.8 Tables

Table 3.1 - Duration of study periods within the ultrasonic repellent experiment.

Study Period	Dates	Repellent devices
Preliminary 1	21 November - 30 November 2020	Off
Preliminary 2	1 December - 9 December 2020	Off
Test Phase	10 December - 19 December 2020	On

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF ORIGINALITY

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of Work	Page number(s)
All text, tables, and figures	50 to 72

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Date

CHAPTER 4 - INVESTIGATING A POTENTIAL SURVEY TOOL FOR IDENTIFYING RAIDED TURTLE NEST SHELL FRAGMENTS: EGGSHELL MICROSTRUCTURAL DIFFERENCES IN FRESHWATER TURTLES

4.1 Abstract

Researchers frequently encounter cryptic turtle nests only after the nest has been raided by a predator. While these nests are obviously unsuccessful, useful information might still be collected from these detections if remains could be confidently assigned to a particular species. However, this can be difficult where multiple turtle species nest within an area at a given time. This study assessed the efficacy of using scanning electron microscopy (SEM) as a diagnostic tool for distinguishing the species of origin of turtle eggshell fragments that are typically recovered post-predation. Eggshell fragments were collected from known sources of four species of turtle native to eastern Australia: Eastern long-necked turtle (*Chelodina longicollis*), Murray River turtle (*Emydura macquarii*), Bell's turtle (*Myuchelys bellii*), and Bellinger River turtle (*M. georgesi*). These fragments were scanned and analysed for visual diagnostic features, and measured for differences in size of microstructural features across species. No obvious diagnostic features emerged from the visual, qualitative analysis. Central plaque diameter emerged as a potential diagnostic feature long-necked, with Murray River turtles having relatively small mean plaque size (19.8 µm ±10.28 SD), while Bell's turtles (43.5 µm ±14.16 SD) and Bellinger River turtles (41.9 µm ±15.41 SD) had relatively large plaques, with little overlap between species. Eastern long-necked turtles had no visible plaques on any samples. Other measured features (shell unit size, basal knob size, and shell unit density) significantly differed in central tendencies among species, however there was also considerable range overlap or a lack of statistical difference among species, preventing the technique being definitive. Some of this observed variance may be due to confounding factors inherent in method, such as the replicability of mounting fragments for scanning, the condition of the eggshell, and the stage of embryonic development at the time of collection. These results suggest that the use of an SEM to measure microstructural features has some promise as a means for distinguishing the provenance of eggshell fragments, and further investigation to develop site specific protocols for the use of SEM as a diagnostic tool for turtle ootaxonomy is warranted.

4.2 Introduction

Turtle nests are notoriously cryptic, and with good reason; turtle eggs make for easy and nutritious meals for those predators that can locate them. Turtle females will bury their eggs in soil, often during periods of high rainfall, which may wash away visual and olfactory cues associated with their digging activities (Bowen and Janzen, 2005). Turtle nests are most vulnerable within the first week after burial, when such cues are still fresh, but nests are at risk of predation throughout the entire incubation period (Riley and Litzgus, 2014). There is a subsequent increase in predation risk just prior to hatchling emergence, possibly due to predators detecting the scent of leaking egg fluids as the nestlings hatch, or from audio cues of vocalizing hatchlings (Ferrera *et al.*, 2014; Riley and Litzgus, 2014; McKenna *et al.*, 2019). The crypsis of turtle nests is a necessary aspect of their reproductive ecology (Wirsing *et al.*, 2011; Voves *et al.*, 2016), and may represent a significant amount of parental investment in their offspring. However, it also presents difficulties for conservation efforts that are required for many turtle species of conservation concern. Locating turtle nests for research or conservation purposes, such as investigating occupancy, productivity, reproductive output, or population density, is challenging for researchers without considerable effort. All too often, a researcher finds only the remains of eggshells after a predator has raided the nest.

While it is preferable for researchers to locate intact eggs, data may still be gleaned from the remains of raided nests. Approximate nest shape and structure, substrate preferences, nest placement relative to habitat features, and estimates of clutch size are all characteristics that may be collected post-predation. Further, in some jurisdictions identifying occupancy of a habitat by a protected species can confer legal protection to a nesting habitat. For example, in Australia it is illegal to disturb critical habitats of threatened species without appropriate permits through the Environmental Protection and Biodiversity Conservation Act (Ministry of the Environment, 1999). However, in regions with multiple sympatric turtle species, it can be problematic to assign these data to a particular species. Consequently, methods to identify the species of origin for eggshells post-predation would be extremely useful for assigning such variables, and for determining the conservation effects of nest predation on a particular species. Determining the provenance of eggshells (ootaxonomy) is a process commonly performed for invertebrate eggs, with most literature focused on insects and crustaceans (e.g. Munuswamy *et al.*, 1985; Gaino *et al.*, 1987; Fausto *et al.*, 1992). Literature on vertebrate ootaxonomy appears to be largely restricted to fossil taxa (e.g. Mikhailov, 1997; Lawver and Jackson, 2017; Lawver and Boyd, 2018), with little to no literature on the ootaxonomy of fresh eggshells from extant vertebrate species (Angoh *et al.*, 2018).

The techniques applied to invertebrates and fossil vertebrates should be broadly applicable to the eggs of living vertebrate taxa. One such technique is the use of scanning electron microscopy (SEM) to describe the microstructures of turtle eggshells, allowing researchers to potentially identify diagnostic features. Examination of reptilian eggshells using an SEM has long been a field of study (Mikhailov, 1997; Gibbons *et al.* 2020), however little to no research has been conducted on the use of SEM images to distinguish eggshells amongst extant species. The eggs of turtle species may be poorly distinguishable based on visual examination or measurements of intact whole eggs. Angoh *et al.* (2018) found that central tendencies of macroscopic measurements of eggshells significantly differed among species but generally had considerable overlap in a range of sizes, making simple measurement of the whole eggshell a poor diagnostic tool. Fragmented eggshells would be even more challenging to measure, and thus to discriminate species.

Eggshell structure has been described using SEM in numerous species of chelonians, including leatherback sea turtles (*Dermochelys coriacea*; Chan and Solomon, 1989; Sikiwat *et al*., 2015), narrow-headed softshell turtles (*Chitra chitra*; Kitimask *et al*., 2003), hawksbill sea turtles (*Eretmochelys imbricata*; Phillott and Parmenter, 2006; Sikiwat *et al*., 2015), flatback turtles (*Natator depressus*; Phillott and Parmenter, 2006), loggerhead sea turtles (*Caretta caretta*; Phillott and Parmenter, 2006), green sea turtles (*Chelonia mydas*; Phillott and Parmenter, 2006; Sikiwat *et al*., 2015), European pond turtles (*Emys orbicularria*; Mitrus, 2003), and painted turtles (*Chrysemys picta*; Gibbons *et al*., 2020). Some of these studies report on characteristics of multiple species, and make note of some differences in the ultrastructure of the eggshells, such as the crystalline matrix within the shell units (Sikiwat *et al*., 2015). Ultrastructure requires specialized equipment and training to examine, while examining the microstructure of an eggshell is a much simpler prospect.

This study was conducted on four species of native Australian side-necked turtles (Chelidae; Pleurodira): the Eastern long-necked turtle (*Chelodina longicollis*), the Murray River turtle (*Emydura macquarii*), the endangered Bell's turtle (*Myuchelys bellii*), and the critically-endangered Bellinger River turtle (*M. georgesi*). Murray River turtles and Eastern long-necked turtles are widespread and common across NSW. Bell's turtles and Bellinger River turtles are sympatric with Eastern long-necked turtles and Murray River turtles, but not with each other. Bell's turtles live only in a few west-flowing, upland streams of the Murray-Darling catchment in the New England Tablelands, while Bellinger River turtles are endemic to the Bellinger River catchment, an east-flowing river that drains into the Pacific Ocean.

Pleurodiran taxonomy is contested. Le *et al*. (2013) listed *Myuchelys* and *Emydura* as sister genera that diverged ca. 25 mya; Pereira *et al*. (2017) place the divergence date as ca. 30 mya. Georges *et al*., (1999) also placed *Emydura* and *Myuchelys* (then listed as part of the genus *Elseya*) as sister genera, but do not speculate on divergence times. Ferreira *et al*. (2018) place the *Myuchelys*/*Emydura* divide much earlier (ca. 125 to 130 mya), with *Myuchelys* more closely allied to *Elseya* than to *Emydura*; however, known instances of hybrid Murray River turtle x Bellinger River turtle individuals (Chessman *et al*., 2019) suggest that *Emydura* and *Myuchelys* have diverged more recently. *Chelodina* is generally considered to be quite distantly-related to the short-necked pleurodiran genera (Georges *et al*., 1999; Pereira *et al*., 2017; Ferreira *et al*., 2018). Pereira *et al*. (2017) place divergence between long-necked and the short-necked Australian genera at ~105 mya. Whole, intact eggs of these turtle species are likely indistinguishable by visual examination, but diagnostic features may have evolved in the eggshell microstructure over time since divergence of the various genera and species from their common ancestors, which would allow the identification of turtle eggshells post-predation.

This Chapter sought to explore the use of SEM imagery to describe the microstructure of eggshells from three species of freshwater turtle found in the New England Tablelands, and one additional species located in the Mid North Coast, ~100 km to the east of the Tablelands. Eggshells of known provenance were acquired from captive breeding programs and compared using an SEM. The microstructures of these eggshells were described and compared for diagnostic features. It was hypothesized that the microstructure of eggshells would differ across species, and that this method could then be used in a diagnostic manner to determine the provenance of an eggshell. If microstructural differences are shown to be diagnostic, the use of scanning electron microscopy could be a powerful tool in conservation and research, and may be broadly applicable amongst other egg-laying species.

4.3 Methods

Egg Collection. —Eggshells from Bell's turtles and Murray River turtles were supplied by captive breeding efforts at the University of New England. The Bell's turtle eggs were all collected post-hatching, except for one clutch which was opportunistically extracted from a dead gravid female. Captive-bred Murray River turtle eggshells were all collected post-hatching. Intact eggshells were opportunistically extracted from a road-killed female Eastern long-necked turtle collected in the New England Tablelands. Post-hatching eggshells from Bellinger River turtles were acquired from a captive breeding program at Taronga Zoo in Sydney, NSW (Chessman *et al.*, 2019).

Post-hatching eggshells of Bell's turtles and Murray River turtles were stored immediately upon collection in a -20°C freezer at the University of New England until examination, as were the whole eggs extracted from dead females. Some eggshells were stored in plastic bags in dry conditions at room temperature, including all eggshells from raided nests and all Bellinger River turtle eggshells, to allow for examination of eggshells that had experienced some deterioration; exact numbers of eggs are difficult to quantify due to fragmentation. Storage method and general condition of each eggshell specimen was noted prior to beginning microscopic analysis on a given eggshell.

Eggshell Anatomy.— The gross morphology of turtle eggshells are highly conserved across taxonomic lines. The eggshell consists of two major structural layers: the hard mineral layer and the flexible membrane. The membrane consists of densely-packed fibres arranged in an apparently-random configuration. This fibrous matrix allows exchange of gases between the interior of the egg and the environment. The inner surface of the egg is a smooth sheet of material, while the outer surface is covered by the mineral layer.

The mineral layer consists of clusters of mineralized nodules called shell units. The base of each shell unit is fused to the membrane beneath it, with an interior hollow at the base of each shell unit called the central plaque. Large gaps between clusters of shell unit serve as pores, facilitiating gaseous exchange across the membrane layer.

Microscopy. — Fragments of eggshell were mounted on steel slug-style mounts using aqueous silver adhesive (ProSciTech, Queensland, Australia) with either the outer or inner surface of the eggshell exposed (Fig. 4.1). Outer surfaces were either left unmodified to examine the eggshell mineral coating in some samples, or the mineral layer was removed for examination of the egg membrane surface in other samples. The mineral layer was removed by gently folding the eggshell fragment in half with forceps to create cracks in the mineral layer, and then carefully peeling or scraping the pieces of mineral away from the membrane. Other samples were mounted with the inner surface of the eggshell exposed to examine that surface. Further, the mounted eggshells regularly curled upwards when contacted by the adhesive, allowing imaging of cross-sections of the membrane.

Samples were coated in gold with a NeoCoater MP-19020NCTR sputter coater (JEOL USA, Massachusetts, USA) for 1 minute. The samples were then re-oriented and coated twice more for a total of 3 minutes. Coated samples were placed in a JEOL JSM-6010LA scanning electron microscope (JEOL USA, Massachusetts, USA), with an accelerating voltage of 10 kV. Images of each fragment were taken at 100x and 500x resolutions, and at least one photo of an eggshell cross-section from each egg was additionally taken at 500x resolution. Seven images were taken per fragment. Image sites within these broad regions were chosen based on lack of imaging artefacts or contamination, such as dirt or dried albumen.

Analysis. — Four major components of turtle eggshells were examined in this study (Fig. 4.2): the outer mineral layer, the outer membrane surface after the mineral layer had been removed, the inner membrane surface, and the fibrous matrix between the two membranes examined via cross section. Central plaque characteristics on the outer membrane surface were a focus of investigation. Pores were too irregular in shape to consistently measure.

Eggshell images were analysed visually and are described using the terminology presented in Mikhailov (1997) and Gibbons *et al*. (2020). Similarities and differences among the four species were first compared qualitatively, with a focus on outer and inner membrane surfaces, the mineral layer, and cross-sectional images from the egg fragment edges (Fig. 4.2). Initial descriptions were then used to identify areas of interest that could be measured and compared quantitatively. These microstructural features were measured with Digimizer version 5.4.4 digital analysis software (MedCalc Software, Ostend, Belgium) as outlined below.

The outer mineral layer of the eggshells were analysed by measuring the diameter of the shell units, (Fig. 4.2). Ten shell units per image of high quality per 100x image were selected randomly. For each selected shell unit, its largest diameter and smallest diameter were measured, and the ratio between these measures was also calculated for comparison of shape regularity. Shell unit density was also measured, by drawing a rectangle of ~500,000 μm^2 across the image, counting the number of shell units contained within the rectangle, and dividing the number of shell units by the enclosed area.

The outer membrane surface was analysed by measuring the diameter of central plaques left after the removal of the mineral layer. Ten plaques per 100x image were selected randomly and the diameter of each plaque was measured. Surrounding each plaque was the remnant of the shell unit's base, the basal knob, and the largest diameter of each basal knob that was paired with the selected plaques was also measured (Fig. 4.2)..

The inner membrane surfaces of eggshells were universally smooth and did not provide measureable features on a consistent basis, so were not quantified for statistical analysis but were described visually. Images of eggshell edges were of inconsistent height above the mounting, which skewed image perspective during the SEM image capture process, and lead to measurements of membrane thickness providing uncertain results. Given this, statistical analyses were not conducted on this metric. The fibrous matrix, most visible in the cross-sectional images, did not provide any obvious features for measurement, and was also not used in statistical analysis.

Measurements of microstructural features (maximum shell unit size, minimum shell unit size, shell unit size ratio, shell unit diameter, basal knob diameter,) were compared across species with ANOVAs, and with post-hoc Tukey's tests if a feature showed significant differences among species. The practical effectiveness of these microstructures as diagnostic features was further tested using a discriminate function analysis (DFA) to determine if the models could distinguish eggshell provenance based on microstructure measures. These DFAs used 3/4 of each dataset as training data, and attempted to identify the remaining 1/4 of the dataset by species using each feature. Separate DFAs were performed for any features that showed significant differences among species. All DFA tests were conducted with all four species and repeated for the three turtle species present in the New England Tablelands, excluding the Bellinger River turtle. The results of DFAs were expressed as confusion matrices, showing the number of successful identifications by the model. These DFA results were then compared with a Chi-squared goodness of fit test. Statistical analyses were calculated by hand for goodness of fit tests, and discriminate function analyses were performed with the 'MASS' package (Venables and Ripley, 2002) conducted using R version 4.03 (R Core Team, 2021).

4.4 Results

Mineral Layer. — All turtle species examined showed a typical turtle eggshell morphology according to Mikhailov (1997), with some exceptions (Fig. 4.3). In particular, the Eastern long-necked turtle eggshells extracted from the road-killed female showed an unusual microstructural morphology, exhibiting no shell units on samples taken from the top or bottom of the eggs, instead showing flat sheets of mineral crystal (Fig. 4.2). Depredated eggshells of Eastern long-necked turtle did exhibit properly formed shell units in samples taken from the same locations on the egg, so this lack of shell units may be due to interrupted and incomplete mineral deposition, pre-laying. No other obvious microstructural differences were observed in the mineral layers among hatched or depredated eggshells from the four species during visual examination. While preparing eggshells for SEM observation, it was noted that the mineral layers of Eastern long-necked turtle eggshells were difficult to separate from the outer membrane surface compared to the eggshells of the other three species.

Largest shell unit diameter differed significantly among species ($F_{(3,86)}$=8.1, p<0.01; Fig. 4.4); Bellinger River turtle shell units were on average smaller than Bell's turtle ($t_{(1)}$=24.5, p<0.01) or *E. macquarii* ($t_{(1)}$=31.2, p<0.01) shell units, but did not differ from long-necked turtle shell units ($t_{(1)}$=8.2, p<0.01). Smallest shell unit diameter differed significantly among species ($F_{(3,86)}$=6.0, p<0.01; Fig. 4.4); Bellinger River turtle ($t_{(1)}$=23.5, p<0.01) and long-necked turtle ($t_{(1)}$=24.5, p<0.01) shell units were smaller than Murray River turtle shell units. Shell unit diameter ratio did not differ among species ($F_{(3,86)}$=1.9, p=0.15; Fig. 4.4). Shell unit density did not differ among species ($F_{(3,18)}$=1.3, p=0.30). While the central tendencies differed in some measures, there was considerable overlap in the range of shell unit size across all species (Fig. 4.4).

The DFA conducted on all species had low success at distinguishing among species for measures of the shell units (Table 4.1). The training data (n=68) was unable to train the test data (n=22) to distinguish from random using largest shell unit diameter (χ^2_3=4.4, p=0.25) or smallest shell unit diameter (χ^2_3=1.2, p=0.75). The DFA conducted on the three New England species had low success at distinguishing among species for measures of the shell units (Table 4.1). The training data (n=53) was unable to train the test data (n=18) to distinguish from random using largest shell unit diameter (χ^2_2=3.7, p=0.10) or smallest shell unit diameter (χ^2_2=1.9, p=0.50).

Outer Membrane Surface.— No central plaques or basal knobs were seen on any images of Eastern long-necked turtle eggshells, so those images were excluded from statistical analyses. This may be related to the difficulty of separating the mineral layer from the membrane in this species' eggshells; the mineral layer may come away completely, taking the outermost portions of the membrane with it and not leaving basal knobs and central plaques behind. The internal fibrous matrix of the membrane was more frequently visible on the surface of Eastern long-necked turtle samples than in other species (Fig. 4.5). For the purpose of species identification this was not an issue, as the results were consistent across samples using this method.

Central plaque diameter differed significantly among species ($F_{(3,87)}=33.9$, $p<0.01$; Fig. 4.6); Murray River turtle plaques were smaller on average than Bell's turtle ($t_{(1)}=23.7$, $p<0.01$) or Bellinger River turtle ($t_{(1)}=22.2$, $p<0.01$) plaques, which did not differ from each other ($t_{(1)}=1.5$, $p=0.91$). Basal knob diameter differed significantly among species ($F_{(3,86)}=7.8$, $p<0.01$; Fig. 4.6); Murray River turtle basal knobs were on average smaller than Bellinger River turtle ($t_{(1)}=18.9$, $p<0.01$) but not Bell's turtle ($t_{(1)}=12.6$, $p=0.07$), and the two *Myuchelys* species did not differ from each other ($t_{(1)}=6.3$, $p=0.54$). However, while the central tendencies differed, there was considerable overlap in the range of basal knob size across all species, and plaque sizes for Murray River turtle overlapped with both *Myuchelys* species (Fig. 4.6).

The DFA conducted on all species had some success at distinguishing among species for measures of the central plaques (Table 4.3). The training data (n=67) was able to train the test data (n=24) to distinguish from random using pore diameter ($\chi^2_2=8.3$, $p<0.05$), but not for basal knob diameter ($\chi^2_2=4.3$, $p=0.10$).

The DFA for Murray River turtle, Bell's turtle, and Bellinger River turtle showed high success at distinguishing among Murray River turtle from the two *Myuchelys* species with measures of central plaque diameter, but not with basal knob diameter (Table 4.3). The training data (n=61) was able to train the test data (n=16) to distinguish from random using plaque diameter ($\chi^2_1=4.1$, $p<0.05$), but not for basal knob diameter ($\chi^2_1=0.3$, $p=0.75$).

Membrane Cross Section. — While not able to be quantified, the fibrous matrix of membranes were visible in cross section in most images. These were often heavily coated with gold and detail could not be distinguished on the structures with the technique used (Fig. 4.7). No obvious diagnostic patterns in the matrix fibres emerged among species.

Inner Membrane. — The inner surfaces of the eggshell membrane did not provide any features for quantified statistical measurement. The eggshell membranes were typically smooth (Fig. 4.8); samples sometimes showed traces of the fibrous matrix through the intact membrane layer; these traces were more obvious in dried rather than frozen samples. The inner surface was also frequently contaminated with soil particles or dried albumen (Fig. 4.8), which sometimes interfered with imaging; the gold coating may have adhered more strongly to the contaminants than to the eggshell, caused imaging artefacts due to uneven reflectance of the electron stream. No obvious qualitative differences emerged among different species during visual examination (Fig. 4.8).

Diagnostic Utility. — The DFAs for 3 turtle species and the DFA for the New England species (Murray River turtle and Bell's turtle only) showed high success at distinguishing the two species with measures of central plaque diameter (Table 4.3; 4.4). As a result, plaque size is considered the most useful of the measured microstructural features for diagnosing provenance of turtle eggshells among the species studied. The two congeneric species in this study, Bell's turtles and Bellinger River turtles, were not distinguishable from each other, however these two species are allopatric. By considering geographic range where samples were collected, plaque size allowed discrimination among the three species found in the New England region, if comparing the upper and lower quartiles of pore diameter using a large number of pores. Thus, the following dichotomous key is proposed (Fig. 4.9): A lack of visible plaques in any samples from a particular eggshell indicates a member of the genus *Chelodina*. Consistent numbers of larger plaques (≥30 µm in diameter) in the outer membrane surface indicate a member of the genus *Myuchelys*, while smaller plaques (≤ 25 µm in diameter) indicate a member of the genus *Emydura* (Fig. 4.6).

4.5 Discussion

These results suggest that scanning electron microscopy has strong potential as a diagnostic tool in turtle ootaxonomy, although further refinement of the technique is required. With the exception of central plaque size, microstructural features of the four different turtle species are highly similar, and no features were discovered that could readily serve for diagnosis, either by qualitative inspection or by quantitative measurement. With plaque size, identification could be made to at least genus level. In the area of eastern Australia in which these four species live, eggshells that lack visible plaques on any eggshell fragments may be assigned to *Chelodina*, as the mineral layer appears to completely separate from the membrane layer during removal, leaving neither basal knobs nor plaques on the sample, although further investigation is needed to determine if this is the case. Eggshells with consistently small plaques (~15 to 25 µm in diameter) may be assigned to *Emydura*, and eggshells with consistently large plaques (~30 to 55 µm in diameter) may be assigned to *Myuchelys*. Given that Bell's turtles and Bellinger River turtles are allopatric, consideration of species' geographic ranges will further aid in identifying samples to species-level in the New England region.

Except for the lack of visible central plaques and basal knobs in Eastern long-necked turtle eggshells, qualitative differences in eggshell microstructure did not emerge among the four species. As noted, separating the mineral layers and outer membranes was particularly difficult for the Eastern long-necked turtle samples, although it seems unlikely that all central plaques were destroyed in this process even if damaging; difficulty in separating the mineral layer and consistent lack of visible plaques in samples from eggshell fragments could themselves serve as diagnostic features. Some outer membrane images from other species in this study (~17%) also did not yield visible plaques, which may be explained by damage to the eggshell during preparation, so consistent lack of central plaques must be considered for multiple eggshell fragments from a single egg to be considered diagnostic. Modification to the sample preparation protocols should be explored for future studies, such as those used for examining bird eggshells (Blankespoor, 1987), to best preserve the integrity of the membranes for examination. Additionally, samples from multiple locations on the eggshell among multiple specimens should be examined to determine if microfeature distribution differs across the membrane. Other qualitative features would be poor features for discriminating among species. The inner membrane surface and the cross sections of the fibrous matrix in particular were nearly identical across species, so would be poor qualitative diagnostic features. Eggshell SEM images at similar magnifications as taken for this study are available for painted turtles (Gibbons *et al*., 2020) and European pond turtles (Mitrus, 2003), and are visually similar to the pleurodiran turtles from this study. Pleurodira and Cryptodira diverged in the lower Jurassic (175 - 200 mya) according to Pereira *et al*. (2017), and if the eggshell microstructure of painted turtles and pond turtles (both cryptodires) are not visually distinct from those of pleurodiran species, there is a low probability that they will be visually distinguishable within Pleurodira. Thus, qualitative examination of eggshells within Pleurodira is unlikely to yield robust diagnostic differences of use in the current context.

Quantitative measurements of microstructural features (shell unit size, central plaque size, and basal knob size) tended to show differences in central tendencies but not in range of values, a result that is similar to the findings of Angoh *et al*. (2018) for macro-scale measurements of eggshells in other species. Of the measured microstructural features, plaque diameter emerged as the most distinctive, as Murray River turtle eggshells had smaller plaques than those of the *Myuchelys* species, in terms of both range and central tendencies. These results were strengthened when species' geographic range was considered; Murray River turtles and Bell's turtles, which are sympatric in some New England streams, were readily distinguished by pore size. A researcher that encounters a raided nest in New England may collect the eggshells, prepare multiple samples from each eggshell for analysis with an SEM, measure the size of central plaques (if any are present), and could assign the eggshells to species using the dichotomous key developed in this study.

Comparison of this study with images from previous SEM literature is confounded by differences in sampling and imaging protocols. For example, Phillott and Parmenter (2006) collected eggs directly from the oviduct of nesting sea turtles, and did not sputter coat the eggshell fragments prior to scanning, while Kitimasak *et al.* (2003) sampled eggs from a single female turtle that were incubated long enough to be determined to be infertile, and they sputter coated their eggshell samples. Similar trials in this study without sputter coating did not produce clear images. Likewise, most published studies are strongly focused on the ultrastructure of the eggshell (e.g. Chan and Solomon, 1989; Sikiwat *et al.*, 2015), rather than the microstructure (e.g. Gibbons *et al.*, 2020). Where most of the images examined in this study were taken at 100x or 500x magnification, several previous studies may have examined images taken as high as 2000x magnification (e.g. Phillott and Parmenter, 2006).

The measurements of shell unit diameter appeared to be in a similar range as painted turtle shell units as reported by Gibbons *et al.* (2020). The shell units measured in this study may be slightly larger than those of painted turtles, but without direct comparison of measurements it is difficult to conclude for certain. Gibbons *et al.* (2020) report that both shell units and pores increase in size throughout incubation. This could be a confounding factor in using quantitative measurements as a diagnostic feature; the eggshells sampled from in this study were mainly from eggs that had already hatched prior to sampling or were taken from the oviduct of dead females, whereas Gibbons *et al.*, (2020) sampled eggshells up to stage 20 of embryonic development. Collecting depredated eggshells *in situ* for scanning, the age of which would be indeterminate, could make calibrating an SEM protocol for species identification via pore size challenging.

The findings of Sikiwat *et al.* (2015) suggest that the using SEM to examine ultrastructure of the eggshell, particularly of the internal crystalline matrix of the shell units, may warrant further investigation as a diagnostic tool. Sikiwat *et al*., (2015) report that the internal structure of the shell unit differs among three species of closely-related sea turtle, suggesting a possible diagnostic feature; however, they did not report sample sizes, so further research is required to determine if the ultrastructural differences are consistent within and amongst species. If ultrastructural differences are consistent, and the equipment is available to fracture shell units in such a way as to reliably expose the internal crystalline structure, using ultrastructure may also be useful as another diagnostic tool to integrate with a SEM diagnosis protocol.

In conclusion, this initial investigation into the use of scanning electron microscopy to distinguish turtle eggshells by species via their microstructural traits has allowed the development of a dichotomous key for the New England region of Australia. Further, this study shows that this technique has scope for future, more intensive exploration into the development of a dichotomous key for other regions in Australia and globally. Turtle eggshells provide a number of microstructural features for comparison, and while there are few obvious qualitative differences across the four species studied, quantitative measurements of central plaque size shows that enough differences exist to warrant continued investigation into the utility of this technique. However, there are requisite developments for the technique that need to be addressed. One condition includes the need to develop a standardized protocol for collecting, preserving, and preparing samples for scanning, and another would be the need to develop a calibrated progression scheme of eggshell feature changes based on embryonic development stage, as an *in situ* nest can be raided at any time and microstructural features can change in the period between laying and depredation. Additionally, exposure time should be considered in future expansions of this study to calibrate for damage to samples caused by air, water, and light. If these challenges are overcome, a true dichotomous key for the ootaxonomy of turtles of turtles in Australia would be attainable in the near future, as this study has done for the New England region specifically.

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4.7 Figures

Figure 4.1 - Example mounting of Bell's turtle (*Myuchelys bellii*) eggshell fragments, prior to being sputter-coated in gold. The three right-hand samples are from eggshell MB014, and the left-hand samples are from eggshell MB019.

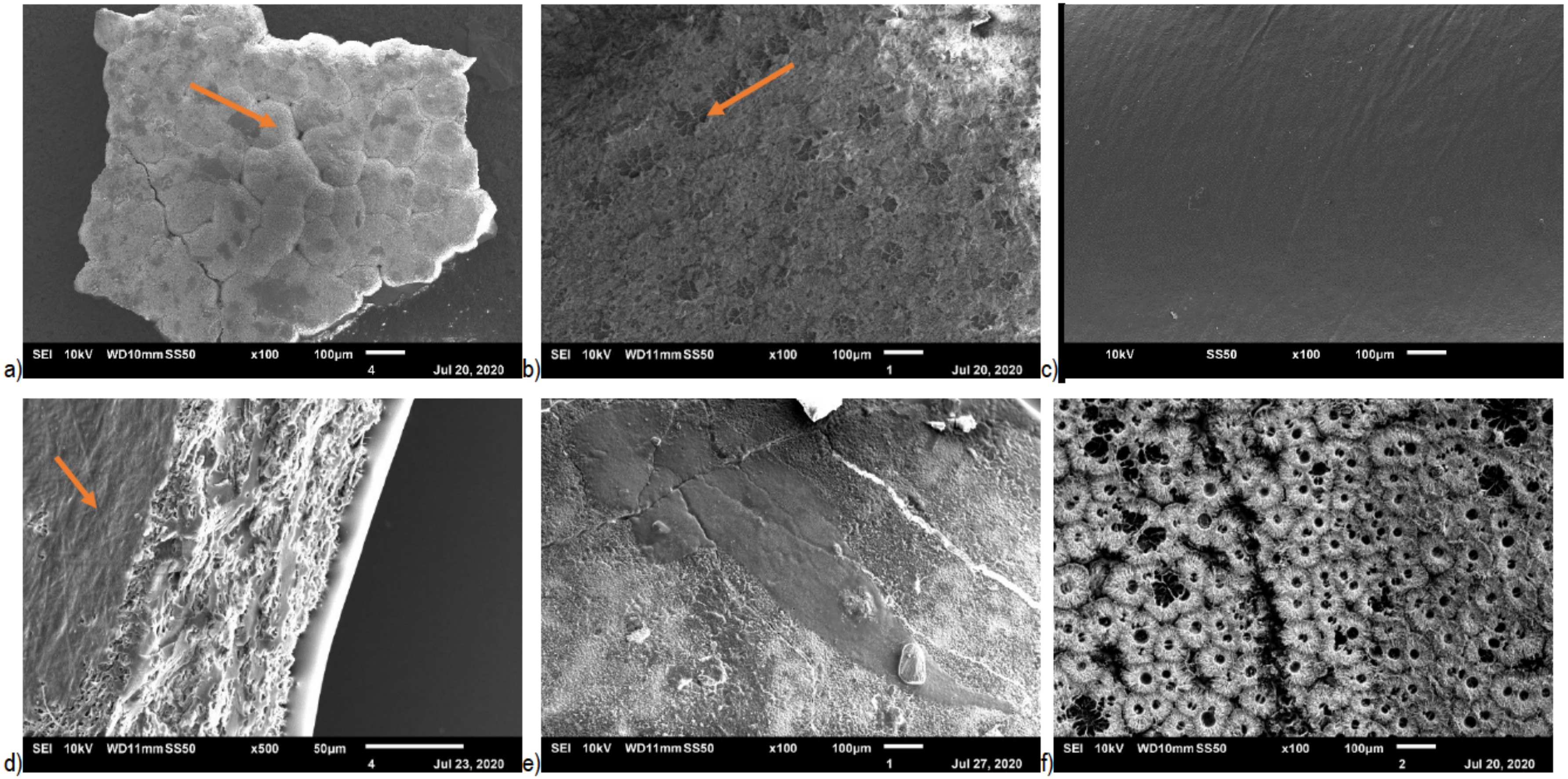


Figure 4.2 - Examples of layers and microstructural features of turtle eggshells used for analysis. Sequence of images: a) outer mineral layer of Murray River turtle (*Emydura macquarii)* eggshell, arrow indicating an individual shell unit, b) outer membrane surface of Murray River turtle eggshell, arrow indicating central plaques and basal knobs, c) inner membrane surface of Bell's turtle (*Myuchelys bellii*) eggshell, d) cross section of membrane layer of Bellinger River turtle (*Myuchelys georgesi*) eggshell, arrow indicating inner surface of the membrane for positional reference, e) surface of outer mineral layer of Eastern long-necked turtle (*Chelodina longicollis*) eggshell taken directly from the oviduct, showing a lack of shell units, and f) interior surface of Murray River turtle mineral layer after having been separated from the membrane. Images at 100 times magnification except for d), which is represented at 500 times magnification.

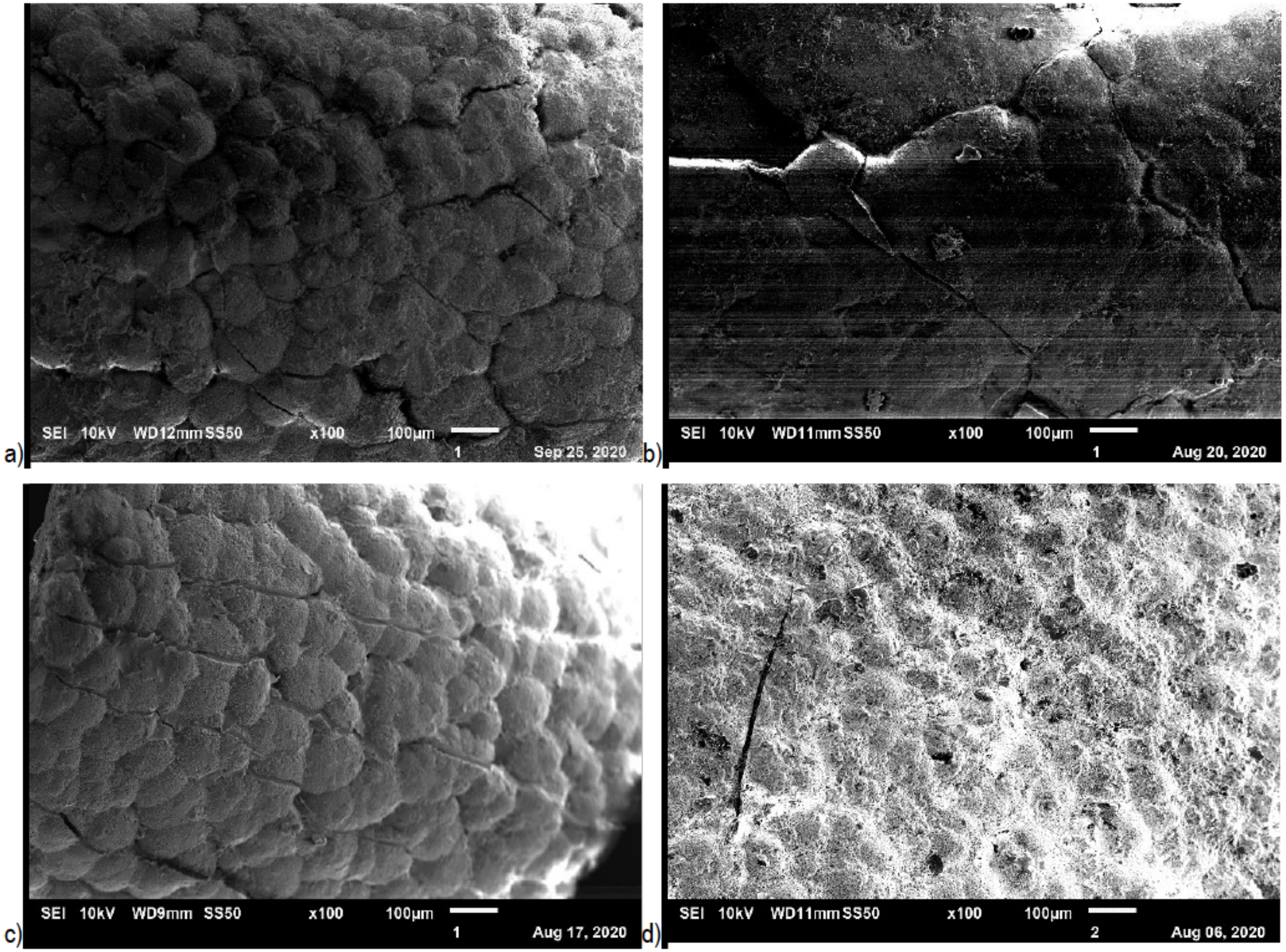


Figure 4.3 - Examples of eggshell mineral layer images from each species: a) Eastern long-necked turtle (*Chelodina longicollis*), b) Murray River turtle (*Emydura macquarii*), c) Bell's turtle (*Myuchelys bellii*), and d) Bellinger River turtle (*M. georgesi*). Note the similarity of shell unit size and shape.

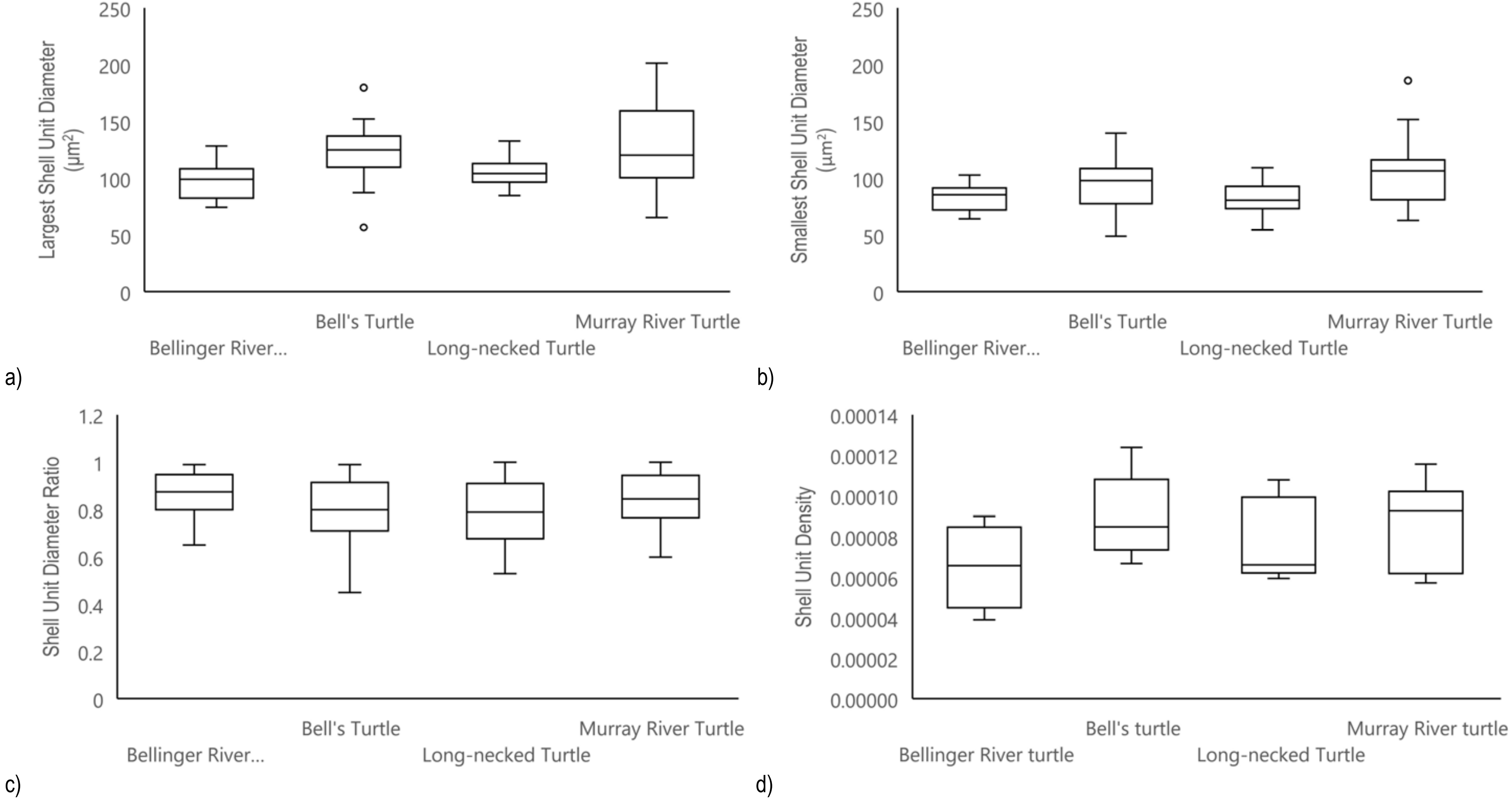


Figure 4.4 - Size comparison of turtle egg shell units across species: a) largest shell unit diameter ($F_{(3,86)}=8.1$, $p<0.01$), b) smallest shell unit diameter ($F_{(3,86)}=6.0$, $p<0.01$), c) ratio of shell unit diameters ($F_{(3,86)}=1.9$, $p=0.15$), and d) shell unit density ($F_{(3,18)}=1.3$, $p=0.30$).

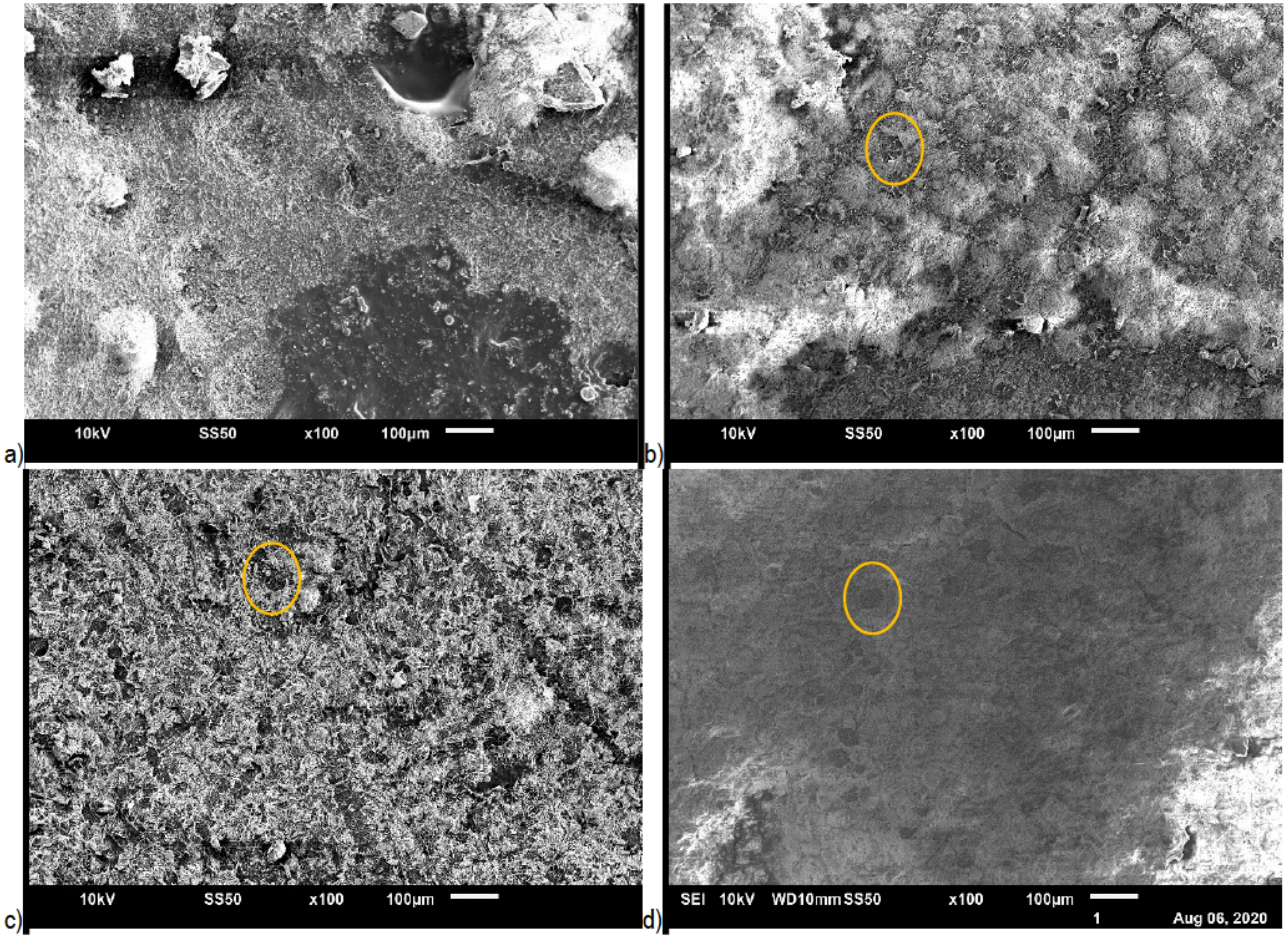
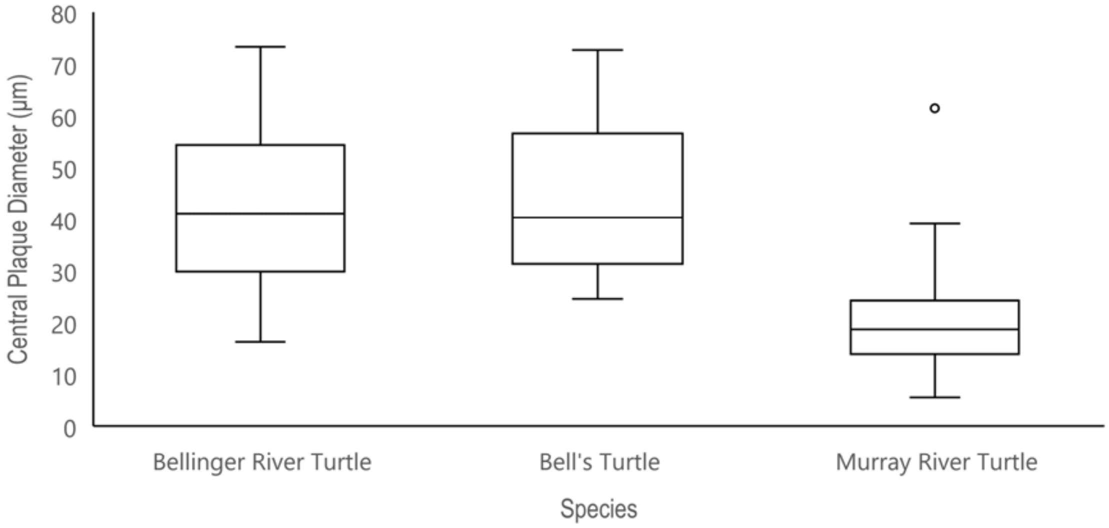
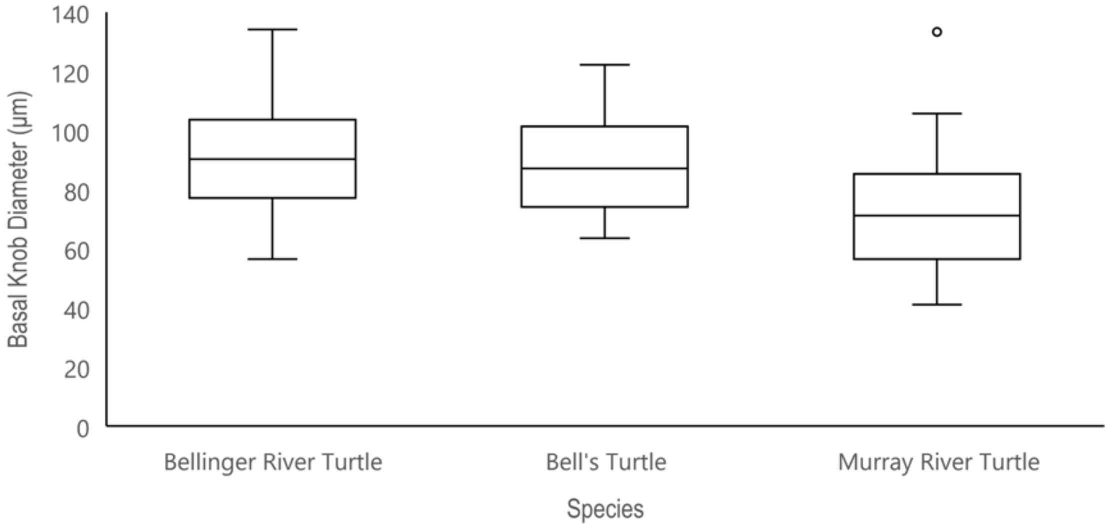


Figure 4.5 - Examples of eggshell outer membrane surface images from each species: a) Eastern long-necked turtle (*Chelodina longicollis*) (dry-preserved) b) Murray River turtle (*Emydura macquarii*) (frozen-preserved), c) Bell's turtle (*Myuchelys bellii*) (frozen-preserved), and d) Bellinger River turtle (*M. georgesi*) (dry-preserved). Circles show examples of central plaques and surrounding basal knobs. Note the lack of visible plaques or basal knobs on the long-necked turtle image. All images taken at 100x magnification.



a)



b)

c)

Figure 4.6 - Size and density comparison of turtle egg pores across species: a) central plaque diameter ($F_{(2,87)}=33.9$, $p<0.01$),and b) basal knob diameter ($F_{(2,87)}=7.9$, $p<0.01$), Note that plaques and basal knobs were not visible on any Eastern long-necked turtle (*Chelodina longicollis*) images, so are not included here**.**

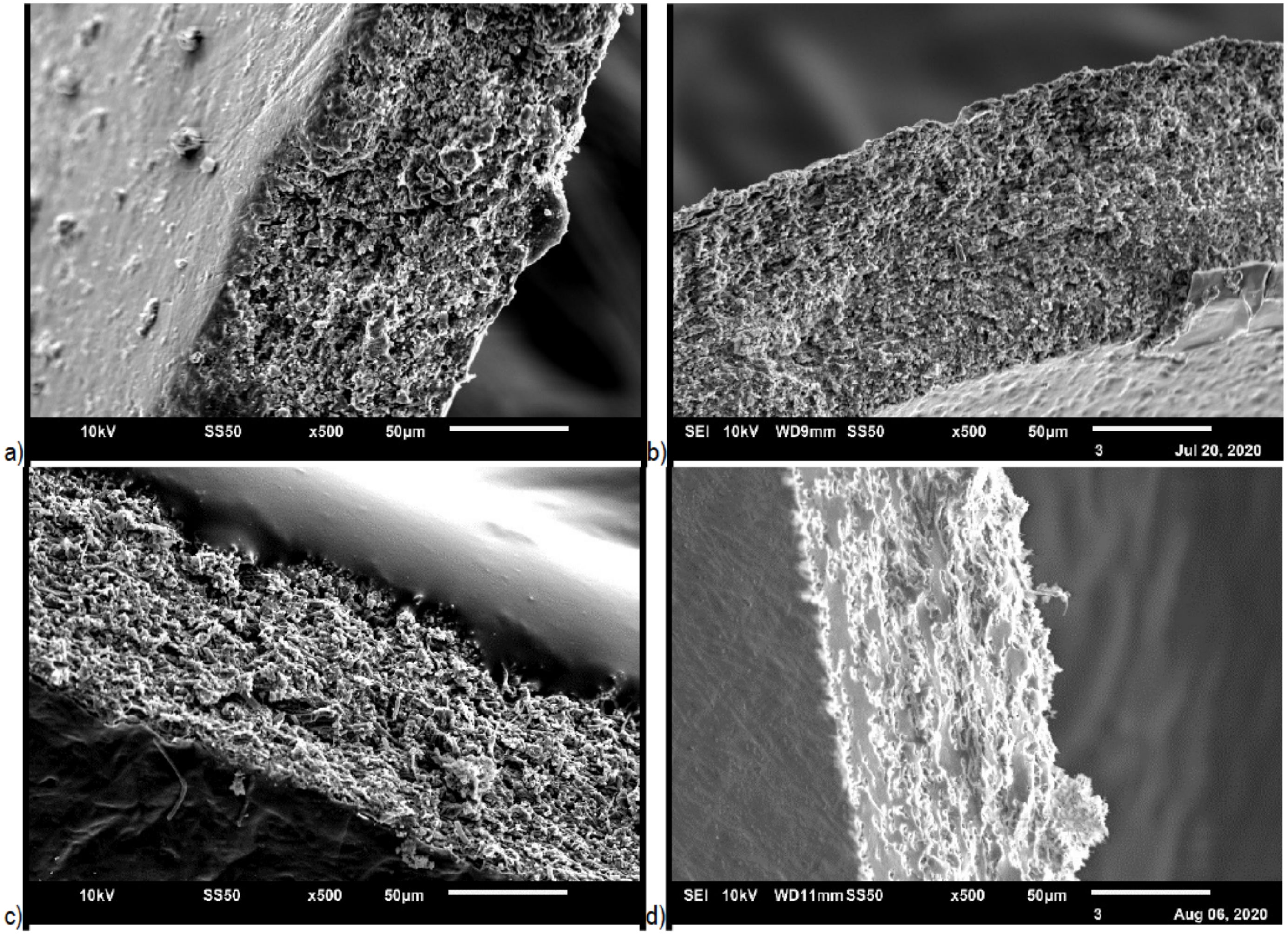


Figure 4.7 - Examples of eggshell cross-sectional images from each species: a) Eastern long-necked turtle (*Chelodina longicollis*), b) Murray River turtle (*Emydura macquarii*), c) Bell's turtle (*Myuchelys bellii*), and d) Bellinger River turtle (*M. georgesi*). Note the similarity in the fibrous matrix in all images. The bands in the Bellinger River turtle image are pooled gold from the sputter coating process.

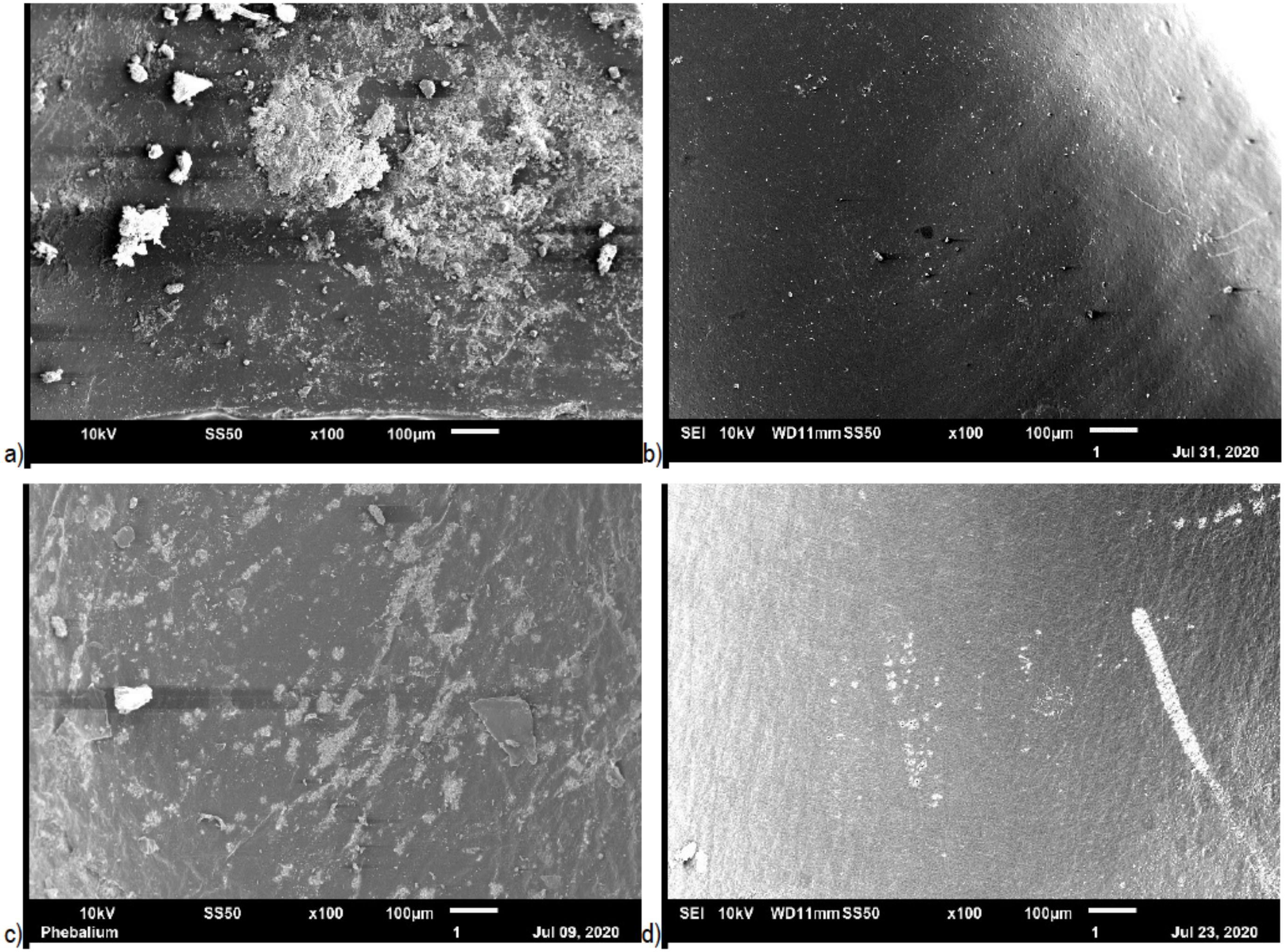


Figure 4.8 - Examples of eggshell inner membrane images from each species: a) Eastern long-necked turtle (*Chelodina longicollis*), b) Murray River turtle (*Emydura macquarii*), c) Bell's turtle (*Myuchelys bellii*), and d) Bellinger River turtle (*M. georgesi*). Samples sometimes show damage or contaminants from dirt or dried albumen. Note the lack of any distinguishing features.

1) Mineral layer readily flakes and separates from outer membrane when dried eggshell fragment is folded in half. Central plaques and basal knobs visible on eggshell fragment outer membranes 2
Mineral layer remains firmly attached to outer membrane when dried eggshell fragment is folded in half. Central plaques and basal knobs not visible on eggshell fragment outer membranes ....Eastern long-necked turtle (*Chelodina longicollis*)

2) Multiple central plaque diameter measurements in outer membrane surface of eggshell consistently measure 15 to 25 µm in size ....Bell's turtle (*Myuchelys bellii*)
Multiple central plaque diameter measurements in outer membrane surface of eggshell consistently measure 30 to 55 µm in size ....Murray River turtle (*Emydura macquarii*)

Figure 4.9 - Proposed dichotomous key for identifying the eggs of three freshwater turtle species in the New England region of NSW, Australia, based on eggshell features. Species are Eastern long-necked turtle (*Chelodina longicollis*), Murray River turtle (*Emydura macquarii*), and Bell's turtle (*Myuchelys bellii*).

4.8 Tables

Table 4.1 - Confusion matrices showing discriminate model predictions with test data on four turtle species found in New South Wales based on shell unit largest diameter (χ^2_3=4.4, p=0.25) and shell unit smallest diameter (χ^2_3=1.2, p=0.75).

Largest diameter (LDA coefficient=0.04)	**E. long-necked turtle**	**Murray River turtle**	**Bell's turtle**	**Bellinger River turtle**	**Prior**	**Success Rate**
E. long-necked turtle	2	1	2	2	25%	29%
Murray River turtle	1	1	0	2	25%	25%
Bell's turtle	0	2	2	2	25%	33%
Bellinger River turtle	2	0	0	4	25%	67%

Smallest diameter (LDA coefficient=0.05)	**E. long-necked turtle**	**Murray River turtle**	**Bell's turtle**	**Bellinger River turtle**	**Prior**	**Success Rate**
E. long-necked turtle	3	1	1	2	25%	33%
Murray River turtle	2	1	1	0	25%	25%
Bell's turtle	3	2	1	0	25%	17%
Bellinger River turtle	3	0	1	2	25%	33%

Table 4.2 - Confusion matrices showing discriminate model predictions with test data three turtle species found in the New England Tablelands, NSW, based on shell unit largest diameter (χ^2_2=3.7, p=0.10) and shell unit smallest diameter (χ^2_2=1.9, p=0.50).

Largest diameter (LDA coefficient=0.04)	**E. long-necked turtle**	**Murray River turtle**	**Bell's turtle**	**Prior**	**Success Rate**
E. long-necked turtle	3	0	1	33%	75%
Murray River turtle	3	1	1	33%	20%
Bell's turtle	2	6	1	33%	11%

Smallest diameter (LDA coefficient=0.04)	**E. long-necked turtle**	**Murray River turtle**	**Bell's turtle**	**Prior**	**Success Rate**
E. long-necked turtle	2	2	0	33%	50%
Murray River turtle	2	1	2	33%	20%
Bell's turtle	3	5	1	33%	11%

Table 4.3 - Confusion matrices showing discriminate model predictions with test data on three turtle species found in New South Wales, based on pore diameter (χ^2_2=8.3, $p<0.05$) and basal knob diameter (χ^2_2=4.3, p=0.10).

Pore Diameter (LDA coefficient=0.08)	**Murray River turtle**	**Bell's turtle**	**Bellinger River turtle**	**Prior**	**Success Rate**
Murray River turtle	10	1	2	33%	77%
Bell's turtle	0	1	4	33%	20%
Bellinger River turtle	3	2	1	33%	17%

Basal Knob Diameter (LDA coefficient=0.05)	**Murray River turtle**	**Bell's turtle**	**Bellinger River turtle**	**Prior**	**Success Rate**
Murray River turtle	4	4	5	33%	31%
Bell's turtle	2	2	1	33%	40%
Bellinger River turtle	2	1	3	33%	50%

Table 4.4 - Confusion matrices showing discriminate model predictions with test data on two turtle species found in the New England Tablelands, NSW, based on pore diameter (χ^2_1=4.1, $p<0.05$) and basal knob diameter (χ^2_1=0.3, p=0.75).

Pore Diameter (LDA coefficient=0.09)	**Murray River turtle**	**Bell's turtle**	**Prior**	**Success Rate**
Murray River turtle	9	1	50%	90%
Bell's turtle	1	4	50%	80%

Basal Knob Diameter (LDA coefficient=0.05)	**Murray River turtle**	**Bell's turtle**	**Prior**	**Success Rate**
Murray River turtle	4	6	50%	40%
Bell's turtle	3	2	50%	40%

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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University of New England

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Type of Work	Page number(s)
All text, tables, and figures	75 to 102

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CHAPTER 5 - INTERSPECIFIC COMPETITION BETWEEN ENDANGERED BELL'S TURTLE (*MYUCHELYS BELLII*) AND THE RANGE-EXPANDING MURRAY RIVER TURTLE (*EMYDURA MACQUARII*): IMPLICATIONS FOR CONSERVATION

5.1 Abstract

Among turtles, interspecies competition is an increasing concern for conservation efforts. On the New England Tablelands of New South Wales, Australia, the endangered Bell's turtle (*Myuchelys bellii*) may be under competition pressure from the expanding range of the more common and widespread Murray River turtle (*Emydura macquarii*). To examine any evidence of competitive suppression between these two species, the biometrics (carapace length, mass, and standardised mass index (SMI)) of captured Bell's turtles were compared between populations that were sympatric and those that were allopatric with Murray River turtles. These biometrics were also compared to local abiotic variables (mean annual temperature, mean annual runoff, and the coefficient of variation for runoff) to determine if presence/absence of Murray River turtles better explained variation in Bell's turtle size metrics than the local environment. Adult Bell's turtles that were sympatric with Murray River turtles were smaller in carapace length and mass than allopatric females, but did not differ in SMI. Presence of Murray River turtles explained more variation in the size and mass of adult female Bell’s turtles than the measured abiotic factors. Presence of Murray River turtles explained variation in the size and mass of adult male Bell's turtles, but less-so than the measured abiotic factors. Presence of Murray River turtles showed no correlation with differences among immature Bell's turtles. The presence of Murray River turtles did correlate with reductions in Bell's turtle size among females in particular, suggesting that further examination of potential causal links to competitive suppression between these species would be beneficial. Focusing on female Bell’s turtles is also recommended, given the importance of mature females to population persistence in freshwater turtles.

5.2 Introduction

Interspecific competition is a concept frequently studied in a native/non-native paradigm, where a completely foreign species invades a naïve system and begins to outcompete an ecologically-similar native species. Well known examples of this phenomenon include North American grey squirrels (*Sciurus carolinensis*) competing with

Eurasian red squirrels (*Sciurus vulgaris*) in Great Britain (Kenward and Holm, 1993), Argentine ants (*Linepithema humile*) displacing native ant species from food resources in California (Human and Gordon, 1996), and Caribbean brown anoles (*Anolis sagrei*) invading the southern United States, competing with the native green anole (*Anolis carolinensis*; Stuart *et al*., 2014). Among turtles, the most infamous invasive species is the red-eared slider (*Trachemys scripta elegans*); native to the Mississippi basin and the coastline of the Gulf of Mexico, this species' popularity as a pet has led to it being introduced across much of the world, including much of North America, and it has colonies in Europe, Africa, Asia, and Australia due to the pet trade (Cadi and Joly, 2003, 2004; Robey *et al*., 2011; Standfuss *et al*., 2016). Red-eared sliders are thought to be particularly effective competitors as they evolved in ecosystems with high turtle species richness. The invasion of systems with much more limited turtle diversity presumably provides ample opportunities for accessing resources whilst under comparatively limited levels of interspecific competition (Lindeman, 2000; Cadi and Joly, 2003; Taniguchi *et al*., 2017).

Competition does not necessarily involve direct aggression. Red-eared sliders monopolised preferred basking sites in competition trials with European pond turtles (*Emys orbicularia*), despite no detection of interspecific agonistic behaviour. Pond turtles appeared reluctant to climb on to a basking site that was already occupied by red-eared sliders (Cadi and Joly, 2003). Another European species, the Mediterranean pond turtle (*Mauremys leprosa*), avoided chemical signals left by red-eared sliders in ponds, even when there were no sliders present in the pond (Polo-Cavia *et al*., 2009). A long-term captive study found increased mortality and weight loss among male and female pond turtles that co-habited with sliders, although the exact cause for either impact was not observed (Cadi and Joly, 2004). However, male sliders engaged in aggressive courtship of the pond turtle females, which may have caused stress and interfered with courtship attempts by male pond turtles (Cadi and Joly, 2004). This monopolization of resources and aggressive mate-chasing may combine with other factors, such as relatively high fecundity and rapid maturation for turtles (Pérez - Santigosa *et al*., 2008), more efficient exploitation of limited food resources (Pearson *et al*., 2015), and being disease and parasite vectors (Meyer *et al*., 2015). Introduced red-eared slider populations are thus considered responsible for declines of native turtle species across the globe (Stadfuss *et al*., 2016; Taniguchi *et al*., 2017).

However, interspecific competition can also be studied in non-foreign systems, such as among species that are naturally sympatric in places, if one taxa is able to outcompete the other in zones of co-occurrence. Freshwater turtle systems provide a good opportunity to study this phenomenon given the available information from red-eared slider incursions. Three species of chelid turtle are known from the streams of the New England Tablelands in northern New South Wales and southern Queensland: the eastern long-necked turtle (*Chelodina longicollis*), the Murray River turtle (*Emydura macquarii*), and the endangered Bell's turtle (*Myuchelys bellii*). The long-necked turtle inhabits all river systems in the Bell's turtle's range, although the two species have different life history strategies. Long-necked turtles are semi-terrestrial and wholly carnivorous (Pritchard, 1984; Georges *et al*., 1986), while Bell's turtles are highly aquatic and generalist omnivores (Fielder *et al*., 2015; Cann and Sadlier, 2017). Such different ecologies, combined with total sympatry, suggests that the two species do not strongly compete. However, Murray River turtles share many life history traits with Bell's turtles, and are only sympatric with Bell's turtles in small portions of their range in the Border Rivers catchment: the Deepwater River in NSW, and the lower parts of Bald Rock Creek in southern Queensland (Fig. 5.1). In these zones of sympatry, Murray River turtles are by far the more abundant species (Cann and Sadlier, 2017). It has thus been hypothesized that the two species directly compete, and that Murray River turtles can out-compete Bell's turtles (Chessman, 2015). For instance, it has been speculated that the Bell's turtles were once found in the Macintyre River (Border Rivers catchment), but have been completely replaced by Murray River turtles (Chessman, 2015; Fig. 5.1).

The diet of the Bell's turtle is considered omnivorous with a bias toward herbivory (Fielder *et al*., 2014), in contrast to its congeners, the common saw-shelled turtle (*M. latisternum*) and the Bellinger River turtle (*M. georgesi*), which are both considered to be principally carnivores (Tucker *et al*., 2012; Spencer *et al*., 2014). Dietary comparisons have not been performed between Bell's turtles and Murray River turtles, but have been performed between Murray River turtles and these other *Myuchelys* species. In both comparisons, Murray river turtles were shown to be more herbivorous, but with a flexible diet depending on local conditions and food availability (Chessman, 1986; Tucker *et al*., 2012; Spencer *et al*., 2014; Petrov *et al*., 2020). The Bell's turtle's diet is less thoroughly understood (see Hughes *et al*., 2020), but the possibility of dietary overlap (for example, Bell's turtles and Murray River turtles both consume large quantities of filamentous green algae; Fielder *et al*., 2015; Petrov *et al*., 2018; 2020), combined with the dietary plasticity of the Murray River turtle may give the latter a competitive edge in foraging competition. This flexibility has elicited concerns that, should Murray River turtles manage to invade the other catchments inhabited by Bell's turtles, they may outcompete their already-endangered relatives (Spencer *et al*., 2014; Chessman *et al*., 2020). A population of Murray River turtles have become established in the Copeton Dam (Chessman, 2015; see Fig. 1.4c in Chapter 1), a reservoir with a 113 m embankment in the New England Tablelands, providing a potential avenue to invade the Gwydir River and possibly putting the Gwydir population of Bell's turtles at risk. Additionally, small numbers of individual Murray River turtles have been captured in recent years in the Gwydir and Severn River catchments, possibly translocated by humans (Chessman and Fielder, pers. comm.).

This chapter sought to explore the potential for competition among these two native turtle species with overlapping ranges on the New England Tablelands, to determine the level of evidence for the existence of hypothesized competition and to identify in what form any competition may manifest. Recent and historical observations of Bell's turtle sub-populations in the Border Rivers system allowed for comparisons in the biometrics and condition indices of Bell's turtle individuals that co-occurred with Murray River turtles and those that did not. It was hypothesized that there would be detectable differences in the biometrics and condition indices of Bell's turtle sub-populations that do and do not co-occur with Murray River turtles. It was predicted that if these differences occur, then sympatric Bell's turtle individuals would show smaller body sizes and exhibit a lower condition than allopatric Bell's turtles, possibly indicating competition due to reduced growth from lower food intake and/or increased stress. The results of these studies will be useful in determining if and how much of a threat introduction of Murray River turtles would be to Bell's turtle population persistence, and will provide avenues to guide future research into this topic.

5.3 Methods

Data Collection. — From 2016 to 2020, Bell's turtles were captured in streams of the New England Tablelands. Turtles were captured with fyke nets, baited cathedral traps, or baited modified crab pot traps (Northside Nets, QLD, Australia; T & L Netmaking, VIC, Australia), or more rarely opportunistically by hand. Older data from 2002 - 2015 were also acquired from previous studies utilising the same techniques (Fielder, 2010; Chessman, 2015; Chessman, Fielder, Spark, and Streeting, unpublished data). Trapping seasons ranged from September to April, as Bell's turtles decrease their activity in the cooler months of the southern hemisphere (Fielder, 2012) and become more difficult to capture.

Once captured, the turtle's carapace length was measured to the nearest 0.1 cm with a pair of callipers (various models) and mass was measured to the nearest gram using electronic scales (various models). Other signs of injury or ill health were also assessed visually and documented for each individual. Turtles were given permanent individualised markings by notching or marking marginal scutes using either a power drill or hacksaw. These marks were treated with antiseptic/antibacterial cream before the turtle was released at the site of capture. Recaptures of the same individual were identified by these markings. Recaptured individuals were re-measured and inspected on each capture occasion across the sampling period.

Bell's turtles are highly sexually-dimorphic (Fielder *et al.*, 2015), with adult females generally being larger than adult males. Secondary sex characteristics begin to show at ~16 cm carapace length, with males developing longer, thicker tails; however, females are not considered sexually mature until their carapaces are ~22 cm long (Fielder *et al.*, 2015). As such, turtle records for this study were divided into four life history categories for these analyses: juveniles (all turtles 9.5 cm to 16 cm carapace length), sub-adult females (16 cm to 22 cm), adult females (>22 cm), and adult males (>16 cm), with sex allocated based on visual assessment of secondary sexual features for all turtles over 16 cm in length (Fielder *et al.*, 2015). There were limited records of turtles smaller than 9.5 cm, so these were excluded from the study. Turtle records were also excluded from analysis if the animal was deceased at time of collection.

Analysis. — All Bell's turtle capture records used in analysis were sorted into stream segments based on the National Environmental Stream Attributes geodatabase (Stein *et al.*, 2014). Each of these segments was assigned to a binary Murray River turtle presence/absence category based on consistent captures of Murray River turtles at those sites. Some sites had sporadic capture records of Murray River turtles that were considered to be translocated individuals, rather than representatives of a breeding population; these sites were categorised as "Murray River turtles absent".

To maintain independence of data within field seasons, an individual turtle's measurements were only used from one capture event per field season. If a turtle was captured multiple times in a given field season, the record closest to 1 January in that summer was used, to represent the "peak" of the field season that ran from September to the following April. If a turtle was captured multiple times in close temporal proximity (e.g. multiple days in a row), the first record was selected to prevent biased mass measurements from consumption of bait.

Abiotic variables were also compared to turtle biometrics for all turtle records (Table 5.1), to serve as comparison points for the effects size that Murray River turtle presence/absence may have on Bell's turtle growth and condition. Data for these variables were drawn from the National Environmental Stream Attributes geodatabase for each stream segment (Stein *et al*., 2014). These abiotic factors were used to compare the correlative strength of Murray River turtle presence/absence against factors that are generally considered to affect turtle growth and condition (Chessman, 2015): mean annual runoff (as an estimate of discharge), coefficient of variation for mean annual runoff ("reliability" of flowing water in a system), and mean annual temperature. Temperature data were collected for the stream segment that the turtle was captured in. Runoff and runoff variation data were collected at the sub-catchment level; that is, the stream segment of capture and all stream segments upstream of it.

Linear mixed models were constructed for analysis of correlation between biometric variables and Murray River turtle presence/absence, using carapace length, mass, or SMI as response variables. These biometrics were used as proxies for growth and condition among the sympatric and allopatric Bell's turtle populations. Fixed effects were Murray River turtle presence/absence, runoff, runoff variation, and temperature (Table 5.1). "Year" (field season rather than calendar year) was randomized to correct for variable capture effort across field seasons. "Day" (a modified Julian calendar treating 1 August as Day 1 and 31 July as Day 365 or 366 in leap years) was randomized to correct for seasonal variation in mass. Significance of the fixed variables was determined using likelihood ratio tests. Model fit was assessed via plotting the distribution of residuals for all models, and deemed adequate for all models.

Statistical analyses were conducted on the 4 life history categories separately. Turtle condition was estimated as scaled mass index (SMI), using the methods outlined in Peig and Green (2009). This method provides a condition index based on relative size and mass rather than absolute size and mass, using overall population trends to calculate a scaling exponent. This analysis used mean carapace length for each life history category as the fixed measurement when calculating SMI.

All analyses were conducted in R (R Core Team, 2021). Mixed models were constructed using the 'lme4' package (Bates *et al*., 2015). The 'smatr' package was used to identify the scaling exponent during SMI calculations (Warton *et al*., 2012).

5.4 Results

Adult Females. — Presence/absence of Murray River turtles had a significant correlation with carapace length (Table 5.2); sympatric female Bell's turtles (n=29) were significantly smaller than allopatric females (n=1593; Fig. 5.2), and the average sympatric female was 1.53 cm (±0.34 SE) shorter than the average allopatric female. Presence/absence of Murray River turtles explained more variation in Bell's turtle carapace length than any abiotic factor (Table 5.2; Appendix II, Fig. A.1).

Presence/absence of Murray River turtles had a significant correlation with mass (Table 5.2); sympatric female Bell's turtles were significantly lighter than allopatric females (Fig. 5.2), and the average sympatric female was 430.51 g (±87.15 SE) lighter than the average allopatric female. Presence/absence of Murray River turtles explained more variation in mass than any abiotic factor (Table 5.2; Appendix II, Fig. A.1).

Presence/absence of Murray River turtles did not correlate with Bell's turtle SMI (Table 5.2). SMI did weakly correlate with the abiotic factors (Table 5.2; Appendix II, Fig. A.1).

Adult Males. — Presence/absence of Murray River turtles had a significant correlation with Bell's turtle carapace length (Table 5.3); sympatric male Bell's turtles (n=30) were significantly smaller than allopatric males (n=1101; Fig. 5.3), and the average sympatric male was 0.56 cm (±0.24 SE) shorter than the average allopatric male. Presence/absence of Murray River turtles explained less variation in carapace length than the abiotic factors (Table 5.3; Appendix II, Fig. A.2).

Presence/absence of Murray River turtles had a significant correlation with mass (Table 5.3); sympatric male Bell's turtles were significantly lighter than allopatric males (Fig. 5.3), and the average sympatric male was 82.94 g (±33.68 SE) lighter than the average allopatric male. Presence/absence of Murray River turtles explained less variation in mass than the abiotic factors (Table 5.3; Appendix II, Fig. A.2).

Neither presence/absence of Murray River turtles nor any of the abiotic factors correlated with SMI (Table 5.3; Figure 5.3; Appendix II, Fig. A.2).

Sub-adult Females. — Sympatric sub-adult female Bell's turtles (n=13) did not differ in carapace length, mass, or SMI from allopatric sub-adult females (n=173; Table 5.4; Fig. 5.4). Mean annual runoff did weakly correlate with these biometrics (Table 5.4; Appendix II, Fig. A.3). The coefficient of variation for mean annual runoff also weakly correlated with carapace length, and mean annual temperature correlated with SMI (Table 5.4; Appendix II, Fig. A.3).

Juveniles. — Sympatric juvenile Bell's turtles (n=8) did not differ in carapace length, mass, or SMI from allopatric juveniles (n=161; Table 5.4; Fig. 5.5). Mean annual runoff did weakly correlate with carapace length and mass (Table 5.5; Appendix II, Fig. A.4).Runoff variation correlated with carapace length, and temperature correlated with SMI Table 5.5; Appendix II, Fig. A.4).

5.5 Discussion

Adult Bell's turtle females that were sympatric with Murray River turtles were smaller than those that were not, although this difference in size did not extend to a difference in body condition (as measured by standardised mass index (SMI)). Female Bell's turtles that were sympatric with Murray River turtles were on-average ~400 g lighter than their counterparts in other streams. The presence of Murray River turtles also correlated with size and mass in adult male Bell's turtles, but less-so than environmental factors, suggesting that for male Bell’s turtles local conditions were a more important factor in shaping male growth and condition than any potential competition. Presence of Murray River turtles did not have a measureable impact on immature life history categories of Bell's turtle for any measurement, suggesting that whatever effect that Murray River turtles may have on sympatric Bell's turtles principally affects mature females. Alternatively, impacts may occur in the immature stages but do not become apparent until maturity, i.e. through long-term expression of the impacts of early reduced growth rates. While SMI may not have differed, the adult Bell's turtles that were sympatric with *Emydura* may not be growing to their full size potential, and as female Bell's turtles have larger maximum sizes than males (Chessman, 2015; *Fielder et al.*, 2015), the effects of reduced growth are more apparent in the females.

Some turtle species show differences in non-reproductive behaviours between sexes. For example, male map turtles (*Graptemys geographica*) consume different prey items than the larger females, showing intraspecific dietary niche partitioning (Richards-Dimitrie *et al.*, 2013). Among Bell's turtles, intersexual differences in behaviour are not well understood, however dietary differences are seen in other members of the genus *Myuchelys*. Male and female common saw-shelled turtles (*M. latisternum*) consume different prey: although primarily carnivorous, females consume relatively more plant matter and scavenge more frequently than males, which preferentially consume aquatic insects (Tucker *et al.*, 2012). Like map turtles, Bell's turtles show female-biased sexual size dimorphism (Fielder *et al.*, 2015), whereas the Murray River turtle's sexes are much more similar in size, and both sexes are larger than male Bell's turtles at full maturity (Chessman, 2015). If the Bell's turtle females are competing for food with adult Murray River turtles of both sexes where they are sympatric, it may explain the reduced average sizes and masses of sympatric female Bell's. If the smaller Bell's turtle males are consuming different food items from the larger turtles, it may also explain the more-limited apparent impacts on the male Bell's turtles. Faecal and gut sampling analyses of sympatric Bell's turtles and Murray River turtles should be performed (see Chessman, 1986; Hughes *et al.*, 2020), to test for dietary differences and similarities among these species/sex categories.

Another possibility is that Murray River turtle males are performing courtship harassment of female Bell's turtles, as was observed with red-eared sliders and European pond turtles (Cadi and Joly, 2004). The courtship behaviours of Murray River turtles have been previously recorded (Murphy and Lamoreaux, 1978); the observed behaviours were non-forceful, characterised by head-bobbing, nosing of the female's cloaca, both animals touching their chins together, and mutual stroking of the head with the forelimbs. One mounting attempt was observed, which the female broke away from; the male attempted to re-engage by initiating more courtship behaviour, not by immediately attempting another mounting (Murphy and Lamoreaux, 1978). Courtship behaviour in Bell's turtles has not been recorded, but Murphy and Lamoreaux (1978) did observe courtship in common saw-shelled turtles, and noted that the behaviours were very similar to those of Murray River turtles.

The findings of Murphy and Lamoreaux (1978) appear to suggest that *Emydura* male harassment of Bell's turtle females is unlikely. However, it should be noted that this was a single observation in a captive setting and Murray River turtle courtship behaviour may be more complex, particularly in the wild. Male painted turtles (*Chrysemys picta*), long used as a model species for "gentle" turtle courtship, have been shown to engage in forceful, even injurious, courtship behaviour (Hawkshaw *et al*., 2019; Moldowan *et al*., 2020). These authors report large male painted turtles biting the necks of females that they are attempting to mate with, using a pair of sharp cusps on their maxillary beak for grip. The aggressor males were also reported to repeatedly strike the female with sharp protruding marginal scutes on the front of their carapaces (Hawkshaw *et al*., 2019). Female painted turtles may exhibit scarring on their necks, quite likely resulting from these encounters (Moldowan *et al*., 2020). While Murray River turtles lack the specialised beak and carapace adaptations used by the male painted turtles (Hawkshaw *et al*., 2019), such structures are not strictly necessary in coercive mating behaviours. For example, male pink-eared turtles (*E. victoriae*), which similarly lack cusps on their beaks, were observed biting the legs and shells of females that they were apparently courting, albeit without inflicting obvious injury (Gaikhorst *et al*., 2011). Female Bell's turtles have not been thus far observed to exhibit injuries consistent with aggressive courtship practices, but the apparent sex-specific bias to the effects of Murray River turtle presence on Bell's turtles mean that attempted mating should be considered as a possibility. Female Bell's turtles and Murray River turtles are of similar sizes when mature, but adult male Murray River turtles are much larger than male Bell's turtles (Chessman, 2015). Harassment by a male turtle that is far larger than her own conspecific males may interfere with foraging or prevent her from mating with other conspecifics, even if copulation does not occur.

If copulation is achieved, hybridization is also a further possible concern for Bell's turtle conservation. Hybrids between Murray River turtles and Bellinger River turtles (*M. georgesi*) have been located, and in some localities, captures of hybrids outnumber the pure Bellinger River turtles (Georges *et al*., 2018). Hybridization is considered a major conservation concern for the critically-endangered Bellinger River turtle (Chessman *et al*., 2020). No apparent *M. bellii* x *E. macquarii* hybrids have been observed to date in the Deepwater River or lower Bald Rock Creek, but *M. georgesi* x *E. macquarii* hybrids captured by Georges *et al.* (2018) resembled one or the other of their parent species, without any "blending" of obvious morphological features. As hybridization does occur with a congener of the Bell's turtle, it may also be possible with Bell's turtles; genetic analyses of individual turtles in the zones of sympatry should be performed as a matter of urgency moving forward, with an eye toward the identification of potential hybrids.

Harassment of mature female Bell's turtles by male Murray River turtles is one possible explanation for the observed patterns in size and mass, and the lack of differences in size/mass due to presence of Murray River turtles that are observed in immature females. Combined with the knowledge gaps about Murray River turtle courtship behaviour, the examples of interspecific harassment seen in other turtle species (Cadi and Joly, 2004), and the possibility for hybridization, behavioural interactions between female Bell's turtles and male Murray River turtles should be more thoroughly explored to support or discard the harassment hypothesis. Additionally, physiological measures of stress such as corticosterone (Baxter-Gilbert *et al.*, 2014) or heterophil/lymphocyte ratios (Selman *et al.*, 2013) should be collected, as high levels of these stress indicators could indicate harassment if stress-levels differ between mature females of sympatric and allopatric populations.

In conclusion, the hypothesis was partially supported: the presence of Murray River turtles does potentially show a negative effect on the size and especially mass of some Bell's turtles, though not in condition as was expected. This effect is most apparent in adult females, explaining more variation in female size and mass than environmental predictors. Mature females are a life history category that is imperative to population persistence in turtles (Dodd *et al.*, 2016; Howell and Seigel, 2019), and as such the potential for competition or other deleterious interactions should not be dismissed lightly. As found in other studies on interspecific competition in turtles, the impacts on mature female Bell's turtles may due to competition for food or key habitat features, or it may be due to harassment of Bell's turtle females by Murray River turtle males. While this correlational study is unable to assign causality to any of the above hypotheses, the results do indicate that further behavioural, ecological, and genetic information should be collected from Bell's turtles *in situ* to determine if the relationships observed are causal. A combination of underwater video cameras, captive behavioural studies, and the use of additional tools such as genetic analysis, stress hormone analysis, and direct assessment of dietary overlap of different sex/size classes should be considered in the future to provide a clearer understanding of the interspecific interactions of these two turtle species.

5.6 Literature Cited

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5.7 Figures

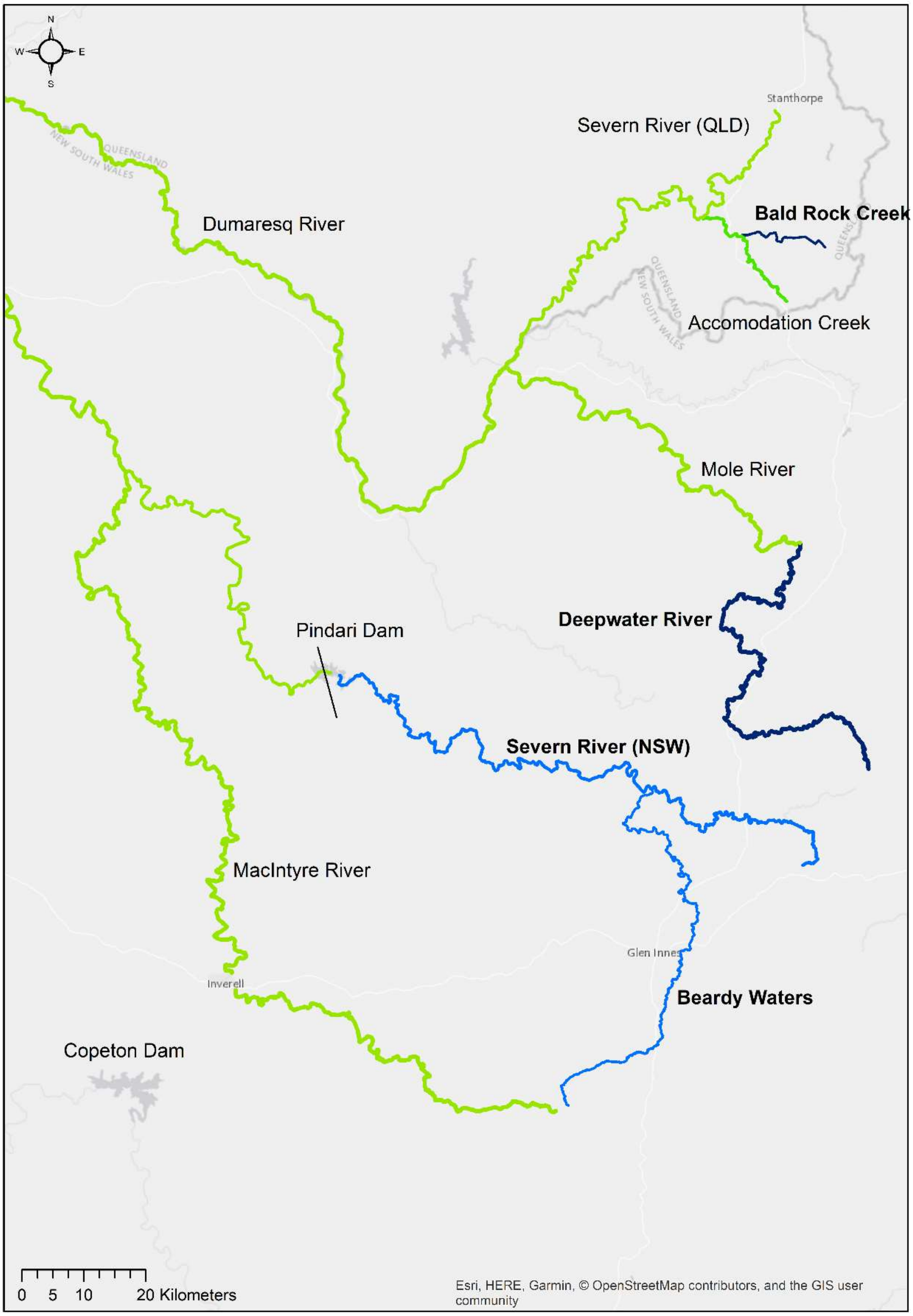

Figure 5.1 - Section of the Border Rivers catchment of northern NSW and southern Queensland inhabited by Bell's turtles (*Myuchelys bellii*). Blue streams (with bolded names) contain only Bell's turtles, green shows connected rivers with only Murray River turtles (*Emydura macquarii*), and purple shows streams where Bell's turtles and Murray River turtles are sympatric. Pindari Dam (black line) is a barrier to Bells' turtle/Murray River turtle interchange in the Severn River.

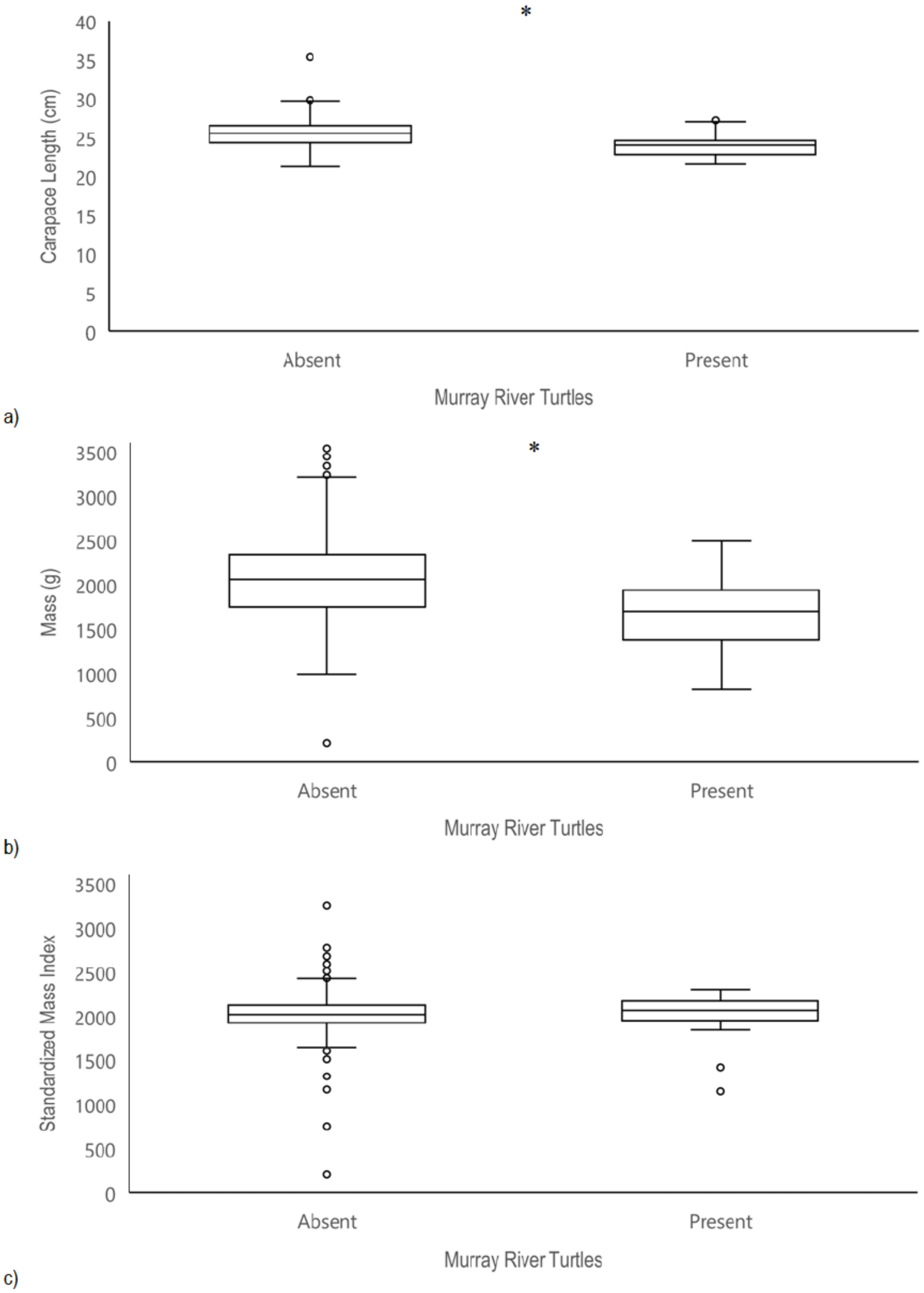

Figure 5.2 - Comparisons of adult female Bell's turtle (*Myuchelys bellii*) a) carapace length (LRT: χ^2_1=20.8, p<0.01), b) mass (LRT: χ^2_1=24.2, p<0.01), and c) standardised mass index (LRT: χ^2_1=1.6, p=0.20) to the presence/absence of Murray River turtles (*Emydura macquarii*). Centre line shows the median value, boxes show 25th to 75th percentiles, whiskers show 5th to 95th percentiles, and points show outliers. An * denotes a significant difference between the two factors.

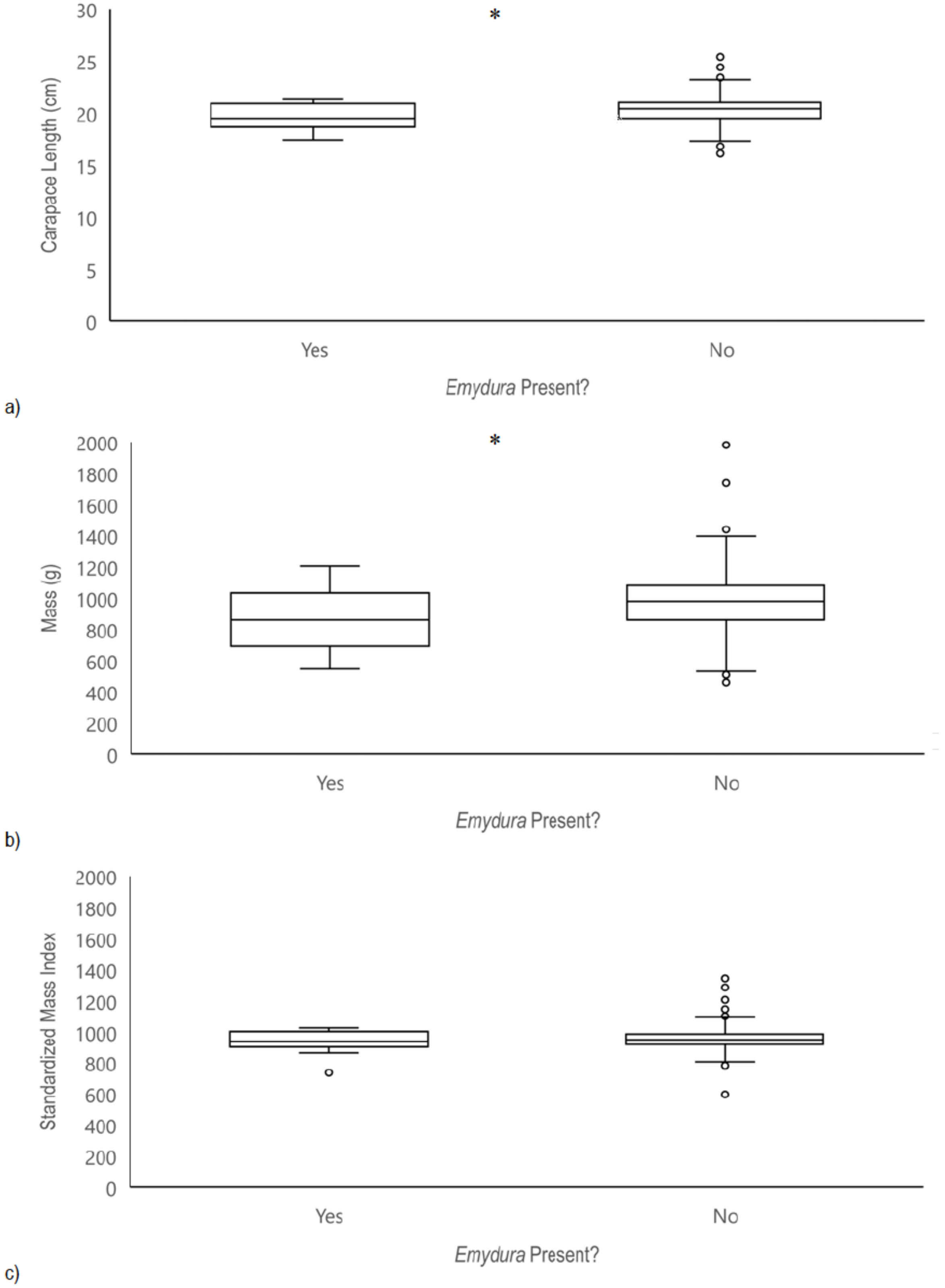

Figure 5.3 - Comparisons of adult male Bell's turtle (*Myuchelys bellii*) a) carapace length (LRT: $\chi^2_1=5.3$, p=0.02), b) mass (LRT: $\chi^2_1=4.2$, p=0.04), and c) standardised mass index (LRT: $\chi^2_1<0.1$, p=0.88) to the presence/absence of Murray River turtles (*Emydura macquarii*). Centre line shows the median value, boxes show 25th to 75th percentiles, whiskers show 5th to 95th percentiles, and points show outliers. An * denotes a significant difference between the two factors.

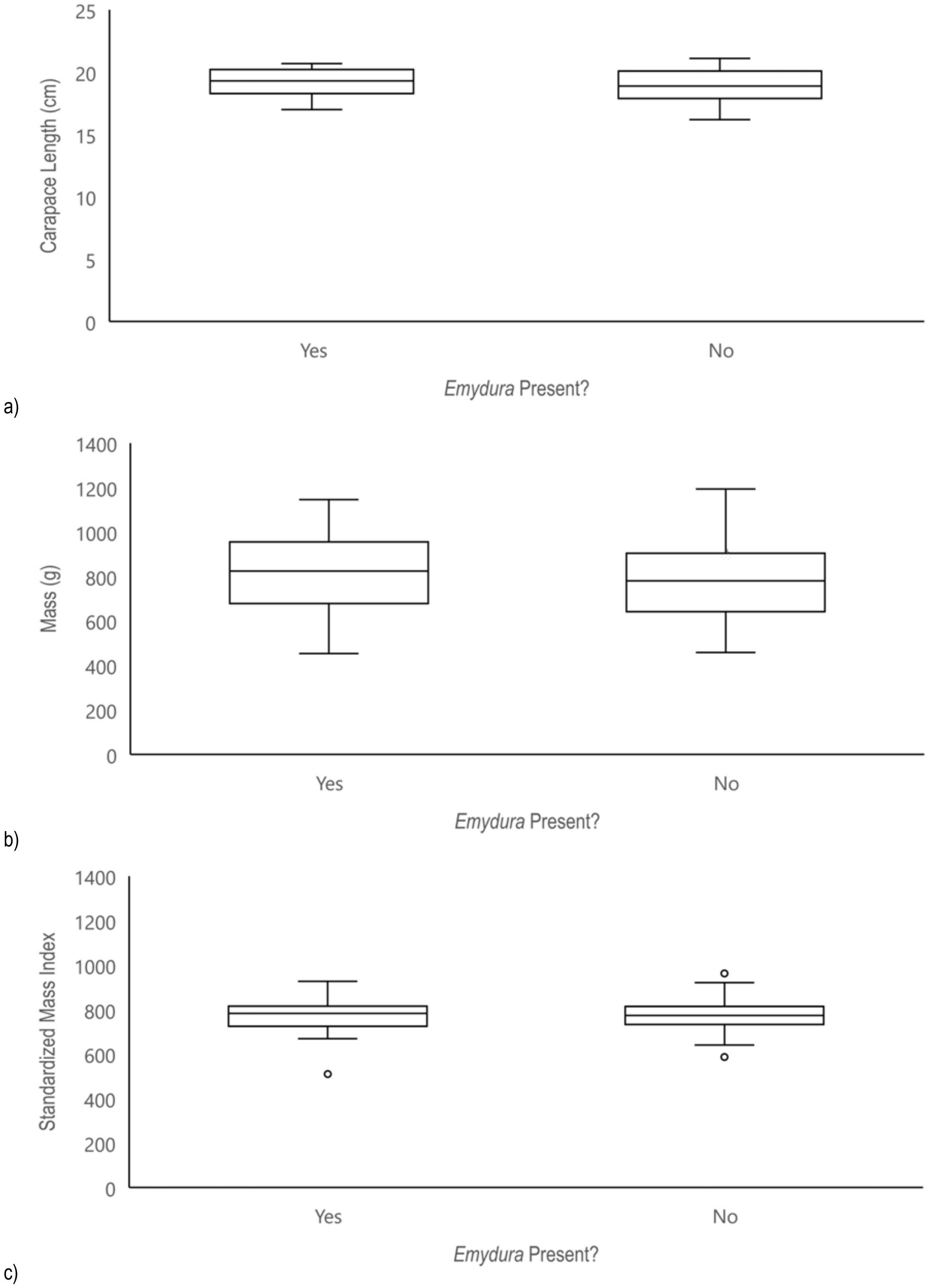

Figure 5.4 - Comparisons of sub-adult female Bell's turtle (*Myuchelys bellii*) a) carapace length (LRT: $\chi^2_1=0.4$, p=0.53), b) mass (LRT: $\chi^2_1<0.1$, p=0.84), and c) standardised mass index (LRT: $\chi^2_1<0.1$, p=0.85) to the presence/absence of Murray River turtles (*Emydura macquarii*). Centre line shows the median value, boxes show 25th to 75th percentiles, whiskers show 5th to 95th percentiles, and points show outliers. No comparisons reached significance.

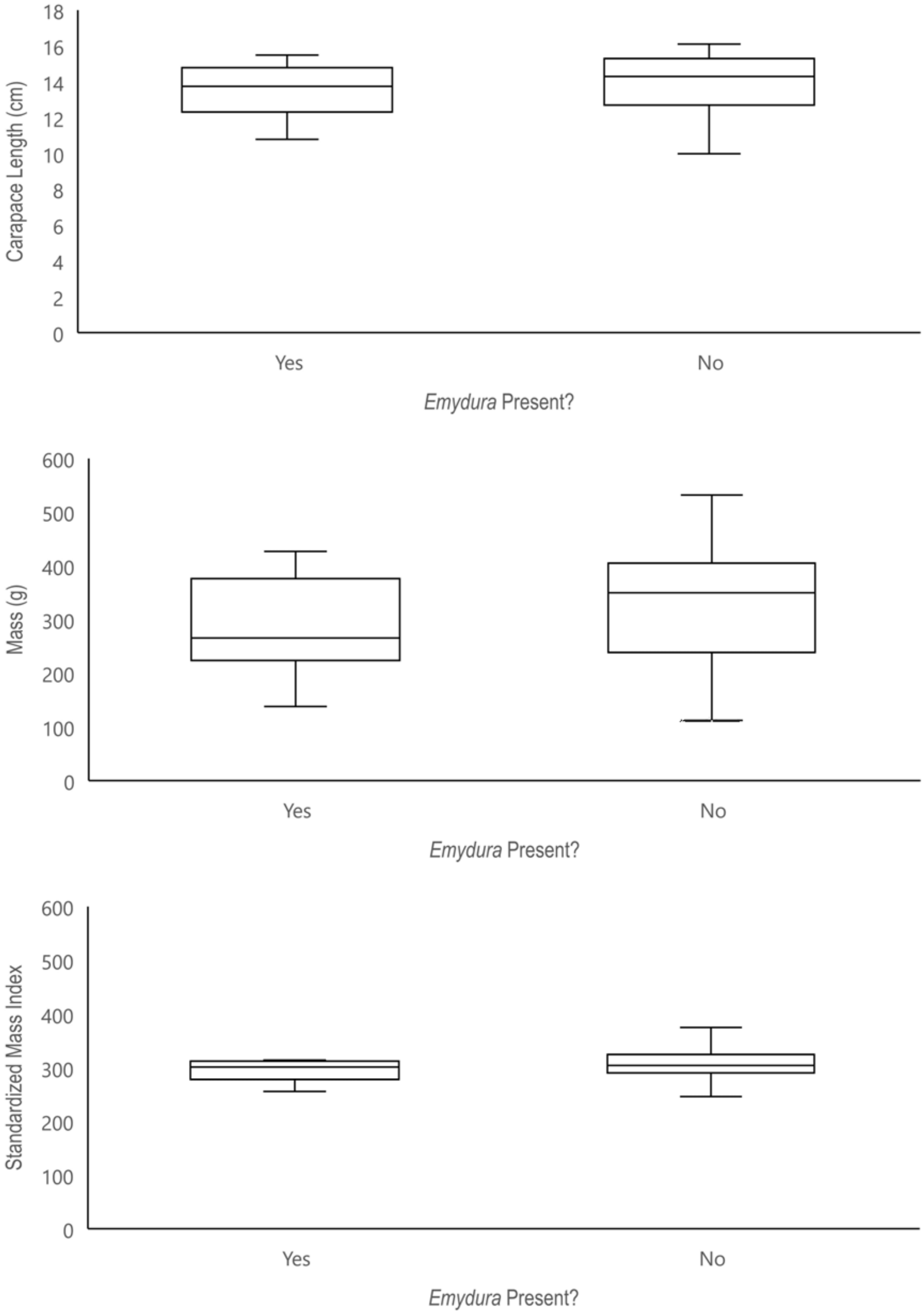

Figure 5.5 - Comparisons of juvenile Bell's turtle (*Myuchelys bellii*) a) carapace length (LRT: $\chi^2_1<0.1$, p=0.97), b) mass (LRT: $\chi^2_1=0.1$, p=0.74), and c) standardised mass index (LRT: $\chi^2_1=1.7$, p=0.19) to the presence/absence of Murray River turtles (*Emydura macquarii*). Centre line shows the median value, boxes show 25th to 75th percentiles, whiskers show 5th to 95th percentiles, and points show outliers. No comparisons reached significance.

5.8 Tables

Table 5.1 - Variables used in abiotic model construction. All data were drawn from the National Environmental Stream Attributes geodatabase (Stein *et al.*, 2014). Descriptions are drawn verbatim from the geodatabase attributes table.

Variable	Unit	Geodatabase Description	Reason for Selection as a Variable
Mean Annual Runoff	ML	Mean of the annual totals of the monthly accumulated soil water surplus values at the stream segment pour-points.	Runoff was selected as a measure of quantity of water in the streams, important for turtle ecology including food availability and movement.
Mean Annual Runoff Coefficient of Variation	n/a	Coefficient of variation of annual totals of accumulated soil water surplus.	Runoff variation was considered important as a measure of reliability/stability of the aquatic environment.
Mean Annual Temperature	˚C	Average value of BIOCLIM parameter "Annual mean Temperature" of all grid cells comprising the stream segment and associated valley bottoms.	Temperature was selected because of its general importance to growth and health in ectothermic animals.

Table 5.2 - Statistical outputs of linear mixed models and liklihood ratio tests comparing adult female Bell's turtle (*Myuchelys bellii*) biometrics by presence/absence of Murray River turtles (*Emydura macquarii*), and by mean annual runoff, coefficient of variation of mean annual runoff, and mean annual temperature. Significant differences are highlighted in grey.

Response Variable	**Predictor Variable**	**LMM Results**		**LRT Results**	**(df=1)**
		Estimate (±SE)	**t-value**	**χ^2-value**	**p-value**
Carapace Length	*Emydura*	-1.53 (±0.34)	-4.56	20.75	<0.01
	Runoff	0.01 (±0.04)	0.29	0.10	0.75
	Runoff Variation	-0.61 (±0.25)	-2.45	5.98	0.02
	Temperature	-2.28 (±0.83)	-2.75	7.54	0.01
Mass	*Emydura*	-430.51 (±87.15)	-4.94	24.23	<0.01
	Runoff	19.73 (±11.51)	1.71	2.83	0.09
	Runoff Variation	-281.79 (±65.00)	-4.34	18.38	<0.01
	Temperature	-935.40 (±217.10)	-4.31	18.34	<0.01
SMI	*Emydura*	-44.48 (±34.62)	-1.29	1.62	0.20
	Runoff	17.86 (±4.33)	4.13	16.96	<0.01
	Runoff Variation	-111.42 (±24.97)	-4.46	19.79	<0.01
	Temperature	-278.28 (±85.39)	-3.26	10.61	0.01

Table 5.3 - Statistical outputs of linear mixed models and liklihood ratio tests comparing adult male Bell's turtle (*Myuchelys bellii*) biometrics by presence/absence of Murray River turtles (*Emydura macquarii*), and by mean annual runoff, coefficient of variation of mean annual runoff, and mean annual temperature. Significant differences are highlighted in grey.

Response Variable	**Predictor Variable**	**LMM Results**		**LRT Results**	**(df=1)**
		Estimate (±SE)	**t-value**	**χ^2-value**	**p-value**
Carapace Length	*Emydura*	-0.56 (±0.24)	-2.31	5.32	0.02
	Runoff	0.14 (±0.04)	3.33	9.74	<0.01
	Runoff Variation	-1.11 (±0.20)	-5.49	26.62	<0.01
	Temperature	-2.28 (±0.69)	-3.28	10.29	<0.01
Mass	*Emydura*	-82.94 (±33.68)	-2.46	6.06	0.01
	Runoff	19.10 (±5.58)	3.42	10.40	<0.01
	Runoff Variation	-167.17 (±27.31)	-6.12	32.79	<0.01
	Temperature	-362.51 (±94.23)	-3.85	14.15	<0.01
SMI	*Emydura*	-13.81 (±12.31)	-1.12	1.29	0.26
	Runoff	2.98 (±1.92)	1.55	2.39	0.12
	Runoff Variation	-16.57 (±9.69)	-1.71	2.97	0.09
	Temperature	-40.77 (±32.74)	-1.25	1.60	0.21

Table 5.4 - Statistical outputs of linear mixed models and liklihood ratio tests comparing sub-adult female Bell's turtle (Myuchelys bellii) biometrics by presence/absence of Murray River turtles (*Emydura macquarii*), and by mean annual runoff, coefficient of variation of mean annual runoff, and mean annual temperature. Significant differences are highlighted in grey.

Response Variable	Predictor Variable	LMM Results		LRT Results	(df=1)
		Estimate (±SE)	t-value	χ^2-value	p-value
Carapace Length	*Emydura*	0.26 (±0.39)	0.67	0.46	0.50
	Runoff	0.20 (±0.07)	2.82	7.84	0.01
	Runoff Variation	-1.12 (±0.52)	-2.15	4.63	0.03
	Temperature	-1.18 (±1.76)	-0.67	0.31	0.58
Mass	*Emydura*	17.82 (±50.80)	0.35	0.15	0.70
	Runoff	30.25 (±9.43)	3.21	8.84	<0.01
	Runoff Variation	-101.15 (±66.91)	-1.51	2.35	0.13
	Temperature	3.60 (±224.36)	0.02	<0.01	0.97
SMI	*Emydura*	-3.87 (±19.00)	-0.20	0.05	0.83
	Runoff	5.16 (±3.57)	1.45	2.08	0.15
	Runoff Variation	12.25 (±23.67)	0.52	0.26	0.61
	Temperature	170.29 (±81.07)	2.10	4.34	0.04

Table 5.5 - Statistical outputs of linear mixed models and liklihood ratio tests comparing juvenile Bell's turtle (*Myuchelys bellii*) biometrics by presence/absence of Murray River turtles (*Emydura macquarii*), and by mean annual runoff, coefficient of variation of mean annual runoff, and mean annual temperature. Significant differences are highlighted in grey.

Response Variable	Predictor Variable	LMM Results Estimate (±SE)	t-value	LRT Results χ^2-value	(df=1) p-value
Carapace Length	*Emydura*	-0.19 (±0.60)	-0.32	0.11	0.74
	Runoff	0.34 (±0.10)	3.22	9.90	<0.01
	Runoff Variation	-1.19 (±0.80)	-1.49	2.24	0.14
	Temperature	3.17 (±2.39)	1.33	1.60	0.21
Mass	*Emydura*	-29.27 (±38.53)	-0.76	0.62	0.43
	Runoff	25.23 (±6.36)	3.97	14.44	<0.01
	Runoff Variation	-103.33 (±50.27)	-2.06	4.07	0.04
	Temperature	193.4 (±152.70)	1.27	1.38	0.24
SMI	*Emydura*	-12.57 (±8.77)	-1.43	2.10	0.15
	Runoff	4.53 (±1.48)	3.07	8.31	<0.01
	Runoff Variation	-18.14 (±11.36)	-1.60	2.45	0.12
	Temperature	18.79 (±33.88)	0.55	0.31	0.58

Higher Degree Research Thesis by Publication
University of New England

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF ORIGINALITY

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Type of Work	Page number(s)
All text, tables, and figures	105 to 128

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CHAPTER 6 - INVESTIGATING THE LANDSCAPE-LEVEL PATTERNS OF AN EYE DISEASE AFFLICTING THE ENDANGERED BELL'S TURTLE (*MYUCHELYS BELLII*)

6.1 Abstract

Emerging infectious diseases remain a growing threat to wildlife across the globe, including herpetofauna. In Australia, major epidemics have severely impacted populations of native turtle species, causing rapid declines of up to 90% in some populations. Given this, the presence of eye abnormalities (mainly presenting as a cataract-like condition) in populations of the endangered Bell's turtle (*Myuchelys bellii*) has led to concerns of a similar outbreak in the future. While it is not clear if these cataracts are caused by a pathogen, or even if the cataracts have significant deleterious effects on afflicted turtles, conservationists cannot be complacent on such matters, as a previously benign disease may rapidly become a lethal epidemic. This study sought to update previous studies on cataract presence with a larger and longer-term dataset. This study examined demographic, spatial, and temporal patterns of eye abnormality presence, and used model selection to determine if a landscape-level predictor of presence could be identified. Demographic and spatial patterns identified in previous studies were largely upheld: eye abnormalities only occurred in adults (≥18.3 cm carapace length), were more prevalent in females (14% of capture records) than in males (4% of capture records), and no males with clinical signs were ever recaptured. Abnormalities were most established in the Macdonald River catchment (19% of capture records), with low incidence in the Gwydir River (1%), Deepwater River (2%), and Severn River (1%) catchments, and no records in the disjunct Bald Rock Creek population. Eye abnormality prevalence greatly increased during the latter years of a severe drought that affected eastern Australia, between 2017 and 2020. Model selection indicated mean annual solar radiation as the most predictive measured variable for eye abnormality presence; the model showed a weak positive relationship with mean solar radiation, indicating that abnormalities may be caused by direct lenticular damage by radiation. While effects' size was relatively weak, this initial study does provide guidance for future investigations in this area moving forward. In particular, the relationship between solar radiation and eye abnormality development should be investigated more thoroughly, as should the fate of afflicted male Bell's turtles.

6.2 Introduction

Emerging infectious diseases are becoming an increasing concern for the conservation of wildlife, including amongst herpetofauna (Harvell *et al*., 1999; Carey, 2000; Dazsak *et al*., 2000; Lesbarrères *et al*., 2014). Most notably, two pathogens are currently responsible for a considerable worldwide decline in amphibian populations: chytrid fungus (*Batrachochytrium* sp.) and *Ranavirus* (Chinchar, 2002; Lesbarrères *et al*., 2012; 2014). Two species of chytrid (*B. dendrobatidis* and *B. salamandrivorans*) affect a wide variety of amphibian species in captive and wild populations (Longcore *et al*., 1999; Martel *et al*., 2013; Olson *et al*., 2013). Amphibian population declines and extinctions have been linked to chytrid outbreaks (Skerratt *et al*., 2007; Olson *et al*., 2013; Stegen *et al*., 2017), although the fungus requires cooler temperatures and is not widespread in the tropics (Piotrowski *et al*., 2004). Ranaviruses have a global distribution, and were named for their initial discovery within leopard frogs (*Lithobates pipiens*, formerly *Rana pipiens*; Granoff *et al*., 1965) but have also shown lethal and sub-lethal effects on a wide variety of taxa including non-ranid frogs (Miller *et al*., 2011; Price *et al*., 2014), salamanders (Souza *et al*., 2012), lizards (Stöhr *et al*., 2013), turtles (Johnson *et al*., 2008; McKenzie *et al*., 2019), and fish (Langdon and Humphrey, 1987; Whitaker *et al*., 2010). Snake fungal disease (*Ophidiomyces ophiodiicola*) is another pathogen that has potential conservation concerns for North American snakes, which is currently being investigated (Lorch *et al*., 2016; Davy *et al*., 2018; McKenzie *et al*., 2020).

Among turtles, the effects of emerging infectious diseases are gaining increasing attention as large-scale potential threats. Examples include ranavirus, which has been reported as causing morbidity and mortality in a number of turtle species, including snapping turtles (*Chelydra serpentina*; McKenzie *et al*., 2019), Eastern box turtles (*Terrapene carolina carolina*; Adamovicz *et al*., 2018), and Hermann's tortoises (*Testudo hermanni*; Marschang *et al*., 1999). Further, a number of herpes viruses have been recorded infecting green sea turtles (*Chelonia mydas*), and less-commonly other marine turtle species, causing fibrous papillomas on the skin that can be debilitating (Herbst *et al*., 1994; Knöbl *et al*., 2011). In Australia, the most infamous case of disease affecting turtles was a mass mortality event affecting an estimated >90% Bellinger River turtles (*Myuchelys georgesi*) in the summer of 2014/2015 (Spencer *et al*., 2018; Chessman *et al*., 2020). The pathogen was identified as a novel nidovirus, named the Bellinger River Virus, which propagated through most of the population within a matter of months (Zhang *et al*., 2018). The Bellinger River turtle is now listed as critically endangered, and intensive conservation efforts are underway to preserve the species

(Chessman *et al.*, 2020). In the same summer, a seemingly unrelated mass mortality event derived from an unknown pathogen also affected the Johnstone River snapping turtle (*Elseya irwini*; Ariel *et al.*, 2017). High levels of the bacterium *Aeromonas hydrophila* were discovered in ulcerated tissues on the turtles and these may have been the cause of the infection, although could also have opportunistically colonized already-diseased tissues (Ariel *et al.*, 2017).

Many Australian turtle species are endemic to single catchments (Cann and Sadlier, 2017), making them particularly vulnerable to highly-infectious diseases (Işik, 2011). The Bellinger River turtle's congener, the endangered Bell's turtle (*M. bellii*), is one such endemic species, found only in three upland river systems of the upper Murray-Darling catchment: the Macdonald River, Gwydir River, and Border Rivers. Mature Bell's turtles are known to suffer from eye abnormalities, most commonly presenting as cataract-like clinical signs which impair vision (Fig. 6.1; Chessman, 2015; Fielder *et al.*, 2015; Cann and Sadlier, 2017). The Bell's turtle populations in the Macdonald River show the highest incidence of this disease, with ~10% of captured adults exhibiting clinical signs in some years, but abnormalities are found in the Gwydir and Border Rivers populations, though notably not in the disjunct Bald Rock Creek population (Chessman, 2015; Fielder *et al.*, 2015). Similar clinical signs are reported in common saw-shelled turtles (*M. latisternum*) and Manning River turtles (*M. purvisi*), though the incidence is much lower than for Bell's turtles (Cann and Sadlier, 2017; Redleaf Environmental, 2019).

Little is known about this condition in Bell’s Turtle. The causative agent for these abnormalities is unclear, and to date no pathogen has been linked with the affliction. Whilst pathogens may cause cataracts or cataract-like clinical signs in some taxa, for example bacteria (Liu *et al.*, 2018) and fungi (Clinch *et al.*, 1989), other possible causes have also been proposed. Known abiotic causes of cataract development include: high levels of radiation causing direct lenticular damage (Balasubramanian, 2000; Adkins *et al.*, 2003; Colitz *et al.*, 2010), a deficit of certain nutrients such as vitamin D or calcium (Large *et al.*, 1984; Jacques *et al.*, 1988; Takahashi, 1994; Brown and Akaichi, 2015), and poor water quality (Woodhouse *et al.*, 2016). Taxonomic effects are also a likely factor; similar eye abnormalities are not reported in Eastern long-necked turtles (*Chelodina longicollis*) or Murray River turtles (*Emydura macquarii*), both of which are sympatric with the Bell's turtles in the New England Tablelands. It remains unknown whether these clinical signs are linked to a direct threat to the condition of individuals or to the species' persistence, as impacts on visual acuity in particular remain unknown. Previous studies have found that body condition did not differ between afflicted and unafflicted turtles, suggesting that the turtles are not strongly debilitated by the cataract-like eye abnormalities (Chessman, 2015). Further, Chessman (2015) reports that one afflicted female was recaptured in 2015 following being first captured with eye abnormalities in 2006. There is also at least one recorded case of an afflicted mature female taken into captivity whose cataracts cleared without any medical intervention (Cann and Sadlier, 2017).

Nonetheless, further investigation into this disease is warranted. The suddenness of the epidemics that have affected the closely related Bellinger River turtle and Johnstone River snapping turtle caution against cavalier attitudes about any potential illnesses, even if they first appear relatively benign. This Chapter sought to explore this disease, first by expanding Chessman's (2015) findings with new data, and also by investigating landscape-level patterns in environmental variables and the occurrence of the disease. This Chapter used model selection to test the correlation of the eye disease occurrence with a number of spatial and environmental variables. Results may be used to guide future research efforts, by providing more specific aims in investigating the causes of eye disease among Bell's turtle.

6.3 Methods

Data Collection. — Bell's turtles were captured in streams of the New England Tablelands, using fyke nets, or baited cathedral traps and modified crab pot traps (Northside Nets, QLD, Australia; T & L Netmaking, VIC, Australia). Older data from 2002 - 2015 were also supplied from previous studies that used the same trapping protocols (Fielder, 2010; Chessman, 2015; Chessman, Fielder, Spark, and Streeting, unpublished data). Trapping seasons ranged from August to May, though most often from October to March, as Bell's turtles become less active in the cooler months of the southern hemisphere (Fielder, 2012).

Captured Bell's turtles were measured to the nearest 0.1 cm (carapace length and width, plastron length and width) with callipers (various models) and mass measured to the nearest gram using electronic scales (various models). Sex was determined visually by tail length and girth; mature male Bell's turtles have noticeably longer and thicker tails than females (Fielder *et al.*, 2015). Other signs of injury or ill health were also assessed visually and documented for each individual, particularly the presence/absence of cataracts or other eye abnormalities. Turtle records that noted "white spots", "cataracts", or "cloudy eyes" were considered to have eye abnormalities for the purposes of this study.

Turtles were given permanent markings in their marginal scutes with individual codes with either a power drill or hacksaw, depending on the collector's preferences. These scute markings were treated with antiseptic/antibacterial cream to prevent infection, and the turtle was released at the site of capture. Recaptures of the same individual were identified by these markings. Recaptured individuals were re-measured and inspected on each capture occasion, however only one capture event from each field season was used in the analysis, to maintain independence of data. If an individual was captured multiple times in one field season, the record closest to 1 January was included in analysis to represent records nearest to the peak of field season activity. If a turtle was captured multiple times in close succession (e.g. multiple trapping days in a row), then the first record was used to prevent mass measurements being skewed by consumption of bait. Turtles below the threshold for sexual dimorphism (<16 cm carapace length; Fielder *et al.* 2015) were not included in statistical analyses, due to Chessman (2015) only finding adult turtles (>16 cm) with eye abnormalities.

For each turtle capture event, a GPS location was taken with a handheld GPS device (various models). These locations were then sorted into stream segments based on the National Environmental Stream Attributes geodatabase (Stein *et al.*, 2014), from which environmental variables were derived for model selection. Stream segments were of variable length and determined by points of stream confluence. Seven abiotic variables were chosen from the National Environmental Stream Attributes geodatabase as being potentially relevant to cataract development, and that also had sufficient non-zero data points within New England to perform analyses on (Table 6.1).

Mean annual solar radiation, mean annual rainfall, and mean annual temperature were examined at the stream segment level. Distance from source, land modification, pesticide use, and mean annual runoff were all examined at the sub-catchment level, that is the stream segment where the turtle was captured plus all stream segments upstream of that location. Land modification (riparian habitat not used for conservation) and pesticide use (riparian habitat where pesticide use is likely) are estimates of land use along the stream course, expressed as percentage, and the accuracy of these variables should be taken with caution

Analysis. — To update Chessman (2015)'s findings with a larger, multi-year dataset, cataract presence was modelled by catchment, sex, carapace length, and standardized mass index (SMI; as outlined in Peig and Green, 2009); additionally, distance from headwaters was also investigated. Sex and catchment were compared against eye disease presence with

Pearson's Chi-square tests, due to the categorical nature of these data. Distance from source, carapace length, and SMI were modelled with general linear mixed models with binomial data structures. The model for distance from source used "catchment" as a random variable to correct for the variable lengths of the different river systems, and the carapace length and SMI models used "sex" as a random variable to correct for the Bell's turtle's female-biased sexual size dimorphism (Fielder *et al.*, 2015).

Temporal variation in eye abnormality prevalence was examined with a Pearson's Chi-square test. Due to high degrees of variation in capture effort across years, eye abnormality prevalence was examined from the 2017/18 field season through the 2020/21 field season only, excluding prior field seasons. During these four field seasons, the Turtles Forever project conducted systematic trapping efforts in all catchments except for Bald Rock Creek, so capture effort was relatively uniform across the different river systems in these years. This period also coincided with a severe drought that began in 2017 and ended in 2020 (Filkov *et al.*, 2020).

Bell's turtles that were recaptured in multiple seasons were examined for changing patterns of reported eye abnormalities over time, excluding records for turtles that had no records of abnormalities for any capture. Recaptured turtles were categorized as "Onset" if captured initially without abnormalities but were then recaptured with abnormalities, "Neutral" if they displayed abnormalities in all captures, and "Healed" if they were initially captured with abnormalities but these were then absent on a subsequent recapture. The length of time between capture events was compared in these groups to estimate how rapidly eye abnormalities could occur, how long a turtle could persist with abnormalities, and how rapidly they might recede.

Modelling and Model Selection. — All models used for model selection were general linear mixed models with binomial data structures. Cataract presence in a capture record was used as the response variable, using a simple yes/no dichotomy; due to differences in notation by a wide variety of researchers across 18 years of study, finer definition of cataract presence and severity was not possible for this analysis.

These variables were used to construct an array of univariate and multivariate models for model selection (Table 6.2). Random variables in all models were "catchment" to correct for differing capture effort across catchments and "year" to correct for differing capture effort across field seasons. Models were tested with Aikake's Information Criterion (AIC), corrected for small sample sizes, and selected top models were averaged to identify which variables were the most explanatory of eye abnormality presence. Models and variables that were considered to be sufficiently predictive were examined for patterns in cataract presence.

All analyses were conducted in R version 4.0.3 (R Core Team, 2021). Mixed models were constructed with the "lme4" package (Bates *et al*., 2015), AIC was performed with the "AICcmodavg" package (Mazerolle, 2020), and model averaging with the "MuMIn" package (Barton, 2020).

6.4 Results

Spatial and Temporal Comparisons. — Eye abnormality prevalence significantly differed among catchment (χ^2_4=252.1, p<0.01); the Macdonald River had the highest prevalence, with 230 capture records out of 1028 (22%) noting eye abnormalities (Fig. 6.2). The Gwydir River had the second-highest incidence rate of 6 out of 1028 records (1%) (Fig. 6.2).The Deepwater and Severn Rivers had one record each, out of 91 (1%) and 82 (1%) records, respectively (Fig. 6.2). Bald Rock Creek had no recorded incidents of eye abnormalities out of 126 records in total. Eye abnormality presence significantly differed by location within catchment (χ^2_1=147.9, p<0.01); eye abnormalities were more common in the lower parts of each stream than the headwaters (Fig. 6.3).

During the Turtles Forever study period (2017 - 2020), eye abnormality prevalence significantly differed across years (χ^2_3=106.4, p<0.01). The 2017/18 field season showed a 5% incidence rate and the 2018/19 field season a 2% incidence rate of eye abnormalities being reported, while the 2019/20 and 2020/21 field seasons showed a significant increase in incidence rates to 22% and 23%, respectively (Fig. 6.2).

Turtles that were captured multiple times between 2002 and 2021 showed changes in eye abnormality patterns (Table 6.3). Eight female turtles were considered "healed" cases, with these individuals captured once with eye abnormalities and subsequently recaptured without abnormalities. Half of these (n=4) sampled 1 year apart, indicating that the abnormalities can recede rapidly (Table 6.3). Twenty-four females and two males were considered "onset" cases, captured without eye abnormalities and subsequently captured with abnormalities, indicating that onset of abnormalities can also be rapid (Table 6.3). Thirty-three females were considered "neutral" cases, captured with eye abnormalities in all captures, including with one female that persisted with ongoing abnormalities for at least a 7-year period (Table 6.3). However, as the abnormalities can apparently appear and recede rapidly, it is unclear if this female was afflicted for all 7 years between captures, or experienced cycle(s) of remission and onset across the sampling period. Any male turtles that were captured once with eye abnormalities (n=43) were never recaptured in subsequent field seasons. Recapture rates of males are typically low (9%); however, even at these recapture ratesat least 3 afflicted male recaptures would have been expected to be observed in the dataset.

Demographic and Biometric Comparisons. — No turtles below the threshold of sexual size dimorphism ($\leq$16 cm carapace length) were recorded with eye abnormalities. The smallest turtle recorded with eye abnormalities was a male that measured 18.3 cm carapace length from the Macdonald River in the 2020/21 field season. The largest was a female that measured 28.2 cm from the Macdonald River in the 2019/21 field season. Both of these turtles were captured at the same site, in the town of Bendemeer, NSW.

Eye abnormality prevalence significantly differed between sexes (χ^2_1=75.5, p<0.01), with abnormalities more prevalent among females (14% incidence rate) than among males (4% incidence rate; Fig. 6.2). Eye abnormality presence significantly differed by carapace length independent of sex (χ^2_1=7.2, p<0.01); larger turtles were more likely to have eye abnormalities than smaller turtles, although the majority of large turtles did not have eye abnormalities (Fig. 6.2). Eye abnormality presence differed by standardized mass index (χ^2_1=40.7, p<0.01), with higher-SMI turtles more likely to have eye abnormalities, although the majority of high-SMI turtles did not have eye abnormalities (Fig. 6.2).

Predictive Modelling. — When all developed models were assessed, model selection based on AIC values chose the "Radiation" model as the most predictive, with "Rainfall", "Climate", and "Spatial + Water" as the next highest models (Table 6.4). All models were more predictive than the null model of random terms alone (Table 6.4).

The four most-predictive models were selected for conditional model averaging, which selected mean solar radiation as the most predictive variable within these four models (Table 6.5), although estimate effects size was weak (4.52 ±1.40 SE). Distance from source was also predictive (Table 6.5; Fig. 6.3), as part of the Spatial + Water model (Table 6.4), however estimated effects' size was extremely weak (0.02 ±0.003 SE), and distance from source was not further considered. All other variables had similarly weak effect sizes, and these overlapped considerably with zero once standard error was associated with the measure (Table 6.5).

Mean annual radiation showed the strongest effect size on eye abnormality presence (χ^2_1=77.3, p<0.01). Prevalence of eye abnormalities peaked at sites that experienced 18.2 MJ/m^2/day annually, occurring at higher than expected levels at those sites compared to captures of turtles without clinical signs (Fig. 6.5). According to the National Environmental Stream Attributes geodatabase (Stein *et al.*, 2014), 18.2 MJ/m^2/day is the median mean annual radiation level for all New England sites, with an overall range of 17.5 to 18.4 MJ/m^2/day. Eye abnormality presence was reduced at radiation levels above 18.2 MJ/m2/day (Fig. 6.5).

6.5 Discussion

The findings by Chessman (2015) on eye abnormalities in Bell's turtles have been largely upheld by the larger sample analysed in this study. Abnormalities were only found in turtles larger than the threshold of sexual dimorphism (*i.e.* approximately >16 cm carapace length), and were more prevalent among females than males. Abnormalities were most prevalent in the Macdonald River (listed as the "Namoi River" in Chessman (2015), see Fig. 1.4b). However, the additional sampling and analysis of this study identified an apparent relationship between a greater probability of eye abnormality presence and both larger size and greater body condition. It is expected that this indicates that eye abnormalities are more likely to develop in larger, and thus older, turtles. Alternatively, smaller/younger turtles that develop abnormalities may suffer high mortality rates such that capture rates for afflicted smaller turtles are non-existent, while larger, older turtles may persist with the clinical signs.

Most concerning was the lack of recaptures of afflicted males. While large females were shown to persist with abnormalities and in some cases recover between field seasons, no male Bell's turtles captured with eye abnormalities have been re-captured to date. This lack of recaptures is below the admittedly low rate of recapture for males in general. It may be that afflicted males suffer from higher mortality rates than afflicted females, which can apparently survive for at least 7 years with eye abnormalities according to these data. If afflicted males do suffer higher mortality rates, it may explain the skewed sex ratios observed in the Macdonald River by previous studies; Fielder *et al.* (2015b) noted that females outnumber males in the Macdonald River, and the Turtles Forever project has continued to note female-biased sex ratios of captures in the Macdonald River throughout the project

(Spark, Streeting, pers. comm.). If eye abnormalities are linked to increased mortality in males or in younger turtles, the eye disease becomes a much more serious threat to population persistence in the Macdonald River.

Landscape-level patterns in eye abnormality presence did emerge from modelling, with mean annual solar radiation appearing to be the most predictive variable of those tested. The relationship between solar radiation and eye abnormality presence followed the predicted pattern of higher levels of radiation potentially causing damage to the eyes of turtles, although presence did reduce at mean annual radiation levels higher than 18.2 MJ/m^2/day. Cataract formation due to lenticular damage from solar radiation exposure (particularly ultraviolet) is a well-documented phenomenon in human medical literature (Hiller *et al.*, 1986; Balasubramanian, 2000) and veterinary literature (Adkins *et al.*, 2003; Colitz *et al.*, 2010).

Another possible link between radiation and cataract formation is the availability of vitamin D, which is a vital component in the metabolism of calcium, and is especially important for the health of heliothermic reptiles, particularly turtles (Millichamp and Jacobsen, 1983; Manning and Grigg, 1997; Hedley, 2012). Calcium deficiencies have been reported as leading to cataract formation in human and veterinary literature (Large *et al.*, 1984; Jacques *et al.*, 1988; Takahashi, 1994; Brown and Akaichi, 2015). Where a negative relationship between eye abnormalities and radiation would be expected if that were the case, vitamin D and calcium remains a potential causal link between radiation levels and cataract development, and thus bears further investigation. Vitamin D and calcium can be easily assessed from blood samples, and the relationship between eye abnormalities and nutrition can be examined simply by taking animals exhibiting clinical signs into captivity and providing nutritional supplements.

Despite support for a link between radiation levels and eye abnormalities, effect sizes were relatively small, and the range of radiation levels available from the geodatabase data were low. Given this, further investigation at a finer-scale into the relationship of solar radiation to eye abnormalities is warranted. However, it must be acknowledged that the cause of eye abnormalities may not have been captured in models, or radiation may be a proxy for other environmental variable(s) that were not directly measured. The lack of similar eye abnormalities recorded in eastern long-necked turtles and Murray River turtles, both of which are sympatric with Bell's turtles in the New England Tablelands, suggests that radiation is only one component of eye abnormality development in Bell's turtle, and other factors are likely important in its mediation and expression in the environment. The surge in reported cases in the latter years of a severe drought in the region and the relative increase in cases in the lower parts of the catchments suggests a suite of causative agents working in tandem, perhaps including the age and sex of an individual turtle, water quality and nutrition, and radiation regimes in the local habitat.

In conclusion, while a causative effect for the cataract-like eye abnormalities in Bell's turtles has yet to be fully elucidated, this preliminary assessment has identified a number of potential avenues for future investigation. Further exploring the effects of the eye disease on males must be a high priority in the future, to determine if this condition may be more heavily impacting males, thereby skewing sex ratios in the Macdonald River population to a female bias. Long-term monitoring of solar radiation at Bell's turtle capture sites should be conducted with a particular focus on the ultraviolet range, as these spectra are most responsible for cataractogenic lenticular damage in other taxa, and in vitamin D synthesis in reptiles. The acquisition of this finer-scale radiation data, with a focus on radiation spectra of biological relevance, would establish appropriate links that were only hinted at by the data available from the National Environmental Stream Attributes geodatabase. Vitamin D and calcium levels in individuals may be determined through simple blood draws, and comparisons of these micronutrient levels between afflicted and unafflicted individuals would be a simple and direct approach to establishing or discarding a link between vitamin D/calcium and cataracts in Bell's turtles. Alternatively, Bell's turtles could be taken into captivity and held under varying UV conditions to determine if cataracts develop. Identifying the causes and patterns of wildlife illnesses is vital for developing solutions to those illnesses, and thus in informing management policy and best practices into the future. Given past catastrophic population declines in closely related species, notably the Bellinger River turtle, fully ruling out a pathogenic origin remains a key future objective.

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6.7 Figures

Figure 6.1 - Bell's turtle (*Myuchelys bellii*) exhibiting a cloudy, cataract-like abnormality in right eye (arrow) and an apparently unafflicted left eye (photo credit: P. McDonald).

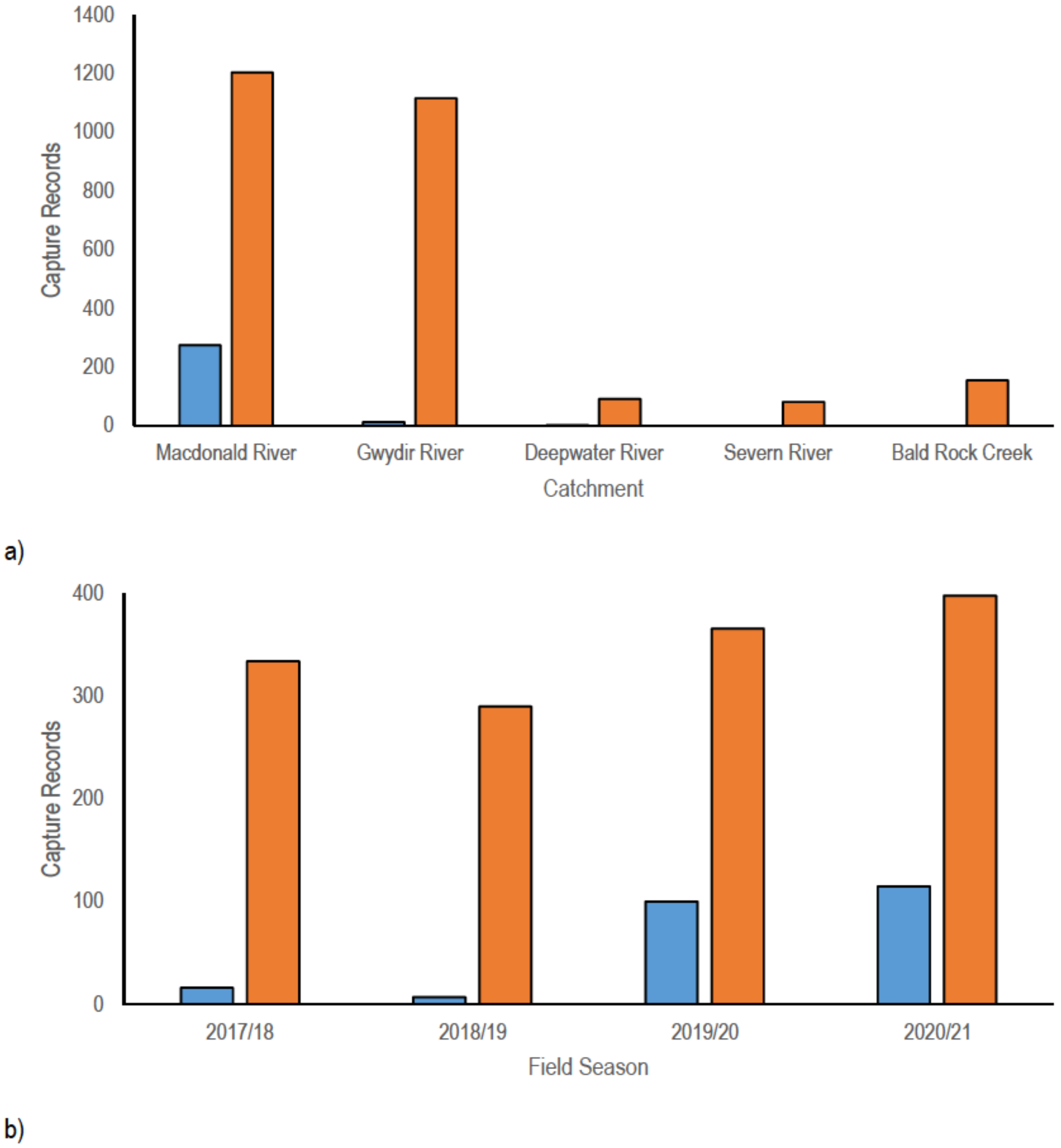

Figure 6.2 - Comparisons of eye abnormality prevalence in Bell's turtles (*Myuchelys bellii*) to spatial and temporal factors: a) catchment (χ^2_4=252.1, p>0.01), and b) field season (χ^2_3=106.4, p<0.01). Blue bars indicate turtles with eye abnormalities, and orange bars indicate turtles without eye abnormalities.

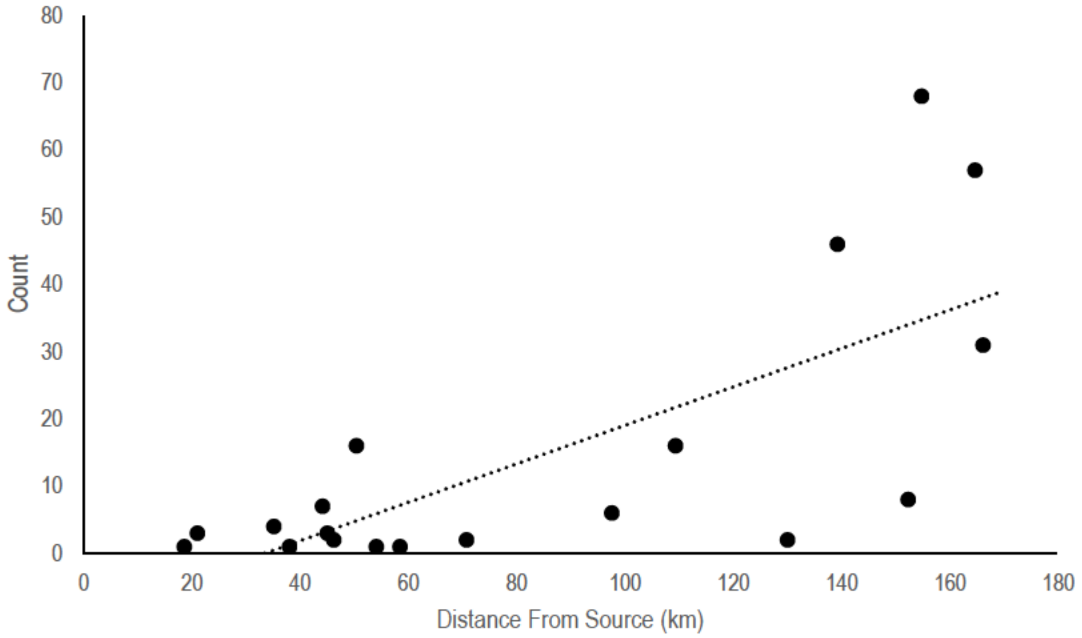

Figure 6.3 - Eye abnormality presence in Bell's turtles (*Myuchelys bellii*) across the length of catchment (χ^2_1=147.9, $p<0.01$).

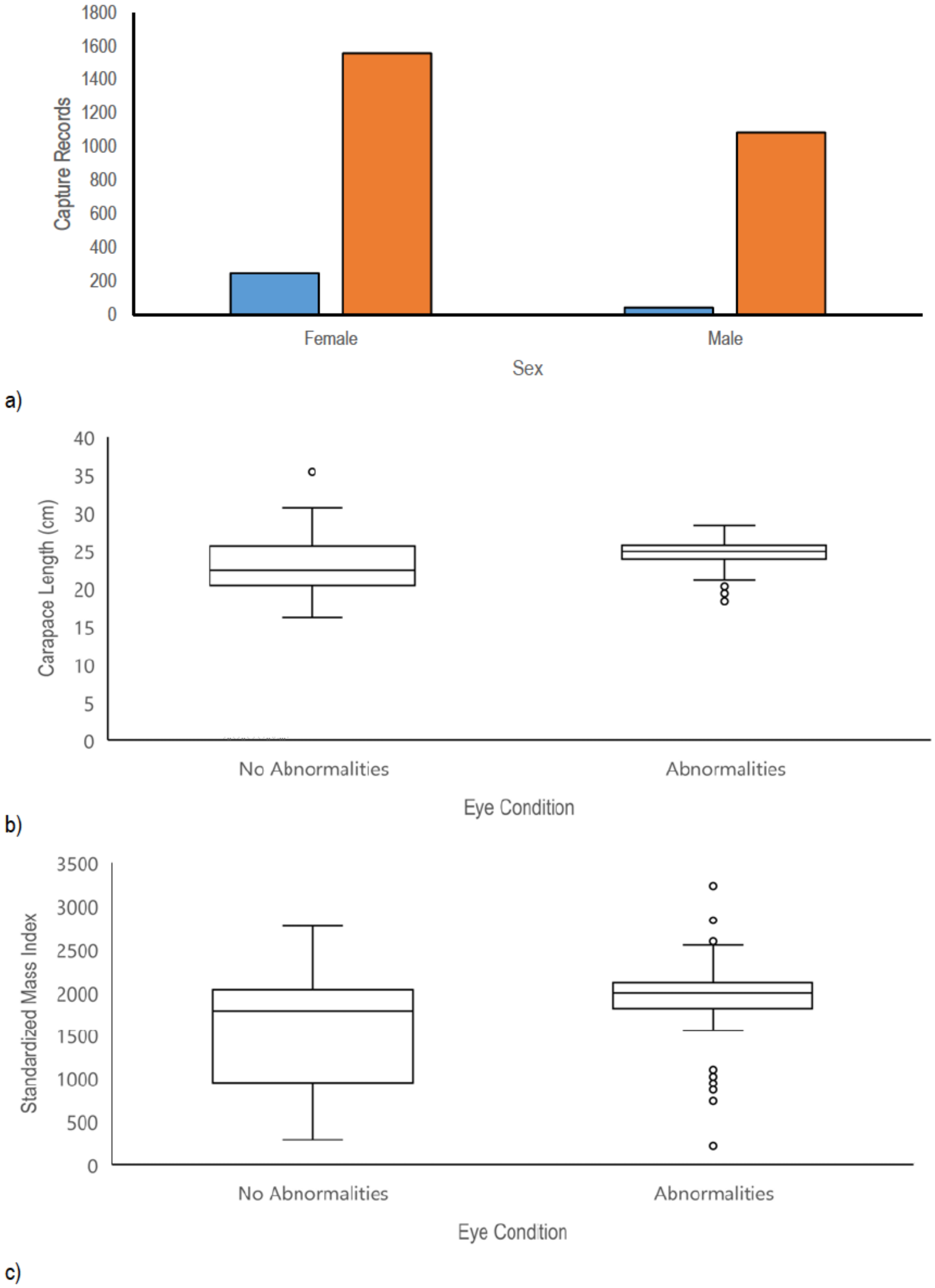

Figure 6.4 - Comparisons of eye abnormality presence in Bell's turtles (*Myuchelys bellii*) to demographic and biometric factors: a) sex ($\chi^2_1=75.5$, $p<0.01$), b) carapace length ($\chi^2_1=7.2$, $p<0.01$), and c) standardized mass index ($\chi^2_1=40.7$, $p<0.01$). In the bar plot, blue bars indicate turtles with eye abnormalities, and orange bars indicate turtles without eye abnormalities. In box plots, the centre line shows median, boxes show 25th to 75th percentiles, whiskers show 5th to 95th percentiles, and points show outliers.

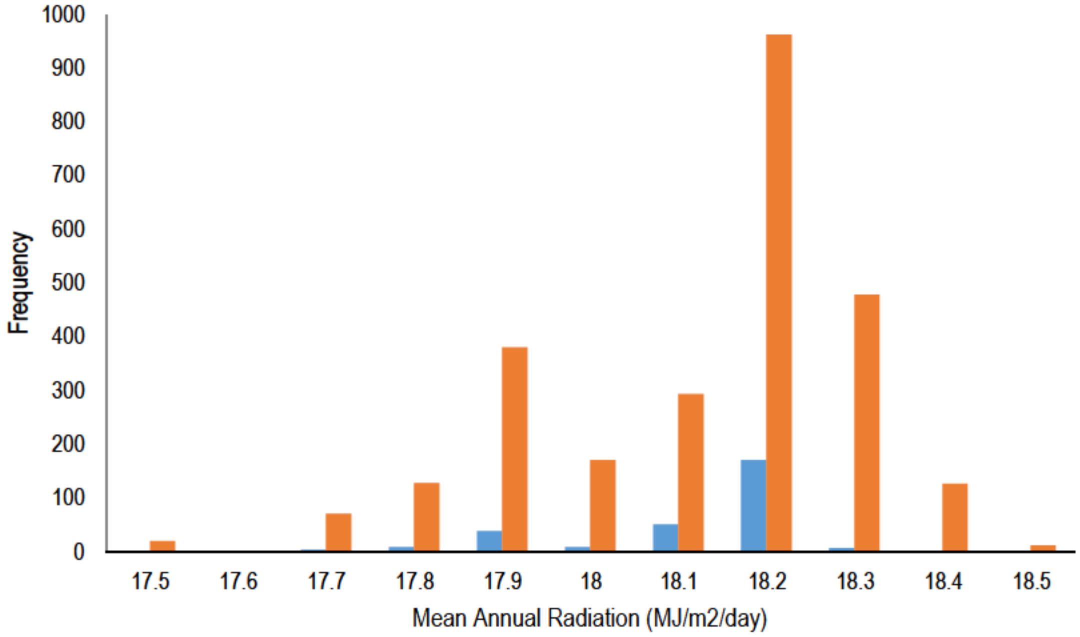

Figure 6.5 - Histogram of Bell's turtle (*Myuchelys bellii*) capture records with eye abnormalities (blue) and without eye abnormalities (orange) compared to mean annual solar radiation (χ^2_1=77.3, $p<0.01$).

6.8 Tables

Table 6.1 - Variables used in model selection, collected from the National Environmental Stream Attributes geodatabase (Stein *et al*., 2014). Descriptions taken verbatim from the geodatabase attributes table.

Variable	**Unit**	**Geodatabase Description**
Distance From Source	km	Maximum flow path length upstream to the segment pour-point, calculated by incrementing the maximum upstream length of neighbouring contributing cells.
Land Modification	%	Proportion of stream and sub-catchment that is modified land (i.e. not conservation)
Pesticide Use	%	Proportion of sub-catchment with landuses where herbicides/pesticides is likely to be used.
Mean Annual Solar Radiation	$MJ/m^2/day$	Stream and environs annual mean solar radiation
Mean Annual Rainfall	mm	Stream and environs average annual mean rainfall
Mean Annual Runoff	ML	Annual mean accumulated soil water surplus
Mean Annual Air Temperature	˚C	Stream and environs annual mean temperature

Table 6.2 - Models used in model selection to determine potential landscape-level causes of cataract presence in Bell's turtles (*Myuchelys bellii*). All models include Catchment and Year as random factors.

Model Name	Model	Justification
Distance From Source	Cataracts ~ Distance From Source	Initial investigations of the spatial patterns of eye abnormality presence showed that the abnormalities were more prevalent lower in the catchments.
Land Modification	Cataracts ~ Land Modification	Human modification to the landscape surrounding the streams may indicate disturbances to the streams, and the addition of effluents to turtle habitat. Correlates strongly with fertilizer use (97%), so likely indicates farmland.
Rainfall	Cataracts ~ Rainfall	Rainfall washing silt/effluent into streams, which may cause mechanical/chemical damage to the turtle's eyes or introduce novel pathogens.
Runoff	Cataracts ~ Runoff	Pathogens/chemicals/silt being transported from higher in the catchment or mobilized from the sediment.
Radiation	Cataracts ~ Radiation	Radiation damage to the eyes of basking/surfacing turtles.
Temperature	Cataracts ~ Temperature	Temperature variation promoting/suppressing pathogen appearance.
Pesticide	Cataracts ~ Pesticide	Pesticide effluent causing chemical damage to the eyes.
Climate	Cataracts ~ Temperature + Rainfall + Radiation	General climatic conditions of streams.
Disturbance	Cataracts ~ Land Modification + Pesticide	General disturbance of landscape surrounding streams.
Disturbance + Water	Cataracts ~ Runoff + Land Modification + Pesticide	Disturbance of landscape surrounding streams interacting with runoff to carry foreign material into the streams.
Pollution	Cataracts ~ Runoff + Pesticide	Pesticide being carried pesticides into the streams.
Spatial + Water	Cataracts ~ Distance From Source + Runoff	Distance from source and the amount of water in the catchments carrying foreign material.

Table 6.3 - Eye abnormality changes over time from Bell's turtles (*Myuchelys bellii*) captured in multiple years. "Healed" were turtles that were captured with abnormalities and subsequently recaptured without abnormalities. "Neutral" were turtles that had abnormalities during all captures. "Onset" were turtles that were captured without abnormalities and subsequently recaptured with abnormalities. Gap refers to the number of years between captures where a change was noted.

Category	n	Range of Gap (years)	Mode of Gaps (years)	Mean Gap (years ±SE)
Healed	8	1 - 5	1	1.9 (±0.48)
Neutral	33	1 - 7	1	1.2 (±0.18)
Onset	26	1 - 4	1	1.8 (±0.19)

Table 6.4 - Statistical outputs from model selection for determining the most predictive model for eye abnormality presence in Bell's turtles (*Myuchelys bellii*). Models highlighted in grey were chosen for model averaging.

Model	ΔAIC	AICc	cum.wt	k	LL
Radiation	0	1382.95	0.57	4	-687.47
Spatial + Water	2.11	1385.06	0.77	5	-687.52
Rainfall	3.32	1386.27	0.88	4	-689.13
Climate	3.50	1386.45	0.99	6	-687.21
Distance From Source	8.54	1391.50	0.99	4	-691.74
Temperature	9.22	1392.17	1.00	4	-692.08
Disturbance + Water	31.99	1414.94	1.00	6	-701.46
Pollution	39.85	1422.80	1.00	5	-706.39
Disturbance	40.73	1423.68	1.00	5	-706.83
Land Modification	50.34	1433.30	1.00	4	-712.64
Pesticide	55.80	1438.75	1.00	4	-715.37
Runoff	60.88	1443.83	1.00	4	-717.91
Random	75.34	1458.29	1.00	3	-726.14

Table 6.5 - Results of model averaging for determining the most predictive variable for eye abnormality presence in Bell's turtles (*Myuchelys bellii*). Variables highlighted in grey did not overlap with zero when SE was taken into account.

Variable	Estimate (±SE)	z-value	p-value
Radiation	4.52 (±1.40)	3.24	<0.01
Distance From Source	0.02 (±<0.01)	7.54	<0.01
Runoff	0.61 (±1.17)	3.16	<0.01
Temperature	0.13 (±0.22)	0.60	0.55
Rainfall	0.01 (±10.15)	0.46	0.64

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

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Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF ORIGINALITY

(To appear at the end of each thesis chapter submitted as an article/paper)

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All text, tables, and figures	131 to 155

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CHAPTER 7 - GENERAL DISCUSSION

7.1 Overview

Bell's turtles (*Myuchelys bellii*) have only recently gained attention from researchers, with few mentions in published literature prior to the year 2000. In the past decade, studies have been performed on their population genetics and taxonomy (Fielder *et al.*, 2012; Fielder, 2013), dive performance (Fielder, 2012), reproductive ecology (Fielder *et al.*, 2015), and disease occurrence (Hall *et al.*, 2020). Recent attention has been paid to the conservation concerns that the species is facing (Chessman, 2015), and the primary focus of this thesis was to clarify and attempt to address some of these concerns through two broad objectives. The first was an applied conservation approach, wherein new methods were trialled to safeguard turtle nests from predators (Chapters 2 and 3), and to identify the provenance of nests post-predation (Chapter 4). The second was an investigative approach, wherein a combination of statistical modelling was used to better understand the potential for threats to the Bell's turtle that had been previously noted by other researchers, namely competition with Murray River turtles (*Emydura macquarii*; Chapter 5) and the occurrence of cataract-like eye abnormalities (Chapter 6). What follows is a summary of the key findings of this research, and how it advances the body of knowledge about Bell's turtle and conservation biology in general.

7.2 Nest Protection Structures and Ultrasonic Animal Repellers Are Ineffective Methods for Protecting Bell's Turtle Nests

Turtle nests are frequently targeted by predators, and in some cases a turtle population may suffer near-total yearly nest losses. Through much of Australia, introduced red foxes (*Vulpes vulpes*) have been devastating to turtle populations of many species through unsustainable nest depredation (Van Dyke *et al.*, 2019), and Bell's turtle populations are facing similar pressure from foxes (NSW Office of the Environment and Heritage (NSW OEH), 2014). Methods for protecting turtle nests are a common factor of conservation strategies for turtles, which can include eradication of local nest predators (Spencer, 2002; Garmestani and Percival, 2005; Engeman *et al.*, 2006), conditioned aversion to negative stimuli such as capsaicin powder or human scent (Burke *et al.*, 2005; Lamarre-DeJesus and Griffin, 2013), and direct protection of individual nests with protective cages (Riley and Litzgus, 2013). Trialling novel methods is an important endeavour, as it adds new tools for conservation organisations. However, techniques that have high efficacy for some species and/or in specific locations and habitats may not achieve a similar success in other scenarios. The turtle species' behaviour, habitat, and the suite of predators that they face must be considered when designing these methods, as much as the method or technology itself. Two methods were trialled during this research that had not previously been attempted in Bell's turtle conservation strategies.

Nesting refuge structures were deployed successfully by Quinn *et al.* (2015) for protecting diamondback terrapin (*Malaclemys terrapin*) nests in the United States, and a similar structure was used for Bell’s turtles herein. Over two summers, Bell's turtles approached the entrances of these nesting refugia, and in some cases began to dig in front of the entrances, although none were recorded completing nesting. One female fully entered the structure, although she also did not nest. Foxes were never shown entering any structures, and no foxes were recorded near the structures in the second year (2020/21). While Bell's turtles commonly nest on riverbanks (NSW OEH, 2014), seasonal flooding is typical of rivers in the New England region. This makes these nesting refuge structures vulnerable to flooding, and over both summers of study, flooding irreparably damaged most of the structures deployed. Given this, it was concluded that nesting refuge structures were not suitable as a method for protecting Bell's turtle nests.

Ultrasonic repellent devices were also tested in Bell's turtle nesting habitat, with the intent of inflicting a negative stimulus on nest-searching foxes and other mammalian predators. However, while foxes were noted on-site during preliminary observations, no foxes were recorded during the study period (summer 2020/21), including before the repellent devices were activated, presumably due to poor conditions in the region in the previous season. Instead, ravens (*Corvus* sp.) were the most common nest raider on the site, which would not have been affected by the repellent devices as most birds cannot hear in the ultrasonic range (Seamans *et al*., 2013). Pigs (*Sus scrofa*), common brushtail possums (*Trichesurus vulpecula*), and various macropods (Macropodidae) were infrequent visitors to the sites, and similarly did not display any aversion to the repellent devices despite being able to hear in the ultrasonic range (Guppy, 1985; Osugi *et al*., 2011). While the ability of the devices to repel foxes could not be directly tested, the lack of aversion by other mammalian visitors, for whom the ultrasonic stimulus would have been audible, suggests that this technique is unlikely to be effective in repelling foxes from turtle nesting beaches.

The lack of foxes recorded during the 2020/21 field season was unexpected, but may be linked to the severe drought and bushfires that occurred in the previous year (Filkov et al., 2020). However, in both trials the lack of foxes was not the primary problem with the methods. Flooding showed that the nesting refuge structures were not a viable protection measure for Bell's turtle nests, and the lack of aversion by other mammal species to the ultrasonic devices is sufficient justification to conclude that foxes would also not be repelled by these devices. These two methods were shown to be ineffective for different reasons, but such null results are nonetheless important in applied conservation. By rigorously trialling these methods and finding them unsuitable in this habitat, or unsuitable for the targeted predators, the limited resources of conservation organizations may be directed away from this unsuccessful method and toward other methods that may be more effective.

7.3 Turtle Eggshells Show Differences in Microstructure among Species, which is Useful for Identification

Turtle nests are frequently raided by predators, but useful information can be gleaned from these nests if the species of origin can be identified. Ootaxonomy, the identification of the species of origin for eggs, has long been employed for invertebrates and fossil taxa (Gaino *et al*., 1987; Fausto *et al*., 1992; Lawver and Jackson, 2017), but less commonly for extant vertebrates. Angoh *et al*. (2018) tested the gross morphology of turtle eggshells among different species and found it a poor diagnostic method; central tendencies differed among species but with considerable range overlap. Analysis of microstructural features was investigated as an alternative ootaxonomic method, and this thesis included an initial assessment of scanning electron microscopy (SEM) as a tool for examining turtle eggshells for diagnostic features.

Eggshells of four turtle species native to north-eastern New South Wales were collected for this study: Bell's turtles, the congeneric Bellinger River turtle (*M. georgesi*), the Murray River turtle, and the Eastern long-necked turtle (*Chelodina longicollis*). Fragments of these eggshells were compared under a SEM, with the aim of identifying and measuring microstructural features that may be diagnostic. Diameter of central plaques on the outer membrane surface showed strong utility as a diagnostic feature. Plaque size showed similarity across taxonomic lines, with *Myuchelys* having larger pores than *Emydura*. No plaques were observed in Eastern long-necked turtle eggshells, as may be the case in other species (e.g. Packard *et al*., 1982; Phillott and Parmenter, 2006). A dichotomous key was developed for the turtle species found in New England based on these results.

By providing a method to determine the provenance of a depredated turtle nest, researchers in New England can now begin to confidently assign data collected from those nests to a species. This will provide conservation efforts with information regarding occupancy, numbers of breeding female turtles on a site, reproductive output, and predation rates on different species of turtle within the region. This method is low cost and requires minimal specialized training to conduct, provided researchers have access to an SEM. Refinement and standardisation of protocols for this method should be conducted, but the methodology trialled herein has been shown to be expedient, and the potential exists to expand it to other regions within Australia and beyond.

7.4 Murray River Turtles May Be Competing With Bell's Turtles

Additional threats may further be impacting Bell’s turtles, and these were also investigated. The Murray River turtle and Bell's turtle are sympatric in two streams within the Bell's turtle range, and the Murray River turtle may be expanding its range into historic Bell's turtle habitat in recent times (Chessman, 2015). Murray River turtles are more commonly captured than Bell's turtles in these zones of sympatry, leading to concern that the Murray River turtles, which are widespread and common in eastern Australia (Cann and Sadlier, 2017), are outcompeting the endangered Bell's turtles. This is particularly concerning, as turtles are translocated by humans, often as discarded pets (Cadi and Joly, 2003; 2004), and individual Murray River turtles have been captured in the Gwydir River and Severn Rivers during the course of this thesis (Fielder, Chessman, pers. comm.). There is no known source populations for these animals to reach these areas unassisted, suggesting that human-assisted movements are an ongoing potential threat.

Sympatric Bell’s turtle adults were on-average smaller and had lower mass than allopatric adults, particularly among adult females. Immature Bell's turtles did not differ in average size between sympatric or allopatric populations; this could indicate that immature turtles do not compete, or that the consequences of competition (i.e. reduced growth) may not become apparent until the turtles reach maturity. While there are other potential explanations for these differences such as geographic variation in size (Judge, 2001) or differing demographic distributions among populations, this thesis identifies the strong possibility that Murray River turtles are outcompeting their endangered relatives, such that further investigation is warranted. The impacts of the red-eared slider (*Trachemys scripta elegans*), a North American turtle species which has become widely established across much of the globe including Australia, show that interspecific competition among turtles must be taken seriously. Introduced sliders have caused population declines and outcompete native turtles where they are introduced (Cadi and Joly, 2003; 2004), and Murray River turtles maybe similarly detrimental to Bell's turtles as they expand their range.

7.5 The Eye Abnormalities Affecting Bell's Turtles May Be Linked With Solar Radiation

The presence of an eye abnormality affecting Bell's turtles was reported more than 20 years ago (Cann and Sadlier, 2017). While preliminary investigations showed no apparent fitness consequences for afflicted turtles (Chessman, 2015), its high prevalence remains a cause for concern, as an infectious disease caused the near-extinction of the congeneric Bellinger River turtle in 2015 (Chessman *et al.*, 2019). With access to a long term mark-recapture dataset, a thorough investigation of landscape-level predictors was undertaken for this thesis to quantify the potential impacts of the condition, and an assessment of the appropriate level of concern.

Prevalence of eye abnormalities was most ubiquitous in the Macdonald/Namoi catchment, with only a few instances in other catchments. Larger, potentially older turtles were more likely to be captured with clinical signs, and it was also more prevalent among females than males. With a larger, multi-year dataset, this thesis was also able to compare changes in eye abnormalities incidence over time. Female turtles were shown to recover from the eye abnormalities in some cases, similar to a single recorded instance of an afflicted female taken into captivity whereby the clinical signs cleared without medical intervention (Cann and Sadlier, 2017). Other females survived for long periods of time with clinical signs, as was reported by Chessman (2015). In contrast, and of most concern is that male turtles captured with the eye abnormalities were never re-captured in later seasons. Overall eye abnormality prevalence showed a marked increase in the latter years of the study, which coincided with a severe drought that affected much of Australia from 2017 to 2020. Finally, modelling showed a possible link between mean solar radiation and eye abnormalities, with turtles that live in higher-radiation areas more likely to develop abnormalities. This potential link was not strong, perhaps owing to the coarseness of the available radiation data, so further studies are warranted to confidently establish or dismiss this link.

7.6 Conclusions and Future Directions

This thesis has achieved its two major aims: to trial novel techniques for applied conservation of Bell's turtles, and to conduct preliminary ecological modelling to clarify questions about unknown and emerging threats to the species. While researchers have begun examining the species in recent times, much of the species' general ecology remained unknown. The Bell's turtle's shy disposition and the turbid water of its habitat make direct, *in situ* observation challenging. Nonetheless, aspects of the species' diet, relationship with their stream habitats, and behavioural interactions with conspecifics (including courtship) and interspecifics (including competition) should be a priority for research moving forward.

It is unfortunate that the exact techniques for nest protection trialled in this thesis were unsuccessful, but valuable lessons have been learned in the broader aspects of protecting Bell's turtle nests. Large, semi-permanent protection structures are likely to be damaged by flooding, and ultrasonic repellers appear ineffective at inducing aversion responses in nest predators. Thus, protection methods that succeed in preventing nest raiding whilst also being unaffected by flooding should be trialled for the protection of Bell's turtle nests. Possibilities include conditioned food aversion, which has shown success in protecting ground-nesting birds from fox depredation (Tobajas *et al.*, 2020), or refinements to existing nest-caging techniques. Further, the species' relationship with seasonal flooding warrants further investigation, to determine if Bell's turtle nests can survive periods of inundation, or if changes in magnitude and frequency of flooding should likewise be considered a conservation concern.

The identification of the provenance of eggshells use of scanning electron microscopy was shown to be a promising avenue for conservation. Future studies should continue to develop this technique with the goal of enhancing the preliminary dichotomous key for turtle eggshell identification for specific sites. Increasing the sample size, including more species to establish similarities and differences across taxonomic lines, and devising standardized protocols for collection, preservation, and analysis of samples are all priorities for this line of research. Nonetheless the technique shows promise as a means for clarifying both nesting site usage, and also predation rates and potential impacts on recruitment on a site-by-site basis. These data will provide critical information required for population viability analyses and similar fields, so are an important tool for understanding the conservation status of species.

Modelling showed that the potential negative effects of interspecific competition from species expanding their range may be problematic for Bell’s turtles where they co-occur with Murray River turtles. Future studies should examine behavioural interactions between the species, particularly for antagonistic behaviour over food items and persistent courtship behaviours that may be detrimental for female Bell’s turtles. Such negative impacts could manifest through slowed growth rates, resulting in smaller average adult Bell's turtles in the Deepwater River and lower Bald Rock Creek catchments. Further, genetic analysis should also be conducted within the zone of sympatry to determine if hybridization with Murray River turtles is occurring, as it is for the closely-related Bellinger River turtle (Georges *et al*., 2018). The importance of the potential threat of hybridization is currently unknown, and given the likely cryptic nature of genetic swamping, may already be impacting turtles in areas of sympatry.

Finally, further investigation into the relationship of solar radiation and eye abnormalities in Bell's turtle should be a priority. Whether caused by direct damage to the lens or mediated through nutritional deficits, the eye disease remains a conservation concern for the species and should be investigated with captive animals in controlled conditions. Similar clinical signs are noted more rarely in common saw-shelled turtles (*M. latisternum*; Cann and Sadlier, 2017) and Manning River turtles (*M. purvisi*; Redleaf Environmental, 2019), but not in turtle species sympatric with Bell's turtles (eastern long-necked turtles and Murray River turtles), so the potential taxonomic provenance of the disease may be an interesting avenue of study. The most pressing issue arising from the results of this thesis, however, is the complete lack of recaptures of afflicted male Bell's turtles. If males are experiencing high mortality from developing eye abnormalities (and thus not being recaptured), the disease would represent a serious threat to the species' persistence, particularly in the Macdonald River. Captive studies investigating the impacts of eye abnormalities on male Bell's turtles must be of the highest priority.

This thesis provides valuable information for both ecology and applied conservation of the Bell's turtle, and provides avenues for future iterations of Bell's turtle research projects and those examining similar freshwater species to proceed. Overall, while this study was able to advance current understanding of aspects of the Bell's turtle's ecology and behaviour *in situ*, future studies should seek to address the key identified knowledge gaps. Further, many of the future directions proposed by this thesis could not be conducted during this study due to the severe climatic conditions experienced in New England in 2018 - 2020, namely severe drought and bushfire seasons, and further due to the global pandemic of 2020-21, which prevented collection of individual turtles for prospective captive studies. Despite these significant challenges, this thesis has made a notable contribution to the body of knowledge around conservation biology in general, and Bell's turtle in particular.

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We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Type of Work	Page number(s)
All text, tables, and figures	158 to 168

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APPENDICES

Appendix I - Consumption of *Daphnia* spp. by Bell's turtles (*Myuchelys bellii*).

A version of this document has been published as a natural history note. It has been re-formatted to follow the same style as the rest of this thesis, and some spelling has been adjusted to Australian English. The published note may be found as:

Hughes, G.N., A. Curtsdottir, P.F. Lagos, and P.G. McDonald. 2020. *Myuchelys bellii* (Bell's turtle): Unexpected dietary contents. Herpetological Review 51(3): 579 - 580.

Freshwater turtles are typically omnivores and dietary generalists, and fill important niches as aquatic grazers and scavengers. Some species may prefer plant material over animal or vice versa, but in general freshwater turtle species will take advantage of all available food sources. The natural diets of a species can be inferred by directly observing feeding behaviour, examining faeces, and by flushing the stomachs of captured individuals. Stomach flushing provides more intact samples than faeces, and is more logistically feasible than observing foraging behaviour, but is also more invasive than these other methods (Legler, 1977).

Previous work on Bell's turtles (*Myuchelys bellii*) posits a typical omnivorous diet, with a bias toward plant matter; these inferences were based on microscope analysis of faecal samples (Fielder *et al.*, 2015). Plant material found in the faecal matter included the fruits of an invasive blackberries (*Rubus* sp.), and unspecified aquatic weeds and filamentous green algae. Animal matter included freshwater sponges, crayfish of the genera *Eustacus* and *Cherax*, and various unspecified insects and carrion. Evidence of smaller invertebrates was not noted in these studies, though they may have been fully digested, such that a microscope analysis did not identify them.

Snorkelling surveys for Bell's turtles were conducted on 27 October 2019 in the Macdonald River, south of Walcha, New South Wales, Australia (-31.114˚, 151.450˚). Local climatic conditions were dominated by a severe two-year drought, and the river was not flowing; surveys took place in a large remnant pool that was approximately 4 m deep at its maximum, and approximately 250 m long by 25 m wide. Water was ~20˚C at the surface and turbid (< 1 m visibility); weather was clear with a slight breeze. Two female Bell's turtles were captured by hand in the late morning; they were underwater but close to shore, and attempted to flee into deeper water. The smaller female (#5011) had a carapace length of 23.7 cm and a mass of 1528 g. The larger female (#5012) had a carapace length of 27.1 cm and a mass of 2695 g. Females at these sizes are considered adults, and #5012 was in the 90th percentile for carapace length and mass for all capture records of female *M. bellii* (B. Chessman, D. Fielder, G. Hughes, P. Spark, L. Streeting, unpubl. data). While Turtle #5011 was seemingly healthy, Turtle #5012 had cataracts in both eyes, a condition which afflicts ~10% of adult *M. bellii* in the Macdonald River catchment (Fielder *et al.*, 2015).

Both turtles were brought to the Armidale campus of the University of New England (UNE) and subjected to stomach flushing within two hours of capture, using the methodology outlined in Chessman (1986). Immediate visual assessment showed a considerable amount of yellow-brown matter in the collected contents, although detail was not distinguishable to the naked eye. Samples were collected, preserved in 70% ethanol solution, and refrigerated for storage. The turtles were held at UNE for 14 days as part of a separate behavioural study, and were released at the site of capture on 9 November 2019.

Stomach contents samples were later examined under a dissecting microscope, revealing that the yellow-brown matter was *Daphnia* spp. No other animal or plant material was found in the samples. The samples were filtered and dried in a drying oven at 60˚C for 24 hours to obtain dry mass. The sample drawn from #5011 contained 0.080 g, and the sample from #5012 was 0.027 g of dry organic matter. Based on estimated dry mass for an individual *Daphnia* from these samples (~25 µg), these dry masses translate to ~3200 *Daphnia* for Turtle #5011 and ~1080 *Daphnia* for Turtle #5012. Notably, the turtle with healthy eyes (#5011) had considerably more prey in her stomach than the much larger turtle afflicted with cataracts (#5012).

To our knowledge, this is the first recorded instance of such large freshwater turtles consuming mass quantities of small, free-swimming invertebrates. Another side-necked turtle,the yellow-spotted river turtle (*Podocnemis unifilis*), has been observed skimming the surface of the water for food (Belkin and Gans, 1968), but its diet appears to be almost exclusively herbivorous (Balensiefer and Vogt, 2006). All Bell's turtles subsequently sampled in this study (n = 5) had empty stomachs, except for a small male that had a single larval caddisfly in its stomach.

This presents a number of possible trophic relationships between Bell's turtles and *Daphnia*. *Daphnia* may represent a regular part of the species' diet, and turtles without this prey item in their gut had simply not foraged successfully prior to capture. *Daphnia* may be a seasonal part of the species' diet, representing a major food source only in spring. Alternatively, consumption of *Daphnia* may have been a "starvation diet" food item, forced by the severe drought. The full pattern of diet for these endangered turtles should be explored in more depth to further elucidate their relationship with *Daphnia*. Further, the method of prey capture employed by large turtles to capture *Daphnia* may be an interesting line of future study, particularly given one of these turtles presumably had impaired visual acuity. Perhaps Bell's turtles can employ a filter feeding strategy, as has been reported in other species of freshwater turtles.

We would like to thank the NSW Environmental Land Trust for funding this project through the Saving Our Species initiative, and the Holsworth Wildlife Research Endowment for their generous funding as well. We would also like to thank the volunteers that made capturing and sampling these turtles possible. All work was conducted in accordance with UNE Animal Ethics protocol AEC18-113 and National Parks and Wildlife permit SL102192.

LITERATURE REVIEW

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All text, tables, and figures	171 to 173

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Appendix II - Supplementary Figures for Chapter 4

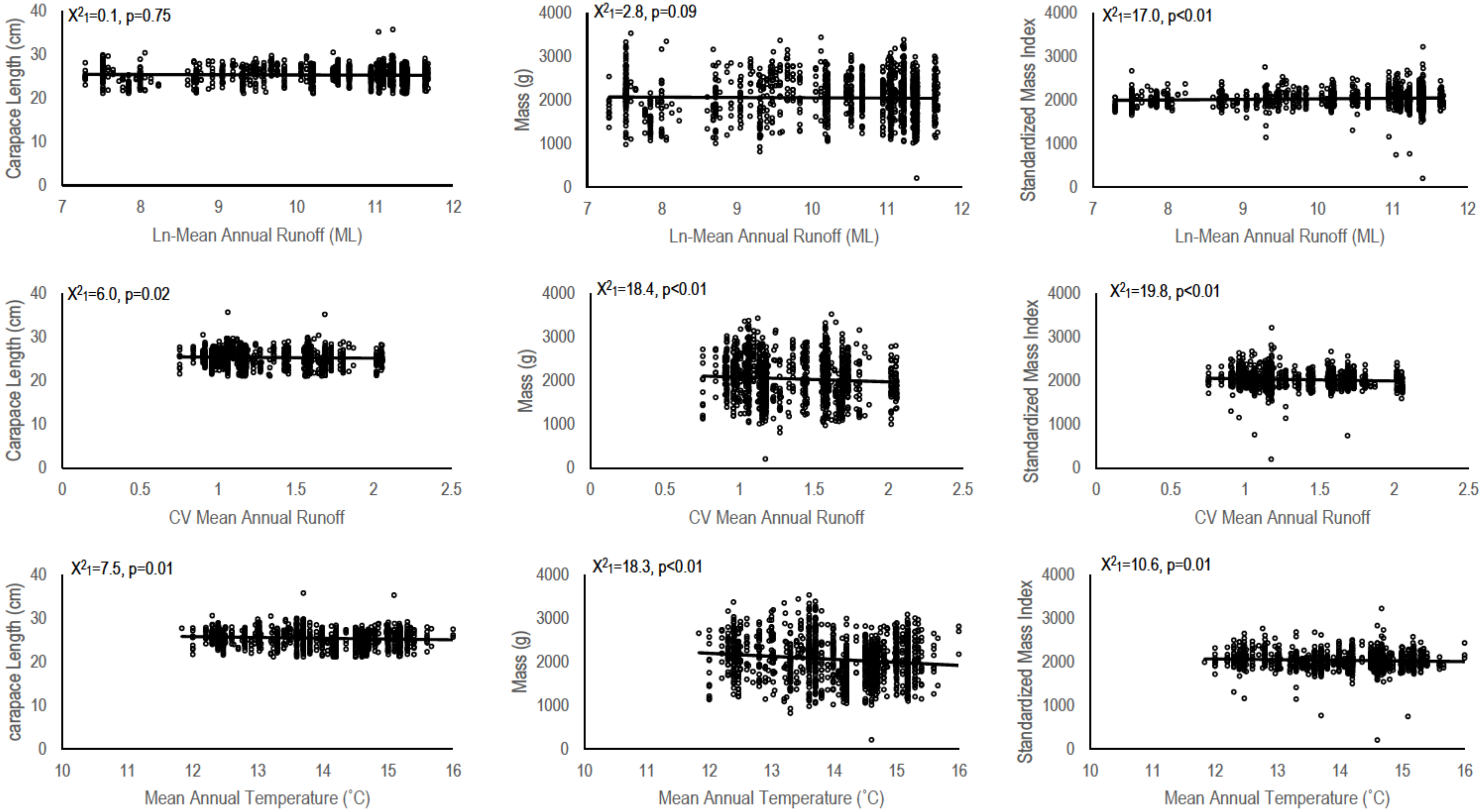

Figure A.1 - Correlations between biometric measurements (carapace length, mass, and standardized mass index) in adult female Bell's turtles (*Myuchelys bellii*) and abiotic environmental factors at site of capture: mean annual runoff (transformed with the natural logarithm), coefficient of variation in annual runoff, and mean annual temperature. Statistical outputs from liklihood ratio tests are embedded in the figures.

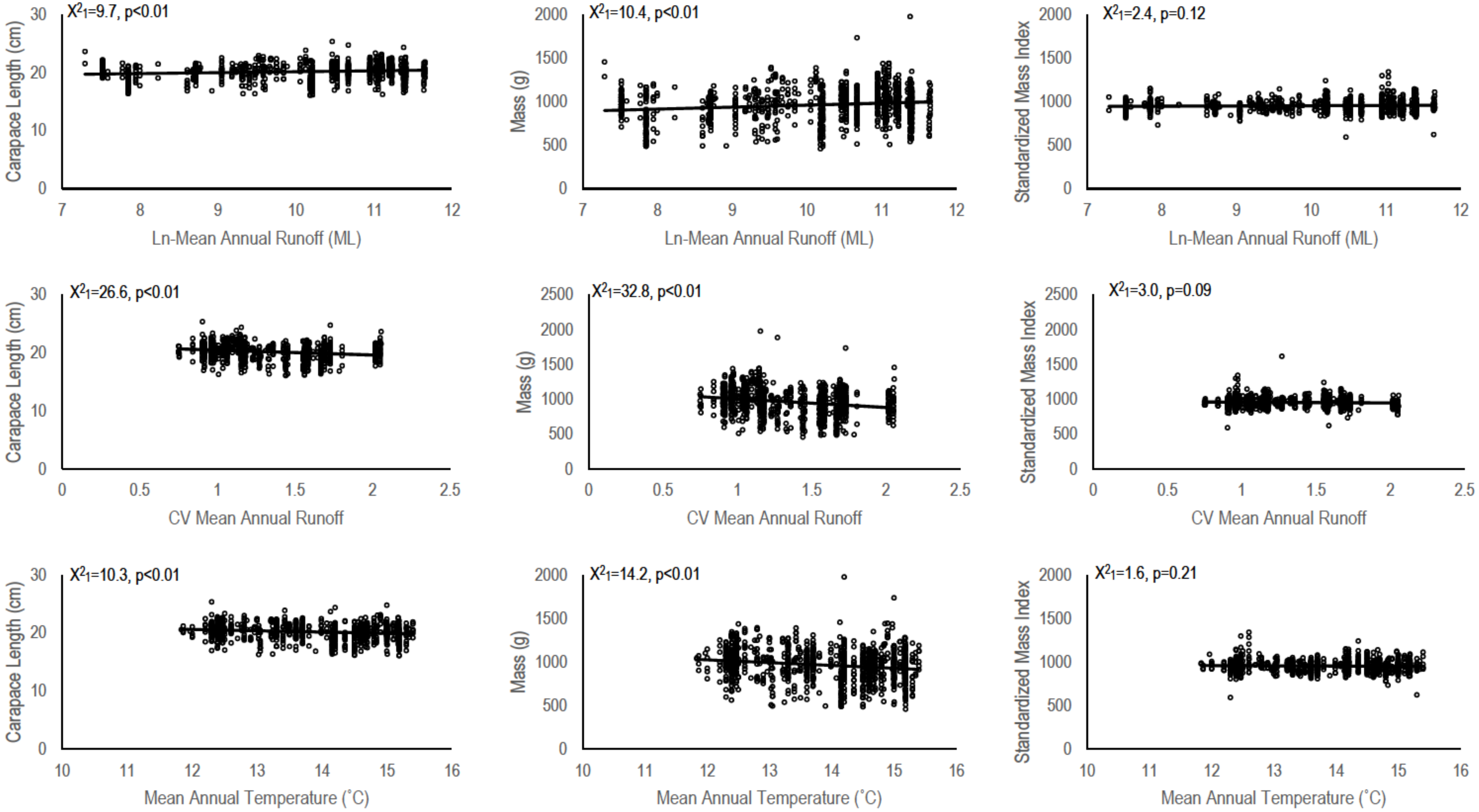

Figure A.2 - Correlations between biometric measurements (carapace length, mass, and standardized mass index) in adult male Bell's turtles (*Myuchelys bellii*) and abiotic environmental factors at site of capture: mean annual runoff (transformed with the natural logarithm), coefficient of variation in annual runoff, and mean annual temperature. Statistical outputs from liklihood ratio tests are embedded in the figures.

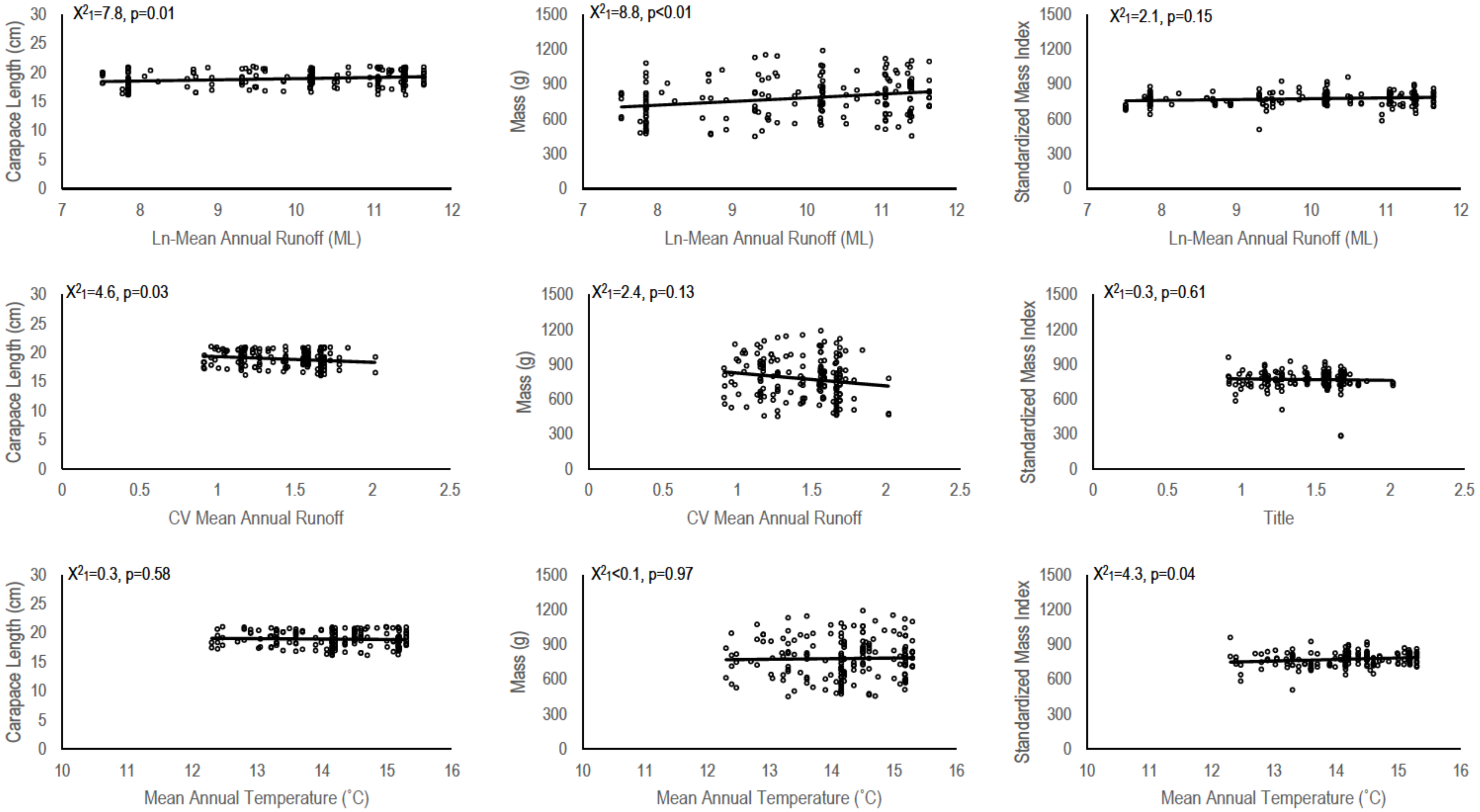

Figure A.3 - Correlations between biometric measurements (carapace length, mass, and standardized mass index) in subadult female Bell's turtles (*Myuchelys bellii*) and abiotic environmental factors at site of capture: mean annual runoff (transformed with the natural logarithm), coefficient of variation in annual runoff, and mean annual temperature. Statistical outputs from liklihood ratio tests are embedded in the figures.

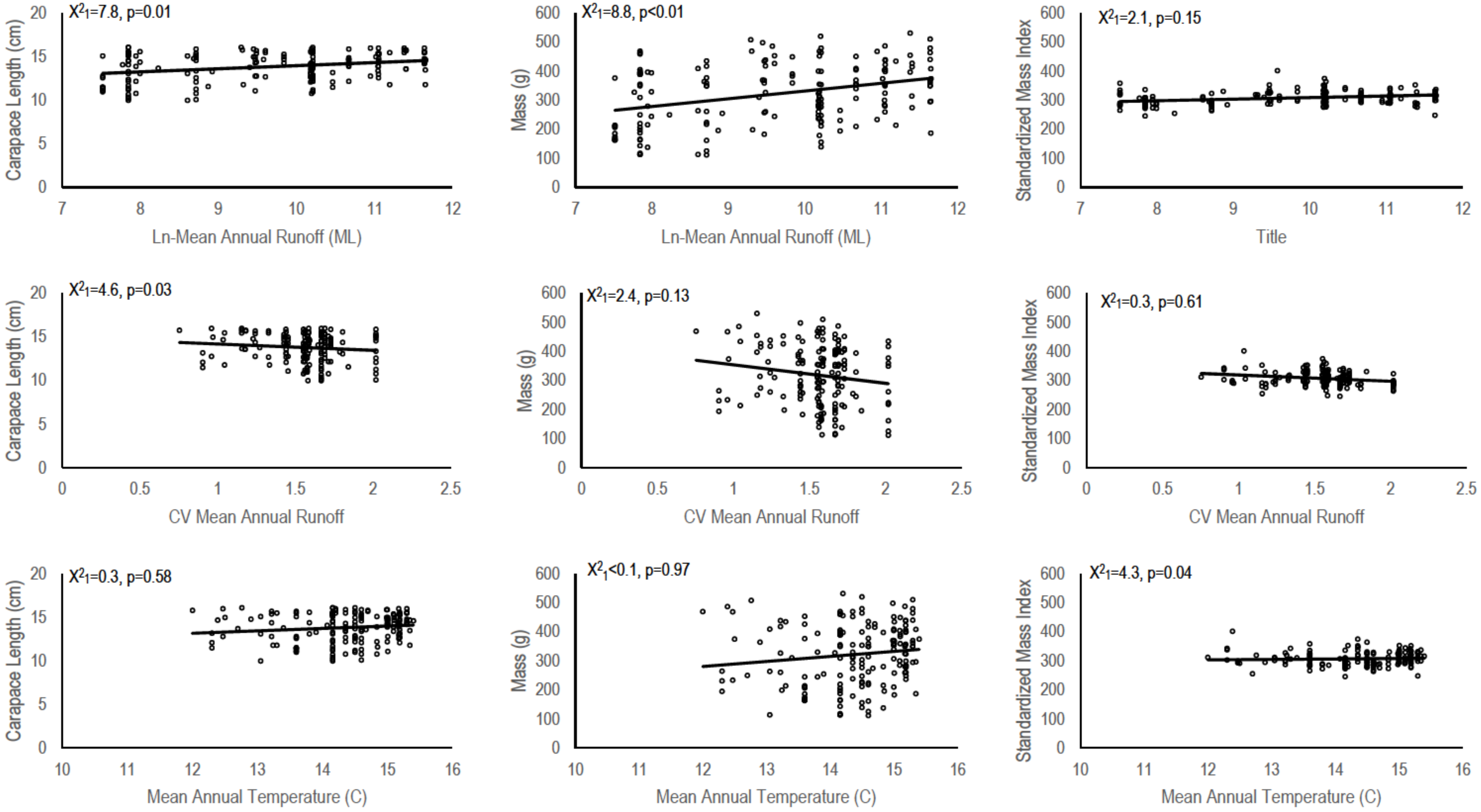

Figure A4 - Correlations between biometric measurements (carapace length, mass, and standardized mass index) in juvenile Bell's turtles (*Myuchelys bellii*) and abiotic environmental factors at site of capture: mean annual runoff (transformed with the natural logarithm), coefficient of variation in annual runoff, and mean annual temperature. Statistical outputs from liklihood ratio tests are embedded in the figures.

A large female Bell's turtle (*Myuchelys bellii*) captured near Uralla in 2019.

A male Bell's turtle (*Myuchelys bellii*) captured near Uralla in 2019.