The ecology, conservation, and management of Australia's ephemeral freshwater granite rock-holes

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School of Biological Sciences

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Contents

Declaration of Originality

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Thesis Summary

Ephemeral freshwater ecosystems are ecologically distinct from their permanent counterparts and are of significant conservation value, yet they are relatively understudied and often overlooked in key policy documents. Furthermore, they are at risk due to the impacts of a series of key threatening processes. I sought to improve understanding of the ecological role and threats to one such ecosystem, the freshwater granite rock-holes found in arid Australia.

I used wildlife camera trapping alongside environmental DNA (eDNA) metabarcoding to record vertebrate visitation at rock-holes to determine the extent of their use as a resource to native and invasive vertebrates, as well as test the validity of eDNA metabarcoding as a biomonitoring tool for recording vertebrate visitation to this ephemeral freshwater ecosystem. Environmental DNA metabarcoding was also used to characterise invertebrate communities in rock-holes, and assess variability at spatial and temporal scales. Rock-hole hydrology was modelled and forecast under a series of emissions scenarios to predict the effect of climate change on this ecosystem. Finally, I applied these scenarios to rock-hole invertebrates to assess the potential impact of climate change on rock-hole inhabitants.

Wildlife camera traps recorded six native mammal, four reptile, and 18 bird species plus four invasive species known to cause ecological harm. The most common native taxa were macropods, emus, crows and ravens. Visitation increased with prolonged periods without local rainfall, and invasive species visitation (primarily by goats) increased with elevation. Twenty-one vertebrate species were identified with freshwater eDNA metabarcoding, and the method was deemed an effective alternative to wildlife camera trapping at detecting mammal visitations. However, it was less well suited for detecting birds.

Rock-hole invertebrate communities mostly comprised crustaceans and insects and varied spatially and temporally, with a peak in species richness in July. Freshwater eDNA metabarcoding was effective for detecting rock-hole invertebrate communities, but an underrepresentation of arid lands species and sequences in public genetic databases limited the taxonomic resolution that could be achieved (i.e. up to Order level).

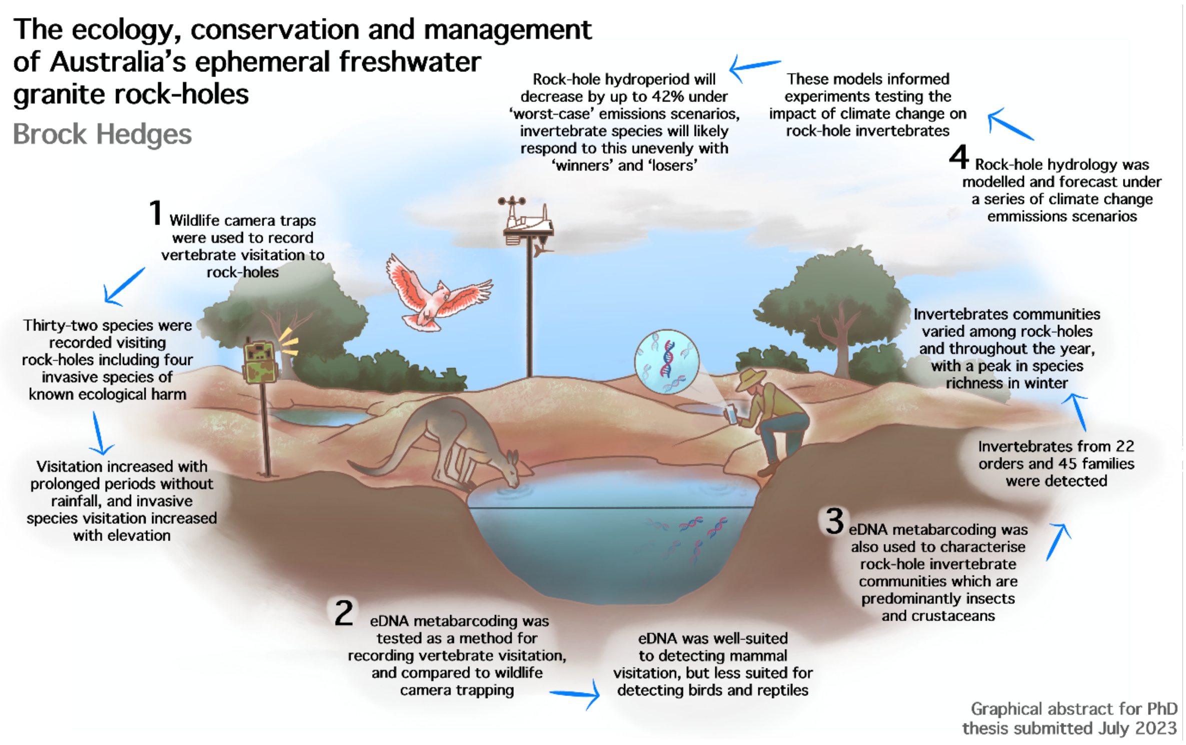
In the arid region of Australia examined here, future changes in climate will result in increases in water temperatures and decreases in hydroperiod under all emissions scenarios. Under the most severe scenarios, modelling showed a 42% decrease in hydroperiod. Experiments that duplicated conditions likely caused by climate change impacted test species *Sarscypridopsis* sp. and *Daphnia clarinata* unevenly. These results indicate that climate change will adversely affect some rock-hole invertebrates, although others will likely have capacity to adapt and compensate by increasing their rates of growth and reproduction.

My research shines a light on often-overlooked freshwater granite rock-holes, and contributes to understanding of the importance of ephemeral freshwater ecosystems more generally. I have demonstrated that the rock-holes provide potable water to native vertebrate communities and are a habitat for invertebrates. However, unmanaged, these habitats face two key threats: fouling and degradation by invasive species and climate change. In the absence of significant local and global efforts to mitigate the impacts of these threats, degradation of these high value ecosystems is certain. Future research should further explore the impacts of climate change on ephemeral freshwater ecosystems more broadly and involve specific experimental studies on target taxa, as well as the establishment of long-term biomonitoring and custom barcode reference libraries, and the application of practical strategies to mitigate degradation.

Abbreviations

eDNA: Environmental DNA
EP: Eyre Peninsula
EWB(s): Ephemeral water body/bodies
GRAC: Gawler Ranges Aboriginal Corporation
GB: Gawler bioregion
GRNP: Gawler Ranges National Park
HNR: Hiltaba Nature Reserve

Graphical Abstract



The ecology, conservation and management of Australia's ephemeral freshwater granite rock-holes

Brock Hedges

1 Wildlife camera traps were used to record vertebrate visitation to rock-holes

Thirty-two species were recorded visiting rock-holes including four invasive species of known ecological harm

Visitation increased with prolonged periods without rainfall, and invasive species visitation increased with elevation

2 eDNA metabarcoding was tested as a method for recording vertebrate visitation, and compared to wildlife camera trapping

eDNA was well-suited to detecting mammal visitation, but less suited for detecting birds and reptiles

3 eDNA metabarcoding was also used to characterise rock-hole invertebrate communities which are predominantly insects and crustaceans

Invertebrates from 22 orders and 45 families were detected

Invertebrates communities varied among rock-holes and throughout the year, with a peak in species richness in winter

4 Rock-hole hydrology was modelled and forecast under a series of climate change emmissions scenarios

These models informed experiments testing the impact of climate change on rock-hole invertebrates

Rock-hole hydroperiod will decrease by up to 42% under 'worst-case' emissions scenarios, invertebrate species will likely respond to this unevenly with 'winners' and 'losers'

Graphical abstract for PhD thesis submitted July 2023

Chapter 1. General Introduction

Arid regions represent important biodiversity hotspots for several iconic organismal groups (e.g. plants (Li et al. 2019, Borrell et al. 2019), mammals (Soultan et al. 2019) and reptiles (Liz et al. 2023)). Water availability is a major limiting factor in these locations, and one that has likely influenced selection through competition (Soultan et al. 2019, Mossop et al. 2023). Water resource availability is often highly contested, integral to arid-land ecosystem function, and typically highly ephemeral, persisting inconsistently throughout the year. In many arid regions, ephemeral water bodies (hereafter EWBs), are the only available sources of surface freshwater providing water to local terrestrial vertebrates and complex communities of freshwater plants and invertebrates. However, EWBs are thought to be in decline globally due to a variety of threatening processes such as the presence of invasive species and modifications due to land use, which negatively impact water quality and the communities associated with them. These threats are also expected to be further exacerbated by the impacts of climate change, which are projected to disrupt climatic regimes and alter rainfall, evaporation, and the hydroperiod of EWBs. Such forces are cause for concern and suggest that these ecosystems require targeted and research-driven management. The freshwater granite rock-holes of Australia are one such habitat clearly deserving focus. In this thesis I sought to characterise these unique ecosystems, quantify their conservation and freshwater resource value, and propose a series of management recommendations to safeguard them into the future.

1.1 Ephemeral freshwater ecosystems are in decline

An EWB is defined here as any source of naturally occurring freshwater that is both accessible at the surface and temporary in its occurrence, including both lentic (flowing) and lotic (still) habitats. These water bodies include but are not limited to ephemeral river and creek-lines (Scott et al. 2003, Steward et al. 2012, Acuña et al. 2017), ephemeral lakes (Karagianni et al. 2018), seasonal wetlands (Strachan et al. 2014, Calhoun et al. 2017), vernal pools and temporary ponds (Andrushchyshyn et al. 2003, Kneitel et al. 2017), claypans (Gibson et al. 2018), and ephemeral rock-holes (Bayly 2001, Timms 2017). Variable in their hydroperiod (the duration for which water is present), some EWBs are recharged from local sources and persist for only days at a time before drying up, while others may persist for years at a time before regional drought conditions result in their drying (Bayly 1999a). Despite their importance as a freshwater source for biodiversity in regions with low annual rainfall and likely high vulnerability with climate change, EWBs are often overlooked in major policy decisions and global reviews (Brooks 2009).

Throughout semi-arid and arid regions, scarcity of water is a challenge that local biota must overcome, frequently requiring them to seek sources of water wherever they are available. Often EWBs are the only source of drinkable freshwater in these regions, and known to be of importance to native species in Australia (Votto et al. 2022, McDonald et al. 2023). Some EWBs provide habitat for vertebrates such as amphibians (Hedges et al. 2021) and water birds (Kingsford et al. 2004) but rarely for fishes (Brendonck et al. 2002). However, changes in landscape management throughout much of Australia's semi-arid and arid regions have modified the availability of freshwater due to the formation of more permanent artificial water points such as dams. These shifts have also led to increased abundance of some native herbivores such as kangaroos and wallabies (Read et al. 2021) and probably increased their visitation to EWBs in adjacent habitats. Australia has also seen increasing abundances of a suite of invasive species of concern including goats (*Capra hircus*), pigs (*Sus scrofa*), water-buffalo (*Bubalis bubalis*), horses (*Equus caballus*) and camels (*Camelus dromedarius*) (Commonwealth of Australia 2008, 2010, 2011a, 2011b, 2017) in arid regions. Prior to these introductions, large-bodied ungulates were not native to Australia and there is evidence that their activity has led to disturbance and degradation of freshwater sites (Brim Box et al. 2016).

In addition to their role as a source of drinkable water for terrestrial vertebrates, EWBs sustain a complex suite of aquatic inhabitants. These communities are highly variable (Hart et al. 1997) and are often distinct from those in more permanent water bodies (Boix et al. 2008, Jurkiewicz-Karnkowska 2009). Due to their disjunct distribution and isolations, EWBs often support endemic, sometimes endangered species (Bagella et al. 2012, Cross et al. 2015a, Kneitel et al. 2017, Herrera et al. 2018), and have higher rarity indices than permanent water bodies (Collinson et al. 1995, Ernandes et al. 2013, Gilbert et al. 2014). The majority of taxa that inhabit EWBs have physiological and reproductive adaptations that allow them to survive long periods of desiccation (Florencio et al. 2013), such as the production of desiccation resistant eggs (Aguilar-Alberola et al. 2011, Datry et al. 2017, Bellin et al. 2021), cryptobiosis (Datry et al. 2017), parthenogenesis (Černý et al. 1993, Dudycha et al. 2013, Coviaga et al. 2015), delayed hatching (Chakri et al. 2010), rapid life cycles (Dudycha et al. 1999, Krieger et al. 2000) and drought resistance (Chapuis et al. 2012). Others have behavioural adaptations that optimise dispersal and colonisation of recently recharged EWBs (Bonada et al. 2007, Deacon et al. 2011, Frisch et al. 2012, Castillo-Escrivà et al. 2017) or to retreat to more suitable locations (DiStefano et al. 2009). Plants associated with EWBs also have traits that facilitate their persistence in this unstable environment. Seed germination in EWBs is often regulated by inundation, temperature, and chemical cues, and seeds can remain viable after being dry for long periods (Lesica 1992, Cross et al. 2015a).

Ephemeral water bodies often lack large predatory species since fishes are mostly unable to access them (Brendonck et al. 2002) and have limited survivability in drought-stressed systems (Bêche et al. 2009) and truly ephemeral habitats (Escalera-Vázquez et al. 2010, García et al. 2018). The absence of large predators means that EWBs can be crucial locations for recruitment for invertebrate species. Whilst fish presence and absence does not always correlate reliably with invertebrate abundance (Jara et al. 2013), amphibian populations in EWBs are often driven primarily by predation (Gibbons et al. 2006, Canals et al. 2011, Hamer et al. 2013). When predatory regulation does occur, it is often the result of smaller invertebrate predators such as dragonfly nymphs or backswimmers (Brendonck et al. 2002, Andrushchyshyn et al. 2003), which have a presence often dependent on factors of habitat structure, such as the occurrence of macrophytes (Hernandez et al. 2006, Carchini et al. 2007, Le Gall et al. 2018).

A range of factors can impact EWB community composition, one of the most important of which is ecosystem history (Fukami 2004, Jeffries 2011). Egg and seed banks are the primary determinant of species richness after wetting (Liu et al. 2006). The duration of the hydroperiod often plays a role in determining community composition (Bodie et al. 2000, Carchini et al. 2007, Jocqué et al. 2007, Boven et al. 2008, Della Bella et al. 2008, Anusa et al. 2012, Bagella et al. 2013, Ernandes et al. 2013, Cepeda-Pizarro et al. 2015, Cross et al. 2015b, Lozada-Gobilard et al. 2019) and is particularly important in shaping predator richness and diversity (Anusa et al. 2012). Other physical characteristics often determine community composition, such as habitat size (Bosiacka et al. 2012), connectivity (Jurkiewicz-Karnkowska 2009), surface area (Eitam et al. 2004, Jurkiewicz-Karnkowska 2009), habitat heterogeneity (Bagella et al. 2009), local environmental features (Hall et al. 2004, Declerck et al. 2011, Deans et al. 2017), elevation (Brose et al. 2005, Herrera et al. 2018), vegetation biomass (Liberto et al. 2012), and geography (Boven et al. 2008, Cucherousset et al. 2008, Bogan et al. 2012, Bosiacka et al. 2012, Datry et al. 2014, Boda et al. 2018).

The physiochemical conditions of an EWB also play a key role in determining community composition and are ultimately derived from water regime (Gascón et al. 2005). Factors that can impact community composition include salinity (Desender et al. 1999, Howard et al. 2000, Álvarez et al. 2006, Anton-Pardo et al. 2012, Mabidi et al. 2018), conductivity (Chappuis et al. 2014), dissolved CO2 (Gilbert et al. 2017), pH and ionic content (Kováč et al. 2001, Iglikowska et al. 2012), and turbidity (Gutiérrez-Estrada et al. 2010, Gutiérrez-Estrada et al. 2013). In small EWBs, these factors can vary greatly even across consecutive days due to short-term variations in weather, temperature, and evaporation. The nutrient availability of a system can also impact community composition (Della Bella et al. 2008, Anusa et al. 2012, Chappuis et al. 2014). Flooding with suspended vegetation can cause trophic shifts (Burdett et al. 2009) and, in extreme cases, eutrophication and complete ecosystem shifts (Kneitel et al. 2010, Daoud-Bouattour et al. 2011).

Despite the biodiversity value of ephemeral freshwater bodies, studies investigating arid-zone freshwater ecology are under-represented in the scientific literature (Dallas et al. 2007, Piña et al. 2022). Permanent freshwater ecosystems, such as rivers and lakes have, to date, attracted far greater research focus and management funding compared to often smaller EWBs that are characteristic of arid regions. However, there is growing evidence that suggests the long-term water security of many EWBs is uncertain (Brooks 2009). **Invasive species**, **changes to land use**, and **climate change** are amongst the key threatening processes that are currently impacting these habitats.

Due to their often small size and isolation, EWBs are particularly susceptible to disturbance by **invasive species**. Invasion events can cause large changes in algal communities (Buchberger et al. 2018), macrophyte biomass (Carreira et al. 2014), and invertebrate populations (Devereaux et al. 2006). Even single-species additions or losses can result in substantial impacts to community composition over time (Jonsson 2006). For example, large-bodied invasive mammals such as camels and feral pigs impact ecosystem health and quality when in proximity to EWBs by causing disturbance and fouling water (Doupe et al. 2010, Brim Box et al. 2016).

As adjacent **land use** affects biotic richness and diversity (Hall et al. 2004, Bouahim et al. 2014), ephemeral water bodies can also be impacted by activities associated with agriculture (Dimitriou et al. 2006, Kerezsy et al. 2014, Bruno et al. 2016). High input of fertilizers and manure into EWBs can result in detrimental effects on biodiversity and can lead to potential trophic cascades (Boggs et al. 2007). Regional water use and excessive abstraction can also negatively impact EWBs (Brim Box 2016). For example, unnatural flow regulation, a practice common in Australian rivers, can lead to complete disruption of EWB water regimes and drastically change ecosystem function (Curtis et al. 1998). Livestock such as cattle also impact water quality, increasing local nitrogen and turbidity (Canals et al. 2011).

Climate change poses a distinct threat to the future persistence of EWBs (Krieger et al. 2003, Kneitel 2016). To date, climate change has already led to shifts in distribution and phenology of species typically associated with EWBs (Ewald et al. 2013). Furthermore, changing climate regimes have been observed to impact plants (Neill et al. 2009, Mohammad et al. 2022), invertebrates (Frisch 2001), and vertebrates (Chessman 2011, Howard et al. 2016) associated with EWBs. Plants associated with EWBs have been shown to be in decline, with populations having become increasingly isolated (Mohammad et al. 2022). Increased abundances and predation rates have been observed in EWB crustacean communities in experiments that simulate the effects of future climate scenarios (Ewald et al. 2013). In Australia, freshwater turtles (*Chelodina longicollis* and *Emydura macquarii*) that live within EWBs have been observed to be in decline due to increasing drought frequency (Chessman 2011, Howard et al. 2016). Furthermore, inter-species interactions such as predation and competition have been shown to differ under increased water temperatures (Ewald et al. 2013), suggesting that the effects of climate change may result in shifts in ecosystem composition for many EWBs. Vertebrate activity around EWBs has been shown to vary seasonally (Dixneuf et al. 2021, Votto et al. 2022), suggesting that their value as a freshwater resource is also linked to climate. These factors all contribute to an emerging narrative that identifies climate change as a key threatening process for semi-arid and arid-lands surface freshwater systems (Beasley-Hall et al. 2023), with EWBs perhaps being amongst the most at risk.

1.2 The freshwater granite rock-hole habitat

Throughout much of the semi-arid and arid regions of southern Australia, sources of freshwater that are accessible at the surface are limited, and in many locations EWBs are amongst the only sources present. Freshwater granite rock-holes are located throughout the northern Eyre Peninsula and Gawler Ranges of South Australia and are one of the main sources of water in this otherwise very dry environment (Figure 1.1). Having formed over millennia through a series of physical and chemical weathering processes (Twidale and Corbin 1963, Twidale and Romani 2005, Timms and Rankin 2016), rock-holes provide temporary storage of rainwater (Jenkin et al. 2011b, Timms 2014, Hedges et al. 2021). This water lasts for only a short period, between a few weeks to months, before drying up entirely due to evaporation. Despite the extreme nature of the habitat, rock-holes have a complex ecosystem associated with them including a suite of plants, crustaceans, insects, other invertebrates, and occasionally vertebrates (Bayly 1997, Pinder et al. 2000, Timms 2014, Timms 2017, Hedges et al. 2021). Rock-hole communities are considered to be behaviourally and physiologically adapted to persist in these rock-holes despite their eventual drying (Jones 1975, Hedges et al. 2021).

Figure 1.1. A) An aerial view of the Photopoint rocky outcrop at Hiltaba Nature Reserve in South Australia (distance across the granite dome = 120 m); B) Rain-filled rock-holes at the Pretty Point outcrop at Hiltaba Nature Reserve; C) A dry rock-hole at the Photopoint outcrop at Hiltaba Nature Reserve (diameter = 1.4 m), pictured: Jenna Draper (left), Oliver Gore (right); D) A rain-filled rock-hole at the Photopoint outcrop at Hiltaba Nature Reserve (Length of the rock-hole = 3 m).

Granite rock-holes are highly variable in their size and shape (Timms 2013b, Timms and Rankin 2016) and these physical characteristics are thought to influence the communities associated with them (Timms 2014, Hedges et al. 2021). They are also known to be attended by vertebrates as a source of freshwater (Jenkin et al. 2011a, Nature Foundation 2023, McDonald et al. 2023). As such, granite rock-holes are likely to be important in the conservation of a range of vertebrates that periodically depend on them as a source of freshwater in an otherwise dry environment.

My PhD specifically focussed on rock-holes in the Gawler bioregion (GB) on a single property, Hiltaba Nature Reserve (HNR) (Figure 1.2A). Hiltaba Nature Reserve was selected because its rock-holes are likely to be amongst the least impacted in the GB due to ambitious conservation initiatives enacted onsite. The GB is diverse in its habitat types, comprising a series of low rolling granite hills, interspersed by sparse woodlands and grasslands (Figure 1.2B, C & D). While the region is currently classified as semi-arid, it is projected to increase in its aridity over the next 100 years, a shift likely to negatively impact the rock-holes (IPCC 2022a). HNR is a large (78,000 ha) property that has been managed for conservation outcomes since 2012. Prior to its acquisition by the Nature Foundation, HNR was a pastoral sheep property for more than a century and all of its nearby neighbours—except for the Gawler Ranges National Park (GRNP)—are still used as sheep enterprises. A series of conservation programs have been implemented at HNR which include targeted removal of invasive species (primarily goats (*Capra hircus*), cats (*Felis catus*) and foxes (*Vulpes vulpes*)), decommissioning of artificial water points, and weed removal. These efforts have led to the recovery of vegetation communities as well as the westernmost population of yellow-footed rock wallaby (*Petrogale xanthopus*), a species in decline elsewhere in the GB and one listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Department of the Environment 2023). As is the case throughout the GB, HNR has a series of exposed granite outcrops, many of which have rock-holes present on them, although no complete inventory of rock-hole locations has been compiled. The rock-holes present at HNR provided the primary sampling and experimental unit of this PhD research.

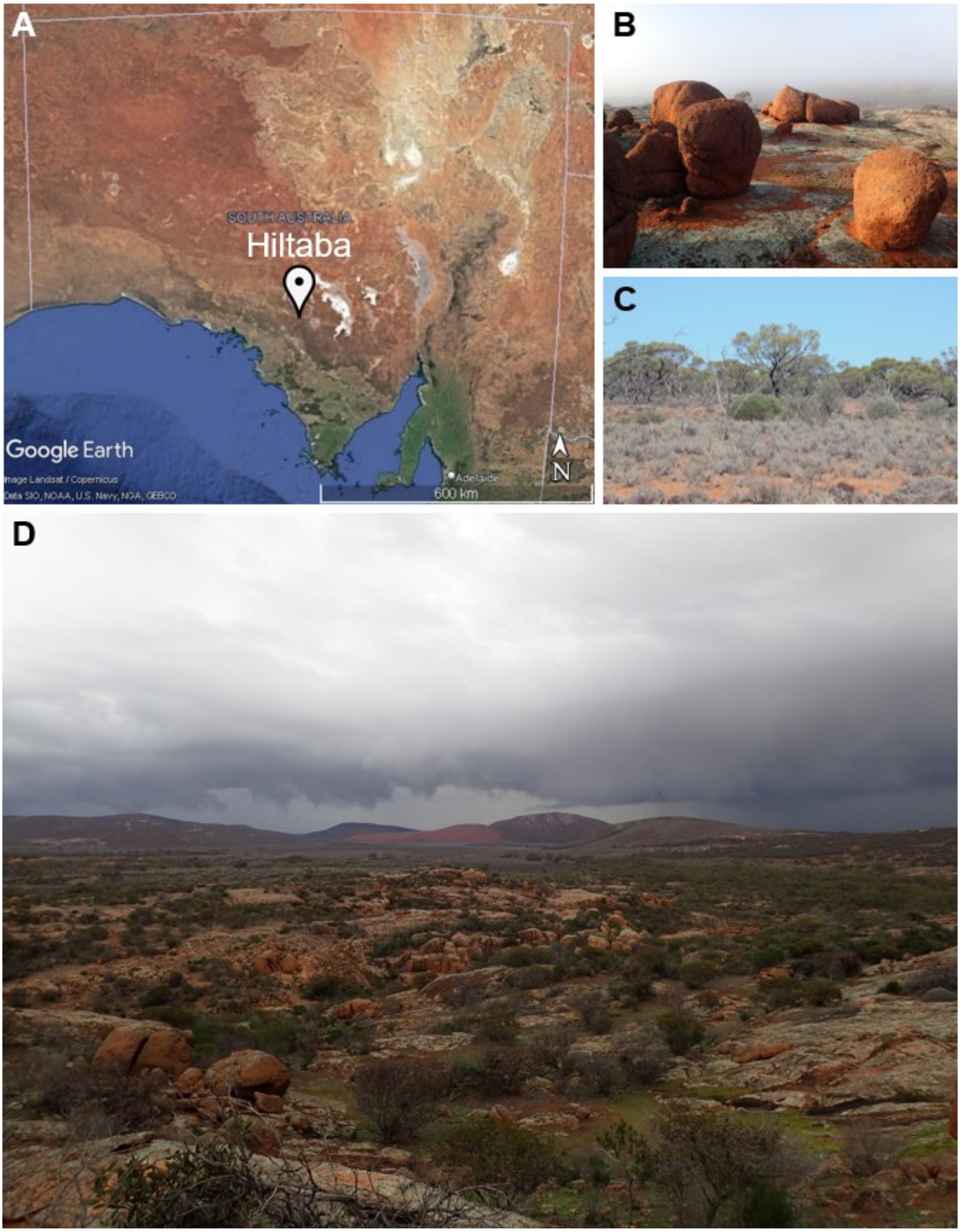


Figure 1.2. A) The location of Hiltaba Nature Reserve in South Australia; B) Rocky outcrops at Hiltaba Nature Reserve; C) Sparse woodlands and grasslands characteristic of the GB; D) A view from the top of an outcrop at Hiltaba Nature Reserve during a Winter storm.

In addition to their biological significance, the granite rock-holes throughout southern Australia are noted to be of great cultural importance to many First Nations Australians (Jenkin et al. 2011b). Since they are typically present in semi-arid and arid regions where accessible freshwater is scarce, the rock-holes have long been used as a reliable, but seasonal, source of drinking water by humans

(Jenkin et al. 2011b). Perhaps some of the Australian rock-holes best known for their cultural and biological significance are the gnammas of south-western Western Australia (Bayly 1997, Pinder et al. 2000). The term gnamma refers to rock-holes present in granite and is a Nyungar word used by the Noongar people of Western Australia with origins in the Western Desert group of languages (Bayly 1997, Jenkin et al. 2011b). In the scientific literature the term gnamma has been commonly used to refer to any freshwater granite rock-hole across Australia, although it is not a term used by First Nations peoples in South Australia, where the term rock-hole is preferred (Jenkin et al. 2011b).

The rock-holes present in the GB are significant to the Traditional Custodians of the land. HNR is situated on Bungala country with connections from the Kokatha and Wirangu peoples into the Gawler bioregion (AIATSIS map of Indigenous Australia 2008). These rock-holes are of immense cultural value to these peoples for their role as a crucial supply of freshwater in an arid landscape that lacks permanent freshwater and flowing rivers (Jenkin et al. 2011b). As such, rock-hole occurrence and distribution has historically dictated routes of travel, and tracks often radiate out from them (Jenkin et al. 2011b). Aboriginal law or *Tjukurrpa* forms a rich cultural landscape, and information regarding the location and management of these rock-holes is passed from generation to generation through story, song and dance (Jenkin et al. 2011b). The rock-holes present at HNR are still used today for cultural purposes and are subjected to ongoing traditional management practices, involving the use of timber and rocks to prevent animals from falling into the water, and regular cleaning (Figure 1.3). Aboriginal law assigns direct responsibility for the management and maintenance of water sources (Jenkin et al. 2011b), which is implemented by the Gawler Ranges Aboriginal Corporation (GRAC), Native Titleholders for the region (Commonwealth Native Title Act 1993).

Figure 1.3. A rock-hole managed using traditional practices involving rows of timber placed across the surface of the rock-hole, and rocks placed around the perimeter. Diameter of the rock-hole at widest point = 2.2 m.

Despite their biological uniqueness and their cultural significance, granite rock-holes have been largely neglected in both research and policy decisions. Only a handful of ecological surveys have addressed the invertebrate communities present in the rock-holes, most from Western Australia (Bayly 1997, 2000, Pinder et al. 2000). The only South Australian rock-holes that have been the focus of similar studies are those along the Eyre Highway in a region with a Mediterranean climate (Timms 2014), while those in the semi-arid and arid reaches of the state have been largely neglected. Assessments of the cultural, hydrological and biological significance, as well as impacts, were proposed in White (2009) and have been undertaken for rock-holes at seven pastoral leases in the Gawler bioregion (Jenkin et al. 2011a), but there is no legislative protection in place to safeguard them, nor have they been the focus of any significant government programs in recent years. Since these systems are rain-fed and are in regions of projected drying under all future emissions scenarios, it is likely that the impacts of climate change will cause deviations from their historical hydrological patterns (IPCC 2022a). Rock-holes are also found in regions with recent proliferations of invasive vertebrate species, many of which are known to detrimentally impact freshwater quality (Brim-Box et al. 2016, Moseby et al. 2021); all of these factors mean that rock-hole conservation security in GB is threatened. In this PhD, I seek to improve understanding of the rock-holes, the role they play in the environment (particularly as a provision of resources to local vertebrates), their invertebrate communities, and the threats that they face. Ultimately, I seek to generate knowledge that can inform conservation and management of this unique ecosystem in Australia's arid and semi-arid zones.

1.3 Techniques for assessing freshwater ecosystems

Methods used to assess freshwater ecosystems and their biota are as diverse as the ecosystems themselves. A long history involving the application of traditional ecological approaches, primarily in Europe, has shaped the field (Fediajevaite et al. 2021). In recent years, emerging technologies have become increasingly common in studies seeking to improve our understanding of these ecosystems: 1) Wildlife cameras are effective tools for recording large animals while they interact with water bodies and move about in the environment (Draper et al. 2022, Votto et al. 2022); 2) Environmental DNA (eDNA) is a recently emergent and versatile tool that can be used to detect species presence in a freshwater environment using only small volumes of water (Klymus et al. 2020, Fediajevaite et al. 2021); and 3) Climate and hydrological modelling can be used to understand the current and future hydrology of a system, allowing for predictions of how climate change will impact systems vulnerable to its impacts (Hanasaki et al. 2010). The application of these three techniques has underpinned my PhD research, and the information gained from comparative analyses provided insights that could not have been gleaned through any one method alone.

Traditional ecological approaches

Historical approaches to surveying freshwater ecosystems are increasingly being eschewed in favour of emerging techniques that are automated or non-invasive (Murray et al. 2021). Visual detection of freshwater species can be challenging and often results in generation of falsely negative data (Thomsen et al. 2012, Baltazar-Soares et al. 2022). Methods of surveying freshwater fauna that rely on trapping or capture for later identification can also be costly and time consuming (Bonar et al. 2019, Shaw et al. 2016, Dal Pont et al. 2021) and may involve ethical concerns regarding animal welfare. Where study sites are remote, critical scientific infrastructure is often unavailable and there can be significant costs associated with travel. Furthermore, the faunal inhabitants of many Australian ecosystems are relatively unknown, with high proportions of the invertebrate fauna yet to be formally described (Taxonomy Decadal Plan Working group 2018). This issue is compounded further by a decline in taxonomic expertise that has resulted in a lack of capacity for specimen identification affecting many groups of freshwater taxa (Taxonomy Decadal Plan Working Group 2018). Wildlife camera trapping, eDNA and hydrological and climate modelling are all techniques that address the shortfalls of well-established traditional techniques (Fediajevaite et al. 2021, Buckland et al. 2023).

Wildlife camera trapping

Accurately observing and recording vertebrate populations has been a major component of ecology for centuries. What once was undertaken primarily by field operatives however has been largely automated in recent decades, with the goal of limiting the presence of researchers in the field and the disturbance to wildlife that results from their presence (Buckland et al. 2023). One method of vertebrate monitoring that has seen widespread use is the application of wildlife cameras (sometimes called camera traps) (Murray et al. 2021, Votto et al. 2022). Wildlife cameras encompass a range of motion-triggered (usually by infrared) or timed cameras that record high-resolution photographs of an environment that can be categorised to generate data regarding local vertebrates (Dytkowicz et al. 2023). Wildlife cameras provide a generally non-invasive method of collecting high-quality data regarding vertebrate distribution (Murray et al. 2021), abundance (Villegas et al. 2023), organism health (Murray et al. 2021), and behaviour (Krauss et al. 2018). Wildlife cameras have been used in a range of vertebrate monitoring surveys including in the Australian arid-lands (Draper et al. 2022) and for recording vertebrate activity at arid-lands freshwater sites (Votto et al. 2022).

Historically, the limiting factor in wildlife camera surveys has been the many hours of researcher time that are often required to correctly categorise photographs and identify taxa captured. False triggers (triggers resulting from movement of vegetation, wind, camera settings) often result in hundreds to thousands of empty photographs which has historically added to the burden of wildlife camera research (Duggan et al. 2021). However, advancement in the fields of machine learning and artificial intelligence has provided a relatively time-efficient method for processing these massive collections of photographs (Sollmann et al. 2018, Farmer et al. 2022). These automated methods have improved the feasibility of ambitious wildlife camera studies, and improved reproducibility of research due to the removal of sometimes subjective photograph assessment (Duggan et al. 2021).

Environmental DNA

In recent years, high-throughput sequencing technologies have increased in accessibility, allowing for assessment of biodiversity through metabarcoding of environmental DNA (eDNA) (Fediajevaite et al. 2021). This rapidly emerging field of research allows for detection of a range of biota across a variety of habitat types via non-invasive and non-destructive sampling (Fediajevaite et al. 2021). The technique involves the collection of bulk genetic material, deposited by organisms through a range of biological mechanisms including by excretion, defecation and general shedding of tissue (White et al. 2020), from an environment such as water or soil. In freshwater habitats, eDNA metabarcoding has been seen to be particularly successful for detection of permanent occupants of ecosystems such as fishes and arthropods (Shaw et al. 2016, Klymus et al. 2020, Johnsen et al. 2020, White et al. 2020). However, in recent years it has also been shown to beuseful for detecting and measuring terrestrial visitation to freshwater bodies (Farrell et al. 2022, McDonald et al. 2023). The manner in which eDNA compares to the more traditional use of wildlife camera traps is a subject of growing research focus (Farrell et al. 2022, Johnson et al. 2023) and one that will be explored here.

In addition to its application for detecting vertebrate visitation to, and use of resources, at freshwater rock-holes (McDonald et al. 2023), eDNA also provides a means for characterising invertebrate communities that live within arid-land freshwater habitats (Perry et al. 2021, Beasley-Hall et al. 2023). Rock-hole invertebrate communities consist of a range of phyla, although the greatest contributors to biomass are crustaceans and insects (Timms 2014, Pinder et al. 2000). Due to the remote nature of the sites and a resulting lack of research, as well as the greater lack of taxonomic resolution for much of Australia's invertebrate biodiversity (Yeates et al. 2003, Austin et al. 2004, Taxonomy Decadal Plan Working group 2018, Engel et al. 2021), it is difficult to reliably identify much of the fauna present in the rock-holes (Timms 2014). As a result, eDNA provides a possible tool for ecological study of the system that is not dependent on morphologically-verified taxonomic assignments. Genes such as cytochrome oxidase I (*COI*) and 16S ribosomal RNA can be used as "barcodes" to identify invertebrates including insects, molluscs, and crustaceans, and tailored primers can be developed to target these groups for ecological assessment of the system (Weigand et al. 2019, Rimet et al. 2021).

Hydrological and climate modelling

Climate change has been increasingly identified as a key threatening process, expected to impact all ecosystems on Earth over the next century (Scheffers et al. 2016). It is expected that these changes will, among others, drive species decline and extinctions (Meireles et al. 2023), shifts in the distribution of species (Chaudhary et al. 2023, Herrera-Feijoo et al. 2023, Istifanus et al. 2023), and the proliferation of invasive species (Healy et al. 2023). The specific mechanisms by which climate change will cause these impacts are often unique to each region, habitat and species (de la Fuente et al. 2023) and generally relate to alterations in temperature ranges and frequency and intensity of rainfall. However, these mechanisms are often poorly understood despite being critical for the planning and implementation of proactive conservation and resilience programs (Araújo, et al. 2005). Climate modelling is one method used to understand how such processes might impact ecosystems and can be used to inform studies that seek to understand the potential effects of climate change.

Climate modelling involves using historical climate data for a region of interest and applying a series of mathematical models and simulations to predict future climatic behaviour under a series of scenarios (van Vuuren et al. 2011, O'Neill et al. 2016, Tebaldi et al. 2021). These scenarios incorporate data regarding the concentration of greenhouse gases that are expected to occur under varying degrees of limitation and mitigation (van Vuuren et al. 2011, O'Neill et al. 2016). Climate modelling allows for predictions to be made regarding the future climatic averages of a region, although they are limited by the temporal span and resolution of historical data (O'Neill et al. 2016).

These data can be used to inform management, or further research that seeks to understand the likely impacts of climate change on an ecosystem. For example, climate modelling can be used to predict changes in the viable range of native or invasive species that result from climate change (Artaev 2023, da Silva et al. 2023, Makki et al. 2023). The findings generated by climate models can be further tailored to the study of freshwater ecosystems when used in conjunction with hydrological models. These allow for simulations of the various inputs, outputs and movement dynamics of freshwater (Sánchez-Gómez et al. 2023) and provide a pathway for assessing the impacts of changes in land use, agriculture and climate change on freshwater systems (Arnold et al. 1998). The synergy of these techniques provides a framework for better understanding the complex effects of climate change on freshwater ecosystems that have been identified as at-risk.

The value of the rock-holes as a model system

An understanding of the impacts of climate change on hydrology, particularly for areas where water availability is already low and expected to decline further, is critical (Sánchez-Gómez et al. 2023, Scanlon et al. 2023). In addition to their own inherent biodiversity value, ephemeral granite rock-hole ecosystems provide an opportunity to better understand the broader impacts of climate change on freshwater ecosystems in arid lands by acting as a model system. Typically, the hydrology of freshwater systems is a function of their inputs and outputs (Hanasaki et al. 2010, see Figure 1.4A for an example). The complexity of these systems often makes hydrological modelling difficult due to the interplay between these factors and the difficulty involved in accurately measuring each of them (Condon et al. 2021). However, the rock-hole system assessed here has comparably few inputs and outputs (Figure 1.4B). Granite rock-holes are primarily fed by rain, with little to no evidence of any link to groundwater (Jenkins et al. 2011b), and water is lost only to evaporation and through occasional drinking by animals. As a result, the hydrological model that predicts rock-hole filling and emptying is much simpler than that for more complex systems. This makes the system ideal for small-scale studies seeking to investigate the likely impacts of climate change on freshwater ecosystems.

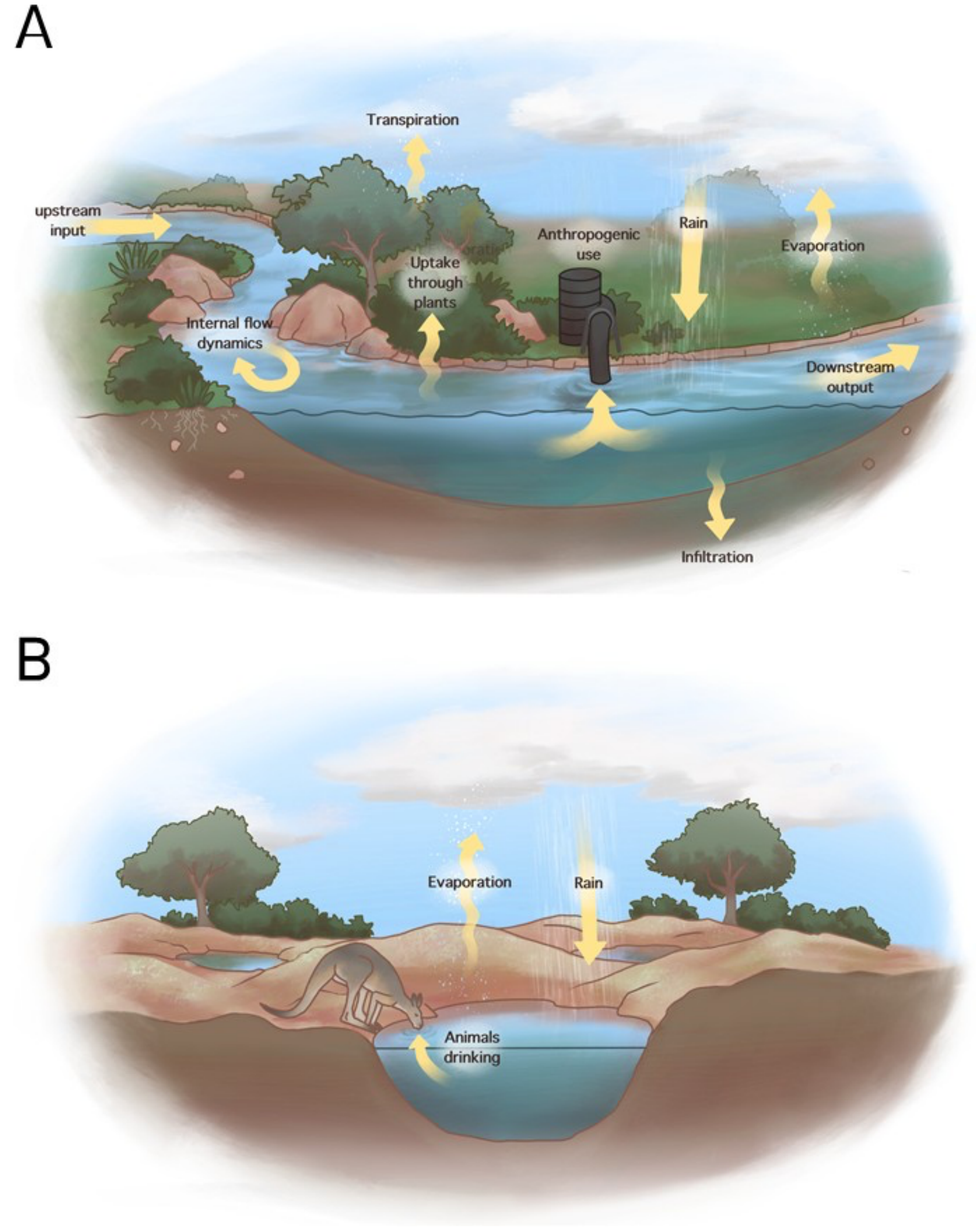


Figure 1.4. The major hydrological inputs and outputs of A) a typical freshwater river system; and B) the ephemeral granite rock-hole system. Illustration by Mollie-Rosae Slater-Baker.

1.4 Aims of the Project

The overarching aim of this PhD was to improve understanding of the ecology of ephemeral freshwater ecosystems and assess the threats they face. To address this aim, I used freshwater granite rock-holes in the Gawler bioregion. As an ecologically distinct and potentially highly valuable ecosystem, a clearer and more detailed understanding of its biodiversity value will facilitate robust conservation and management efforts, as well as potentially yielding greater biodiversity conservation outcomes in areas of the GB, in which proactive efforts are already being taken to manage these unique habitats.

The first aim of this thesis was to assess the extent to which the freshwater rock-holes are currently utilised by local vertebrates in an effort to better understand the resource value of this system. I addressed this aim using two complementary methods. Firstly, a traditional wildlife camera trapping approach was used to detect and measure vertebrate attendance at rock-holes (**Chapter 2**). Secondly, eDNA metabarcoding was used to detect vertebrate attendance at rock-holes, and its success as a monitoring technique was compared with the use of data from wildlife camera trapping (**Chapter 3**).

The second aim of this project was to document the invertebrate communities associated with the rock-hole ecosystem at Hiltaba Nature Reserve (**Chapter 4**). This has been identified as a priority by the South Australian government in White (2009), where it is suggested that sampling of rock-hole macroinvertebrates may be used to determine ecological value of rock-holes. Here I also applied eDNA metabarcoding techniques to generate broad inventories of the taxa present and assess how they varied spatially and temporally in their occurrence within rock-holes.

The third aim of this project was to investigate the potential future impacts of climate change on the rock-hole ecosystem (**Chapter 5**). This was achieved initially through hydrological and climate modelling to project the likely hydrological states of the ecosystem under a series of future climate scenarios. The results were then used to inform a series of experiments that applied these future scenarios to a culture of invertebrates sourced from rock-holes and recording the impacts on their survival and generation.

Finally, the fourth aim of this thesis involved the dissemination of findings to a series of stakeholders and the general public through a range of outreach efforts. A summary of the presentations given and resources produced throughout the PhD research has been included.

The structure of this thesis

Apart from the general introduction, the thesis that follows consists of four data chapters, a general discussion, and supplementary material. The data chapters have been formatted for publication in a scientific journal, and as such there is some repetition of content in the introductions, and to a lesser extent, the methods and discussion sections. Furthermore, the reference lists for all six chapters of this thesis have been combined into a single reference list and is included at the end of this thesis, so as to avoid unnecessary repetition. Statement of authorship forms for all chapters have been included in Appendix H.

The impact of COVID-19 on my research

The project summarised by this thesis commenced in March of 2019 and, as such, much of my research took place during the global pandemic that resulted from the spread of the COVID-19 virus. This pandemic caused many disruptions to general life and to research, particularly research that involved travel to vulnerable communities, as mine did. Apart from travel restrictions imposed by the South Australian Government, The University of Adelaide imposed additional restrictions to travel. For many months, it was possible to travel to field sites as a single individual, but this was not possible for remote area work which under health and safety rules required at least one other person to be involved. As a result, both the timing of my field trips, and the temporal span at which I could apply my methods, were more limited than I originally envisioned.

Statement of Authorship

Title of Paper	The only free drink in the desert vertebrate visitation to freshwater rock-holes 1how1 dependence on ephemeral water bodies
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Currently under review with Ecology and Evolution

Principal Author

Name of Principal Author (Candidate)	Brock A Hedges
Contribution to the Paper	Led data curation, methodological design, data analysis and production of manuscript
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1'1-/07-/ 02.3

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii permission is granted for the candidate in include the publication in the thesis; *and*

iii the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2. The only free drink in the desert: vertebrate visitation to freshwater rock-holes shows dependence on ephemeral water bodies

2.1 Abstract

Throughout semi-arid and arid Australia, accessible freshwater is often a limiting resource and an important consideration in the conservation of native species, and the management of invasive species. With climate change, many rain-fed freshwater systems are likely to experience divergences from historical norms. In the Gawler Ranges of South Australia, freshwater granite rock-holes represent an important and persistent source of water which we investigated as a resource for vertebrate communities. Using sites at Hiltaba Nature Reserve in the Gawler bioregion a suite of vertebrate species (n = 32) were recorded visiting a series of freshwater granite rock-holes employing motion-triggered wildlife cameras (eight rock-holes, 205 trapping days). This study examines their visitation patterns and whether species visitation varied in temporal and spatial frequency. Total visitation events increased with prolonged periods without local rainfall. Different species displayed distinct patterns in the time-of-day of visit. Whilst species richness was relatively consistent across rock-holes, those at higher elevations displayed a significantly higher proportion of visitation events by invasive species, namely goats, compared with rock-holes at lower elevations. Under future climate scenarios of warming and drying, we suggest that vertebrate dependence on freshwater available at granite rock-holes will increase. A series of management recommendations including increased invasive species management in the region to alleviate pressure on the available water are proposed.

2.2 Introduction

Southern Australia's granite rock-holes are a unique ephemeral freshwater system of biological and cultural value (Bayly 1997, Bayly 1999a, Timms 2014). Having formed over millennia through chemical weathering processes (Twidale and Corbin 1963, Twidale and Romani 2005, Timms and Rankin 2016), they provide locations of freshwater storage following seasonal rainfall events. The confined water is only present temporarily, evaporating entirely over the weeks and months following rainfall events. Sometimes referred to by the indigenous term 'gnammas,' a Nyungar word originally used by the Noongar people of south-west Western Australia, these rock-holes are culturally significant to many Australian First Nations peoples (Bayly 1999a). When wet, the rock-holes are a habitat for a complex community of freshwater phytoplankton, aquatic crustaceans and insects (Bayly 1997, Pinder et al. 2000, Timms 2014, Hedges et al. 2021), aquatic plants, and occasionally freshwater vertebrates such as frogs (Hedges et al. 2021). Much of Australia is characterised by arid and semi-arid climates (Jenkin et al. 2011b), and as a result, these rock-holes are a source of freshwater in arid landscapes that are typically very limited in surface freshwater (Bayly 1999a). Due to surface freshwater often being a key limiting resource for arid-zone animals (Noy-Meir 1973, Porporato et al. 2002, Votto et al. 2022), and a factor in constraining distribution and abundance (Fisher, Lindgren et al. 1972, Abdu et al. 2018), it is likely that many native Australian species are behaviourally adapted to capitalise on this resource when it is available. A suite of local vertebrate species is anecdotally known to attend these rock-holes throughout much of southern Australia (Nature Foundation 2023), however, the true diversity of species that access this freshwater is not currently known.

As ephemeral sources of freshwater, rock-holes are entirely rain-fed with little/no geological evidence of groundwater input (Jenkin et al. 2011b). Thus, they are entirely dependent on local weather patterns for their persistence. Any deviation from historical average annual rainfall, temperature and humidity are therefore likely to impact the wet-dry regime of the rock-holes. The Gawler bioregion, South Australia, contains many rock-holes (Figure 2.1), although most are likely undocumented. Characteristically arid, this is an area of projected drying climate over the next 100 years, with decreases in average annual rainfall predicted under all emission scenarios (IPCC 2022a). It is likely that a change in the seasonality of rainfall will also occur, with shifts from predominantly winter rainfall events, to less frequent but higher intensity summer rainfall (CSIRO 2018). Disruption to the historical wetting-drying regime may also impact water quality as a resource for the arid-zone ecosystem, including vertebrates (Votto et al. 2022). Since water quality is regulated by the filter feeding activity of invertebrates (Coughlan 1969, Atkinson et al. 2013, Buelow and Waltham 2020, Simeone et al. 2021), any change in the composition of the communities associated with climate change are likely to impact provision of critical ecosystem services. Therefore, the long-term security of the rock-hole ecosystem, its biodiversity (including endemic species), and its resource value is of concern with projected climate change.

Over the last 200 years, a large number of invasive terrestrial vertebrate species have been introduced into Australia. Indeed, predatory species, primarily feral cats (*Felis catus*) and red foxes (*Vulpes vulpes*), have spread to occupy up to 99% and 80% of mainland Australia, respectively (Commonwealth of Australia 2008, 2015b, Stobo-Wilson et al. 2022). Immediately prior to introduction, Australia had no native predators that were equivalent to cats and foxes. These species have drastically altered the composition and abundance of small vertebrate species present in mainland Australia (Woinarski 2015) and are implicated in the extinction of at least 20 native mammal species with a mass between 0.35 and 5.5 kg (Johnson and Isaac 2009, Woinarski 2015). Similarly, invasive herbivores have been successful at colonizing Australia's interior. Medium and large-bodied ungulates including goats (*Capra hircus*), pigs (*Sus scrofa*), water-buffalo (*Bubalis bubalis*), horses (*Equus caballus*) and camels (*Camelus dromedarius*) have been documented throughout Australia (Commonwealth of Australia 2008, 2010, 2011a, 2011b, 2017). The European rabbit (*Oryctolagus cuniculus*) has also become well established, and is another source of ecological pressure throughout Australia's semi-arid and arid zones (Commonwealth of Australia 2016, Cooke 2020, Finlayson et al. 2022). It is likely that invasive species have increased their access to arid-lands surface freshwater systems, accompanied by increases in disturbance and degradation of freshwater sites, as documented for invasive camels (Brim Box et al. 2016).

Dramatic changes in landscape management have also led to increases in the abundance of many native herbivore species such as the western grey kangaroo (*Macropus fuliginosus*) and the red kangaroo (*Osphranter rufus*) (Read et al. 2021). Artificial water points such as farm dams and bores have increased the accessibility and availability of freshwater, and the widespread removal of the dingo (*Canis lupus dingo*) from southern and eastern Australia has removed top-down predator pressure for small to medium-sized vertebrates. Rare native small mammals and birds are thought to be in decline throughout much of arid Australia, but a lack of high-quality, long-term datasets has led to uncertainty on the extent of this issue (Wilson et al. 2017). Freshwater sites in the Australian arid-zone are known to be of importance to native species (Votto et al. 2022), however, information on visitation rates of native and non-native species to such sites are largely absent. For effective management of arid-land ephemeral freshwater systems, it is important that a better understanding is developed of the degrees to which these sites are currently accessed by vertebrate species, both native and introduced/invasive.

Wildlife cameras can document the presence, movement and behaviour of terrestrial vertebrate species (Krauss et al. 2018), including in arid-regions (Bragato et al. 2022, Votto et al. 2022). Advancements in the field of machine learning and automation have also advanced the field by improving the feasibility of processing large wildlife camera datasets without laborious manual image processing (Sollmann 2018, Farmer et al. 2022). Wildlife cameras are therefore a viable option for understanding how vertebrates use arid-lands resources and have successfully been used to measure bird visitation to potential food sources in the Gawler bioregion (Draper et al. 2022).

Using wildlife cameras for image capture and machine learning automation to quantify vertebrate species diversity and attendance at rock-holes in the Gawler bioregion we aimed to a) record species visitation to the granite rock-holes and determine whether species vary in their visitation habits; b) determine whether visitations were influenced by recent weather events such as rainfall; c) identify the impact of rock-hole elevation upon species richness and the proportion of visitation events attributed to invasive species; d) investigate patterns in the visitation of invasive and introduced species such as goats, foxes, feral cats and rabbits; e) explore the validity of machine learning for processing wildlife camera datasets; and f) provide recommendations regarding the conservation and management of the freshwater granite rock-holes at Hiltaba Nature Reserve.

2.3 Methods

Site description

Hiltaba Nature Reserve (HNR) is a large ex-pastoral property situated near the Gawler Ranges National Park, to the north of South Australia's Eyre Peninsula (Figure 2.1C). Previously a pastoral sheep property, approximately 78,000 ha in area, HNR has been managed for conservation outcomes by the Nature Foundation since its acquisition in 2012. Primarily composed of a series of rolling granite hills, HNR comprises numerous habitats, with grasslands, woodlands, and rocky outcrops supporting local plants and animals known to be in decline elsewhere. The Nature Foundation has enacted a series of conservation programs that aim to improve biodiversity management at HNR. This has included the removal of pastoral species (primarily sheep), the implementation of pest control measures (targeting goats, cats, and foxes), and other small-scale projects such as decommissioning of artificial water points and weed removal.

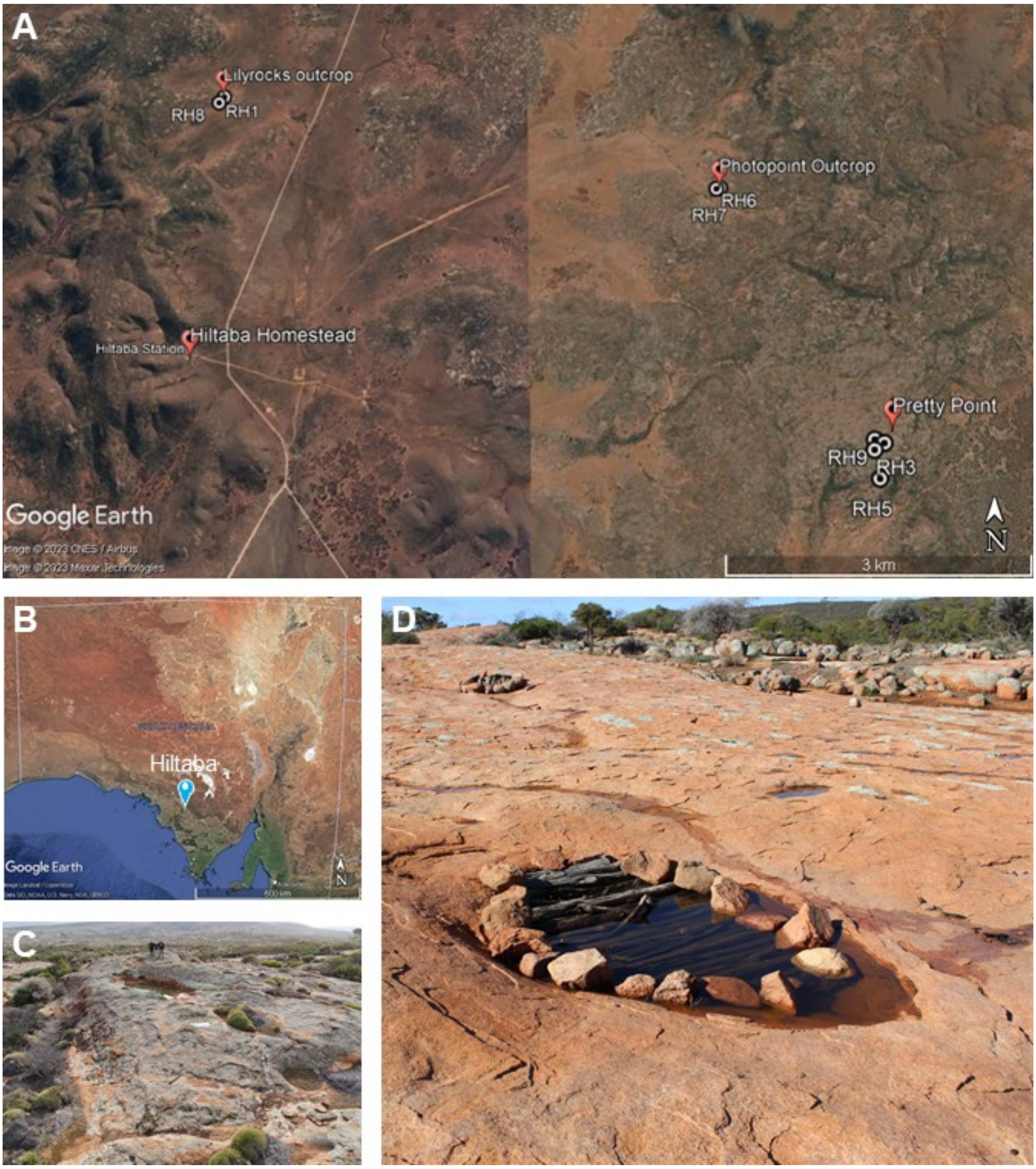

Figure 2.1. A) The locations of the eight rock-holes surveyed with wildlife cameras during 2020; B) The location of Hiltaba Nature Reserve in South Australia; C) Rain-filled granite rock-holes at the ‘Pretty Point’ outcrop at Hiltaba Nature Reserve; D) A rain-filled granite rock-hole on the ‘photopoint outcrop’, diameter of the rock-hole at widest point = 2.2 m.

The granite hills present throughout much of the northern Eyre Peninsula, and in the landscape at HNR, are entirely exposed in many locations, resulting in surface-level granite outcrops of varying morphology. Generally, the flat outcrops contain depressions which provide an impermeable location for storage of water and sediments, following rain.

Wildlife cameras

During November 2019, 70 rock-holes were observed across nine outcrops at HNR. A subset of eight rock-holes was selected across three outcrops for further study (Table 2.1). Rock-holes were selected on the basis of accessibility and to account for varying sizes, types: deep pits with near vertical walls or shallow pans with gently sloped walls (as classified in Timms (2014)) and elevation. Eight motion-triggered wildlife cameras (Browning Dark Ops Pro XD) were deployed at each of the eight rock-holes with standing surface freshwater, strapped to a series of star-droppers hammered into bare soil surrounding each outcrop. Cameras were arranged approximately 1 m away from the outcrop edge, and 1 m above the ground. The cameras were deployed between 8–11 July 2020, and were collected between 8–9 June 2021. Two of the eight rock-holes had been subjected to ongoing management practices by the Traditional Owners and the Gawler Ranges Aboriginal Corporation (Figure 2.1D), which involve the placement of a ring of large rocks (of the same granite as the outcrop) around the lip of the rock-hole, as well as a series of dry logs placed uniformly parallel across the surface of the water. Wildlife cameras were programmed to take a high-resolution photograph immediately following movement sufficient enough to trigger an infra-red sensor. Visitation was recorded as a series of images from which vertebrate species could be identified. Field methodologies were reviewed and approved by the University of Adelaide animal ethics committee.

Table 2.1. Summary of the HNR granite rock-holes surveyed with wildlife cameras between July 2020 and June 2021

ID	Latitude	Longitude	Elevation	Type	Camera orientation
RH1	-32.12802354	135.0721868	217	Pan	S
RH2	-32.16615383	135.148955	312	Pit	N
RH3	-32.167106	135.1487941	310	Pan	N
RH5	-32.16967785	135.1490754	268	Pan	S
RH6	-32.13945114	135.1349829	237	Pit	W
RH7	-32.13954573	135.1345149	235	Pit	N
RH8	-32.12880215	135.0716203	216	Pan	S
RH9	-32.16648104	135.1499659	311	pan	N

Data processing and analysis

Wildlife camera photographs were processed using two methods. To separate false triggers from images with animals, the MegaDetector machine learning algorithm (Microsoft AI for Earth 2020) was used to bulk process and categorize images through the MegaDetector desktop application v0.0.2 (Gyurov 2022). Confidence thresholds were set to 75%, and all other settings were left at default. Subsequent assignment of species identifications was undertaken manually. A subset of the data (five wildlife cameras) were assessed manually by four student volunteers (see Acknowledgements) using Camelot version 1.6.16 (Hendry and Mann 2018), as part of an undergraduate project to test the validity of the MegaDetector identification method.

All statistical analyses were performed in R version 4.2.2 (RCoreTeam 2013). Animals observed in the vicinity of rock-holes were assumed to be visiting them to access water, regardless of whether the photograph displayed an animal drinking from a rock-hole. A trap event window of 30 min was applied to the dataset (Votto et al. 2022) using *Lubridate* (Grolemund 2011). This assumed that successive photographs containing the same species of animal near a rock-hole within 30 min of the first photograph at that rock-hole were recording the same visitation event in order to limit the impact of temporal pseudoreplication. Relative abundance index (RAI) (Sollmann 2018) was calculated using the following equation:

$$RAI = \frac{D}{TN} x\ 100$$

Where D was equal to the number of detections for a species and TN was equal to the total number of days during which the wildlife camera was deployed at the site in question. RAI as an index of abundance is widely used where it is preferred for its greater accuracy in indicating site use compared to number of photographs alone (Sollmann 2018, Farmer et al. 2022). An assumption was made that animals not detected beyond the field of view of the camera were not engaging in rock-hole visitation and could be discounted from subsequent analyses. This decision was based on the parameters that the wildlife cameras used in this study captured the entirety of our study area and that RAI does not incorporate a measure of detectability. Rarefaction plots were generated using the *iNEXT* package (Chao et al. 2014) to visualise the degree to which sampling effort allowed us to estimate the likely total number of species that were visiting rock-holes at HNR. Density plots and circular distribution plots were generated using *ggplot2* (Wickham 2016). To explore the relationship between the number of days since a significant rainfall event and visitation, a series of models were tested including Poisson generalised linear models and negative binomial generalised linear models. A generalised additive model (GAM) (Hastie and Tibshirani 1990) with a cubic spline smoother was selected as the relationship observed was nonlinear. These models were fitted using the *mgcv* package (Wood 2001). Rainfall data were extracted from the Bureau of Meteorology (Australian Government - Bureau of Meteorology, 2023). A significant rainfall event was defined as any day with rainfall greater than 0.9 mm.

2.4 Results

Wildlife camera data

Over the one-year period of this study, the eight wildlife cameras deployed at rock-holes generated a total of 331 trapping days (Table 2.2). Data for 205 days were used for statistical analyses, with all cameras after 30 January 2021 excluded due to camera failures resulting in some lost data beyond this period. In total, 50,269 photographs were included. After initial filtering using MegaDetector, 43,136 photographs were discarded as having resulted from false triggers with no animals present. Of the remaining 7,128 photographs, 4,855 contained animals, with 32 species of animals being observed: 18 bird species in 5,557 photographs, 10 mammal species in 4,134 photographs, and four squamate reptile species in 20 photographs (see Figure 2.2 for some example photographs).

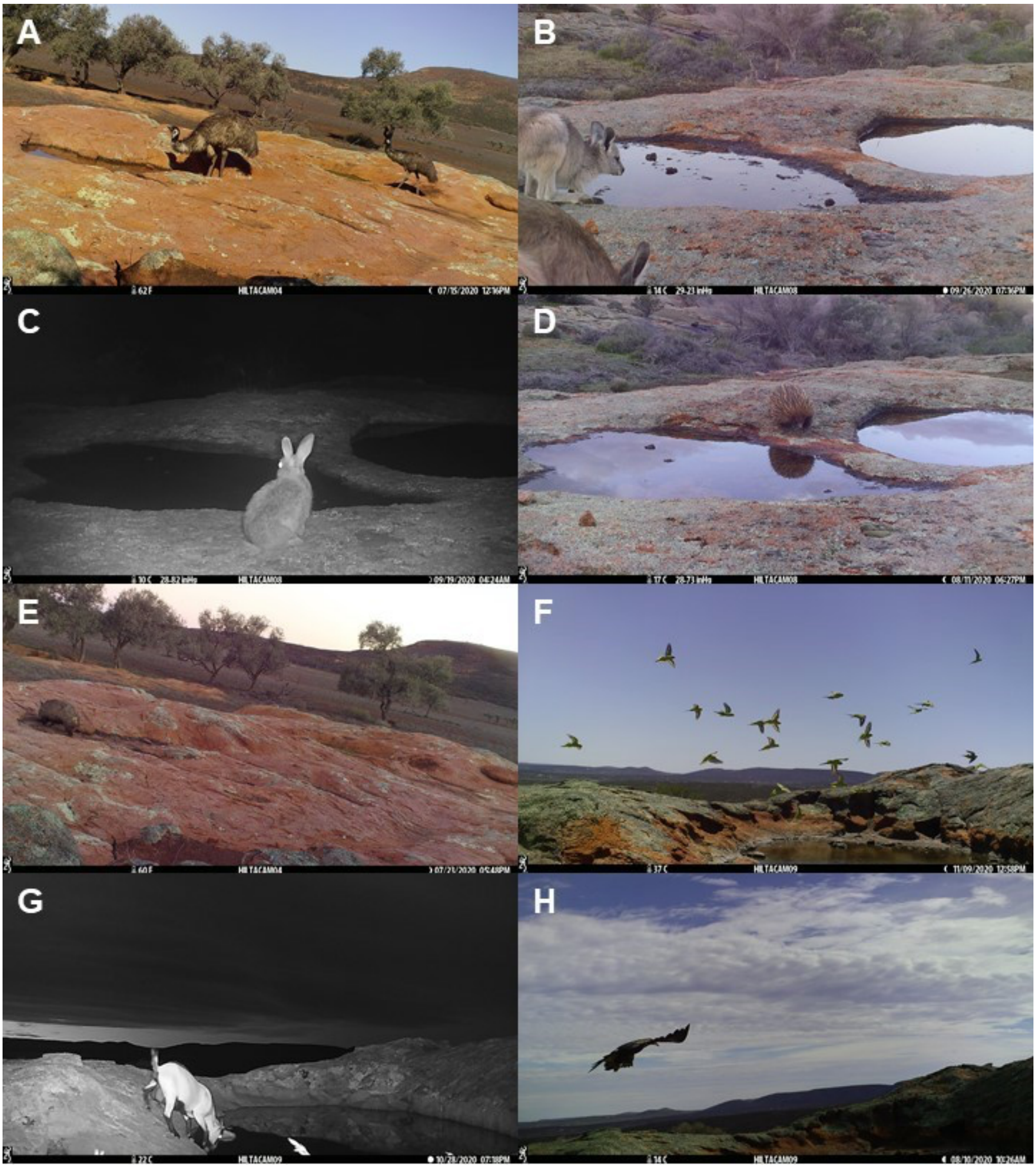

Figure 2.2. Vertebrates captured by wildlife camera photographs visiting freshwater granite rock-holes at Hiltaba Nature Reserve. A) emu (*Dromaius novaehollandiae*); B) common wallaroo (*Macropus robustus*); C) European rabbit (*Oryctolagus cuniculus*); D) short-beaked echidna (*Tachyglossus aculeatus*); E) southern hairy-nosed wombat (*Lasiorhinus latifrons*); F) budgerigars (*Melopsittacus undalatas*); G) feral goats (*Capra hircus*); H) wedge-tailed eagle (*Aquila audax*).

Table 2.2. Summary statistics of MegaDetector output vs. subsampled dataset. RH refers to rock-hole, followed by each rock-hole's numerical identifier. Negative numbers indicate where MegaDetector discarded images which included animals in addition to false triggers. * denote cameras that were subsampled and processed manually.

	RH1	**RH2***	**RH3**	**RH5**	**RH6***	**RH7***	**RH8***	**RH9***	**Total**
n. photos recorded	8,528	747	9994	17212	4439	2491	1884	4969	50,264
n. photos excluded by MegaDetector	8154	359	9548	16435	2773	968	1075	3824	43,136
n. photos excluded manually	76	170	308	731	-31	-182	80	1121	2,273
n. photos with animals present	298	218	138	46	1697	1705	729	24	4,855

Identification to species level was possible for most photographs (Table 2.3.). Red kangaroos (*O. rufus*), western grey kangaroos (*M. fuliginosus*) and common wallaroos (*M. robustus*) were distinct in day-time photos, but in low-light and night-time photos, differentiation between the three species was often difficult. Australian ravens (*Corvus coronoides*) and little ravens (*Corvus mellori*) were distinct in most photos, with a limited number of photos where confidence in species differentiation was low. In these cases, further analyses were undertaken with the data combined, rather than as separate species. Replication across HNR was sufficient to capture an estimated 86% of the true detectable visitation to rock-holes (Figure 2.3).

Table 2.3. Number of wildlife camera photographs recorded per rock-hole site and species. * denotes non-native species.

Species	RH1	RH2	RH3	RH5	RH6	RH7	RH8	RH9	Total	RAI
Birds										
Wedge-tailed eagle (*Aquila audax*)	1	8	0	7	0	3	0	0	19	9.3
White-winged chough (*Corcorax melanorhamphos*)	0	0	0	0	0	11	0	0	11	5.4
Australian raven (*Corvus coronoides*) and little raven (*Corvus mellori*)	12	15	4	13	44	381	3	0	472	230.2
Australian magpie (*Gymnorhina tibicen*)	7	10	10	3	1	11	28	3	73	35.6
Grey butcherbird (*Cracticus torquatus*)	0	0	0	0	0	0	2	0	2	1.0
Emu (*Dromaius novaehollandiae*)	132	3	1	0	917	749	290	0	2092	1020
Galah (*Eolophus roseicapilla*)	0	0	0	0	10	70	0	0	80	39.0
Brown falcon (*Falco berigora*)	0	0	0	1	0	0	0	0	1	0.5
Magpie-lark (*Grallina cyanoleuca*)	0	0	0	0	4	2	0	0	6	2.9
Pink cockatoo (*Lophochroa leadbeateri*)	0	0	0	0	0	3	0	0	3	1.5
Yellow-throated miner (*Manorina flavigula*)	0	0	0	0	3	0	0	0	3	1.5
Budgerigar (*Melopsittacus undalatas*)	0	2	0	0	0	0	0	0	2	1.0
Crested pigeon (*Ocyphaps lophotes*)	0	0	0	0	6	3	0	0	9	4.4
White-browed babbler (*Pomatostomus superciliosus*)	0	0	1	0	0	0	0	0	0	0.5
Fairy martin (*Petrochelidon ariel*)	0	1	0	0	0	0	0	0	1	0.5
Tree martin (*Petrochelidon nigricans*)	0	2	0	0	0	0	0	0	2	1.0
Common bronzewing (*Phaps calcoptera*)	0	0	0	0	0	2	0	0	2	1.0
Mammals										
Feral goat (*Capra hircus*) *	30	127	68	10	68	32	12	15	362	176.6
Chocolate wattled bat (*Chalinolobus morio*)	0	2	0	0	20	3	1	0	26	12.7
Cat (*Felis catus*) *	0	1	0	0	2	1	0	0	4	2.0
Southern hairy-nosed wombat (*Lasiorhinus latifrons*)	0	0	0	0	16	4	30	0	50	24.4
Kangaroos and wallaroos (*Macropus fuliginosus*, *Macropus robustus*, *Osphranter rufus*)	116	47	48	12	569	429	357	4	1582	771.7
Domestic rabbit (*Oryctolagus cuniculus*) *	0	0	2	0	33	0	2	0	37	18.1
Short-beaked echidna (*tachyglossus aculeatus*)	0	0	3	0	0	0	0	1	4	2.0
Red fox (*Vulpes vulpes*) *	0	0	0	0	1	1	0	0	2	1.0
Reptiles										
Peninsula dragon (*Ctenophorus fionni*)	0	0	1	0	0	0	0	0	1	0.5
Shingleback lizard (*Tiliqua rugosa*)	0	0	1	0	0	0	4	1	6	2.9
Sand Goanna (*Varanus gouldii*)	0	0	0	0	2	0	0	0	2	1.0
Unidentified snake species	0	0	0	0	1	0	0	0	1	0.7
Total detections	298	218	138	46	1697	1705	729	24	4855	

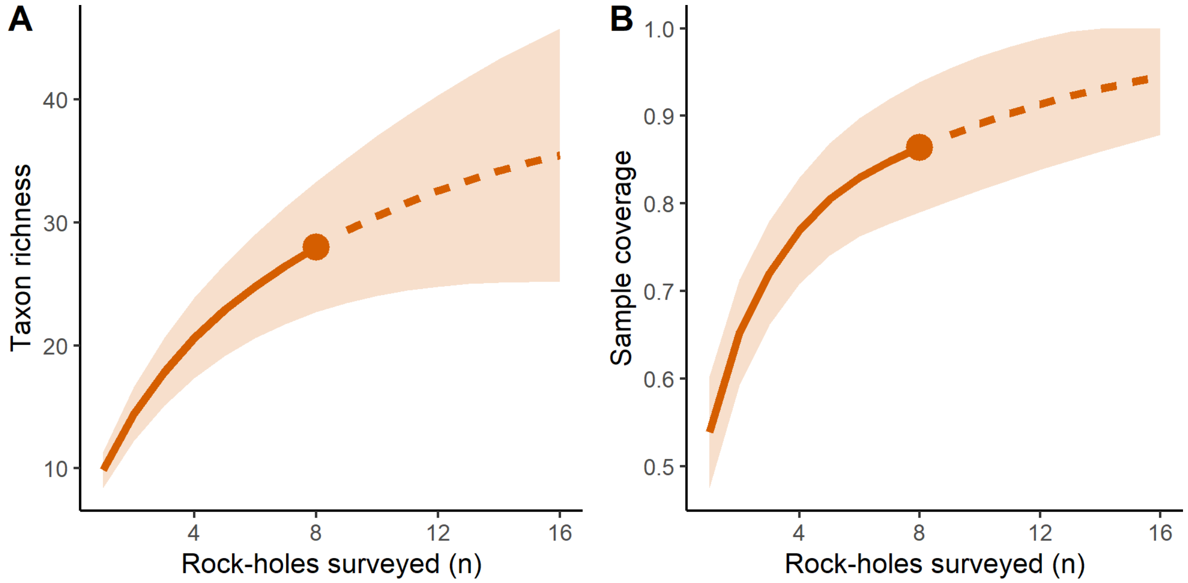

Figure 2.3. A) rarefaction curves with increasing number of rock-holes surveyed with wildlife cameras showing observed taxon richness (unbroken line, maximum = 28), extrapolated taxon richness (broken line) and 95% confidence intervals (shading); B) rarefaction curves with increasing number of rock-holes surveyed with wildlife cameras showing sample coverage (unbroken line, maximum = 0.8643), extrapolated taxon richness (broken line) and 95% confidence intervals (shading).

Visitation patterns

The four most abundant groups observed in the wildlife camera photographs were macropods (comprising three species of kangaroo/wallaroo), *Dromaius novaehollandiae* (emus) *Corvus* spp. (crows and ravens) and *Capra hircus* (feral goats). These species were observed throughout the study period, but exhibited notable peaks in their visitation in early-August, late-September, and mid-November (Figure 2.4). These peaks corresponded with periods of notable dryness, with each being preceded by >2 weeks of little to no local rainfall.

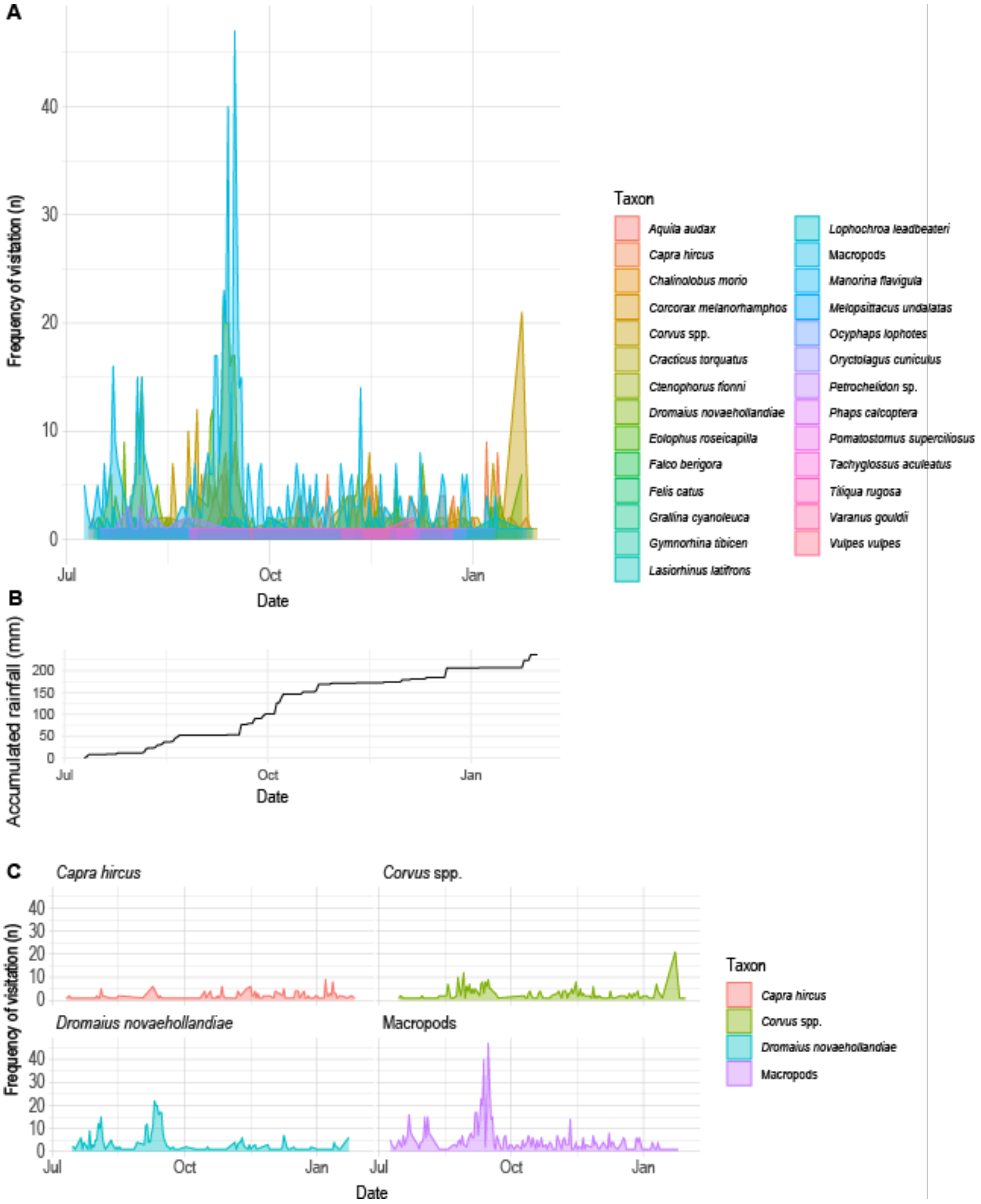

Figure 2.4. A) Density plots indicating frequency of visitation throughout the study period for 27 genera of vertebrates; B) accumulated rainfall over the same period; C) density plots indicating frequency of visitation for the four most frequently photographed vertebrate groups.

Generalised additive models showed that there was a positive correlation between the number of days since a significant rainfall event and the number of visitation events for crows and ravens (p-value 6.33e-06 ***), emus (p-value <2e-16 ***) and macropods (p-value <2e-16 ***). No effect was detected for goats (p-value = 0.681) (Figure 2.5, see appendix Table D1 for summary statistics).

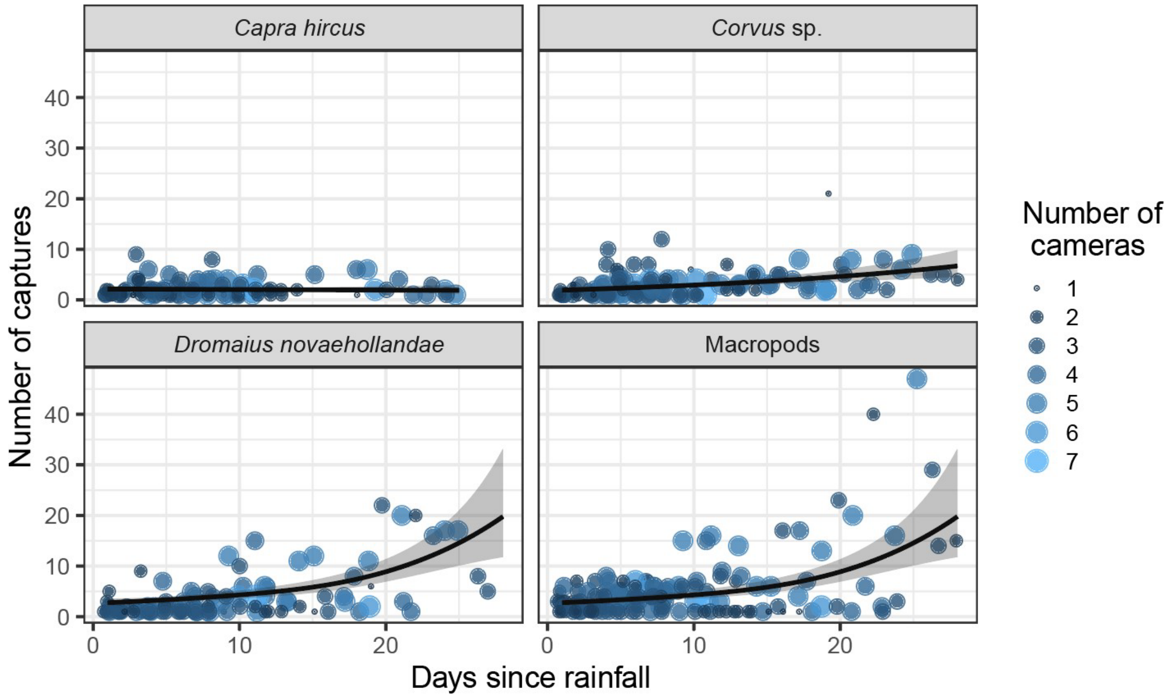

Figure 2.5. Estimated response in visitation (number of captures) by goats (*C. hircus*), crows and ravens (*Corvus* spp.), emus (*D. novaehollandae*), and kangaroos and wallaroos (*macropods*) to the number of days since a significant rainfall event (0.9 > mm) modelled with generalised additive models. The grey bar around each curve denotes the 95% confidence interval. A summary of GAM model statistics can be seen in appendix Table D1. Residual plots for each model can be seen in appendix Figure D1.

The time of day at which animals visited rock-holes varied notably between genera (Figure 2.6). Kangaroos and wallaroos (macropods*)* were observed more frequently at dawn and dusk than throughout the day and overnight. Crows and ravens (*Corvus* spp.) were observed almost exclusively during daylight hours. Goats (*C. hircus*) were mostly observed during the day, with occasional overnight occurences and emus (*D. novaehollandiae*) showed no pattern in their attendance.

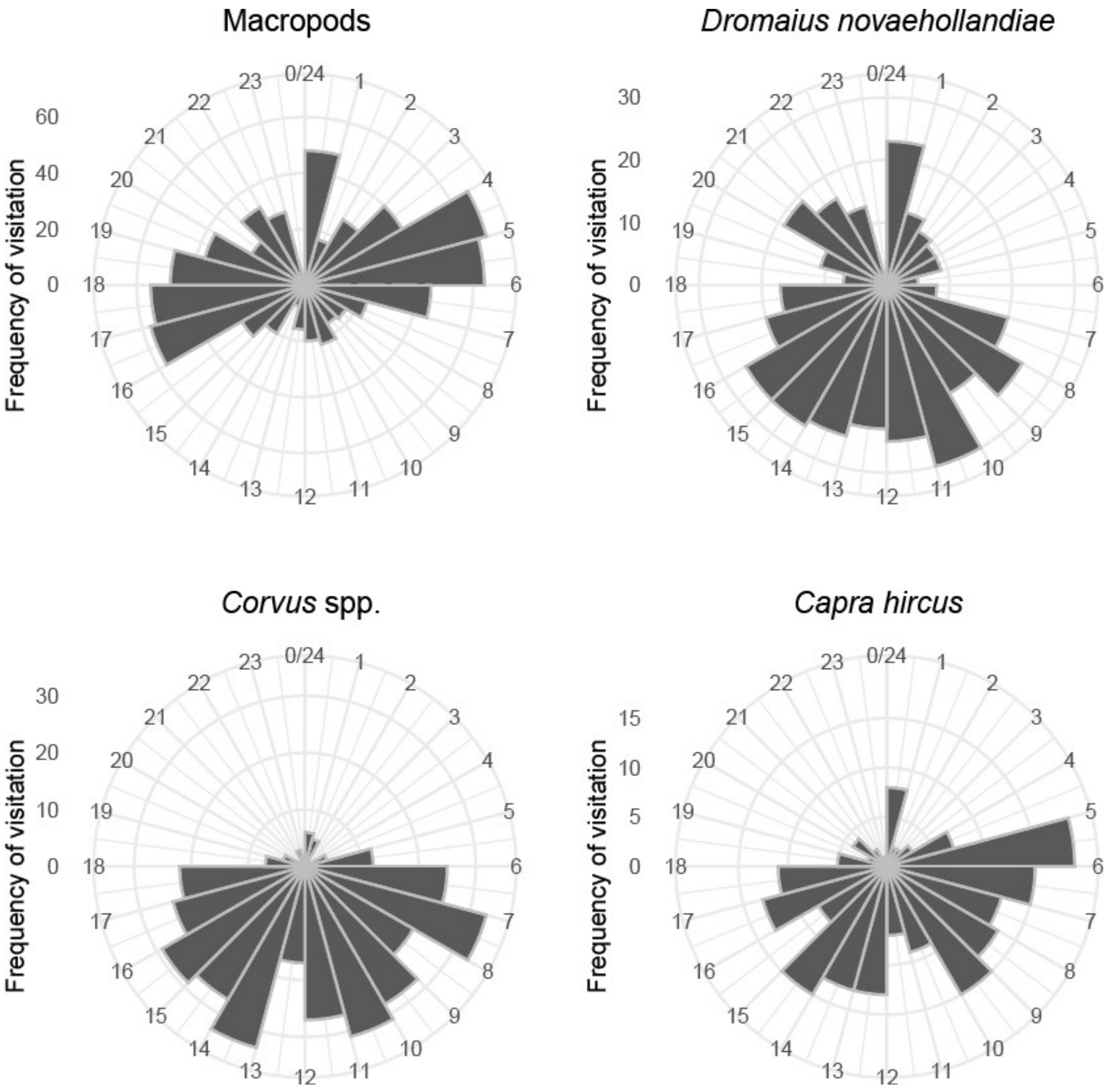

Figure 2.6. Circular distribution of vertebrate visitation patterns for the four most frequently observed animal groups attending rock-holes at Hiltaba Nature Reserve: A) *Macropus* (comprising three species of kangaroo/wallaroo); B) *Dromaius novaehollandiae* (emus); C) *Corvus* spp. (crows and ravens); and D) *Capra hircus* (feral goats). Bar lengths represent cumulative number of visitation events for each hour across the entire wildlife camera study period. See appendix Figure D2 for circular distribution patterns for all other taxa.

There was no relationship between the number of species detected visiting a rock-hole and the altitude of the rock-hole (Figure 2.7A). However, the proportion of events that involved visitation by invasive species sharply increased with altitude (Figure 2.7B). This was due primarily to a very high proportion of high-altitude records including feral goats.

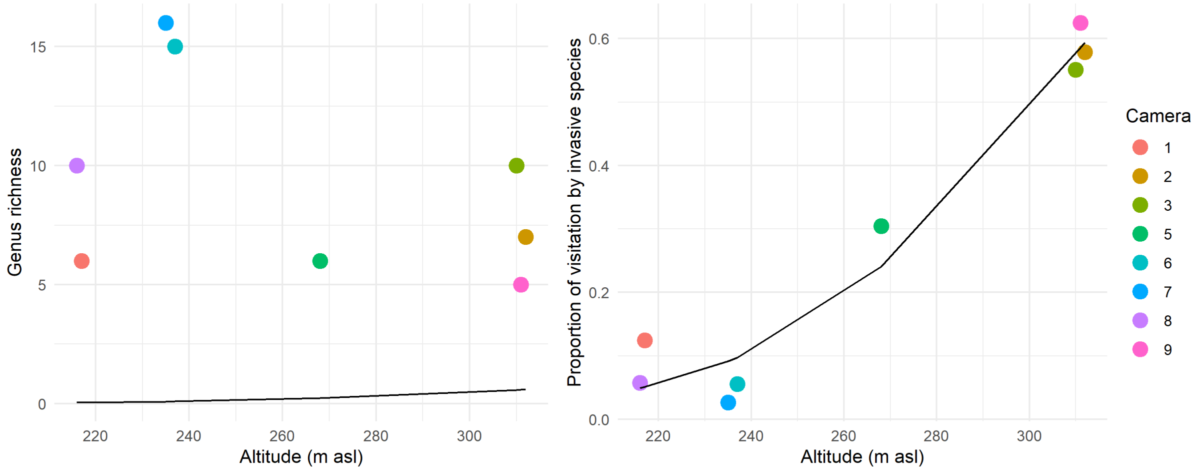

Figure 2.7. A) Species richness as observed at each site via wildlife camera photographs along an altitudinal gradient; B) Proportion of records that involved visitation by invasive species along an altitudinal gradient. Black lines indicate a Beta regression fit to altitude and genus richness/proportion of visitation by invasive species.

2.5 Discussion

Here we present the first detailed analysis of the suite of vertebrates that access freshwater granite rock-holes in the Gawler bioregion. Kangaroos, wallaroos, emus, crows, ravens and goats accounted for the majority of visitation, with temporal visitation patterns varying among taxa. Whilst species richness was relatively consistent across rock-hole locations, the proportion of visitation events that were attributed to invasive species increased with elevation. Further, the frequency of visitation increased with prolonged periods without local rainfall, while species displayed distinct patterns in the time-of-day of visitation. With projected scenarios for arid Australia involving warming and drying as a result of climate change, it is likely that vertebrate dependence on the rock-holes and similar ephemeral freshwater bodies will increase. Our study design captured 86% of the species that were likely to be detected through the use of wildlife cameras (Figure 2.3), but greater spatial replication would likely have allowed for improved confidence in detectability of the full suite of vertebrates that attend the rock-holes at HNR. The years immediately prior to the commencement of this study had been characterised by a drought throughout most of Australia, with average rainfall in the region measuring 150.6 mm p/a. However, this drought ended during 2020 and the years of 2020-2021 occurred over a multi-year La Niña (Gillet and Taschetto 2022), which resulted in greater than average rainfall across much of the continent (298.5 mm in 2020 and 382.8 mm in 2021). As such, it is likely that our results were influenced by these conditions. A transition from a water-stressed environment to a period of relatively high water availability and resulting improvement of ecosystem health may be contributing to trends observed here. Temporal replication over successive years would improve the validity of analyses in predicting the impacts that climate change might have on this and similar ecosystems.

Patterns of attendance at the sites varied notably among taxa. Macropods are known to be most active at dawn and dusk (with some variability in activity between seasons) (Southwell 1987, Hill et al.

1988), and this was reflected in their visitation frequency. No temporal pattern could be detected for emu attendance, although emus were noted to spend the greatest duration at rock-holes, with many consecutive triggers (i.e. images) containing emus having been excluded when filtering data temporally to exclude potential pseudoreplication. In addition to attendance by native species, granite rock-holes were also frequently visited by invasive vertebrates, with feral goats being recorded at all rock-holes as well as rarer occurrences of feral cats, foxes and rabbits.

Whilst the results of our study showed no correlation between the elevation of a rock-hole and the number of species that attended it, the proportion of detections that included invasive species (primarily goats) was greater for rock-holes at a high elevation (>280). This is likely due to goats having a high dispersive capacity, their preference for rocky range habitats, as well as their ability to climb steep slopes that are a disadvantage to other species less adapted to climbing (Letnic et al. 2015). McDonald et al. (2023) speculated that some native Australian mammals may avoid higher altitude rock-holes due to increased risk of injury and/or predation, and our findings may support this. Invasive mammals (primarily ungulates) negatively impact surface freshwater bodies throughout much of Australia via disturbance and by dramatically increasing nutrient input through their faeces (Doupe et al. 2010, Brim Box et al. 2016). Increased feral goat activity is often closely linked to artificial watering points on pastoral properties, but they have also been recorded visiting natural freshwater bodies, such as rock-holes (Moseby et al. 2021). Additionally, goats will congregate at these natural freshwater bodies, and when unmanaged, populations can reach unsustainable densities. High densities of goats can have a significant impact on sensitive cultural heritage sites and surrounding vegetation by heavy browsing, preventing regeneration of palatable species resulting in altered plant communities (Lethbridge 2016, Moseby et al. 2021). Although intensive goat control has occurred at Hiltaba, resulting in the removal of more than 12,000 goats in 10 years and significant improvement in vegetation and rock-hole water quality, the impacts caused by goats throughout the Gawler bioregion in areas receiving less intensive management are likely to contribute to a further decline in the viability of the rock-hole habitat through threatening processes such as water fouling. This will likely cause further stress on the distinct invertebrate and plant communities that inhabit the rock-holes (Timms 2014, Timms 2017), and as a freshwater resource for the native vertebrates that visit them.

As mentioned above, we found that attendance at rock-holes increased during periods where there had been little to no recent rainfall (< 5 mm for > 2 weeks). This suggests animals move across the landscape in search of sources of potable drinking water, with the granite rock-holes at Hiltaba being some of the only sources of surface freshwater in an otherwise dry landscape. No notable temporal pattern could be detected in goat attendance, likely due to their herd migration behaviour, which sees large groups moving continuously throughout the landscape (Moseby et al. 2021). We suggest that their visitation at rock-holes is relatively stochastic in its temporal occurrence. Crows and ravens

(*Corvus* spp.) are diurnal in their scavenging activity (Bragato et al. 2022), and as such their visitations occurred almost exclusively during daylight hours. Assessment of temporal visitation were limited for rarer taxonomic groups due to smaller sample sizes.

Recent research suggests that 1 in 6 species will face extinction due to the impacts of unmitigated climate change, with Australia noted as being amongst those regions most at risk (Urban 2015). It is likely that the value of freshwater at granite rock-holes will increase in its conservation value during such periods. As the impacts of climate change are felt over the coming century, drier conditions across southern Australia are expected (IPCC 2022a), and it is likely that periods of low or no rainfall will increase in frequency. This will result in further stress to unique habitats such as the rock-holes examined here, and the diversity of animals that depend on them, as local rainfall accounts for 100% of their hydrological input. Significant global action to mitigate against climate change as outlined in IPCC (2022b) is likely the only management option that may alleviate this stress. Artificial irrigation of rock-holes could yield benefits to the conservation of relevant species by providing standing water throughout periods of extended drought, allowing historical hydrological norms to continue. However, irrigation is not feasible at an operational level throughout most of semi-arid and arid Australia due to low water availability, high infrastructure costs, and vast distances.

Management implications

The research presented here demonstrates the conservation value of the granite rock-holes present at Hiltaba Nature Reserve, due to their role in providing potable freshwater to a suite of at least 32 local vertebrate species, comprising both native and invasive taxa. A notable proportion (19.48%) of the vertebrate fauna previously recorded from the property (Commonwealth of Australia 2015a, see Table D2 for comparison) was observed visiting the eight granite rock-holes surveyed over the course of this study, with rare and less dispersive species (such as the short-beaked echidna) found to attend the site. Spatial replication appeared sufficient to capture > 75% of the taxa likely to be detected using wildlife cameras (Figure 2.3A). Although rock-holes within the known home range of the vulnerable yellow-footed rock-wallaby (*Petrogale xanthopus*) were not surveyed here, it is possible that the freshwater resources supplied by rock-holes are of value to the population at HNR. However, current evidence suggests that the wallabies do not rely heavily on accessible surface freshwater except at sites where impact by other herbivores (feral goats and overabundant wallaroos) is great (M Lethbridge, pers comm.). The relative lack of other sources of accessible freshwater throughout the landscape has likely resulted in dependence on sites like the rock-holes by local vertebrates, when compared with other regions with more readily available surface freshwater. We suggest that management of granite rock-holes should be considered when devising conservation and management strategies targeted at promoting native vertebrate taxa of interest at HNR and throughout the GB. Efforts to maintain the sites in terms of both hydrology and water quality, and suppression of feral populations, would likely result in benefits to vertebrates of conservation importance.

Machine learning technologies such as those used in this study, are improving the feasibility of wide-scale wildlife camera efforts (Sollmann 2018, Farmer et al. 2022). Our results confirm the successful application of machine learning technologies in handling large datasets that have historically been difficult due to the time burden associated with manually processing. As machine learning algorithms such as MegaDetector (Microsoft AI for Earth 2020, Gyurov 2022) improve, application will become even more accurate. Machine learning also improves reproducibility of analyses, and potentially limits the impacts of observer bias. Subsampling datasets for manual processing provides confidence in the efficacy of identification and allows characterisation and reporting of error rates.

In addition to their use in post-survey analysis, developments in the fields of machine learning and artificial intelligence have also led to improvements in real-time conservation and management tools. The deployment of wildlife camera arrays using artificial intelligence image recognition and 4G mobile connectivity allows for immediate detection of species that are the focus of management programs. Such detections can then be acted upon by conservation managers. At HNR, the use of these arrays, deployed at rock-holes could rapidly identify goats and other invasive species that are known to attend the sites. This would allow for real-time notifications to be delivered to on-site managers, allowing for an immediate management response. These techniques are already being utilised successfully in Australia to inform feral pig (Western Downs Regional Council 2021) and feral cat (Landscape South Australia 2021, Outdoor cameras Australia 2023) management.

Fencing sensitive habitats is often considered a practical solution (Fensham et al. 2010). In the case of the freshwater rock-holes, vertebrate-driven water draw-down pressure could be alleviated with such an exclusion management approach (Smith et al. 2020). Fences with mesh of selective size would allow for exclusion of both over-abundant native species of low conservation concern (such as macropods), as well as potentially harmful invasive species (such as goats), without excluding other smaller species from accessing rock-holes. Although fencing efforts are expensive and difficult to implement throughout much of arid Australia, selective fencing of smaller areas containing rock-holes of conservation significance may be of great benefit (Bode et al. 2013, Helmstedt et al. 2014). Additionally, limiting access to freshwater sources by goats, as well as culling, is likely critical in efforts to manage the species and its impacts throughout Australia’s rangelands (Russell et al. 2011). Improved feral goat management is likely to yield additional benefits for yellow-footed rock-wallabies with whom they share preferred habitat (Hayward et al. 2011).

Frequent attendance at rock-holes by invasive species may also provide an opportunity for targeted management efforts. Federal and state government-led baiting and culling efforts are routinely undertaken in the region for goats, foxes and rabbits (Commonwealth of Australia 2008, Government of South Australia - Department for Environment and Water 2023). The Nature Foundation has also implemented management procedures targeting goats, cats and foxes at Hiltaba Nature Reserve (Nature Foundation 2023). We suggest that high rates of attendance at the rock-holes particularly by goats make the sites suitable locations for management efforts, especially due to their large herd sizes (>10). Judas goats—sterilized goats fitted with radio collars or GPS units and released into the environment—are widely used in eradication efforts (Moseby et al. 2021, Southgate et al. 2022) and could be used to track goat herds to improve understanding of how they move throughout the region.

2.6 Conclusions

High rates of visitation to freshwater granite rock-holes in the Gawler bioregion suggest that these habitats are an important source of water for local vertebrates. The management of these rock-holes is critical for the conservation of numerous species. Three species of macropods, two species of corvids, emus and goats (all species that are common in highly modified semi-arid Australian landscapes) were the most frequent rock-hole visitors, but the sites were important to a diverse suite (32 species) of native and invasive vertebrates. Whilst species richness was relatively similar across rock-holes, the proportion of visitation by invasive species was higher for rock-holes at higher elevations. It is likely that goats are more effective at capitalising on the resources available at these higher elevation sites, and efforts to reduce their numbers will likely benefit the conservation of an array of native taxa.

Statement of Authorship

Title of Paper	Environmental DNA and wildlife camera traps uncover complimentary vertebrate visitation patterns at freshwater granite rock-holes
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Principal Author

Name of Principal Author (Candidate)	Brock A Hedges
Contribution to the Paper	Led data curation, methodological design, data analysis and production of manuscript
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party_t t would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date /2 /07 /2.o2 5

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that

i. the candidate's stated contribution to the publication is accurate (as detailed above);
n. permission is granted for the candidate in include the publication In the thesis; and
iii. the sum of all co-author contributions Is equal to 100% less the candidate's stated contribution.

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Chapter 3. Environmental DNA and wildlife camera traps uncover complimentary vertebrate visitation patterns at freshwater granite rock-holes

3.1 Abstract

Freshwater ecosystems are in decline globally, and in Australia due to a range of threatening processes including changes to land use, invasive species, increasing drought frequency and climate change. In Australia's arid interior terrestrial vertebrate diversity is also in decline. Monitoring tools are needed that allow for assessment of freshwater ecosystem health, as well as freshwater resource use by vertebrate communities. Environmental DNA metabarcoding is one tool that shows promise for monitoring these systems, but understanding of how eDNA data compares to more traditional techniques is limited. We sampled a series (n = 7) of freshwater granite rock-holes at three timepoints and amplified vertebrate eDNA to measure visitation, and compared our findings to camera trapping data. Our results demonstrate the success of eDNA metabarcoding as a tool for monitoring vertebrate visitation to arid-lands freshwater ecosystems, and its compatibility with more traditional survey methods such as wildlife camera trapping. We recovered a suite (n = 19) of vertebrate taxa, both Australian native species and invasive species known to cause ecological harm. These communities varied both spatially and temporally. We provide conservation recommendations and discuss the efficacy of freshwater eDNA for monitoring arid-lands freshwater resource use.

3.2 Introduction

Biodiversity of freshwater ecosystems and their riparian zones is experiencing some of the most rapid habitat declines globally, and the rate of this decline has increased in recent decades (Albert et al. 2021). Most impacts can be attributed to human activities, including changes in land use, pollution, and the effect of climate change (Albert et al. 2021, Mulero et al. 2021). In Australia, droughts are increasing in both their frequency and severity due to climate change (Ndehedehe et al. 2021), and are projected to continue to do so under all future emissions scenarios (IPCC 2022a). Freshwater ecosystems are particularly vulnerable as they are often dependent on thermal and hydrological regimes which are expected to be impacted by climate change (Filipe et al. 2012, da Silva et al. 2023). Freshwater ecosystems are of especially high value in semi-arid and arid regions due to the relative scarcity of accessible surface water (Noy-Meir 1973, Porporato et al. 2002), and correspondingly are amongst the most vulnerable ecosystems in the face of environmental change. In such regions small bodies of water are often present but are commonly ephemeral, being only sporadically wet throughout the year due to their reliance on rainfall (Bayly 1997, Bayly 1999b, Bayly 2001). For declining freshwater habitats, potential recovery in some cases and slowing of losses in others is possible but these are likely to require coordination of efforts at local, regional, national and international levels (Albert et al. 2021, IPCC 2022b). For such measures to be devised and enacted, surveying and ongoing monitoring of biodiversity reliant on these habitats is essential. However, targeted monitoring programs, particularly those with long-term data, are rare, particularly in Australia where accurate distribution and abundance data is lacking or non-existent for many declining taxa (Bino et al. 2020, Scheele et al. 2019). Monitoring effort is frequently hampered by accessibility issues in remote areas as well as their sparsely populated nature and a lack of funding. Monitoring tools are required that will allow for cost-effective determination of both freshwater ecosystem and community health, and characterisation of the resource value to Australian terrestrial vertebrates of any given water body (Beasley-Hall et al. 2023).

Australia has experienced a native mammal extinction crisis since the arrival of Europeans, with terrestrial mammal losses accounting for >10% of species lost over the last 200 years (Woinarski et al. 2015). A third of all mammal extinctions after the year 1500 CE have occurred in Australia (Fisher et al. 2014). Mammals within the ‘critical weight range’ of 35 g – 5.5 kg account for the vast majority of these extinctions (Short and Smith 1994, Johnson and Isaac 2009, Moseby et al. 2009, Murphy and Davies 2014). The loss of small to medium-sized species in Australia, has led to a decline in particular taxonomic groups, many of which are endemic to arid regions. Mammalian losses have also reduced the provision of various ecosystem services, such as bioturbation by small burrowing mammals (Fleming et al. 2014) and predation by marsupials (Moseby et al. 2021). The extinction crisis is not limited to arid ecosystems with similar losses observed for forests (Bradshaw 2012, Bilney 2014), islands (Burbidge and Manly 2002, Hanna and Cardillo 2014), and tropical ecosystems (Fisher et al. 2014, Murphy and Davies 2014).

Invasive predators such as cats and foxes are often considered to be the primary drivers of small mammal decline (Burbidge and Manly 2002, Kutt 2012, Fisher et al. 2014, Frank et al. 2014, Hanna and Cardillo 2014), although environmental change (McKenzie et al. 2007), non-predatory invasive species (Woinarski et al. 2011) and epizootic diseases (Abbott 2006) are also contributors. Whilst the global conservation effort to preserve vertebrate biodiversity is currently insufficient (Butchart et al. 2010, Hoffmann et al. 2010), some success has been achieved in Australia. For example, fenced exclosures in arid regions have improved survival of many threatened and reintroduced species in small pockets across the landscape (Moseby et al. 2009). A clear need exists to understand the changes in mammalian community composition, both native and invasive, in arid lands to inform management of populations, especially in the face of substantive impacts from climatic change. To date, monitoring efforts in these regions are frequently limited in their scope, and often target specific taxa rather than the broader Australian landscape (Moseby et al. 2009, Beasley-Hall et al. 2023).

Monitoring of Australian mammals can be biased and inconsistent, in part due to the non-transferability and difficulty in repeatability and standardisation of sampling techniques between projects and ecosystem types. A clear need has been identified, for interchangeable tools that will allow for comparison between disparate programs (Beasley-Hall et al. 2023, Zhang et al. 2023). Environmental DNA (eDNA) metabarcoding is an emerging method with great potential as a transferable biodiversity monitoring tool across a range of ecosystems. Metabarcoding approaches represent a high-throughput DNA sequencing (HTS) technique that allows for the identification of multiple taxa in a single sample, and can be used to detect multiple species at once in a single freshwater sample (Miya et al. 2020). This approach can characterise entire communities in addition to targeting specific species. Relying on the fundamental and inherited unit of life, eDNA metabarcoding is particularly useful in detecting and potentially monitoring biodiversity in freshwater substrates irrespective of location and species present (e.g. Kuehne et al. 2020, White et al. 2020, West et al. 2021) and identifying of abiotic stressors (Fan et al. 2020) which can facilitate informed conservation management. Environmental DNA lends itself to a standardised and repeatable approach and shows promise as a method for landscape or national level biomonitoring efforts (Sales et al. 2020, Miya et al. 2020). These approaches may also allow for early detection of invasive animal and plant species to inform the need for targeted removal and/or control (Kuehne et al. 2020).

To date, freshwater eDNA metabarcoding studies have focussed predominantly on the species that live within the freshwater system, such as fishes (Hänfling et al. 2016), amphibians (Valentini et al. 2016), and macroinvertebrates (Johnsen et al. 2020, Klymus et al. 2020, Rodgers et al. 2020). However, these methods also show promise for the detection of terrestrial vertebrates that have recently interacted with relevant water bodies (McDonald et al. 2023). Successes in the detection of aquatic, semi-aquatic, and terrestrial reptiles (West et al. 2021) and terrestrial mammals (Harper et al. 2019, Sales et al. 2020b) suggest freshwater eDNA metabarcoding is viable and comparable to traditional methods (wildlife camera trapping, field surveys, etc.) per unit effort, with the potential to improve distribution maps of mammalian diversity at a landscape level (Sales et al. 2020b). Whilst the presence of a species as detected by eDNA has not been linked to behaviour or activity (Harper et al. 2019), it is likely that visitation frequency should drive eDNA signal (Ushio et al. 2017), particularly in regions with low rainfall and for water bodies with low connectivity as is the case for many freshwater habitats in the Australian arid-zone.

Despite being an emerging method, freshwater eDNA metabarcoding performs well compared to traditional monitoring and detection techniques. In freshwater ecosystems, eDNA based methods have been shown to be more successful at detecting fish presence than a combination of four traditional capture techniques for both rare and common species (Piggott et al. 2021). Similarly, eDNA biomonitoring at inland springs has allowed detection of more terrestrial vertebrates than were observed with wildlife cameras, in person or from indirect evidence (e.g. scats or tracks) (Parker et al. 2021). In natural ponds, terrestrial vertebrate eDNA is known to be distributed more unevenly than in experimental artificial water bodies (Harper et al. 2019), which may limit confidence in results where replication is limited. Whilst the correlation between eDNA and abundance and biomass is not yet entirely understood, the approach shows promise in drawing estimates of fish abundance and biomass (Di Muri et al. 2020), and may eventually be a viable tool for assessing other aspects of population structure. Freshwater eDNA surveys are yet to provide complete reflections of a local ecosystem due to the occurrence of false negatives in detecting species (Sales et al. 2020a, Sales et al. 2020b). As such, it has been suggested that freshwater eDNA be used alongside more traditional surveying methods such as the use of wildlife cameras (Sales et al. 2020b), a comparison that has only recently begun to be explored (Farrell et al. 2022, Johnson et al. 2023).

As mentioned above, eDNA metabarcoding has been shown to be effective in detecting vertebrate communities from freshwater systems (Harper et al. 2019, Lyet et al. 2021) and has been compared to camera trapping of terrestrial vertebrates (Leempoel et al. 2020, Sales et al. 2020b) including with samples collected from freshwater systems (Harper et al. 2019, Lyet et al. 2021). A recent study of arid land water sources in Western Australia by Mcdonald et al. (2023) (for which I was a co-author) demonstrated that eDNA metabarcoding can effectively detect vertebrate visitation. Indeed, they were able to detect variation in visitation rates among species between natural and non-natural semi-arid freshwater bodies, and the efficacy of various eDNA sampling methodologies (Mcdonald et al. 2023). How these eDNA results compare to traditional methods of survey was not investigated.

Here we aimed to extend the research in the aforementioned studies by comparing eDNA metabarcoding to camera trap survey data at arid lands freshwater sources in order to assess the relationship between visitation and eDNA deposition (Mas-Carrió et al. 2022). In doing so, we investigated the applicability of eDNA metabarcoding as a conservation management tool. Our specific aims were to a) test the application of eDNA metabarcoding in detecting native and invasive vertebrate visitation to the Australian arid-lands freshwater granite rock-holes system and b) compare detection of vertebrates with eDNA metabarcoding to conventional wildlife camera trapping techniques, c) determine whether vertebrate communities detected using eDNA varied spatially and temporally, d) make a series of conservation and management recommendations for the freshwater granite rock-holes in the Gawler bioregion. Based on previous findings of the efficacy of eDNA metabarcoding in detecting vertebrate species from freshwater, it was predicted that detection of both native and invasive vertebrate communities at the freshwater rock-holes and recovery of the majority of taxa detected using wildlife camera trapping was likely. It was also predicted that eDNA metabarcoding would be sensitive enough to detect spatial and temporal community variation.

3.3 Methods

Site description

The study was located at Hiltaba Nature Reserve (HNR), a large ex-pastoral property that borders the Gawler Ranges National Park to the north of South Australia's Eyre Peninsula (Figure 3.1). Approximately 78,000 ha in area, HNR has been managed for conservation outcomes by the Nature Foundation since it was acquired by the organisation in 2012. Primarily composed of a series of rolling granite hills and interspersed with woodland and grassland, HNR possesses many relictual habitats utilised by local plants and animals known to be in decline elsewhere (Nature Foundation 2023). The Nature Foundation has enacted a series of conservation programs to improve biodiversity management at the reserve.

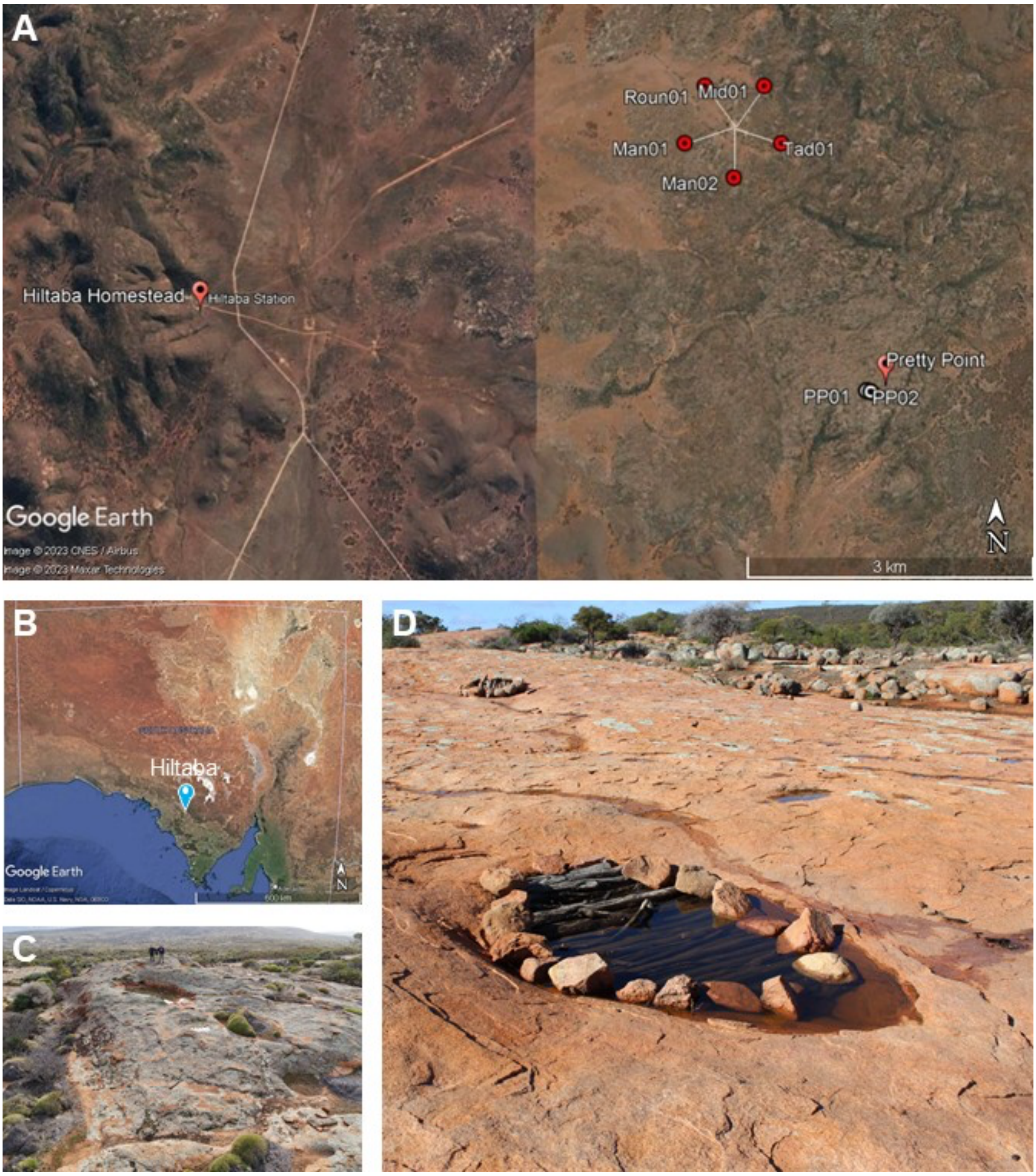

Figure 3.1. A) The location of Hiltaba Nature Reserve in South Australia; B) the locations of the seven rock-holes sampled for freshwater eDNA during 2020; C) a rain-filled granite rock-hole at the 'Pretty Point' outcrop at Hiltaba Nature Reserve; D) a rain-filled granite rock-hole on the 'photopoint outcrop' at Hiltaba Nature Reserve that is actively managed by members of the Gawler Ranges Aboriginal Corporation, diameter of the rock-hole at widest point = 2.2 m.

The granite hills present throughout much of the reserve are entirely exposed in many locations, forming granite outcrops of varying morphologies. Certain areas of these outcrops serve as impermeable locations for storage of water and sediments at depressed points. These depressions or 'rock-holes' provide habitat for a suite of aquatic invertebrates and plants. As many vertebrate species have been observed visiting these rock-holes as a source of accessible freshwater, the granite rock-holes at HNR have been identified by Nature Foundation as a location of critical conservation interest with respect to local vertebrate biodiversity.

eDNA sampling

Water was sampled from five rock-holes in February 2020, and seven rock-holes in each of July and October 2020 (Table 3.1). Five 1 L replicates were collected from each rock-hole. One negative control was implemented for each rock-hole using one litre of bottled water. Replicates were collected using 1 L wide mouth bottles (NALGENE^{TM}). During February, only sites at outcrop 1 were sampled due to inaccessibility of rock-holes at outcrop 2 during the hot summer months. During October, rock-holes Man02 and PP02 were not sampled due to low water levels. Samples were collected whilst wearing disposable latex gloves, stored in and transported in clean plastic tubs to minimise contamination. Sampling bottles were cleaned first with bleach solution, and then ethanol before reuse. Negative field controls (prepared in the field but using RO water) and equipment controls (prepared in the filtering room using RO water) were used to test for contamination.

Replicates and blanks were filtered through glass-fibre membranes with 0.44 µm pores using a vacuum pump (JAVAC, model CC-45) connected to a series of conical flasks. The first flask was filled with silica beads and the second was attached to a magnetic filter funnel. For the July and October samples, a Pall Sentino microbiology pump was used. A pore size of 0.44 µm was selected due to the high concentration of suspended solids present in rock-hole freshwater samples. Pump equipment was wiped down with bleach solution and then ethanol between filtering for each rock-hole.

Membranes were stored on ice while in the field before being transferred to -20°C pending DNA extraction.

Table 3.1. Granite rock-holes sampled for freshwater eDNA during February, July and October trips (green checkmark). Rock-holes not sampled (red cross) were either empty or had only a small volume of water present. Rock-hole type assignments adapted from Timms (2013b).

Rock-hole features						Sample period		
Rock-hole ID	**Latitude**	**Longitude**	**Elevation (m)**	**Type**	**Outcrop**	**Feb**	**July**	**Oct**
Man01	-32.13941	135.13461	216	Pit	1	✓	✓	✓
Man02	-32.13943	135.13478	216	Pit	1	✓	✓	✗
Tad01	-32.1395	135.1348	216	Pit	1	✓	✓	✓
Roun01	-32.13931	135.13492	217	Pan	1	✓	✓	✓
Mid01	-32.13941	135.13461	217	Pit	1	✓	✓	✓
PP01	-32.165521	135.1488	312	Pan	2	✗	✓	✓
PP02	-32.165521	135.1488	310	Pan	2	✗	✓	✗

eDNA laboratory methods

DNA was extracted from half of each filter paper using a modified Qiagen DNeasy blood and tissue kit protocol (Qiagen, Germany) and an automated QIAcube extraction platform (Qiagen). Where more than one filter paper was used for a sample, equal portions of each paper were taken to total a half filter paper. All extractions were undertaken in a dedicated PCR-free laboratory, and extraction controls were processed alongside samples. Extractions were eluted in a final volume of 100 µL AE buffer.

To determine the required dilution for optimal amplification, PCR reactions were performed in duplicate on each extraction by adding DNA template directly to the PCR master mix (neat), then performing a serial dilution (1:10). The PCRs were performed at a final volume of 25 µL where each reaction comprised of: 1× PCR Gold Buffer (Applied Biosystems), 0.25 mM dNTP mix (Astral Scientific, Australia), 2 mM $MgCl_2$ (Applied Biosystems), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.4 mg/mL bovine serum albumin (Fisher Biotec), 0.4 µM forward and reverse primers, 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies), and 2 µL template DNA. PCRs were performed on StepOne Plus instruments (Applied Biosystems) with the following cycling conditions: 95°C for 5 min, followed by 50 cycles of: 95°C for 30 sec, 49°C for 30 sec, 72°C for 45 sec, then a melt-curve analysis of: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, finishing with a final extension stage at 72°C for 10 min.

After selection of the optimal dilution (neat or 1:10), PCRs were repeated in duplicate as described above but instead using unique, single use combinations of 8 bp multiplex identifier-tagged (MID-tag) primers as described in Koziol et al. (2019) and van der Heyde et al. (2020). Master mixes were prepared using a QIAgility instrument (Qiagen) in an ultra-clean lab facility, with negative and positive PCR controls included on every plate to ensure the validity of results. A sequencing library was created by combining samples into mini-pools based on the PCR amplification results from each sample. The mini-pools were then analysed using a QIAxcel (Qiagen) and combined in roughly equimolar concentrations to form libraries. Libraries were then size selected (250 - 600 bp cut-off) using a Pippin Prep instrument (Sage Sciences) with 2% dye-free cassettes, cleaned using a QIAquick PCR purification kit, quantified on a Qubit (Thermo Fisher), and diluted to 2 nM. The libraries were sequenced on an Illumina MiSeq instrument using a 500-cycle V2 kit with custom sequencing primers.

Bioinformatics and statistical analyses

Processing and analysis of eDNA sequence data was performed with QIIME2 v.2021.11 (Bolyen et al. 2019). Raw sequences were demultiplexed, trimmed by quality scores (a subset of samples were visualised in QIIME2.view and a consensus trim length was selected at position 120), filtered to remove chimeric sequences, and denoised (which accounts for amplification errors), using DADA2 (Callahan et al. 2016). Sequences were then tabulated to construct 100% OTUs and to derive representative sequences for each OTU. Representative sequences were aligned using MAFFT (Katoh et al. 2002) and a midpoint-rooted phylogeny was generated using FastTree 2 for downstream calculation of diversity metrics (Price et al. 2010). A custom database was constructed using BLAST from all vertebrate 16S rRNA sequences available on the 9th of June 2022 on NCBI’s GenBank repository (National Center for Biotechnology Information) using the search query ((("16S ribosomal RNA" OR "16S rRNA" OR mitochondrion) AND Vertebrata[Organism]) NOT Homo sapiens[Organism]). BLASTN (Camacho et al. 2009) was then used to query our representative sequences against the custom database, with the maximum number of target sequences set to 1 per query. Representative sequences with non-vertebrate top hits, or those without a top hit with a corresponding species-level assignment, were excluded from further analyses. Alpha diversity metrics were then calculated including evenness (Pielou 1966), Faith’s phylogenetic diversity (Faith 1992), and Shannon’s diversity index (Shannon and Weaver 1949). Beta diversity analyses were examined including Bray-Curtis (Bray and Curtis 1957), Jaccard (Jaccard 1901), and weighted and unweighted Unifrac distances (Lozupone et al. 2011) against metadata columns such as rock-hole location. PCoA plots of these metrics were exported to R version 4.2.2 (RCoreTeam 2013) using *QIIME2R* v0.99.6 for further visualisation (Bisanz 2018). Final PCoA plots were generated using *ggplot2* v3.4.0 (Wickham 2016). Rarefaction curves were generated using *iNext* v3.0.0 (Hsieh et al. 2022).

3.4 Results

Environmental DNA data

We successfully recovered vertebrate eDNA from 79 of 91 samples using the 16S rRNA assay. Sequencing yielded 9,107,435 sequences in total with 90,173 mean sequences per sample. A total of 19 unique vertebrate taxa were detected, including 11 mammals, five birds, three reptiles, and two amphibians (Figure 3.2).

We recovered 80% of the mammal species observed using wildlife cameras (Chapter 2), with three new mammal records using eDNA that were not recorded using wildlife cameras (Figure 3.2). We also recovered 25% of the reptile species with freshwater eDNA that were recorded with wildlife cameras (chapter 2), and generated two new reptile records and two new amphibian records. The application of eDNA was less effective for birds, with less than 11% of species recorded by camera traps in chapter 2 also having been recorded with freshwater eDNA, and three new records.

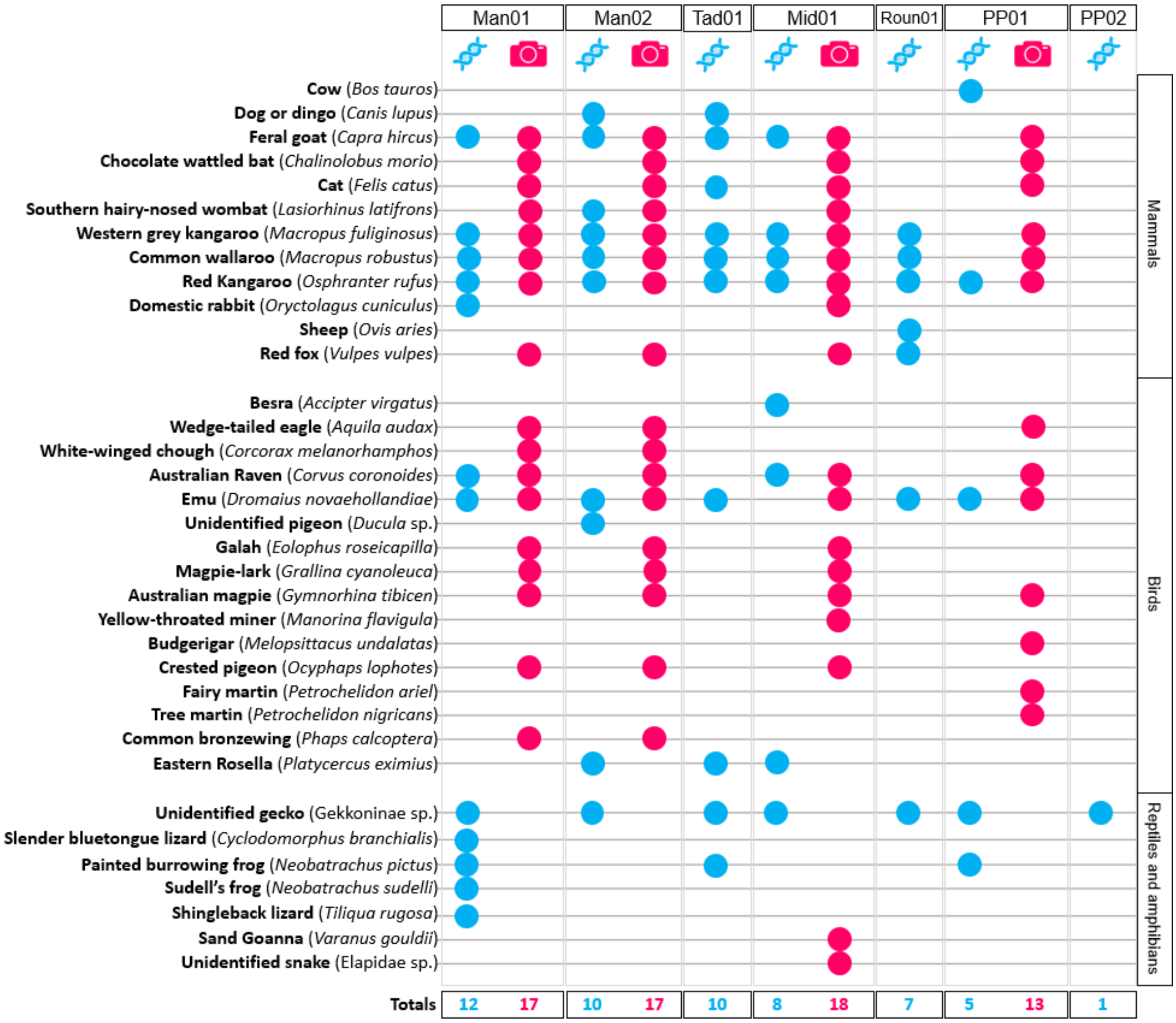

Figure 3.2. A comparison of the vertebrate taxa identification from freshwater eDNA collected from seven rock-holes at HNR throughout 2020 and through wildlife camera trapping at the same rock-holes as presented in chapter 2. Blue circles indicate when a taxon was observed in the eDNA dataset, red circles indicate when a taxon was observed in the wildlife camera trap dataset. The complete dataset of presence absence records can be seen in Table E1, and a comparison between eDNA data, camera trapping data from Chapter 2 and bush blitz survey data from Commonwealth of Australia (2015a) can be seen in Table D2.

A comparison between eDNA and the complete wildlife camera dataset (Chapter 2), which included rock-holes not surveyed with eDNA, showed that only 11 taxa were recovered by both approaches, another 10 taxa were only observed in the eDNA dataset, and 21 were only observed in the wildlife camera dataset (Figure 3.3).

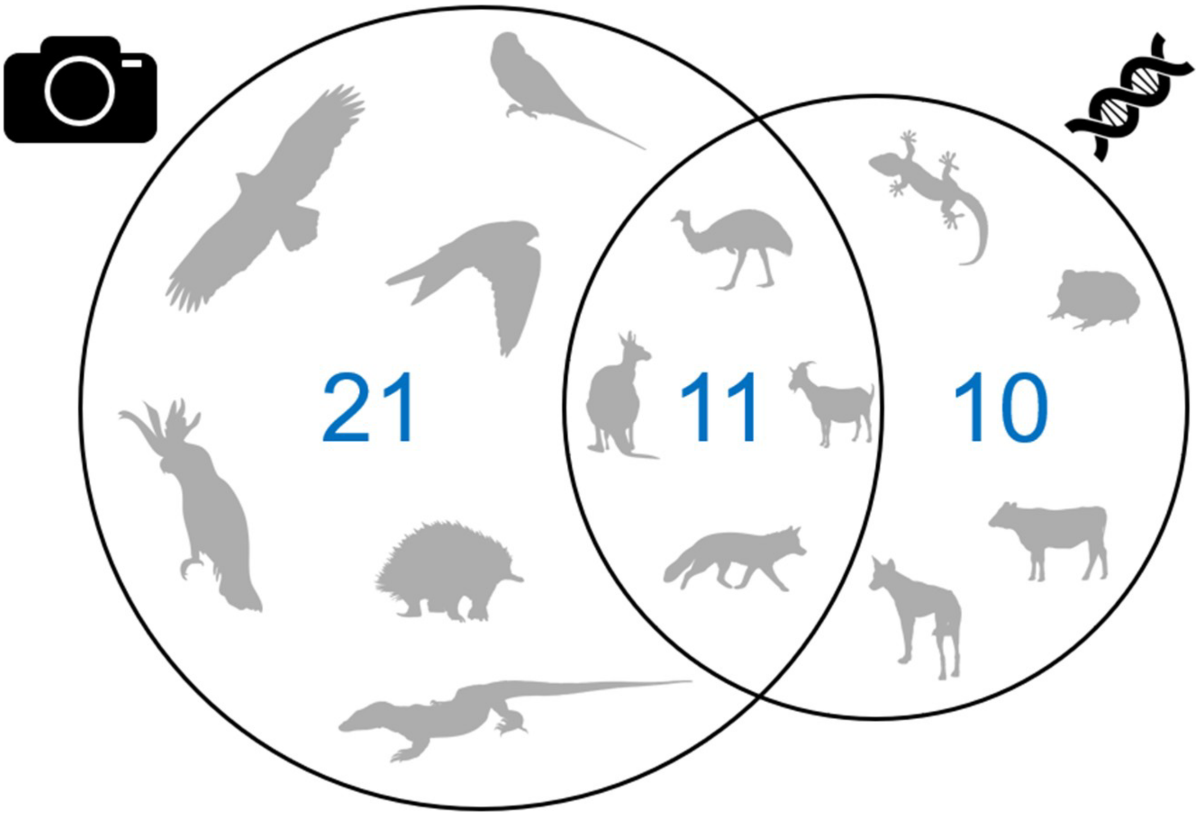

Figure 3.3. Comparison of species identifications between all wildlife camera trap records (left circle) and eDNA (right circle) datasets. 11 species were detected in both datasets. A full summary of taxa observed using eDNA, camera trapping data from Chapter 2 and bush blitz survey data from Commonwealth of Australia (2015a) can be seen in Table D2.

Rarefaction plots suggest that the number of rock-holes sampled was insufficient to capture 90% of the total vertebrate taxa that attend rock-holes at HNR (Figure 3.4). However, within each rock-hole and at each timepoint rarefaction curves varied, with five replicates shown to be sufficient to capture >90% for some rock-holes and timepoints, whilst insufficient to capture 90% of taxa at others (Figure 3.5).

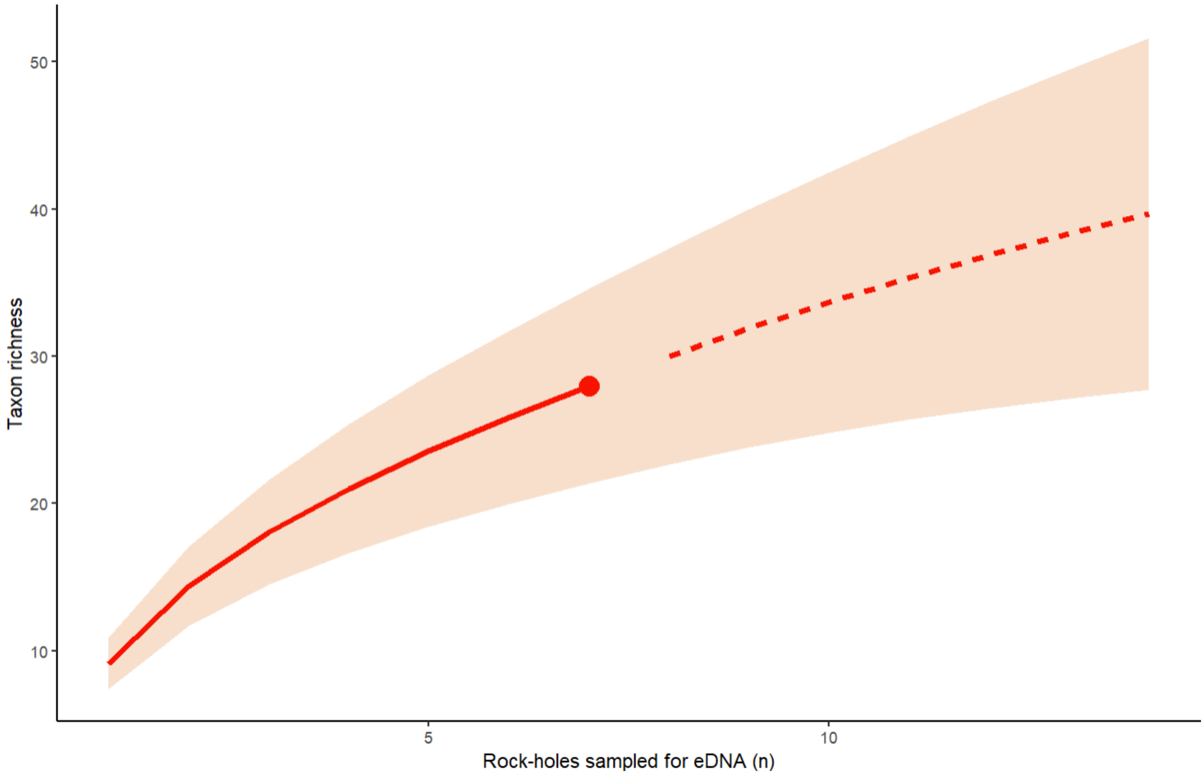

Figure 3.4. Species discovery curve showing accumulation of new records with each added rock-hole. Asymptote indicates that seven rock-holes was sufficient to recover 95 % of OTUs that are recoverable in filtered freshwater eDNA samples

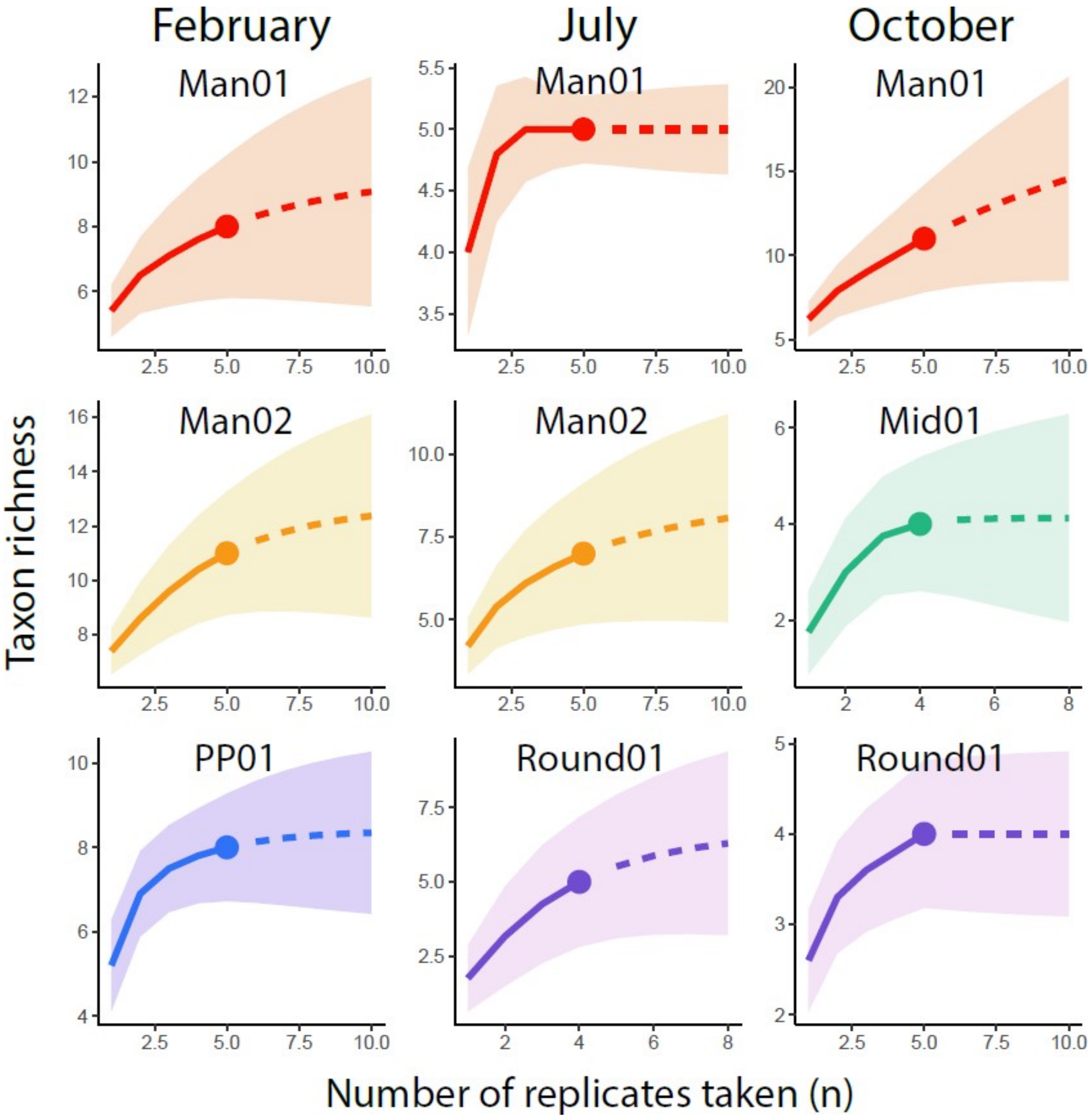

Figure 3.5. Species discovery curve showing accumulation of new records with each added replicate. Asymptote indicates that five replicates plus one blank is sufficient to recover 95 % of OTUs that are recoverable in filtered freshwater eDNA samples

PCoA plots showed that samples collected in July and October were more similar to one another than either were to samples collected in February (Figure 3.6A). However, communities detected from individual rock-holes were generally distinct from one another (Figure 3.6B).

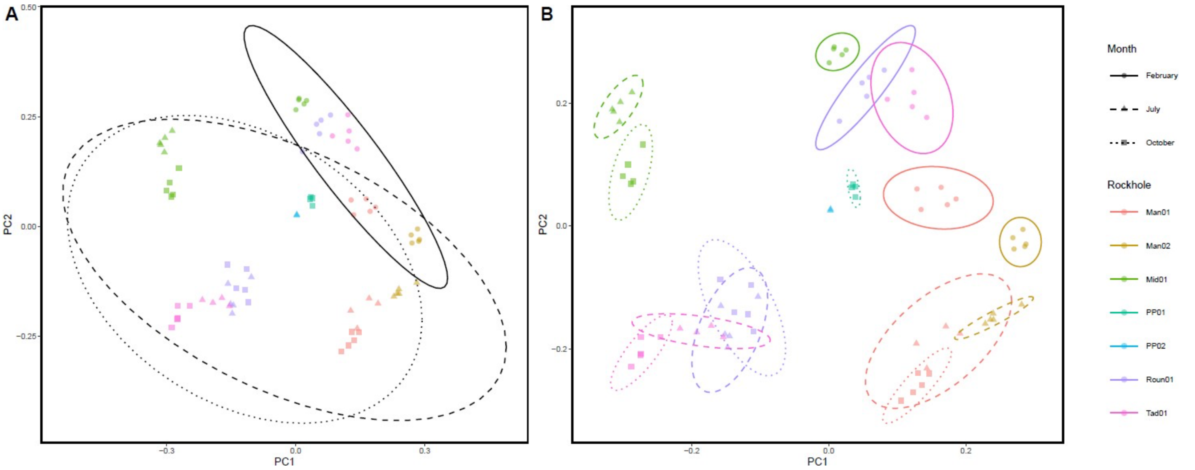

Figure 3.6. PCoA plots showing maximum dissimilarity for vertebrate eDNA samples based on Jaccard distances. PC1 and PC2 represent maximum dissimilarity between samples in order of magnitude. Colours represent individual rock-holes and shapes represent the month during which eDNA sampling was undertaken. Ellipses have been applied to show the similarity between A) sample collection month; and B) individual rock-holes.

3.5 Discussion

Here we have contributed to an emerging narrative regarding the detection of vertebrate visitors to freshwater ecosystems using eDNA methods versus traditional monitoring methods (Farrell et al. 2022, Croose et al. 2023, Johnson et al. 2023 Mcdonald et al. 2023). Our results demonstrate the efficacy of freshwater eDNA as a tool suitable for monitoring vertebrate visitation to arid-lands freshwater ecosystems, and its compatibility with more traditional techniques such as wildlife camera trapping. We present the first comparative analysis of vertebrate communities for the Gawler Ranges rock-holes using freshwater eDNA and wildlife camera trapping. Vertebrate eDNA was successfully recovered from freshwater samples collected from rock-holes at Hiltaba Nature Reserve and was deemed to be a suitable alternative to the use of wildlife cameras for monitoring mammals and amphibians, although for birds and reptiles a combined approach is recommended to capture the complete detectable community (McDonald et al. 2023).

Our findings provide new evidence that the freshwater rock-holes at Hiltaba Nature Reserve are of conservation value to local vertebrates. A suite of 21 vertebrates were recovered from freshwater eDNA samples from seven rock-holes during the three collection periods. The community composition of vertebrates recovered using eDNA was similar to that recovered through wildlife camera trapping in Chapter 2. The taxa that were recorded visiting at the highest frequency using wildlife camera traps, macropods and emus, were amongst those detected in the most eDNA samples. The three mammals detected using eDNA that were not detected by wildlife cameras, cows, sheep and dogs, are also noted as having a higher likelihood of occurring through sequence contamination than other taxa present. Contamination is a challenge faced during eDNA metabarcoding studies, with livestock species frequently appearing in surveys of regions where they should not be present (Zhang et al. 2023).

Invasive species known to cause ecological harm in the region accounted for 20% of the species detected with eDNA, with feral goats being recorded in the greatest number of samples, but with detections of cats, foxes and rabbits also observed. Such a substantial proportion of the community being invasive, further supports findings presented in Chapter 2 that these species are likely capitalising on the resources provided by the freshwater rock-hole ecosystem. Invasive species detection using eDNA metabarcoding is an emerging discipline that is showing promise in facilitating early detection (Rishan et al. 2023, Salis et al. 2023, Zhang et al. 2023) and the findings of our study extend these techniques into semi-arid Australia.

Communities recovered using eDNA metabarcoding showed that replicates within a single rock-hole had high similarity, and that individual rock-holes were distinct from one another (Figure 3.6). Environmental DNA was variable in its performance when compared to vertebrate visitation recorded from wildlife cameras throughout the same period. For mammals, detection was high in comparison to camera traps but substantially lower for birds. Zhang et al. (2023) found that water birds were easier to detect using eDNA than forest birds, and so it is likely that reduced detection of birds may be linked to taxon-specific physiology or behaviour. Less frequent visitation by birds, which was observed for many bird species in Chapter 2 may be driving this decreased detectability. Similarly, lower detectability may also be due to lower rates of eDNA deposition by birds, which may be a function of physiology. Keratinised beaks and feathers are likely poor sources of eDNA (Turcu et al. 2023) and this may have impacted thee capacity for detection of birds from freshwater eDNA samples. Conversely, behaviour may drive this decreased detection rate, as birds were recorded to visit rock-holes at much lower frequencies than terrestrial mammals (Chapter 2). These results suggest a combined approach may be favourable when characterising vertebrate communities accessing desert freshwater habitats. For birds, we recommend the use of more targeted freshwater eDNA approaches such as species-specific assays (e.g. White et al. (2020)).

Vertebrate visitation to rock-holes at HNR varied spatially based on our eDNA data. Rock-holes within an outcrop were more similar to one another than those on other outcrops, with absences of multiple macropod species and emus driving this variability (Figure 3.6). Consistent with findings in Chapter 2, it was observed that elevation impacted species access to rock-holes. However, access limitations at multiple outcrops and an absence of water in some rock-holes during sampling times meant replication was insufficient to accurately test the impact of outcrop location on vertebrate visitation community. These findings were consistent with Chapter 2, where it was shown that various rock-holes attracted unique vertebrate communities and that rock-hole elevation was a likely driver of this dissimilarity, as at higher elevations (>250 m) they are less accessible to taxa which are unable to climb the steep slopes characteristic of the Gawler bioregion. McDonald et al. (2023) explored visitation to similar rock-holes and suggested that some taxa may avoid certain rock-holes due to increased risk of injury or predation. Similarly, Votto et al. (2022) recorded visitation to arid-lands waterholes, and found that visitation rates were impacted by fringing vegetation.

Based on eDNA metabarcoding results, vertebrate visitation to freshwater rock-holes at HNR varied temporally over the year. Whilst communities recovered at the beginning (July) and end (October) of the winter wet period were relatively similar, both of these communities were distinct from those recovered during the summer wet period in February (Figure 3.2). These findings were consistent with findings presented in Chapter 2, where species visitation was inconsistent throughout a continuous study period. Native species (primarily macropods, emus, crows and ravens) dependence on freshwater available at rock-holes was likely to increase with time elapsed since rainfall events, with rock-holes increasing in their resource value with every successive week without rain, although this relationship was not observed for invasive species (primarily feral goats) (Chapter 2). Freshwater visitation by vertebrates is often temporally variable (Eliades et al. 2022, Kassara et al. 2023) and, as such, it is likely that future biomonitoring efforts using eDNA will need to take time of sampling into consideration, as periods when visitation peaks are likely to provide the greatest indication of resource use (Sales et al. 2020b).

Assay success and sampling

The successes in recovery of mammal sequences, and comparison with camera trapping data suggest that the 16S vertebrate assay used here is suitable for detecting Australian mammals in the arid-zone. The technique was notably less successful at detecting birds than wildlife camera trapping. This decrease in detection success may suggest the 16S vertebrates assay is less well suited for detecting birds than for mammals when compared to wildlife camera trapping data.

We found that five one-litre replicates per rock-hole were sufficient to characterise the vertebrate communities that were likely to be recovered from filtered freshwater eDNA samples (Figure 3.4). This is significantly more water than that collected and filtered in a previous study by McDonald et al. (2023), where it was found that five 50 mL samples per rock-hole was insufficient to recover all possible vertebrate sequences. However, due to the unpredictable nature of rock-hole water volumes, it is not always possible to collect these volumes. Our results demonstrate the efficacy of eDNA as a tool for monitoring vertebrate visitation to freshwater systems and provide a foundation for further use of the technique in monitoring semi-arid and arid freshwater ecosystems. Visitation frequency should be a major driver in eDNA signal (Ushio et al. 2017, Sales et al. 2020b), so future research should test the relationship between vertebrate visitation frequency and resulting eDNA concentration.

Implications for management at Hiltaba Nature Reserve

The frequent occurrence of invasive species (primarily feral goats) in our eDNA metabarcoding results suggest such species are capitalising on an unmanaged freshwater resource and that rock-hole management may influence the curbing of invasive species activity throughout the Gawler Ranges. As such, managing their access to this resource may allow for population suppression and improve native species outcomes for the broader landscape. Invasive species visitation is also likely to negatively impact the rock-holes themselves, the communities of non-invasives reliant on these water sources, and the quality of the water therein as a resource for native species of conservation interest. Ungulates can cause degradation of Australian freshwater ecosystems through disturbance and the input of faeces, which dramatically increases nutrient loads (Doupe 2010, Brim Box et al. 2016). Efforts to limit invasive species access to the rock-holes are therefore likely to benefit both broad invasive species management programs, alleviate their impacts in the Gawler Ranges, and benefit the rock-hole ecosystem itself. Fencing to prevent access to rock-holes by invasive species (as well as overabundant native species such as kangaroos) would allow these resources to be preferentially accessed by smaller mammals, reptiles, and birds. However, fencing efforts are expensive to implement and maintain, particularly in remote Australia (Bode et al. 2013) and so fencing of smaller areas that encompass rock-holes of greater conservation and resource value would represent targeted and cost-effective efforts.

Invasive species are also routinely baited and culled in the Gawler Ranges (Commonwealth of Australia 2008, Government of South Australia - Department for Environment and Water 2023) via the targeting of foxes, goats and rabbits. Additional culling efforts are also implemented at HNR that target feral cats, foxes, and goats (Nature Foundation 2023). High invasive visitation rates to rock-holes suggest that these locations may be viable for targeted and intensive culling efforts. Future and broader rock-hole eDNA studies may provide further clarity on the rock-holes that are most frequently attended by invasive species, facilitating more effective culling in the region.

Environmental DNA as a biomonitoring tool

Our results demonstrate the efficacy of eDNA as a tool for monitoring freshwater rock-holes in Australia’s semi-arid and arid lands, performing well compared to more traditional survey methods such as typical human-led field surveys and wildlife camera trapping. Multiple advantages exist in the use of eDNA as a tool for monitoring freshwater rock-holes. The collection of freshwater eDNA samples requires no specialised scientific training and can therefore be carried out by landholders, conservation workers, volunteers, and other stakeholders with ease (Prie et al. 2021, 2023). Specialised equipment is required for on-site filtering of samples, but such equipment is relatively affordable. Additionally, passive freshwater eDNA collection has been trialled in similar rock-holes elsewhere in Australia through the submergence of filter papers in the sampling medium (McDonald et al. 2023), which removes the filtration step used here, further cutting costs and effort related to sample collection. However, communities recovered from passively collected samples have been shown to be capture certain components of the freshwater communities compared to those recovered from filtered samples (McDonald et al. 2023). Once filtered, samples may be easily stored in a conventional freezer, and can be transported on ice. Sequencing costs can be high, particularly for eDNA samples which are highly sensitive to contamination (Zhang et al. 2023). However, it is likely that costs will continue to decrease as advancements are made in the field and eDNA becomes a more widely applied tool (Rishan et al. 2023).

Limitations in the availability of high-quality, taxonomically-verified reference libraries are an obstacle that will need to be overcome before eDNA can be widely applied as a biomonitoring tool, including at the ephemeral rock-holes of the GB (Beasley-Hall et al. 2023). Remote semi-arid and arid Australia is broadly understudied and for many taxonomic groups, including some vertebrates, very little work has been done to generate sequences corresponding to barcoding regions commonly used in eDNA studies. Greater sampling of semi-arid and arid Australian taxa is therefore a critical research priority and would facilitate more effective use of eDNA methods for monitoring the biodiversity of those regions. Primers specifically designed for detection and identification of terrestrial mammals will improve the efficacy of such studies (Ushio et al. 2017, Leempoel et al. 2020). Assays targeting birds and reptiles may allow for greater success in detection of these taxa from freshwater eDNA samples (Neice and McRae 2021, Mousavi-Derazmahalleh et al. 2023). Furthermore, an improved understanding of how organism size, physiology, and ecology impacts eDNA deposition would allow greater confidence in determining true species absences in instances when eDNA has not been recovered for a particular taxon.

3.6 Conclusions

Our findings demonstrate the viability of freshwater eDNA metabarcoding as a method for monitoring vertebrate visitation to granite rock-holes in the Gawler bioregion, with both native and invasive species successfully detected. We found that vertebrate visitation is variable between rock-holes at HNR, with those on different outcrops displaying different communities. Furthermore, vertebrate visitation was variable throughout time, with communities recovered during different months being distinct from one another. With eDNA metabarcoding, we recovered sequences that indicate that invasive species are visiting rock-holes throughout the region, and are likely impacting them and their value as a freshwater resource to vertebrates of conservation interest. We recommend that future studies improve reference databases to allow better taxonomic assignment within eDNA datasets.

Statement of Authorship

Title of Paper	Environmental DNA reveals temporal and spatial variability of invertebrate communities in arid-lands ephemeral water bodies		
Publication Status	☐ Published	☐ Accepted for Publication	
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Principal Author

Name of Principal Author (Candidate)	Brock A Hedges		
Contribution to the Paper	Led data curation, methodological design, data analysis and production of manuscript		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature	[redacted]	Date	12/07/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate in include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 4. Environmental DNA reveals temporal and spatial variability of invertebrate communities in arid-lands ephemeral water bodies

4.1 Abstract

Throughout much of semi-arid and arid Australia surface freshwater is sporadic in its occurrence and where it does occur, it is often ephemeral. This is the case for freshwater granite rock-holes that occur throughout much of southern Australia. Rock-holes support freshwater invertebrate communities which possess behavioural and physiological adaptations to the ephemeral nature of these systems. The ongoing threat of climate change means that this ecosystem is likely to experience disruptions to its historical hydrological patterns which may negatively impact these invertebrate communities. The rock-holes are also likely to be heavily impacted by various invasive vertebrate species that are commonly recorded visiting them. However, the ecology of this ecosystem is poorly understood despite its relative ecological significance and the extent of its associated threats. To provide a baseline ecological understanding of this ecosystem we documented species richness and variability at a series of rock-holes in the Gawler bioregion in South Australia using an environmental DNA approach. Metabarcoding recorded invertebrates from 22 orders and 45 families including Copepoda, Diptera, Ephemeroptera, Hemiptera, Laevicaudata, Odonata and Spinicaudata. Community composition varied among rock-holes and throughout the year, with a peak in species richness in winter. These findings demonstrate the importance of these ecosystems to a wide range of endemic taxa. We propose a series of management recommendations to safeguard the biodiversity of these systems, including establishing monitoring programs, the development of custom barcode reference libraries for the rock-hole ecosystem and future research into the likely impacts of climate change on the communities associated with them.

4.2 Introduction

The interior of Australia is largely characterised by semi-arid and arid climates that have low annual rainfall and high rates of evaporation, resulting in a scarcity of freshwater sources (Bayly 1997). The few water bodies that are present are often highly ephemeral in their nature and inconsistent in both their occurrence and persistence (Bayly 1999a, Bayly 2001). Salt lakes (May et al. 2022), ephemeral creek-lines (Reich et al. 2022), clay-pans (Gibson et al. 2018), and various freshwater water-holes and rock-holes (Timms 2013a, Timms 2014) are often among the only sources of freshwater accessible above-ground in these regions (Bayly 1999a). Freshwater granite rock-holes present throughout much of southern Australia are an understudied habitat distinct from other ephemeral bodies due to a lack of connectivity and having an impermeable substrate (Timms 2013b, Timms 2014). These indentations in granite outcrops formed as a result of chemical weathering processes and provide a location for temporary storage of rainwater (Twidale and Corbin 1963). Periodic shifts in the water levels of rock-holes are caused by the seasonality of rainfall and high evaporation rates, meaning rock-holes may shift from being inundated to entirely dry multiple times in a single year (Timms 2014). As such, rock-holes have long been utilised as a source of potable freshwater for First Nations Australians (Bayly 1999a). Rock-holes are also of great value to local vertebrate species, many of which move long distances across the landscape to access this freshwater (Chapter 2). Freshwater rock-holes also act as critical refugia for a range of relictual freshwater organisms, including plants and invertebrates, that have largely disappeared from throughout the drier regions of Australia (Bayly 1997, Pinder et al. 2000, Bayly 2001). However, despite their high cultural and ecological importance, freshwater granite rock-holes are generally understudied and little is known of their role in the wider environment.

Invertebrate communities associated with southern Australia's granite rock-holes are reliable in their presence despite the extreme seasonal fluctuations in habitat conditions (Bayly 1997, Pinder et al. 2000, Timms 2014). The majority of occupants are crustaceans and insects, which persist through dry periods using a series of adaptations likely gained during Australia's long period of aridification; including desiccation-resistant eggs which can survive for several years without water (Bayly 1997, Chapter 5). Several taxa also display this adaptation in their free-living life stages, such as the chironomid *Paraborniella tonnoiri*, which can survive dry periods in a semi-desiccated larval state (Jones 1975). Other inhabitants of the rock-holes are less physiologically adapted to desiccation and instead rely on repeated colonisation events after each rainfall event (Bayly 1997). Insects with strong flight behaviour, such as damselflies, water-bugs, and diving beetles, regularly either recolonise the system after inundation or oviposit directly into the water column (Hedges et al. 2021). These invertebrate communities are thought to provide a range of crucial ecosystem services to rock-holes through filter-feeding, which is often associated with water quality (Coughlan 1969, Atkinson et al. 2013, Buelow and Waltham 2020, Simeone et al. 2021). It is likely that there is an association between the overall health of the system and the success of this community of invertebrates.

Southern Australia is a region projected to experience significant drying over the next century, with all future emissions scenarios suggesting a decline in rainfall and shifts in the seasonality of rainfall (Chapter 5, IPCC 2022a). As the freshwater granite rock-holes are primarily filled by rainfall and emptied by evaporation they will almost certainly be impacted by climate change and considerable disruptions to their historical wetting-drying cycles are expected. It is unclear how this may impact the invertebrates, many of which already possess adaptations to drought and desiccation. Invasive species also pose a threat to the rock-holes due to the disturbance and associated decline in water quality resulting from their visitation (Doupe et al. 2010, Brim Box et al. 2016). Invasive species activity is associated with declines in freshwater ecosystem health throughout arid and semi-arid Australia and is likely contributing to the decline of overall ecosystem health continent-wide (Brim Box at al. 2016, Chapters 2, 3). Such taxa impact water quality by increasing turbidity and nitrogen concentrations (Canals 2011). Similarly, ephemeral freshwater ecosystems such as rock-holes are also particularly susceptible to invasion by algal communities (Buchberger and Stockenreiter 2018), macrophytes (Carreira et al. 2014), and macroinvertebrates (Devereaux and Mokany 2006), with even single-species additions or subtractions capable of causing large and potentially detrimental changes to community composition over time (Jonsson 2006). Furthermore, land use and agriculture are also known to dramatically impact ephemeral water body communities (Hall et al. 2004, Dimitriou et al. 2006, Bouahim et al. 2014, Kerezsy et al. 2014, Bruno et al. 2016). All of these factors are cause for concern and are likely contributing to an overall decline in habitat security and ecosystem health of rock-holes. However, the scope of monitoring of freshwater ecosystems in semi-arid and arid Australia is currently limited, and the freshwater rock-holes are an ecosystem that have received very little study.

The Government of South Australia has identified sampling of the macroinvertebrate communities associated with the rock-holes in the Gawler bioregion (GB) as a means to assess their ecological value (White 2009). However, the remoteness of semi-arid and arid Australia and a lack of infrastructure, means that targeted biomonitoring programs are difficult to undertake. In cases where surveys have previously occurred, traditional ecological techniques involving direct specimen collection and morphological identification have been used to estimate diversity and abundance (Timms 2014, Pinder et al. 2000). Since taxonomic expertise is increasingly rare, particularly for invertebrates (Yeates et al. 2003, Austin et al. 2004, Engel et al. 2021), emerging technologies have become the focus of considerable interest as viable options for assessing ecosystem health. Environmental DNA (eDNA) is one such tool which involves collection of environmental samples, such as soil or water, and bulk amplification of shed genetic material that may be from organisms that are not physically present in the sample. The technique is relatively non-destructive and non-invasive as communities can be sampled and assessed with minimal disturbance and removal of material (White et al. 2020). Environmental DNA has recently been used to improve detection, monitoring, management, and conservation of endangered freshwater taxa (Rodgers et al. 2020), as well as characterising community composition (Holman et al. 2019). It has been used to detect species present at low densities (Johnsen et al. 2020), non-indigenous and invasive invertebrate species (Holman et al. 2019), and ecosystem stressors (Fan et al. 2020). Environmental DNA methods are underpinned by barcode reference libraries (BRLs), which assign taxonomy to DNA sequences and therefore provide biological meaning (Rimet et al. 2021). Metabarcoding is one common method of characterising eDNA and involves the amplification of a single genetic marker, or "barcode" and sequencing using high-throughput sequencing technology. When BRLs are robust and contain extensive information on target taxa, eDNA metabarcoding can be more accurate in identifying species than traditional approaches based on morphological assessment (Galimberti et al. 2021). Environmental DNA therefore represents a rapid and robust method for identifying species for which taxonomic expertise may be lacking, such as the invertebrate communities found in Australian freshwater rock-holes. To date, eDNA has only been used to monitor vertebrates that occur in association with Australian rock-holes (McDonald et al. 2023, Chapter 3) and no studies to date have used this method to characterise invertebrate communities from this environment. To date, the invertebrate communities associated with the granite rock-hole ecosystems of southern Australia have only been surveyed using traditional methods (Bayly 1997, Pinder et al. 2000, Timms 2014).

Here, we tested the application of eDNA metabarcoding for detecting ephemeral freshwater body invertebrates. The specific aims of this research were to a) test eDNA metabarcoding as a tool for detecting invertebrates in granite rock-holes of South Australia; b) characterise the composition of invertebrate communities in these ecosystems; c) determine whether these communities varied spatially and temporally; and d) provide recommendations regarding community conservation and management. It is predicted that eDNA metabarcoding will allow detection of a suite of invertebrate taxa, including crustaceans and insects, and that the communities recovered with eDNA metabarcoding will vary spatially and temporally.

4.3 Methods

Site description

Hiltaba Nature Reserve (hereafter HNR) is a large ex-pastoral property that borders the Gawler Ranges National Park to the north of the Eyre Peninsula in South Australia (Figure 4.1). The Reserve is approximately 78,000 ha in area and has been managed for conservation outcomes by Nature Foundation since its acquisition by the organisation in 2012. The Reserve is primarily composed of a series of rolling granite hills interspersed with woodland and grassland and contains unique habitats utilised by local plants and animals known to be in decline elsewhere (Nature Foundation 2023). The Nature Foundation has enacted a series of conservation programs that aim to improve biodiversity management at HNR.

The granite hills present throughout much of HNR are entirely exposed in many locations, resulting in granite outcrops of varying slope and morphology. These outcrops are generally flat with slopes of <20° and often display depressions which, following rain, provide an impermeable location for storage of water and sediments. These depressions or 'rock-holes' support a suite of vertebrates (Chapter 2), aquatic invertebrates and plants. In Chapters 2 and 3 the rock-holes were identified as locations of high ecological value for local vertebrate biodiversity.

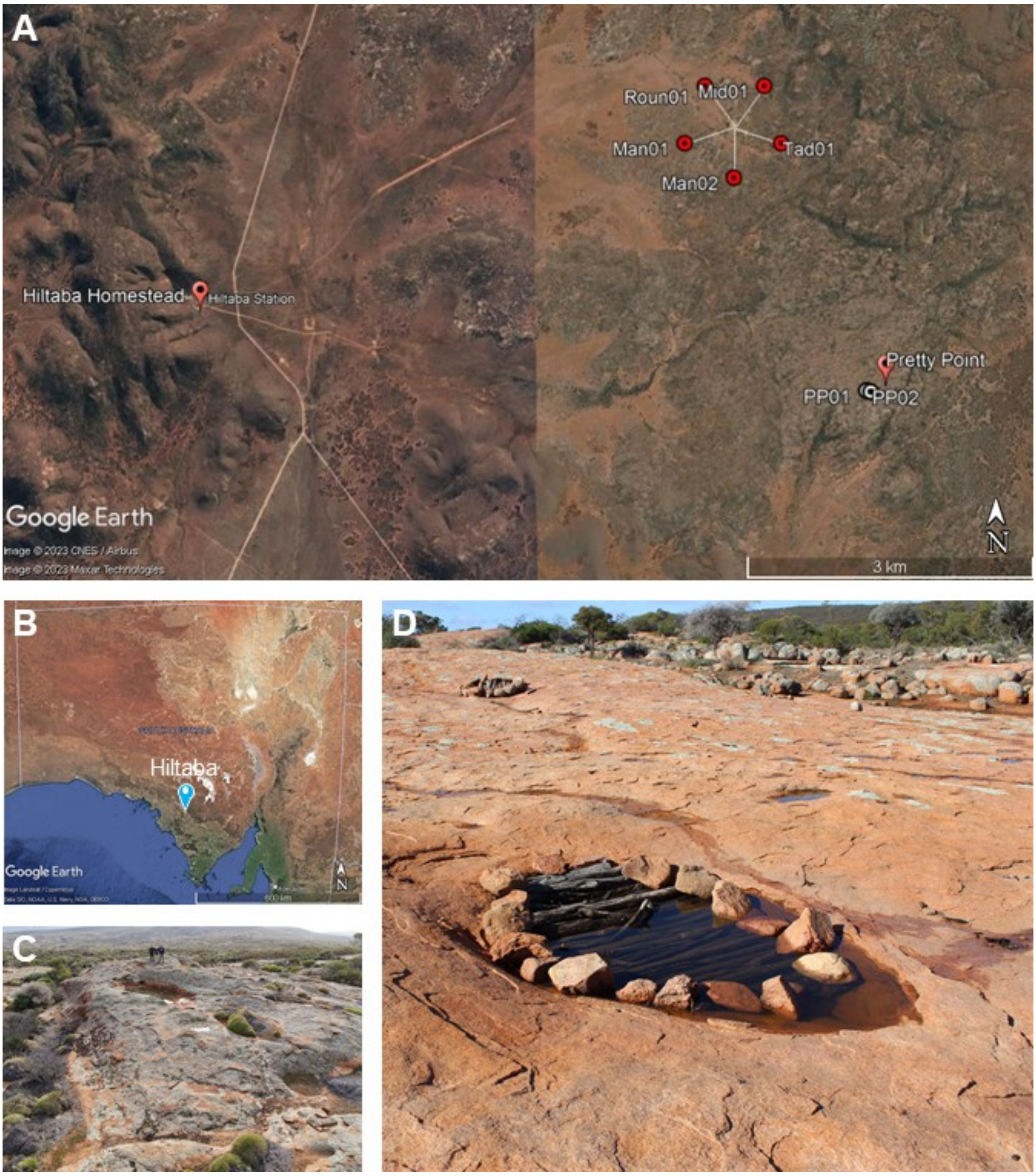

Figure 4.1. Study location of the present study. A) The location of Hiltaba Nature Reserve in South Australia; B) locations of the seven rock-holes sampled for freshwater eDNA; C) a rain-filled granite rock-hole at the 'Pretty Point' outcrop at Hiltaba Nature Reserve; D) a rain-filled granite rock-hole on the 'photopoint outcrop' at Hiltaba Nature Reserve, diameter of the rock-hole at widest point = 2.2 m.

eDNA sampling

Freshwater eDNA sampling was undertaken in 2020 at seven rock-holes in HNR on February 11–12 (summer), July 9–11 (winter), and October 2–3 (spring) (Table 4.1). Five one-litre replicates of water were collected from each rock-hole. Five replicates have previously been shown to be sufficient for detection within a range of freshwater systems (Shaw et al. 2016). One blank sample was taken at each rock-hole using one-litre of store-bought bottled water. Replicates were collected using one-litre, wide mouth NALGENE$^{\text{TM}}$ bottles that were washed with 20% bleach and dried prior to use.

Sampling was undertaken across two outcrops separated by approximately three kilometres. Four of these rock-holes were characterised as pit rock-holes and three were pan rock-holes following the classification scheme by Timms (2013b). During the summer time point (February), only rock-holes at outcrop 1 were sampled due to safety considerations associated with summer sampling of rock-holes at outcrop 2. During the spring time point (October), rock-holes ‘Man02’ and ‘PP02’ were not sampled due an insufficient volume of standing water. Samples were collected whilst wearing disposable latex gloves, stored in and transported in clean plastic tubs to minimise contamination. Sampling bottles were cleaned first with bleach solution, and then ethanol before reuse. Negative field controls (prepared in the field but using RO water) and equipment controls (prepared in the filtering room using RO water) were used to test for contamination.

Replicates and blanks were filtered on-site through membranes with 0.44 µm pores using a JAVAC vacuum pump (model CC-45) in February 2020. The pump was connected to a series of conical flasks, the first filled with silica beads. The second conical flask was attached to a magnetic filter funnel. A Pall Sentino microbiology pump was used in July and October 2020. Membranes were transported on ice and stored at -20°C prior to DNA extraction. A pore size of 0.44 µm was selected due to the high concentration of suspended solids present in rock-hole freshwater samples. Pump equipment was wiped down with bleach solution and then ethanol between filtering for each rock-hole.

Table 4.1. Summary of the rock-holes sampled for freshwater during February, July and October of 2020 (green checkmark). Rock-holes not sampled at certain time points (red cross) were either empty or had only a small volume of water present. Rock-hole type assignments have been adapted from Timms (2013b).

Rock-hole features						Sampled during		
Rock-hole ID	**Latitude**	**Longitude**	**Elevation (m)**	**Type**	**Outcrop**	**February**	**July**	**October**
Man01	-32.13941	135.13461	216	Pit	1	✓	✓	✓
Man02	-32.13943	135.13478	216	Pit	1	✓	✓	✗
Tad01	-32.1395	135.1348	216	Pit	1	✓	✓	✓
Roun01	-32.13931	135.13492	217	Pan	1	✓	✓	✓
Mid01	-32.13941	135.13461	217	Pit	1	✓	✓	✓
PP01	-32.165521	135.1488	312	Pan	2	✗	✓	✓
PP02	-32.165521	135.1488	310	Pan	2	✗	✓	✗

eDNA laboratory methods

DNA was extracted from half of each filter paper using a modified Qiagen DNeasy blood and tissue kit protocol (Qiagen, Germany) and an automated QIAcube extraction platform (Qiagen). Where more than one filter paper was used for a sample, equal portions of each paper were taken to total a half filter paper. All extractions were undertaken in a dedicated PCR-free laboratory, and extraction controls were processed alongside samples. Extractions were eluted in a final volume of 100 µL AE buffer.

To determine the required dilution for optimal amplification, PCR reactions were performed in duplicate on each extraction by adding DNA template directly to the PCR master mix (neat), then performing a serial dilution (1:10). The PCRs were performed at a final volume of 25 µL where each reaction comprised of: 1× PCR Gold Buffer (Applied Biosystems), 0.25 mM dNTP mix (Astral Scientific, Australia), 2 mM MgCl2 (Applied Biosystems), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.4 mg/mL bovine serum albumin (Fisher Biotec), 0.4 µM forward and reverse primers, 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies), and 2 µL template DNA. PCRs were performed on StepOne Plus instruments (Applied Biosystems) with the following cycling conditions: 95°C for 5 min, followed by 50 cycles of: 95°C for 30 sec, 49°C for 30 sec, 72°C for 45 sec, then a melt-curve analysis of: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, finishing with a final extension stage at 72°C for 10 min.

After selection of the optimal dilution (neat or 1:10), PCRs were repeated in duplicate as described above but instead using unique, single-use combinations of 8 bp multiplex identifier-tagged (MID-tag) primers as described in Koziol et al. (2019) and van der Heyde et al. (2020). Master mixes were prepared using a QIAgility instrument (Qiagen) in an ultra-clean lab facility, with negative and positive PCR controls included on every plate to ensure the validity of results. A sequencing library was created by combining samples into mini-pools based on the PCR amplification results from each sample. The mini-pools were then analysed using a QIAxcel (Qiagen) and combined in roughly equimolar concentrations to form libraries. Libraries were then size selected (250 - 600 bp cut-off) using a Pippin Prep instrument (Sage Sciences) with 2% dye-free cassettes, cleaned using a QIAquick PCR purification kit, quantified on a Qubit (Thermo Fisher), and diluted to 2 nM. The libraries were sequenced on an Illumina MiSeq instrument using a 500-cycle V2 kit with custom sequencing primers.

Bioinformatics and statistical analyses

Metabarcoding data were processed using QIIME2 v.2021.11 (Bolyen et al. 2019). Raw sequences were demultiplexed, trimmed by quality scores (a subset of samples were visualised in QIIME2.view and a consensus trim length was selected at position 120), filtered to remove chimeric sequences, and denoised using DADA2 (which accounts for amplification errors) (Callahan et al. 2016). Sequences were then tabulated to construct ZOTUs (zero-radius operational taxonomic units, a proxy for species) and to derive representative sequences for each OTU. Representative sequences were aligned using MAFFT (Katoh et al. 2002) and a midpoint-rooted phylogeny was generated using FastTree 2 for downstream calculation of diversity metrics (Price et al. 2010). A custom database was constructed from all invertebrate 16S rRNA and cytochrome oxidase subunit I (COI) sequences available on 8th June 2022 via the online GenBank repository (National Center for Biotechnology Information) using the following search strings: ((("16S ribosomal RNA" OR "16S rRNA" OR mitochondrion OR “CO1” OR “COI” OR “cytochrome oxidase subunit I” OR “cytochrome oxidase subunit 1”) AND (Mollusca[Organism] OR Arthropoda[Organism]) NOT Vertebrata[Organism] OR Archaea[Organism] OR Fungi[Organism]). BLASTN (Camacho et al. 2009) was then used to query our representative sequences against the custom database, with the maximum number of target sequences set to 1 per query. Representative sequences with non-invertebrate top hits, or those without a top hit that had a corresponding species-level assignment, were excluded from further analysis. The filtered dataset was then subjected to alpha diversity analyses using evenness (Pielou 1966), Faith’s phylogenetic diversity (Faith 1992), Shannon’s diversity index (Shannon and Weaver 1949) and beta diversity analyses using Bray-Curtis (Bray and Curtis 1957), Jaccard (Jaccard 1901), and weighted and unweighted Unifrac distances (Lozupone et al. 2011) against the metadata columns for rock-hole location and sample collection date. Principle coordinate analysis (PCoA) plots of these metrics were exported to R version 4.2.2 (RCoreTeam 2013) using QIIME2R v0.99.6 for further visualisation (Bisanz 2018). Stacked bar plots, violin plots, and PCoA plots were generated using *ggplot2* v3.4.0 (Wickham 2016). Rarefaction curves were generated using *iNext* v3.0.0 (Hsieh et al. 2022). Upset plots were generated using *UpSetR* v1.4.0 (Conway et al. 2017).

4.4 Results

Environmental DNA data

We successfully recovered invertebrate eDNA from 40 of 91 samples using the assay developed for insect and mollusc COI (see Methods). In total, we generated 5,379,229 sequences with 116,939 mean sequences per sample. A total of 179 ZOTUs were detected using BLASTN hits from GenBank. Of these, seven were present only in blanks and positive controls and a further 113 were considered unlikely to belong to freshwater invertebrate taxa after cross-referencing with online repositories (Atlas of Living Australia, World Register of Marine Species) and were excluded from downstream analysis. The remaining 59 ZOTUs belonged to 20 orders and 41 families (Figure 4.2A).

In contrast, the 16S rRNA crustacean assay recovered invertebrate eDNA from 41 of 91 samples, yielding 5,758,543 sequences with 122,522 mean sequences per sample. A total of 35 ZOTUs were detected using BLASTN hits from GenBank. Of these, seven were present only in blanks and positive controls and 16 were considered unlikely to belong to freshwater invertebrate taxa and were excluded from downstream analysis. The remaining 13 ZOTUs belonged to 4 orders and 4 families (Figure 4.2B). Between both datasets, invertebrates from 22 orders, 45 families were recorded, with an average BLASTN pairwise identity match of 89%.

Through comparison with the species lists presented by Timms (2014) we found that fourteen orders and 30 families that had not previously been recovered from South Australian freshwater granite rock-holes were detected using eDNA metabarcoding (see Table F3 for the complete comparison). Notable new order and suborder records included stoneflies (Plecoptera) Harpacticoid copepods (Harpacticoida) and various mites (Sarcoptiformes, Trombidiformes, Prostigmata, Endeostigmata).

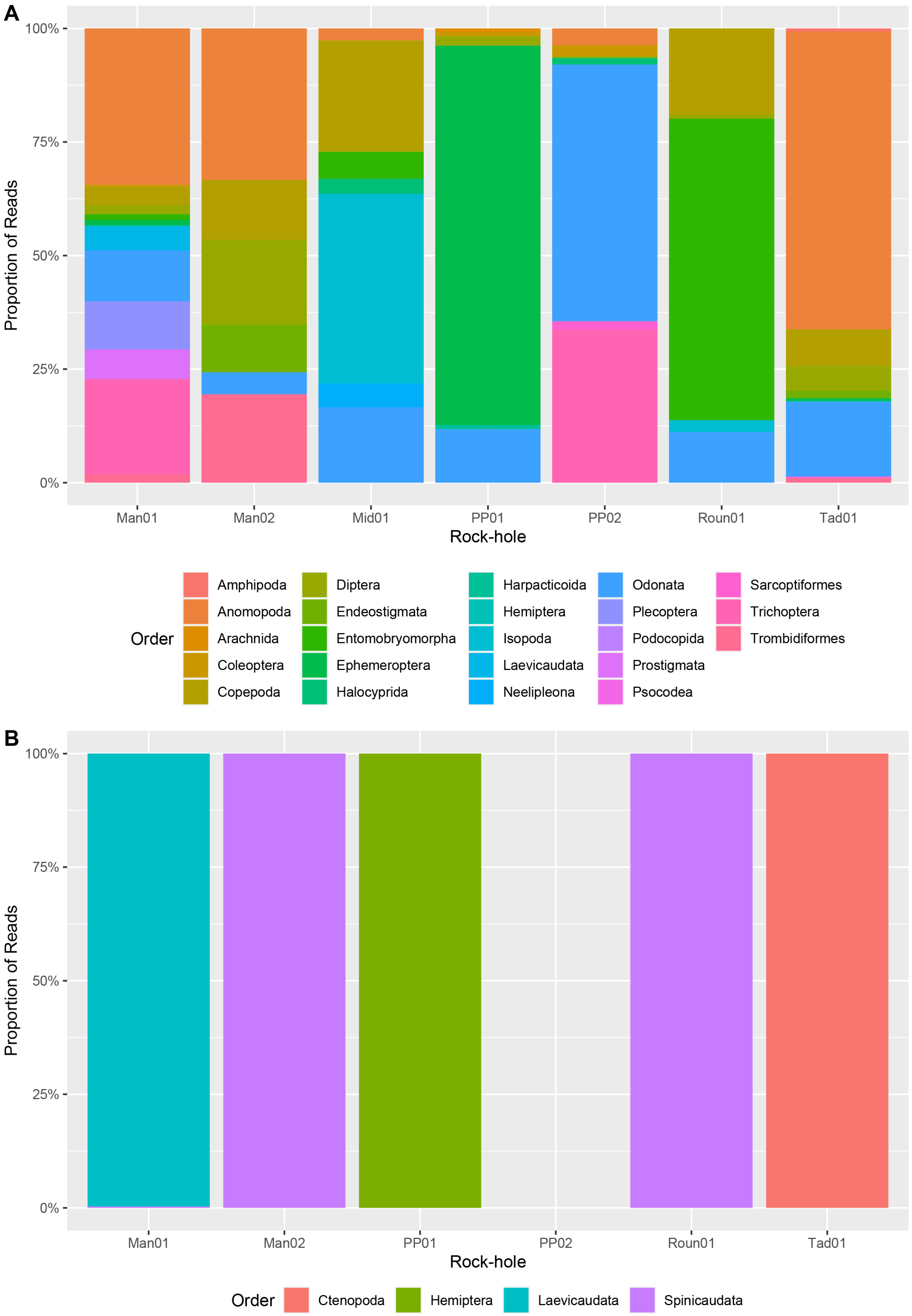

Figure 4.2. Stacked bar plots depicting the proportion of sequences assigned to family for each rock-hole for A) COI; and B) 16S rRNA. The full dataset is presented in appendix Tables F1 and F2, and stacked bar charts for the distribution of reads by family can be seen in appendix Figure F1.

Rarefaction could only be performed for five timepoints for the COI dataset and none of the replicates for 16S rRNA due to an insufficient number of data-rich replicates. Rarefaction of species richness suggested further sampling would likely capture over 75% of invertebrate diversity (Figure 4.3).

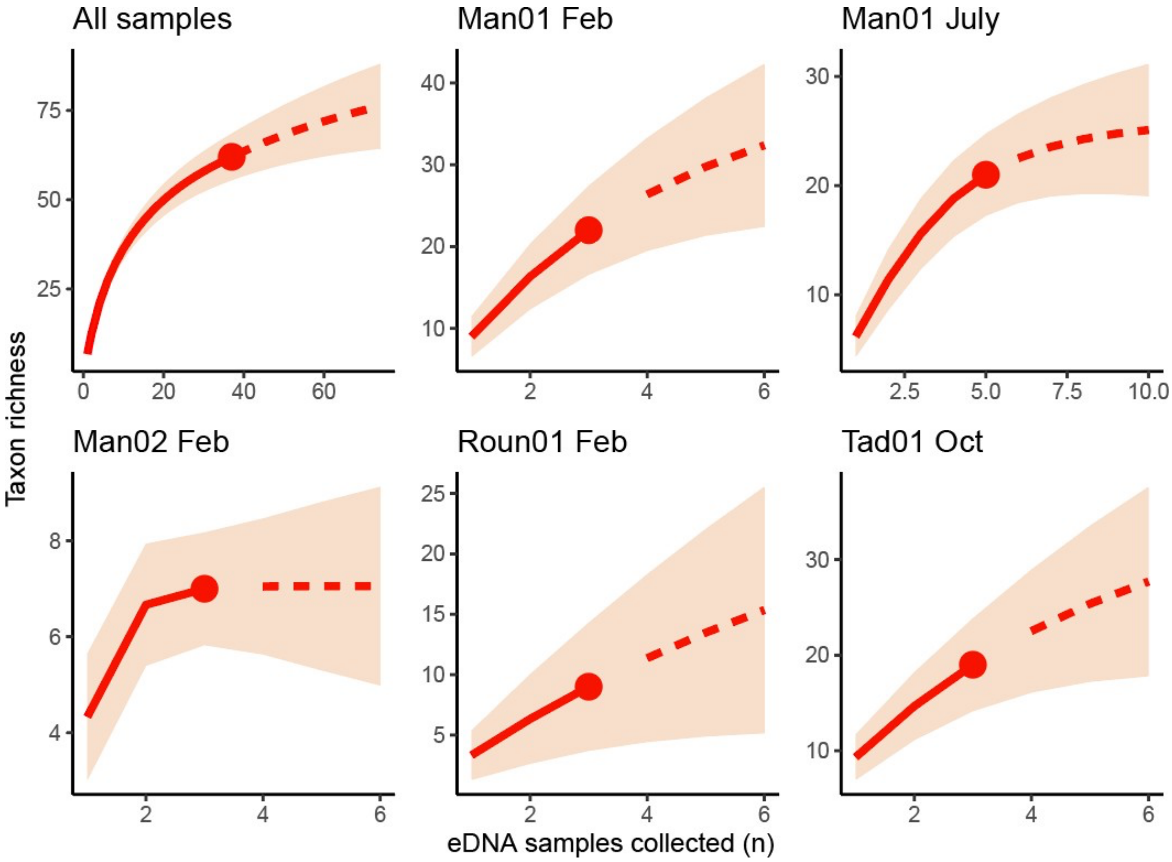

Figure 4.3. Rarefaction plots showing the impact of replication of freshwater eDNA samples for the entire dataset (top left) and at different rock-holes.

Overall ZOTU species richness for the sequences with top hits to GenBank records matches did not vary noticeably among sites (Figure 4.4A) and varied more considerably by sampling time, with winter/spring (July/October) collections displaying greater ZOTU richness than the summer collection period (Figure 4.4B).

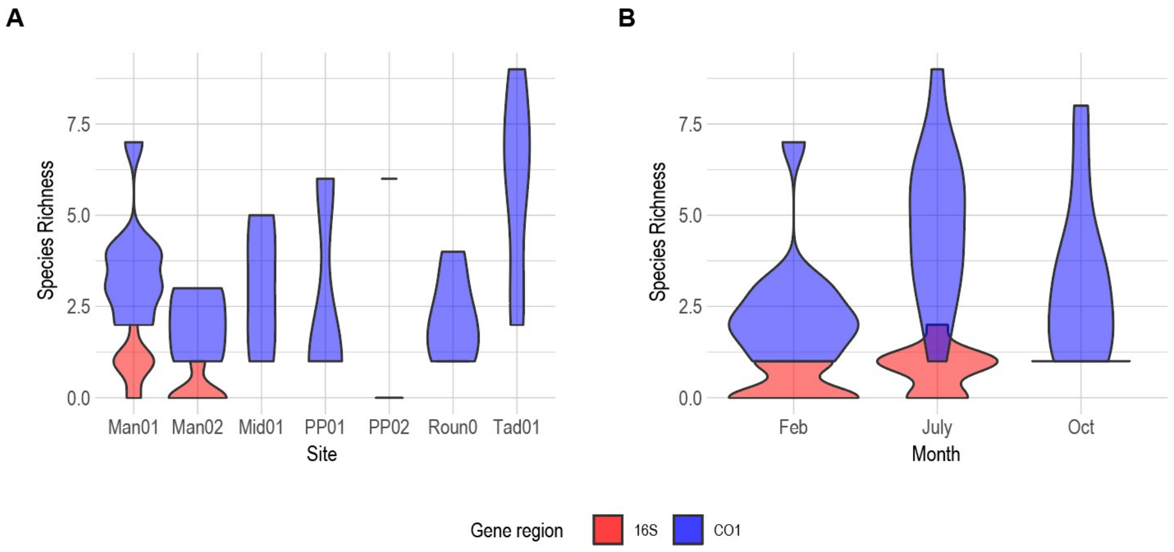

Figure 4.4. Violin plots showing variation in species richness among A) rock-holes; and B) sample collection month for COI (blue) and 16S rRNA (red).

Whilst species richness was relatively consistent across sites, ZOTUs were highly variable, with the majority of taxa (37 ZOTUs for COI and 3 ZOTUs for 16S rRNA) occurring only in a single rock-hole (Figure 4.5).

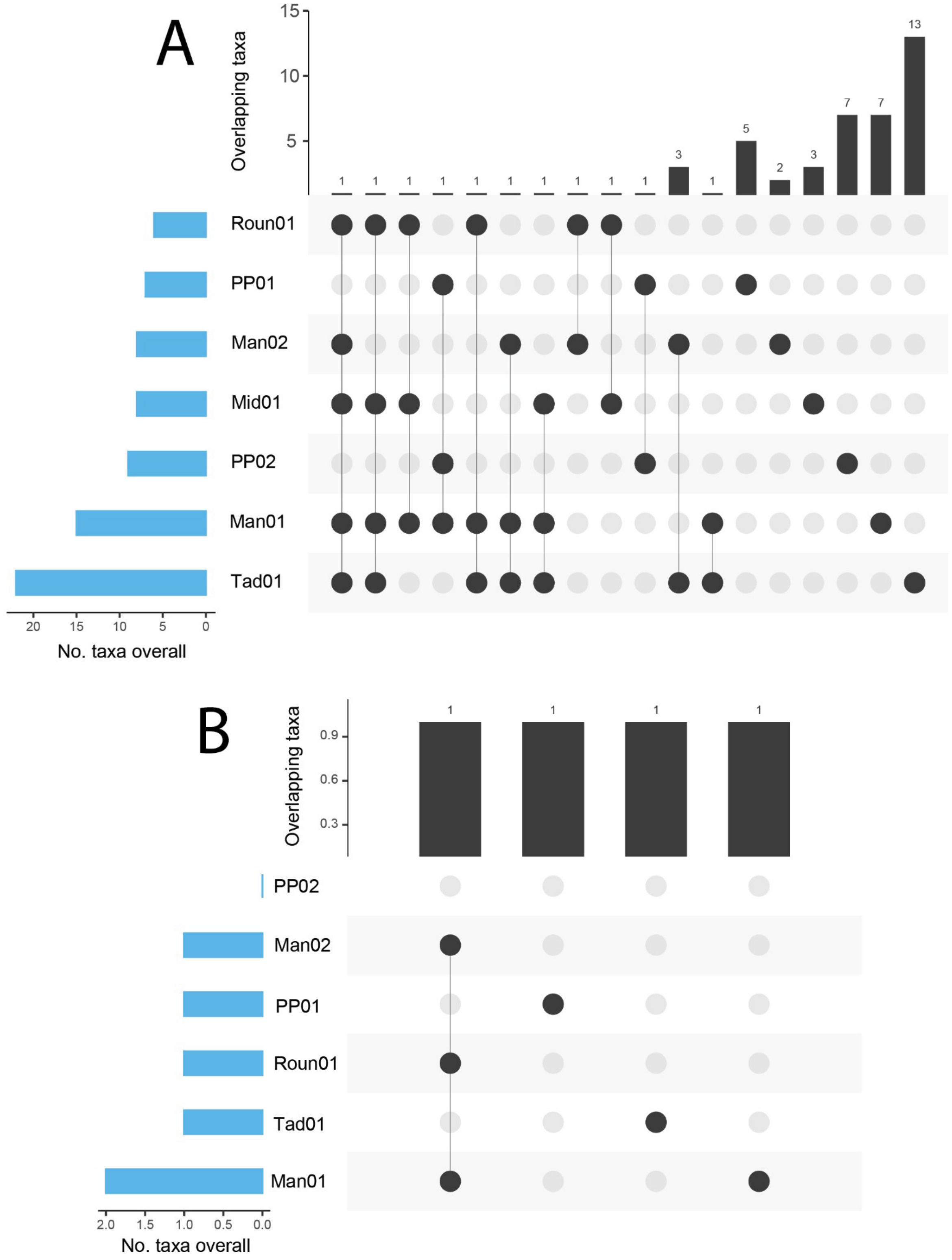

Figure 4.5. UpSet plots showing the number of ZOTUs shared among rock-holes for A) CO1 data; and B) 16S rRNA data. Blue bars represent the total number of taxa within a single site. Black bars represent taxa that are shared between each site with black circles indicating which sites these taxa are shared with. UpSet plots showing the data without replicates collapsed can be seen in Figures F2 and F3.

Principle coordinate analysis showed that replicates tended to cluster closest to other replicates from the same rock-hole (Figure 4.6). Collection month had a slight effect, although this was most pronounced for 16S samples before the taxonomic filter was applied. In the COI dataset and for 16S rRNA after the taxonomic filter had been applied, there was a degree of overlap across the collection months (Figure 4.6).

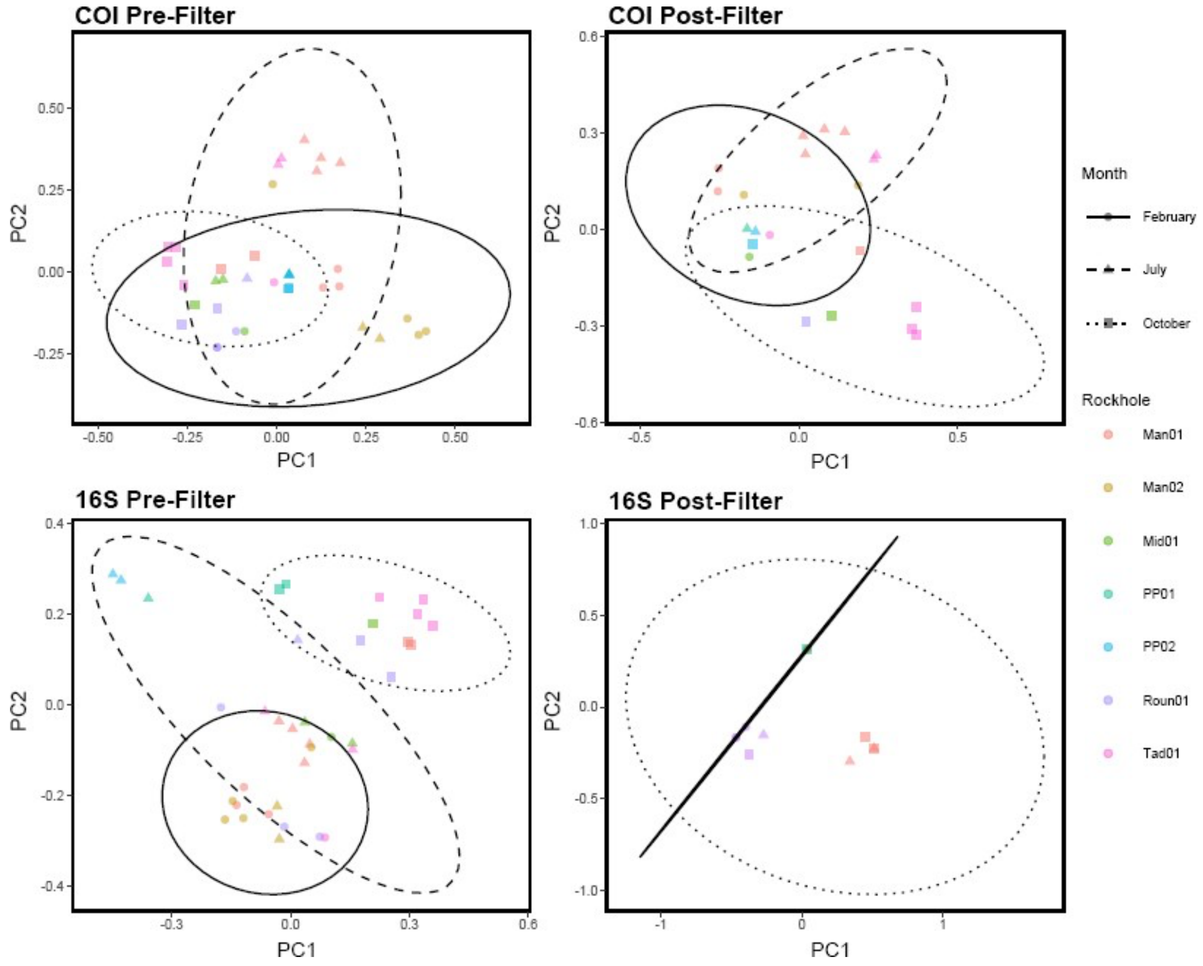

Figure 4.6. PCoA plots showing maximum dissimilarity for pre-filter (all sequences included) and post-filter (non-invertebrate sequences excluded) eDNA samples based on Jaccard distances. PC1 and PC2 represent maximum dissimilarity between samples in order of magnitude. Colours represent individual rock-holes and shapes represent the month during which eDNA sampling was undertaken. Ellipses have been applied to show the similarity between sample collection months.

4.5 Discussion

Here we present the first ecological assessment of the invertebrate communities within rock-hole ecosystems using eDNA metabarcoding approaches. Freshwater invertebrate species from 22 orders and 45 families in seven freshwater granite rock-holes at HNR in southern Australia were detected. Invertebrate communities of rock-holes throughout southern Australia have been the focus of a series of surveys using more traditional ecological methods (Bayly 1997, Pinder et al. 2000, Timms 2014, Hedges et al. 2021), but the present study is the first to employ eDNA as a more rapid tool for assessing community composition. Identification of lineages within rock-hole samples was generally possible, but barcode reference libraries generated from GenBank records were insufficient for species-level identification due to a lack of barcodes deposited from closely-related taxa. Invertebrate communities did not vary dramatically across HNR, although they did shift throughout the year with season and species richness peaking in winter. Below we discuss the suitability of environmental DNA as a tool for biomonitoring the freshwater granite rock-hole ecosystem and present a series of conservation recommendations.

Studies undertaken in granite rock-holes elsewhere in southern Australia have historically discovered high rates of rarity and short-range endemism among invertebrates (Bayly 1997, Pinder et al. 2000). The rock-holes at HNR are likely to be amongst the most pristine in the region as other areas of of the Gawler bioregion are still actively stocked with sheep, with only HNR and the neighbouring Gawler Ranges National Park having been entirely destocked (Nature Foundation 2023), and as such it is likely that rock-holes elsewhere are highly impacted. Understanding the changes in invertebrate community structure of rock-holes, in particular indicator species such as chironomids (Czechowski et al. 2020, Jones 1975, Pinder et al. 2000), are critical to estimating the ecological and biodiversity significance of rock-holes throughout the Gawler bioregion (Jenkin et al. 2011a). Here, the first robust assessment of invertebrate communities within rock-holes of the Gawler bioregion was undertaken using eDNA metabarcoding as a possible assesment tool. Of the orders and families identified using BlastN (Camacho et al. 2009) from sequences available on the online GenBank repository (National Center for Biotechnology Information), a number of common freshwater bioindicator groups (Firmiano et al. 2017, Czechowski et al. 2020, Schröder et al. 2020, Vilenica et al. 2021) were detected including chironomids (Diptera: Chironomidae), mayflies (Ephemeroptera: Baetidae) and dragonflies (Odonata) (see Figure 4.2, F1, Table F1). These groups occurred frequently across samples, but feature counts (the number of sequences that mapped to a species) varied dramatically. Interestingly, the most dominant organismal groups in terms of relative read abundance were Anomopoda (water fleas) for COI and Spinicaudata (clam shrimps) for 16S rRNA. Fourteen orders and 30 families had not previously been recovered from South Australian freshwater granite rock-holes (Timms 2014, see Table F3 for the complete comparison). Notable new order and suborder records included stoneflies (Plecoptera) Harpacticoid copepods (Harpacticoida) and various mites (Sarcoptiformes, Trombidiformes, Prostigmata, Endeostigmata). These records contribute new information to understanding of the composition of rock-hole invertebrate communities in semi-arid and arid Australia. Several of these groups are known to occur in the freshwater rock-hole ecosystems in Western Australia (Pinder et al. 2000, Bayly 1997), a system thought to have been isolated from the South Australian rock-holes by the biogeographic barrier of the Nullarbor Plains (Timms 2014). Validation of these records through targeted field surveys would allow for further identification and incorporation into barcode reference libraries to facilitate future metabarcoding work.

Rock-holes such as those discussed here have previously been proposed as being ecosystems of significant evolutionary value, both as refuges (locations that allow biota to persist despite short-term conditions of disturbance such as drought or flooding), and refugias (locations that allow biota to persist despite environmental change occurring across longer evolutionary time periods) (Davis et al. 2013). Davis et al. (2013) qualify this by suggesting that temporary and ephemeral water bodies are likely to act as refuges for only highly mobile taxa, such as dragonflies and water beetles, but may serve as refugia for less mobile taxa such as less dispersive freshwater crustaceans. The rock-holes of the Gawler Bioregion may therefore represent locations from which individuals present in source populations may disperse to replenish the egg (and seed) banks of nearby water bodies (Sheldon et al. 2010, Davis et al. 2013). Our characterisation of these communities using eDNA metabarcoding builds upon the work of Davis et al. (2013) by improving our understanding of both the composition of these communities, and their variability at spatial and temporal scales.

Insights into spatial and temporal community variability

Our results obtained with freshwater eDNA metabarcoding provide insights into the community dynamics of the invertebrate communities of the freshwater rock-holes system. By collecting samples from seven rock-holes across two outcrops separated by approximately 3 km, and at three different time points over a year, we were able to examine both spatial and temporal patterns. Overall species richness was not observed to vary much among rock-holes (Figure 4.3), but the community composition varied significantly (Figures 4.2, 4.6). The rock-holes also showed a degree of spatial variability regarding the composition of invertebrate communities, with replicates collected from the same rock-hole often displaying high similarity (Figure 4.6). However, we observed no effect of outcrop location, with rock-holes at the Pretty Point outcrop nested within the variability of the rock-holes at the Photopoint outcrop. This finding is consistent with that of Pinder et al. (2000) and Timms (2014) where only slight clustering of rock-holes by outcrop was observed based on invertebrate assemblages. Due to logistical limitations, we were only able to survey two outcrops on one property, but rock-holes have been located throughout the GB (Jenkin et al. 2011a). Sampling of these additional rock-holes spanning the entirety of the GB may improve confidence in assessments of spatial variability.

Rock-hole invertebrate communities were varied temporally in their composition, with species richness across all samples peaking in winter (July) (Figure 4.4). Community composition measured with Jaccard distances varied by sampling month, with some overlap in the communities occurring in both the pre- and post-filter dataset (Figure 4.6). Whilst it is likely that sampling throughout a single year is insufficient to accurately capture temporal variation in the rock-hole invertebrate communities, these results support findings of previous research of temporal variability in rock-hole communities and ephemeral communities (Bayly 2001, Timms 2014). In particular, Timms (2014) showed that five visits over two to three years were needed for species accumulation curves to reach a plateau. Similarly, Bayly (2001) observed gradual change in species composition with time within a freshwater rock-hole, as well as maturation of taxa detected. As such, we suggest successive sampling over consecutive years would improve confidence that the entire community has been adequately sampled. This inference is supported by the rarefaction curves, suggesting that additional field seasons would detect further taxa not recorded here (Figure 4.3).

Environmental DNA as a biomonitoring tool

Environmental DNA generally performs well when compared to more traditional methods for assessing freshwater invertebrate communities (Robson et al. 2016, van der Heyde et al. 2023, Keck et al. 2022, Villacorta-Rath et al. 2022, Davis et al. 2023). Indeed, McDonald (2023) used freshwater eDNA deposited by vertebrates to assess rates of visitation to freshwater granite rock-holes, and in Chapter 3 I provide a comparison and validation of this technique with a more conventional camera trapping approach. Here we show the common taxonomic groups present at HNR rock-holes including Anomopoda, Diptera, Hemiptera, Odonata and Sarcoptiformes. Traditional techniques based on morphology often rely on removal of material from the system, including in many cases live organisms (Timms 2014, Hedges et al. 2021). For systems of relatively small volume, and with slow recharge of water, such removal can be disruptive and destructive. Conversely, sampling for freshwater eDNA results in only a small volume of water being removed from the system (McDonald et al. 2023), though such volumes of water are not always available which may impact capacity to apply methods at times. Furthermore, sampling is easy and can be undertaken by field operatives (e.g. landholders, conservation workers, volunteers) without substantial scientific training (Prie et al. 2021, 2023). As such, we suggest that eDNA metabarcoding is a good solution to sampling invertebrates associated with Australian ephemeral freshwater bodies such as the rock-holes as discussed here.

In our study, incomplete barcode reference libraries substantially limited the extent to which metabarcoding could determine robust species names for ZOTU's. For even comparatively well-studied freshwater invertebrate taxa, such as Odonata, reference datasets are often lacking (Galimberti et al. 2021), and only allow identification to higher taxonomic levels (Mulero et al. 2021). As a result, inferences made regarding the functional roles of species are limited by low confidence in species identities. More robust barcode reference libraries would improve this confidence and allow for assessments of functional groups (Rimet et al. 2021). Comprehensive barcode reference libraries improve confidence in species assignments and in eDNA metabarcoding studies generally (Ekrem et al. 2007, Weigand et al. 2019, Saccò et al. 2022), and as such we recommend future research to develop them for the granite rock-hole ecosystem.

Future work

Applications of eDNA metabarcoding are currently limited in the scope of ecologically informative data that can be gathered, with difficulties in understanding the link between eDNA sequence abundance and organismal abundance and biomass (Johnsen et al. 2020). Studies requiring these data often need to pair eDNA collections with other more traditional analyses such as isotopic and radiocarbon analysis which allow inference of diet regimes, food webs and ecosystem function (Saccò et al. 2019) However, this study demonstrated the validity of eDNA as a broader tool, suited to assessing the various communities associated with the ecosystem. In future, more robust assessment of specific taxa, more targeted freshwater eDNA approaches making use taxon-specific assays, would be ideal. We also recommend that such surveys be used as an opportunity to generate specimens to be used to assemble more robust barcode reference libraries as mentioned above (Rimet et al. 2021).

In some cases, eDNA metabarcoding allows for assessment of the impact of environmental factors such as dissolved oxygen and heavy metals on invertebrate communities (Wang et al. 2023), as well as in making inferences regarding potability of drinking water (Shim et al. 2023). As the rock-holes are a source of drinking water to local vertebrate communities (Chapters 2, 3), adaptation of these techniques may allow for assessment of resource quality in conservation programs.

4.6 Conclusions

Our findings demonstrate the suitability of freshwater eDNA metabarcoding as a method for determining the composition of communities associated with the freshwater rock-holes present in the Gawler bioregion. We were able to identify the key taxonomic groups living in these communities, and the variability within them at both spatial and temporal scales, which saw a peak in species richness during winter. We recommend that eDNA metabarcoding be used as a method for monitoring these communities and that future research be undertaken to improve barcode reference libraries to facilitate such work.

Statement of Authorship

Title of Paper	Climate change will drive asymmetrical species responses in arid-lands ephemeral freshwater rock-holes
Publication Status	I Published t Submitted for Publication t Accepted for Pubfication f7 Unpublished and Unsubrnitted work W"itten in manuscript style
Publication Details	Unsubmitted but with intention to submit.

Principal Author

Name of Principal Author (Candidate)	Brock A Hedges
Contribution to the Paper	Led methodological design and production of manuscript, supported data curation and data analysis.
Overall percentage (%)	65%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and Is not subject to any obligatfons or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	/ Date 1 VL/ 07-/ 1..02-5

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
li. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contributlon.

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Contribution to the Paper	Led data curation and data analysis and edited the manuscript
Signature	Date 4/7/2023

Name of Co-Author	Stuart C Brown
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Nme of Co-Author	Dillon Campbell		
Contribution to the Paper	Provided project guidance, methodological support and edited the manuscript		
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ame of Co-Author	Damien A Fordham		
Contribution lo the Paper	Provided project guidance, supervision and edited the manuscript		
Signature		Date	18/07/2023

Chapter 5. Climate change will drive asymmetrical species responses in arid-lands ephemeral freshwater rock-holes

5.1 Abstract

Throughout much of arid Australia, surface freshwater bodies are rare, disconnected, and often ephemeral in nature. The freshwater granite rock-hole ecosystem present throughout much of Southern Australia is one such ecosystem. These granite rock-holes are entirely rain-fed and as such are likely vulnerable to projected drying and warming resulting from climate change. We investigated the impacts of climate change on this ecosystem, and its associated invertebrate biodiversity. Using unmanned aerial vehicle photogrammetry, we measured the volume of a series of rock-holes ($n = 5$) at Hiltaba Nature Reserve (South Australia) and characterised their current hydrological trends. We then made projections as to the deviations from these trends under a series of emissions scenarios (RCPs 4.5 and 8.5). Finally, we undertook a laboratory experiment to observe the demographic responses of two invertebrate species associated with the system. We predict that the ecosystem will increase in average water temperature and decrease in hydroperiod under all future emissions scenarios, with an up to 42% decrease in hydroperiod under severe emissions scenarios. We observed demographic responses in invertebrate populations that suggest some capacity to compensate for these changes, although under severe emissions scenarios maximum population size will likely decline. Furthermore, we observed that the two species studied here responded asymmetrically to the stress of shortened hydroperiod and increased temperature. We suggest that future conservation and management programs that seek to safeguard the invertebrate biodiversity of the rock-holes consider the asymmetry in species responses to climate stressors. Furthermore, when considering the impacts of climate change on the entire rock-hole invertebrate community, alleviation of other threatening processes to the ecosystem should be a priority.

5.2 Introduction

The impacts of climate change are being recorded on all continents, in all environments and in all habitat types (Scheffers et al. 2016, IPCC 2022). Although understanding of the impacts, extent and mechanisms of these changes are critical for informing proactive management (Araújo et al. 2005), Australian freshwater ecosystems are amongst the least well studied (IPCC 2022). Yet Australian freshwater bodies, particularly those that are dependent exclusively on local weather patterns with no groundwater input are clearly at risk. Those in areas where significant drying is projected will experience disruption to their historical hydrological regimes, with potentially cascading effects to their ecology (Gillespie et al. 2020). These freshwater bodies may decrease in their absolute size or increase in their ephemerality. In southern Australia, a unique freshwater habitat utilised by a diversity of organisms has formed in exposed granite outcrops through physical and chemical weathering processes over vast periods of geological time (Twidale and Corbin 1963, Twidale and Romani 2005). Known as rock-holes, this geological weathering provides a location for the temporary storage of rainfall that is ephemeral in occurrence (Bayly 1997, Timms 2014). Since rainfall throughout much of Southern Australia is infrequent, unpredictable and totals vary greatly between years (King et al. 2020, Imteaz and Hossain 2022), storage of freshwater can be maintained for one week to four mounts in duration (Timms 2017).

Despite the extreme transitions from an aquatic habitat to an entirely dry habitat, a complex community of plants, invertebrates and some vertebrates lives within the rock-holes (Pinder et al. 2000, Timms 2014, Hedges et al. 2021). These organisms, particularly the invertebrates, are thought to possess a suite of adaptations that allow them to persist, despite the highly ephemeral nature of the habitat. Many predatory species, such as frogs, damselflies and diving beetles exhibit behavioural adaptations involving repeated colonisation events after wetting (Bayly 1997, Hedges et al. 2021). Herbivorous and algivorous invertebrates, which are much more abundant in the system are commonly recorded to persist by exhibiting dormancy phases as an egg or in some cases as a live animal (Jones 1975, Bayly 1997, Timms 2014, 2017). These adaptations likely allow such organisms to persist throughout the regular dry periods. These filter-feeding herbivores and algivores likely provide ecosystem services by maintaining and regulating water quality and potability (Coughlan 1969, Atkinson, Vaughn et al. 2013, Buelow and Waltham 2020, Simeone, Tagliaro et al. 2021). In environments where accessible surface freshwater sources are rare and separated by large distances, these rock-holes provide a source of freshwater of likely high ecological and conservation value. Native Australian vertebrates from all four terrestrial classes have also been recorded visiting the rock-holes to access the water resources made available here (Chapters 2, 3). A series of invasive vertebrate species (both herbivores and predators) have also been recorded attending these sites (Chapters 2, 3). As such it is likely that these sites are implicated in the conservation and management of arid-zone vertebrates, as well as efforts to supress harmful invasive species (Chapter 2).

The freshwater rock-holes across southern Australia are in a region of projected drying climate (IPCC 2022), and are already highly ephemeral in their occurrence (Bayly 1997, Timms 2014). With climate change, decreases in the average duration of the period during which water is present in the system (hydroperiod) are likely to occur. Decreased hydroperiods may impact the fitness of the invertebrate communities (Bellin et al. 2021). Furthermore, it is possible that these decreased hydroperiods will be insufficient for some proportion of the invertebrate communities to achieve their historical reproductive output, although increased temperatures may also reduce developmental time for crustaceans (Bellin et al. 2021). Climate projections also suggest that a shift in seasonality of this rainfall is likely to occur (Imteaz and Hossain 2022). Where the rock-holes have historically received the greatest proportion of rainfall during the Australian Winter (June – August), a shift towards less predictable and more intense Summer rainfall is likely. Such changes may cause further stress to invertebrate communities due to higher rates of hatching during less optimal Summer conditions, periods where evaporation is more rapid due to higher ambient temperatures. The impacts of climate change are cause for concern regarding the security of the rock-hole invertebrate communities, and to the ecosystem services that they provide. If these communities are impacted substantially there may also be cascading impacts to local vertebrate communities that are dependent on the system (Chapters 2 & 3).

To better understand the impacts that climate change is likely to have on freshwater granite rock-hole ecosystems, a baseline understanding of current day hydrological dynamics is needed. Current knowledge is limited to anecdotal estimates as to how many weeks the rock-holes often remain wet for (Timms 2014) and to using rock-hole volume as a surrogate for hydroperiod (Timms 2017). A better understanding of current hydrological trends is necessary if the effects of climate change are to be understood. For highly complex systems such as freshwater rivers, flood-plains, lakes and even static ponds, these dynamics are a complex network of inputs and outputs (Devia et al. 2015, Alizadeh et al. 2017, Durgut and Tamer Ayvaz 2023). It can be difficult to measure any one factor due to the additive, synergistic and confounding impacts of factors (see Figure 5.1A for a graphical summary of the interplay between these factors). Conversely, Australia's ephemeral freshwater granite rock-holes provide a system where many of these inputs and outputs don't occur and as such can be excluded from hydrological calculations (Figure 5.1B), simplifying these equations (Jocque et al. 2010). They are disconnected from other waterways, so there are no upstream or downstream flow dynamics, and due to their impermeable granite floors, there is no loss of water through infiltration, nor input from groundwater (Jenkin et al. 2011b). Due to the surrounding granite inhibiting plant establishment, their riparian zones are generally unvegetated (Jenkin et al. 2011a). Similarly, their shallow sediments rarely allow establishment of significant communities of aquatic macrophytes (Hedges et al. 2021), so loss of water to plant uptake and evapotranspiration is usually low. All of these factors make the rock-holes an ideal model system for observational and experimental studies into how climate change will impact Australian ephemeral freshwater bodies (Jocque et al. 2010).

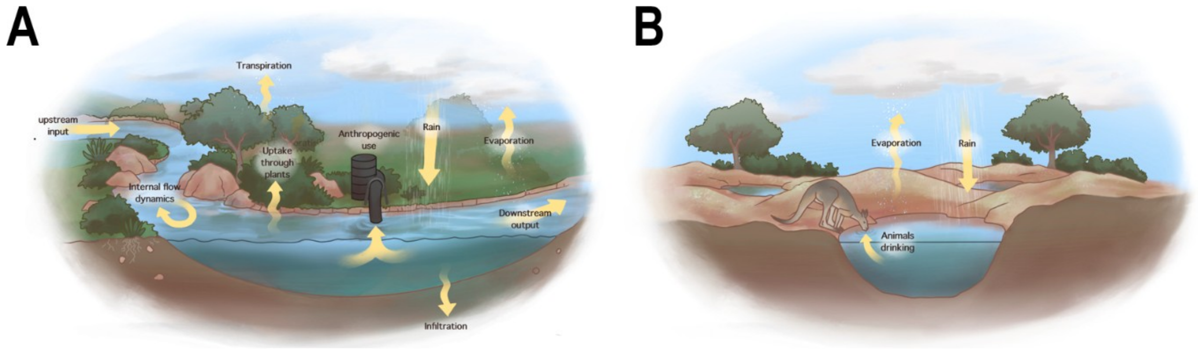

Figure 5.1. A summary of the major hydrological inputs and outputs in A) a typical Australian freshwater riverway; and B) an ephemeral freshwater granite rock-hole. Images illustrated by Mollie-Rosae Slater-Baker.

Using Unmanned Aerial Vehicle (UAV) photogrammetry, hydrological models and a series of microcosm experiments to measure responses among communities of freshwater rock-hole invertebrates, we aimed to a) characterise the current-day hydrological trends of these rock-holes using a combination of field-collected data and local weather data, b) perform projective climate modelling to determine the likelihood of significant changes to rock-hole hydrology under two emissions scenarios and at two timepoints, and c) determine whether future emissions scenarios are likely to result in demographic responses for invertebrate species associated with the rock-hole ecosystem. Based on our findings, we discuss the vulnerability of these populations and potential management strategies for the ephemeral granite rock-hole ecosystem.

5.3 Methods

Site description

Hiltaba Nature Reserve (HNR) is a vast (78,000 hectares in area) ex-pastoral property located adjacent to Gawler Ranges National Park in the northern region of South Australia's Eyre Peninsula (as shown in Figure 5.2C). The reserve sits within the Gawler bioregion (GB) and is characterised by low rolling granite hills, flat grass plains, and open woodlands. Formerly a sheep pastoral property, HNR has been under management by Nature Foundation since its acquisition in 2011 and has since been the site of a number of ambitious conservation efforts (Nature Foundation 2023). Due to these efforts, it exhibits generally higher vegetation quality than is seen throughout the surrounding landscape, much of which is still actively stocked with sheep and grazed. The granite hills that are characteristic of the region are in many places exposed, and exhibit indentations that provide a location for the temporary storage of rainfall. A series of five of these rock-holes at the ‘photopoint outcrop’ were selected for the field component of this research. Research was undertaken with permission and support from Nature Foundation.

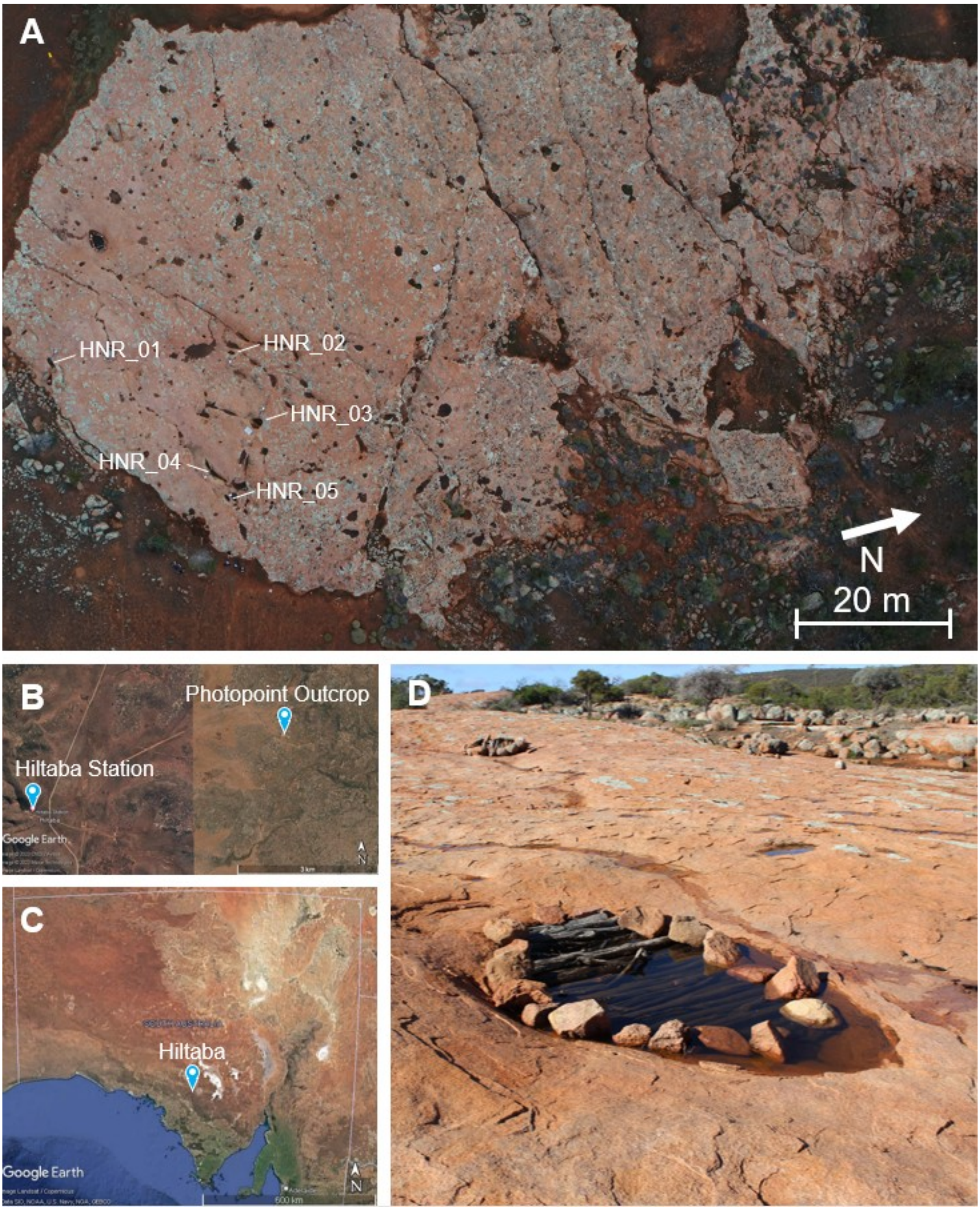

Figure 5.2. A) The locations of the five rock-holes at the photopoint outcrop imaged during 2021; B) the location of the photopoint outcrop in relation to the Hiltaba Station Homestead; C) the location of Hiltaba Nature Reserve in South Australia, Australia; D) a rain-filled, managed granite rock-hole on the photopoint outcrop, diameter of the rock-hole at it's widest point = 2.2 m.

UAV photogrammetry, volumetric and hydrological modelling

A series of 5 granite rock-holes at the photopoint outcrop at HNR (Figure 5.2A and 5.2B) were visited from 8–9 June 2021. Three-dimensional measuring tapes were woven through a wire grid, placed within each rock-hole, and weighed down using 5 and 10 kilogram cast-iron bench-press weights. Scale-boards of known size were deployed adjacent to each rock-hole.

Dedicated missions were performed for each rock-hole using a DJI Matrice 210 RTK carrying a Zenmuse X7 camera with a 50mm lens. Drone missions were planned in-situ using DJI Pilot by 'drawing' a 10 x 10m square polygon around the centre of the rock-hole. This polygon was used to define the position and extent of the flight transects.

Each rock-hole was covered by an automated (~10 by 10m) quadrat mission at an altitude of 17m, providing a ground sampling distance (GSD) of 0.13cm/pixel. The flight path followed a 'lawnmower' pattern made up of parallel flight transects and captured overlapping images in the direction of movement (forward overlap) as well as capturing overlap between parallel transects (side overlap). To ensure enough overlap for precise 3D reconstruction, 85% side overlap was used, with 50% forward overlap, resulting in approximately 50 images per rock-hole.

Raw images of rock-holes were processed into 3D models using commercial photogrammetry software – Agisoft Metashape. During this process the 3D models were scaled using the known distances on the scale-boards (Hodgson et al. 2020) set up around each rock-hole to ensure high spatial accuracy. In Metashape, the 3D models were used to retrieve altitude (MSL) values for every increment on the tape measure. The 3D models were then exported as digital elevation models (DEMs) in raster format.

Volumetric calculations were performed on the rock-hole DEMs using the 'Surface Volume' geoprocessing tool in ArcGIS Pro which calculates the volume of a DEM below a reference plane height. In our case, the volume was calculated at multiple reference plane heights per rock-hole, corresponding to each increment on the 3D tape measure.

Exploratory regression for relationships between weather and volume of water in each rock-hole were inconclusive. This was likely due to complex rainfall-runoff dynamics as a result of site topography and the patchiness of rainfall across the site. As such, we opted to use the average draw-down time of each rock-hole as estimated by monitoring of the rock-holes by rangers at HNR (see Figure G1). The average draw-down (from full) across all rock-holes was estimated at X days (+/- SD). Consequently, our lab experiment was controlled to ensure that our microcosms had a similar drawdown period.

Sediment collection.

Bulk sediment samples containing egg-stage specimens of a series of rock-hole resident crustacean taxa *Sarscypridopsis* sp. and *Daphnia* sp. were collected from rock-holes in the Gawler bioregion in SA (Figure 5.2). These genera are regular residents of ephemeral freshwater granite rock-holes and are frequently recorded in surveys of the habitat (Bayly 1997, Pinder 2000, Timms 2014). A series of

5 rock-holes of greater than 50 L capacity were selected and approximately 100 grams of dry-weight sediment was collected from each. Sediment samples were homogenised and transported to the lab.

Future climate simulation experiment

A series of 500 mL plastic containers were placed within reptile egg incubators (Exoterra incubator pro). Incubators were set to maintain temperature at 5 temperatures, 4 temperatures informed by future climate projections and 1 control temperature chosen to simulate present day field conditions (see Table 5.1). Incubators were placed within constant temperature cabinets programmed to hold at 2°C lower than each experimental scenario. These constant temperature cabinets were in turn placed within a temperature-control room that held at between 10°C and 15°C. The room was then subjected to a 12:12 hour day:night lighting cycle.

Rock-hole sediments (1 gram per tank) were placed within each tank, and then inundated with 135 mL of RO water on day 1 of the experiment. Tanks were then slowly dried so that each tank emptied in accordance within the projected average hydroperiod for each scenario (Table 5.2). Algal feed was added to each tank once every 3 weeks so as to ensure that resources were neither limiting, nor substantial enough to cause eutrophication. Tanks were monitored twice per week (usually every two to three days). Species present were first observed under a stereo microscope, and then the number of individuals were counted using the naked eye. A transparent grid was placed beneath the tank to improve accuracy and limit accidental recounting of individuals. The count was repeated multiple times until a consensus was reached.

Data analysis

Statistical analyses were performed using R version 4.2.2 (RCoreTeam 2013) and PRIMER (PRIMER-e 2017), using the package *PERMANOVA* (Anderson 2001). The maximum rate of population growth for each species in each tank was determined using the formula:

$$\log\left[\frac{!(\#\$\%)}{!\,(\#)}\right],$$

where *N* was the number of individuals at time *t*. Maximum relative abundances for each species were calculated for each tank by dividing the maximum by the current population size to standardise abundance comparisons between tanks and across emissions scenarios. Generalised linear models (McCullagh and Nelder 1989) and permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) were used to explore the effect of emissions scenario on demographic response variables.

5.4 Results

Rock-hole characteristics

We used UAV photogrammetry to generate 3-Dimensional models of five granite rock-holes at Hiltaba Nature Reserve. These rock-holes were all present on a single outcrop (Figure 5.2A) at an elevation of approximately 230 m asl. Volume measurements for these rock-holes ranged from 0.090476 m^3 (90.5 L) to 0.5487705 m^3 (548.8 L), with a mean volume of 0.2663681 m^3 (266.4 L) (Table 5.1).

Table 5.1. Physical characteristics of rock-holes surveyed with UAV photogrammetry

Rock-hole	Latitude	Longitude	Elevation (m asl)	Rock-hole volume (m^3)
HNR_01	135.134726	-32.13949741	229.80	0.1303555
HNR_02	135.1349253	-32.13935653	232.46	0.090476
HNR_03	135.1349614	-32.13934893	231.52	0.5487705
HNR_04	135.1348834	-32.13929287	232.02	0.428262
HNR_05	135.1347888	-32.13929884	231.58	0.1339765

Hydrological and climate modelling

Our hydrological modelling showed that rock-holes currently experience, on average, an 85-day hydroperiod (s.d. = 3.9) for the Winter-Spring period at HNR. Water temperature within the rock-holes for this period is on average 15.5 ± 0.5°C. We estimate that these temperatures will increase under all future emissions scenarios, and that rock-hole hydroperiod will decrease by up to 42% (Table 5.2).

Table 5.2. Summary of emissions scenarios and their expected impacts to rock-hole temperature and hydroperiod.

Laboratory Scenario	**Emission scenario**	**Year**	**Temperature (°C)**	**Hydroperiod (days)**
1	Present Day	2020	15.5	85
2	RCP 4.5	2050	16.9	73
3	RCP 8.5	2050	17.3	66
4	RCP 4.5	2090	17.5	63
5	RCP 8.5	2090	19.2	49

Demographic response to climate change experiment

Two species hatched from sediments collected from rock-holes at HNR. These taxa were tentatively assigned as *Daphnia clarinata* (Crustacea: Cladocera) and *Sarscypridopsis* sp. (Crustacea: Ostracoda) (Figure 5.3A). Hatching success was achieved for almost all tanks for *Sarscypridopsis* sp., however hatching success of *D. clarinata* was determined to be affected by emissions scenario (Figure 5.3B, see appendix Table G1 for summary of a generalised linear model testing the effect of emissions scenario on *D. clarinata* hatching success).

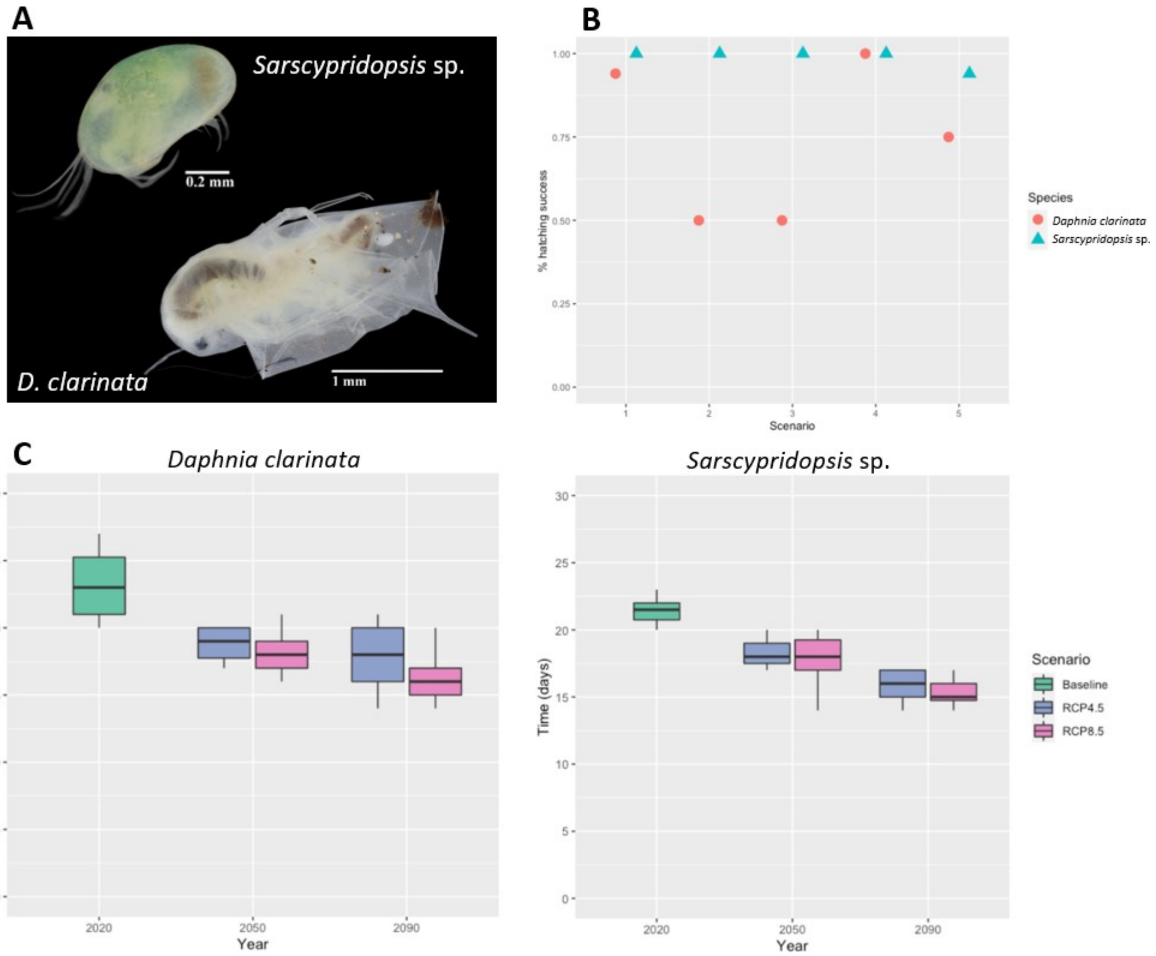

Figure 5.3. A) Photographs of rock-hole invertebrates hatched from sediments collected at HNR; B) the rate of hatching success for both species; and C) time to hatching for left: *Daphnia clarinata* and right: *Sarscypridopsis* sp., at current-day (2020) and future conditions (2050 and 2090) under two emissions scenarios (RCP 4.5 and RCP 8.5).

The number of days to hatching was greatest for both species in scenario 1 (current day), and declined in warmer scenarios (Figure 5.3C). A univariate PERMANOVA showed that emissions scenario (pseudo-F = 45.91 (2,51), p = 0.001) and year within emissions scenario (pseudo-F = 7.90 (2,51), p = 0.006) had strong to moderate effects on hatching date for *D. clarinata* (Table 5.3). A univariate PERMANOVA showed that emissions scenario (pseudo-F = 83.45 (2,71), p = 0.001) and year within emissions scenario (pseudo-F = 34.53 (2,71), p = 0.001) had strong to moderate effects on hatching date for *Sarscypridopsis* sp. (Table 5.3). This was supported by pair-wise tests (see appendix Table G2).

Table 5.3. Univariate PERMANOVA results of the main effect for *Daphnia clarinata* and *Sarscypridopsis* sp.

		Days to hatching		**Max. population growth rate**		**Max. relative abundance**	
Test	Species	Pseudo-F	p (perm)	Pseudo-F	p (perm)	Pseudo-F	p (perm)
Scenario	*Daphnia clarinata*	45.91 (2,51)	0.001	1.23 (2,51)	0.294	0.18 (2,51)	0.859
	Sarscypridopsis sp.	83.45 (2,71)	0.001	9.92 (2,71)	0.001	0.11 (2,71)	0.888
Year (within scenario)	*Daphnia clarinata*	7.90 (2,71)	0.006	1.50 (2,51)	0.225	2.52 (2,51)	0.089
	Sarscypridopsis sp.	34.52 (2,71)	0.001	8.64 (2,71)	0.001	4.29 (2,71)	0.012

Mean abundance of *D. clarinata* was lowest in current day scenarios and highest in 2050 emissions scenarios (Figure 5.4A left). Mean abundance of Sarscypridopsis sp. was also highest under 2050 emissions scenarios, but lowest under 2090 emissions scenarios (Figure 5.4A left). *D. clarinata* showed a distinctive pattern of having an abundance peak, followed by a gradual decline in abundance that coincided with the evaporation of water within tanks (Figure 5.4A left). This was not observed for *Sarscypridopsis* sp. which was observed to increase in abundance right up until the end day of each emissions scenario (Figure 5.4A right).

The maximum relative abundance of *D. clarinata* was lower in 2090 (Figure 5.4B), however differences were small and non-significant (Table 5.3). The maximum relative abundance of *Sarscypridopsis* sp. tended to be lower in 2090 for both emissions scenarios (Figure 5.4B right), however only differences between years within emissions scenarios were significant (pseudo-$F = 4.29$ (2,71), $p = 0.012$). Pair-wise tests indicated that this within emissions scenario difference was only present within the RCP 8.5 emissions scenario (see appendix Table G2).

The maximum population growth rate for both *D. clarinata* and *Sarscypridopsis* sp. was slightly lower in 2090 emissions scenarios (Figure 5.4C). Univariate PERMANOVA results (Table 5.3) suggested there was no significant difference in population growth rates of *D. clarinata* between current day and future emissions scenarios, nor between years within emissions scenarios were (Table 5.3). However, a significant difference in population growth rate was detected for *Sarscypridopsis* sp. both between emissions scenarios (pseudo-$F = 9.92$ (2,71), $p = 0.001$) and between years within emissions scenarios (pseudo-$F = 8.64$ (2,71), $p = 0.001$). This was supported by pair-wise tests (see appendix Table G2).

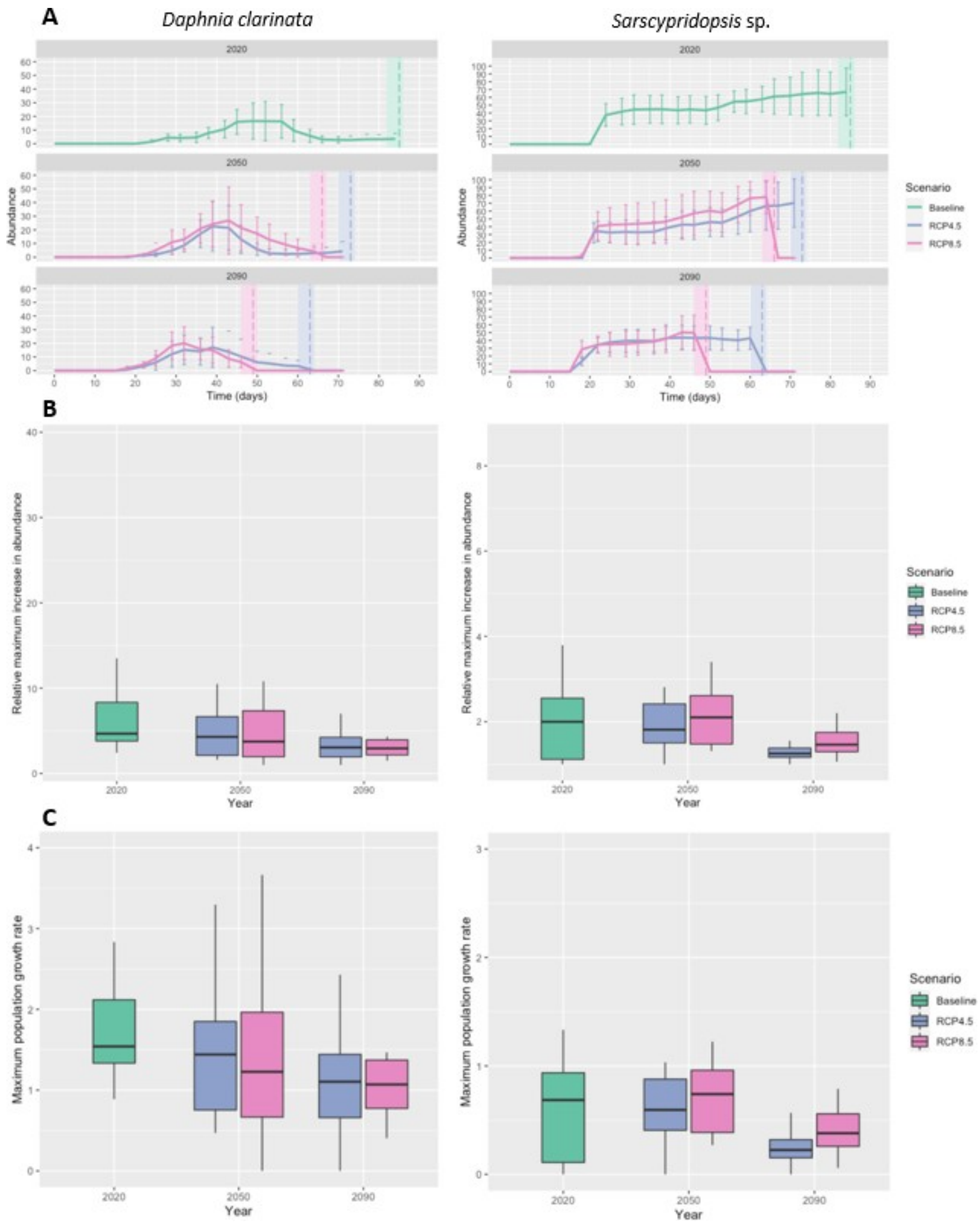

Figure 5.4. A) the mean observed abundance through time; B) the maximum relative abundance (relative to population size one week after hatching); and C) the maximum population growth rate of left: *Daphnia clarinata* and right: *Sarscypridopsis* sp. at current-day (2020) and future conditions (2050 and 2090) under two emissions scenarios (RCP 4.5 and RCP 8.5). Dashed lines in 5A indicate when the experiment ended, with coloured bands indicating the date of the last observation record.

5.5 Discussion

Ours is the first study investigating the likely effects of climate change on the Australian freshwater granite rock-hole ecosystem. Climate change is now expected to impact all habitats, across all continents globally (Scheffers et al. 2016), but the mechanisms and extent of these effects are often not well understood (Araújo et al. 2005). Our findings provide the first step in constructing a research framework to facilitate well-informed and proactive management of this unique ecosystem. Our modelling shows that decreases in the duration of hydroperiod for the ecosystem are likely to occur under all future emission scenarios. We also observed responses in the community dynamics of the invertebrates associated with this ecosystem. *Daphnia clarinata* potentially shows a resilience to the effects of climate change, and to a point may be likely to compensate for the decrease in hydroperiod, but these dynamics will be insufficient to compensate for the change expected under severe emissions scenarios. *Sarscypridopsis* sp. however did not display a similarly plastic response to the experimental stresses. We provide a series of recommendations regarding the conservation of these invertebrates, the rock-hole ecosystem, and its role as a freshwater resource for vertebrates in the broader environment.

To the best of our knowledge, we provide the first data-driven estimates of the current-day hydrology of the Australian freshwater rock-hole system. Whilst this ecosystem has been the focus of a number of comprehensive ecological studies, the hydroperiod is often only roughly described in terms of weeks (Timms 2014, Pinder et al. 2000), not presented at all (Bayly 1982, Bayly 1997) or inferred using habitat size as a surrogate (Timms 2017). Hydrological modelling work has been undertaken for rock-hole systems in arid and semi-arid environments overseas, where it was also observed that climate change would impact rock-hole invertebrate communities, although the effect was determined to be dependent on the dispersal capacity of taxa (Vanschoenwinkel *et al.* 2009). Our understandings of current-day hydrological trends therefore provide a foundation for future research in Australia that seeks to better understand the interplay between environmental factors and community composition.

The freshwater rock-hole ecosystem in the GB is one that is entirely fed by local rainfall, with no geological evidence of groundwater impact (Jocque et al. 2010, Jenkin et al. 2011b). The region they occur in is one expected to experience significant drying over the next 100 years (IPCC 2022a), with changes to local rainfall patterns, ambient temperatures and evaporation rates projected to occur as a result of anthropogenic climate change. Whilst these changes will result in deviations from historical norms for ephemeral freshwater ecosystems, particularly those that are exclusively rain-fed (Jocque et al. 2010), they are often overlooked in local, national and international climate assessments (Brooks 2009). Other EWBs, such as ephemeral rivers and creek-lines (Scott et al. 2003, Steward et al. 2012, Acuña et al. 2017), ephemeral lakes (Karagianni et al. 2018), seasonal wetlands (Strachan et al. 2014, Calhoun et al. 2017), vernal pools and temporary ponds (Andrushchyshyn et al. 2003, Kneitel et al. 2017), and claypans (Gibson et al. 2018), are similarly likely to experience deviations from their historical hydrological norms and associated increases in water temperature, and climate change has already led to shifts in the distribution and phenology of species (Ewald et al. 2013), impacts to plants, invertebrates (Frisch 2001) and vertebrates (Chessman 2011, Howard et al. 2016). Our study addresses this research gap and models the fine-scale impacts of climate change on this unique ecosystem. We show that the average hydroperiod of the ecosystem may decrease by up to half under 'worst-case' emissions scenarios (RCP 8.5). Global research has found that many ephemeral freshwater ecosystems will be similarly impacted, with decreases in hydroperiod being commonly projected (Pyke 2005, Hulsmans et al. 2008). As such, our findings contribute to this emerging narrative illustrating the likely impacts of climate change on ephemeral freshwater bodies.

Here we specifically investigated the effects of increased ambient temperatures and decreased hydroperiod on members of the rock-hole invertebrate communities. Our findings suggest that some members of these invertebrate communities, which consist of a mix of opportunistic occupants and ephemeral habitat specialists (Bayly 1997, Timms 2014), have a natural resilience to increased water temperatures and decreased hydroperiod. More rapid recruitment within populations of the water-flea *Daphnia clarinata* occurred under future climate simulations involving increased temperatures and decreased hydroperiod. However, this increase in recruitment rate was insufficient to compensate for the dramatically shortened hydroperiod under the severe future emissions scenario (RCP 8.5). Furthermore, the two species studied here were observed to vary in their response. *Daphnia clarinata* populations reaching a peak before declining as water dried up. Whereas populations of *Sarscypridopsis* sp. continuing to recruit until the simulated end date of each scenario. This asymmetrical response suggests that assessments of the impacts of climate change on arid-lands freshwater invertebrates need to be undertaken at the species-level, rather than the community-level. Rock-hole invertebrates are adapted to ephemerality (Jones 1975, Bayly 1997, Timms 2014) and as such they are likely to be capable of resisting and responding to the degradative impacts of climate change to some degree. Asymmetry in responses to climate change-driven deviations from historical hydroperiod duration and temperature norms has been observed for other freshwater invertebrates (Sardiña et al. 2017), including ephemeral freshwater body species (Tylianakis et al. 2008, Tasker et al. 2022). Our research contributes to an increasing base of evidence in the unevenness of responses to the stresses that will result from climate change.

Here, two species were used as models for how rock-hole species might respond to changes in hydroperiod. Given the life history differences among species in these ecosystems (Bayly 1997, Bayly 2001, Timms 2014, Chapter 4) we recognise that individual species responses are likely to vary. However, water fleas and ostracods are commonly the most abundant invertebrates in rock-hole communities, contributing significantly to the overall biomass within the system (Bayly 1997, Pinder et al. 2000, Timms 2014), and as such we suggest that are data provide insight into likely community-level changes. Furthermore, we have demonstrated that as a whole, invertebrate communities are likely to be very dynamic, supported by eDNA results that show that invertebrate community composition changes throughout a single year (Chapter 4). Similarly, rock-holes approximately 70 km south of our study site required five visits over a period of two to three years to capture the variability within rock-hole communities and see species accumulation curves asymptote (Timms 2014). These rock-hole ecosystems often comprise substantial functional complexity, with herbivorous and predatory species present (Timms 2014, Hedges et al. 2021, Chapter 4) with community structure driven by predation (Gibbons et al. 2006, Canals et al. 2011, Hamer and Parris 2013). Here, our study, representing only two algivorous species over a period of three months, whilst likely insufficient to capture the real temporal variability in the system, was an important first step in assessing community responses to climate change over a broader temporal span in an arid lands ephemeral freshwater habitat.

The rock-holes in the GB have been repeatedly noted to be of importance to local vertebrates (Jenkin et al. 2011a, Hedges et al. 2021), as is the case for similar rock-holes (gnammas) in Western Australia (McDonald et al. 2023). A suite of vertebrates present in the region have been shown to regularly visit the rock-holes as an accessible source of freshwater (Chapters 2, 3), and as such, any decreases in the average hydroperiod of the system resulting from climate change will likely result in a decrease in the availability of freshwater as a resource to these vertebrate communities. It is likely that this decrease in availability will be unequal in its impact to vertebrates, with vertebrates with lower capacity for dispersal being less capable of compensating for the decreased resource availability. More dispersive species, which include invasive species such as feral goats, are likely to be more resilient, due to their ability to move long distances through the landscape in search of freshwater (Moseby et al. 2021).

Implications for management

In the face of the broad impacts to various habitats resulting from climate change, it is critical that strategies are developed to slow biodiversity loss and achieve restoration wherever possible. Our findings provide additional evidence to the immense volume of data illustrating the harmful impacts of climate change that will progressively intensify over the next century. Specifically, the warmer and drier conditions expected under sever emissions scenarios (RCP 8.5) will result in warmer water temperatures and decreased hydroperiods. As such, we emphasise that to prevent such impacts, a global effort to limit and mitigate further greenhouse gas outputs must be made. Locally however, the management of the rock-hole ecosystem must address the variability between species in their responses to the effects of climate change. Where capacity for compensation varies across a community, an unequal response to the impacts of climate change may result in a trophic cascade effect (Mair et al. 2014, Biswas et al. 2017, Bowler et al. 2017). This could result in shifts in evenness and abundances in the community (Shin and Kneitel 2019) and altered food web dynamics. If left unmanaged, this may lead to loss of species and functional diversity (Covich et al. 1999, Cuenca Cambronero et al. 2018) and eventually loss of ecosystem service provision (Winfree et al. 2015) and resource value. We recommend that monitoring programs involving standardised sampling be implemented to accurately record the composition and relative abundances of these invertebrate communities.

We also emphasise the need for management of the other threats to the ecosystem that have been explored in Chapters 2, 3 and 4. Invasive species management, would decrease drawdown resulting from large herds of goats that frequent rock-holes (Chapter 2). Similarly, further destocking throughout the Gawler bioregion would decrease the impacts of pastoral species (namely sheep) on ephemeral freshwater bodies. As is the case in many Australian ecosystems (Taxonomy Decadal Plan Working group 2018), comprehensive inventories of the invertebrate communities of the granite rock-holes in the GB are sadly lacking. We recommend that targeted surveys such as those done for gnammas in Western Australia (Bayly 1997, Pinder et al. 2000), and for rock-holes to the south of the GB (Timms 2014) be undertaken for those at HNR and on neighbouring properties. These efforts should be accompanied by barcoding, and where necessary alpha taxonomy be to ensure that the appropriate framework exists to facilitate future research.

5.6 Conclusions

Our findings demonstrate the vulnerability of the Australian freshwater granite rock-hole ecosystem to the impacts of climate change. We found that under at present day conditions, the rock-holes experience an average hydroperiod of 85 days, and exhibit an average water temperature of 15.5°C. However, we predict decreases to this hydroperiod and increases in water temperature under all future emissions scenarios. We also observed responses to these future emissions scenarios under experimental conditions within populations of the water flea *D. clarinata* and the seed shrimp *Sarscypridopsis* sp. Whilst these invertebrates exhibit a compensatory response to the stressors that future emissions scenarios place upon them, this response was insufficient to account for the dramatically reduced hydroperiod under severe emissions scenarios (RCP 8.5). Furthermore, the responses observed for the two species studied was asymmetrical, with *Sarscypridopsis* sp. displaying continued recruitment right until the experiment end date, whereas *D. clarinata* declined as the experiment reached its end date. We suggest further research extend our study to assess the impacts of climate change on the entire rock-hole ecosystem. Furthermore, we recommend that conservation and management of the rock-hole ecosystem take the asymmetry of species responses into consideration, and that efforts be made to alleviate the other threatening processes impacting the system, such as high visitation rates by invasive species.

Chapter 6. General Discussion

In this thesis I set out to improve understanding of the ecology of ephemeral freshwater ecosystems in Australia and assess the threats they face. To address this broad aim, I undertook a series of studies on freshwater granite rock-holes in the Gawler Bioregion (GB). Firstly, I assessed the resource value of the freshwater rock-hole ecosystem to local vertebrates using wildlife cameras (Chapter 2). Secondly, I examined the robustness of environmental DNA (eDNA) metabarcoding for recording and documenting vertebrate visitation to rock-holes and compared this method to a more traditional camera trapping approach (Chapter 3). Thirdly, I used eDNA metabarcoding to monitor invertebrate communities associated with the granite rock-hole ecosystem and examined patterns in the spatial and temporal variations of these communities (Chapter 4). Finally, I studied contemporary hydrological trends for the rock-hole system, forecasted changes under a series of future emissions scenarios, and examined how these changes may impact invertebrates associated with the rock-hole ecosystem via variations in demographic responses (Chapter 5). Ultimately, the sum of these chapters has contributed to an emerging narrative regarding the impacts of climate change on ephemeral freshwater ecosystems and their respective ecological communities. This work not only significantly improves understandings of these ecosystems from an ecological standpoint, but also provides a research framework that hopefully will facilitate future proactive conservation and management of ephemeral freshwater habitats in the GB and beyond.

6.1 The aims of this thesis

Aim 1: The resource value of rock-holes to vertebrates

The first aim of this project (as outlined in Chapter 1) was to assess the extent to which the freshwater rock-holes in the GB are currently utilised by local vertebrates to better understand the resource value of this system. I addressed this aim by employing two biomonitoring techniques: wildlife camera trapping (Chapter 2) and eDNA metabarcoding methods (Chapter 3). The freshwater granite rock-holes at Hiltaba Nature Reserve (HNR) were selected as the study sites. In doing so, I generated the first comprehensive inventory of GB rock-hole visitations by vertebrates with a validation of the data using genetic methods. Together, these biomonitoring methods detected 42 vertebrate species attending the rock-holes, including 13 mammals, 21 birds, six squamate reptiles, and two amphibians. These data built upon work undertaken as a parallel study by McDonald et al. (2023) (for which I was a co-author) which recorded vertebrate visitation to similar granite rock-holes in Western Australia using eDNA, and validated by wildlife cameras. I also assessed trends in vertebrate visitation at spatial and temporal scales, and observed increased visitation during prolonged periods without rainfall. Votto et al. (2022) explored the relationship between frequency of visitation to arid-zone waterholes and fringing vegetation by birds, and my research has greatly expanded on this by incorporating analyses of all vertebrate taxa visiting an ephemeral habitat and revealing the impact of short- (time of day) and long-term (seasonal) trends.

Aim 2: Documentation of invertebrate communities

The second aim of this project was to assess the composition of invertebrate communities associated with freshwater rock-holes in the GB (Chapter 4). This aim was identified as a priority of the Government of South Australia by White (2009), where it was suggested that the ecological value of rock-holes may be determined by sampling macroinvertebrates. In this part of the project (Chapter 4) I also applied eDNA metabarcoding to document the taxa present in the ecosystem and assess trends in spatial and temporal variation. In doing so, I have generated the first inventory of invertebrate communities reliant on rock-holes of the GB, detecting taxa from 22 orders and 45 families. Species richness was greatest in July, and whilst richness was relatively consistent across rock-holes, actual community composition was highly variable. Previously, such inventories have been restricted to rock-holes on the Eyre Peninsula to the south of the GB (Timms 2014), in central Australia (Bayly 2001), or further afield in regions disconnected from the GB (Bayly 1997, Pinder et al. 2000, Timms 2017). My findings provide a foundation for future research that seeks to understand the composition of ephemeral freshwater ecosystem invertebrate communities using eDNA metabarcoding. They also generate critical baseline data against which future eDNA metabarcoding surveys can be compared.

Aim 3: Climate change risk

The third aim was to investigate the potential future impacts of climate change on ephemeral freshwater ecosystems by using the GB rock-holes as a model system (Chapter 5). I achieved this through hydrological and climate modelling to predict the deviations from historical norms under a series of emissions scenarios. In doing so, I predicted an up to 42% decrease in the average duration of hydroperiod within the GB under severe emissions scenarios. I then experimentally applied these future conditions to two invertebrate species (the water flea *Daphnia carinata* and the seed shrimp *Sarscypridopsis* sp.) cultured from the rock-holes at HNR, and measured their demographic responses. The response was asymmetrical, with *D. carinata* potentially displaying an adaptive response to the stressors. This was not observed for *Sarscypridopsis* sp. which showed continued population growth even as experimental scenarios ended. These results indicate that the impacts of climate change will be uneven among members of the rock-hole communities, with the potential for trophic cascades to occur. This may lead to dramatically altered invertebrate diversity and loss of ecosystem function. My findings improve our understanding of how climate change is likely to impact ephemeral freshwater ecosystems, and result in potential detrimental impacts, particularly in semi-arid and arid Australia. Further, this work represents a pilot study from which future climate change research can be developed.

Cultural significance

Whilst it was not a specific aim of this PhD to address the cultural significance of the freshwater granite rock-hole ecosystem in South Australia, it is important to note, that this ecosystem is one of immense historical and current importance to Australian First Nations peoples. A continuous history of presence in a landscape characterised by prolonged periods of aridity has resulted in a deep relationship between First Nations people and ephemeral sources of freshwater (Bayly 1997, Jenkin et al. 2011). The Bungala, Kokatha, and Wirangu peoples all have connections with the freshwater granite rock-hole ecosystem and at HNR these connections are maintained through the ongoing management of the system (Figure 6.1, AIATSIS map of Indigenous Australia 2008, Jenkin et al. 2011). In future, any management procedures placed upon the rock-holes to safeguard their biological significance should be developed in collaboration with the Traditional Custodians of the land and with appropriate recognition of this connection.

Figure 6.1. A managed rock-hole at Hiltaba Nature Reserve in the GB. Photograph taken from directly above with an unmanned aerial vehicle by Dillon Campbell. Traditional management practices involve rows of timber placed across the surface of the rock-hole and the placing of rocks around the perimeter.

6.2 Threats to Australian ephemeral freshwater ecosystems

Invasive species

I undertook eight research field trips to Hiltaba Nature Reserve during my PhD, spent collectively over 30 days on-site, and drove approximately 13,000 km to, from, and around the Reserve. During these trips, one thing that became abundantly clear, before the first spreadsheets of scientific data were compiled, was the impact that feral goats were having in the region. I encountered them almost daily, and when I did not see them I heard them, bleating over the hills (Figure 6.2). Nature

Foundation has an active program suppressing goat populations, which has involved the removal of over 12,000 individuals since acquiring the property in 2012 (Nankivell, A. pers comms). However, as this effort is not matched uniformly throughout the GB and due to the great dispersal capacity of feral goats (Lethbridge 2016, Moseby et al. 2021), these efforts have not eradicated goat populations completely. Indeed, both of the vertebrate biomonitoring methods employed in this project detected goats in frequent attendance at rock-holes, often at high densities, with them accounting for 7.5% of all camera trap events (Chapter 2) and 4.2% of all eDNA sequences (Chapter 3).

Feral goats are not the only invasive mammals causing deterioration of the Australian landscape. Feral populations of deer (Bradshaw et al. 2023), camels (Brim Box et al. 2016), pigs (Gentle et al. 2022) and horses (Giles et al. 2023), are all present in other areas of the arid-zone and are known to cause ecological harm to vegetation and freshwater systems. The degrading impact of invasive species on the quality and potability of freshwater resources in Australia is well document (Doupe et al. 2010, Brim Box et al. 2016, Lethbridge 2016) and, whilst it was not a specific aim of this project to assess the impacts that invasive species may have on rock-hole ecosystem health, it is likely that frequent goat visitation is immensely harmful to these and other ephemeral water bodies (EWBs). As such, it will be critical to exclude them from accessing rock-holes in the future, wherever possible. Small-scale exclusion fencing may allow for some mitigation of the impacts of goats on rock-holes (Smith et al. 2020). However, a concerted effort to remove goats from the region more broadly through culling is likely the only successful solution to manage this species and its impacts on Australian rangelands (Russell et al. 2011).

Figure 6.2. Feral goats (*Capra hircus*) encountered whilst on field work over a three-and-a-half-year period at Hiltaba Nature Reserve in the GB.

Limiting the access of invasive species to freshwater rock-holes and other currently unmanaged freshwater resources in the GB such as farm dams, bores and claypans may also result in broader conservation benefits. Invasive species cause a range of degrading effects, such as the alteration of plant communities and the prevention of the regeneration of palatable plant species via heavy grazing (Lethbridge 2016, Moseby et al. 2021, Giles et al. 2023). In the GB, goats can also impact populations of yellow-footed rock wallabies (*Petrogale xanthopus*), a species of conservation concern in the GB (Hayward et al. 2011). Feral goat activity is often closely linked with the management of artificial watering points on pastoral properties, as well as naturally occurring sources of freshwater, such as rock-holes (Russel et al. 2011, Moseby et al. 2021). As such, preventing populations from capitalising on these resources may yield broad benefits in efforts to control them. However, it is unlikely that preventing access to freshwater resources alone will yield the desired effect, without other targeted control methods (Moseby et al. 2021, Russel et al. 2011). A landscape-wide problem needs a landscape-wide solution, and as such efforts to control goats throughout the GB need to be intensified and incorporate modern techniques for control.

Climate change

The other key threatening process likely to be a major driver of change in Australian freshwater EWBs over the next century is climate change. The impacts of climate change are evident on all continents, environments, and habitat types (Scheffers et al. 2016, IPCC 2022) and rock-holes are no exception. As I explore in Chapter 5, climate change will decrease the average hydroperiod of rock-holes and increase water temperature. These deviations from historical norms are likely to result in altered life cycles for rock-hole invertebrates, with preliminary data produced here suggesting species may have differing responses to such changes (Chapter 5). However, such deviations will almost certainly not be restricted to rock-hole ecosystems (Gillespie et al. 2020). Other EWBs, such as ephemeral rivers and creek-lines (Scott et al. 2003, Steward et al. 2012, Acuña et al. 2017), ephemeral lakes (Karagianni et al. 2018), seasonal wetlands (Strachan et al. 2014, Calhoun et al. 2017), vernal pools and temporary ponds (Andrushchyshyn et al. 2003, Kneitel et al. 2017), and claypans (Gibson et al. 2018), are all likely to experience deviations from their historical norms with respect to hydroperiod and water temperature. Shifts in distribution and phenology of species (Ewald et al. 2013), and other impacts to plants, invertebrates (Frisch 2001) and vertebrates (Chessman 2011, Howard et al. 2016) have all been observed for EWBs because of climate change. Asymmetry in species responses is an emerging prediction from recent studies (Tylianakis et al. 2008, Sardiña et al. 2017, Bellin et al. 2021, Tasker et al. 2022), and one of for concern due to its potential in causing multi-trophic cascades. Furthermore, decreases in EWB hydroperiod are likely to result in declining availability of freshwater as a resource to local vertebrates. As shown in Chapters 2 and 3, the GB rock-holes are likely important in supporting populations of local native vertebrates, and the same is likely true for EWBs throughout Australia, as is the case overseas (Dixneuf et al. 2021). Significant global efforts are needed to mitigate the impacts of climate change (IPCC 2022b) but in their absence, more localised efforts are needed to alleviate the impacts of other threatening processes that will be exacerbated by climate change.

Synergistic effects of climate change and invasive species

Whilst the impacts of invasive species and climate change are enough cause for action regarding rock-hole conservation when considered separately, it is likely that these two threatening processes will also interact synergistically. Invasive species often demonstrate greater phenotypic plasticity and adaptive potential than native species (Dybdahl et al. 2005, Tasker et al. 2022), and often have higher tolerances to environmental stresses than native taxa (Lenz et al. 2011, Zerebecki et al. 2011). This can make invasive species more suited to successfully responding to climate change than native species (Tasker et al. 2022). As such, it is possible that the shortened hydroperiods and higher average water temperatures expected to occur in ephemeral freshwater ecosystems in semi-arid and arid Australia may facilitate new invasions and promote persistence of invaders (Tasker et al. 2022). Capacity to respond to species invasions quickly and effectively is dependent on early detection of the threat (Sepulveda et al. 2023), and as such, biomonitoring tools that facilitate early detection, such as those implemented here, are critical. Below, I detail a strategy for biomonitoring of the freshwater rock-hole ecosystem that will facilitate effective and informed management in the face of these threatening processes.

6.3 Biomonitoring techniques for ephemeral freshwater ecosystems

The world currently faces a century during which the impacts of a series of threatening processes will be felt. It is critical that strategies are developed to slow biodiversity loss and achieve restoration where possible. For this to be achieved, a robust toolkit of biomonitoring technologies is required (Sepulveda et al. 2023). As part of the research presented here, I tested two biomonitoring methodologies in an ephemeral freshwater system and compared their suitability for characterising EWB biodiversity and detecting any change in real time. Wildlife camera trapping and eDNA metabarcoding both show promise as biomonitoring techniques for freshwater rock-holes (Chapters 2, 3) and other ecosystems (Hänfling et al. 2016, Valentini et al. 2016, Johnsen et al. 2020, Klymus et al. 2020, Rodgers et al. 2020, Farrell et al. 20022, Votto et al. 2022). However, due to time limitations, as well as disruptions caused by the COVID-19 pandemic, the temporal span of the biomonitoring efforts employed here were a snapshot of possible sampling design. Long-term biomonitoring programs are rare, yet when attempting to make inferences about processes that occur at broad temporal scales, climate change and invasive species dispersals, long term monitoring data are critical (Wilson et al. 2017). Establishment of long-term monitoring programs in the GB would allow for detection of responses to the impacts of climate change, early detection of invasive species establishment (Sandercock et al. 2023, Sepulveda et al. 2023), and measurement of the efficacy of any applied conservation efforts. I recommend that both camera trapping and eDNA metabarcoding be integrated into long-term biomonitoring programs in the region, as outlined in Chapters 2 and 3 (see schematic of these recommendations in Figure 6.3). Given the long-term, on-site management of HNR, this may be a possibility. Additionally, automated weather stations should be deployed in close proximity to rock-holes to record weather conditions that may be used to facilitate future climate change research (as discussed below).

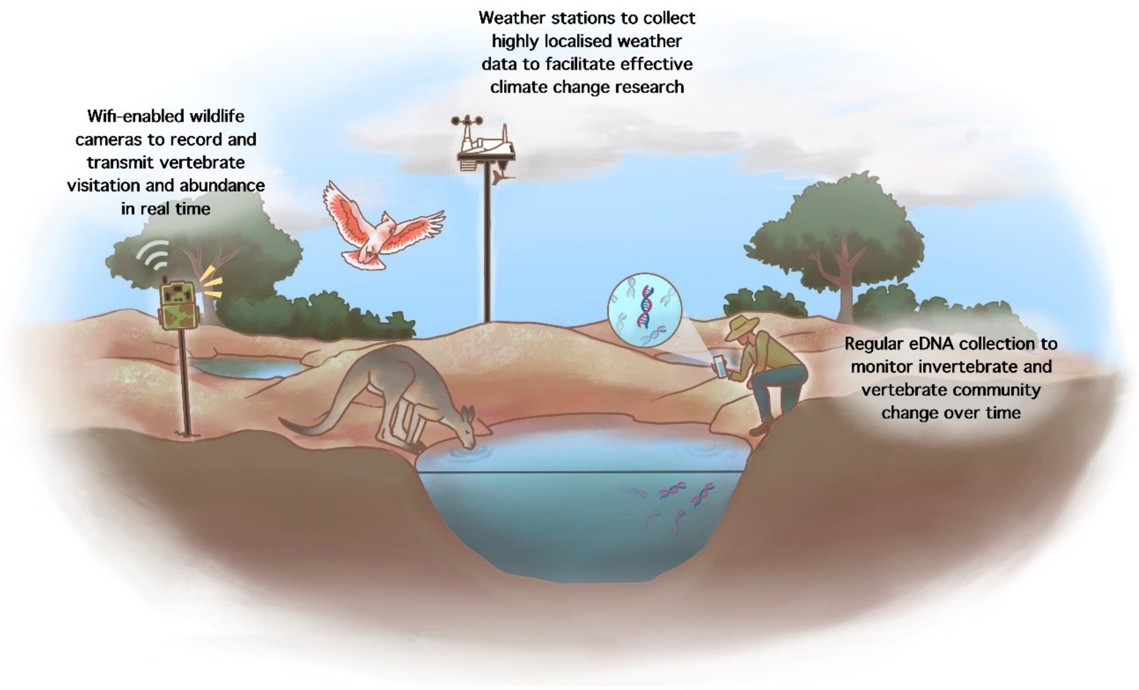

Figure 6.3. Schematic illustrating a recommended integrated approach to ephemeral freshwater body monitoring in the GB.

Wildlife camera trapping

Wildlife cameras have commonly been used in the ecological and animal behavioural sciences to document presence, movement, and behaviour of terrestrial vertebrates (Krauss et al. 2018). Use of wildlife cameras is common in remote regions (Bragato et al. 2022, Draper et al. 2022, Votto et al. 2022), and the technology is increasing in its accessibility with the advance of machine learning and automation (Sollmann et al. 2018, Farmer et al. 2022). My research demonstrates the efficacy of wildlife camera trapping as a biomonitoring tool, particularly for detection of invasive species (Chapter 2). However, my work goes further and utilises the technique in a critical evaluation step to validate eDNA metabarcoding data. I recommend that a long-term biomonitoring program utilising wildlife cameras be implemented at Hiltaba Nature Reserve (Figure 6.4). Such a program could incorporate real-time artificial intelligence-driven image recognition (Western Downs Regional Council 2021, Landscape South Australia 2021) and 4G mobile connectivity to notify managers and facilitate rapid responses, as discussed in Chapter 2.

Figure 6.4. A wildlife camera overlooking a freshwater granite rock-hole at Hiltaba Nature Reserve, South Australia. Pictured: project volunteer Johanna Kuhne.

Environmental DNA metabarcoding

In Chapters 3 and 4, I explored the application of eDNA metabarcoding, for documenting and monitoring of an arid-lands freshwater ecosystem. This method has seen rapid uptake in use in recent years and has been employed to detect permanent occupants of freshwater ecosystems (Shaw et al. 2016, Klymus et al. 2020, Johnsen et al. 2020, White et al. 2020, van der Heyde et al. 2023). The use of freshwater eDNA for detecting visitation to freshwater ecosystems by terrestrial vertebrates, in contrast, is less established (Harper et al. 2019, Lyet et al. 2021, Farrell et al. 2022). As such, my study provides a foundation for future research using eDNA metabarcoding to detect vertebrate visitation to freshwater ecosystems, including the detection of invasive species (Sepulveda et al. 2023). An additional component that makes this research significant is the use of wildlife camera trapping to validate eDNA data. Comparisons between the two methods are still in their infancy and much remains unknown regarding the relationship between these two methods and their respective biases (Harper et al. 2019, Leempoel et al. 2020, Sales et al. 2020). The application of these two techniques in the same study area and during the same period provides an opportunity to interrogate the validity of eDNA metabarcoding that would not have been possible otherwise.

Environmental DNA metabarcoding also allowed for assessment of the composition and variability of the invertebrate communities associated with the GB rock-holes. My research lays the groundwork for documenting rock-hole species composition that may be revisited as barcode reference libraries improve. These data also provide a baseline against which future eDNA surveys may be compared. This will allow for identification and measurement of any declines within these communities that occur because of the above-mentioned threatening processes. Going forwards, I recommend that eDNA metabarcoding be utilised in conjunction with wildlife camera trapping as a biomonitoring tool for the rock-hole ecosystem, and ephemeral freshwater bodies more broadly (Figure 6.3).

6.4 Future research directions

Barcode reference libraries and species inventories

In Chapters 3 and 4, I demonstrated the efficacy of eDNA metabarcoding as a tool suited for both recording vertebrate visitation to, and communities within, freshwater rock-holes. However, both of these studies were limited by the incompleteness of barcode reference libraries for taxa that occur in remote Australian regions. The lack of genomic data associated with taxonomically verified samples meant that in many cases, it was not possible to associate reliable species identities with the eDNA data. Sequences deposited within databases such as GenBank (National Center for Biotechnology Information), whilst a useful tool for a “first pass” at eDNA data, are often insufficient in studies such as these (Weigand et al. 2019). Custom barcode reference libraries allow for more accurate species assignments and lead to greater confidence in eDNA surveys (Ekrem et al. 2007, Weigand et al. 2019, Saccò et al. 2022). In addition to the benefits to future research, such confidence could also potentially improve translation of research findings into practical conservation and management outcomes. As such, I recommend future work be undertaken to sample rock-hole invertebrates (Figure 6.5) and assemble a custom barcode reference library for both the invertebrate fauna of the rock-hole ecosystem and the vertebrate fauna of the GB more broadly. Such a library should be assembled following best practices, including the incorporation of detailed and verified metadata (Rimet et al. 2021), and be publically available.

Figure 6.5. Project volunteers sorting invertebrate specimens collected from freshwater granite rock-holes at Hiltaba Nature Reserve, South Australia. Pictured: Johanna Kuhne (left) and Adam Toomes (right).

Extensions to my climate change research

In Chapter 5 I began the process of investigating the effects of climate change on the hydrology and ecology of the freshwater rock-hole ecosystem and showed that species responses will likely be asymmetrical. However, many questions remain unanswered. Whilst we used two rock-hole species as models, the ecosystem is highly diverse, with many more inhabitants that are likely to be impacted by climate change (Chapter 4). Future research should investigate the impacts of, and responses to, changing conditions from a more holistic viewpoint and consider the full extent of biodiversity supported by the system. Furthermore, due to the highly variable nature of rainfall in the arid interior of Australia (Wright et al. 2023), use of weather data from the closest weather station to a field site is often insufficient for accurate measurement of local conditions. Indeed, only 10 km separates the study site discussed in Chapter 5 and the weather station at the HNR homestead, yet rainfall data collected there was often different to that observed at the rock-holes. Deployment of weather stations immediately adjacent to rock-holes would allow for more accurate data collection, and ultimately facilitate more robust climate change research (Figure 6.3).

6.5 Conclusions

Ephemeral freshwater ecosystems in Australia are greatly understudied, both as habitats supporting their own fauna and flora and as a resource in the broader environment to animals such as large-bodied native vertebrates. The effective management of such ecosystems may be critical to safeguarding Australia’s unique biodiversity, especially in the face of key threatening processes such as overabundant invasive species and climate change. My research has drawn attention to the value of a specific ephemeral freshwater ecosystem—the freshwater granite rock-holes of the Gawler bioregion—and to a suite of taxa including local vertebrates and aquatic invertebrates. This thesis has established a strong foundation upon which future taxonomic and ecological work can build. I have also outlined a series of conservation and management recommendations to limit the impacts of invasive species and to improve biomonitoring efforts and taxonomic resolution in the face of climate change. I believe these recommendations will improve conservation outcomes in the GB and my research can be used as a case study for broader efforts to improve the management of ephemeral freshwater ecosystems in Australia in the future.

7. Appendices

What follows are a series of appendices and supplementary material to this thesis. Appendices A-C contain three publications that I worked and collaborated on during my PhD. Whilst they do not contribute to the primary narrative of my thesis, they provide additional background on content and themes covered within the thesis.

The first publication titled “A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*, in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat” covers a novel association that we observed between two organisms associated with the granite rock-hole ecosystem, and provides a synthesis of, and discussion of, the Australian rock-hole literature up to 2021.

The second publication titled “Detection of vertebrates from natural and artificial inland water bodies in a semi-arid habitat using eDNA from filtered, swept, and sediment samples” explores methodological considerations regarding the use of eDNA metabarcoding in the freshwater rock-hole ecosystem, and provided a research foundation upon which both my vertebrate visitation monitoring and eDNA chapters developed.

The third publication titled “Time capsules of biodiversity: Future research directions for groundwater-dependent ecosystems of the Great Artesian Basin” explores research priorities for groundwater-fed springs that occur throughout the Great Artesian Basin, Australia. Whilst these springs are structurally and functionally distinct from the rock-holes discussed in this thesis, they also have much in common with them, particularly with regard to the threatening processes that are causing habitat degradation and deterioration. The establishment of a strong foundational research framework as a pathway to better ecosystem management is an initiative that I feel should also be applied to the freshwater rock-hole ecosystem too.

Appendices D-G contain supplementary material for each of my four data chapters, including summary statistics and diagnostics for models, additional supporting figures, and tables containing metabarcoding identity data.

Appendix H contains Statement of Authorship forms for all publications included in this thesis, with signatures from all contributing authors.

Appendix A. Supporting publication 1

Publication: A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*, in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat.

Transactions of the Royal Society of South Australia

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/trss20

A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*, in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat

Brock A. Hedges, Andy D. Austin, John G. Conran, Gary S. Taylor, Chris P. Madden & Philip Weinstein

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Statement of Authorship

Title of Paper	A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*, in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Hedges, B. A. Austin, A. D., Conran, J. G., Taylor, G. S., Madden. C. P., Weinstein, P. (2021) 'A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*. in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat.' *Transactions of the Royal Society of South Australia* 145(2): 152-167.

Principal Author

Name of Principal Author (Candidate)	Brock A Hedges
Contribution to the Paper	Led data curation, methodological design, data analysis and production of manuscript
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date t 2/0 7/2.02-3

Co Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andy D Austin
Contribution to the Paper	Provided project guidance and edited the manuscript
Signature	Date 4 7 23

Name of Co-Author	John G Conran
Contribution to the Paper	Provided project guidance and edited the manuscript
Signature	4 Jul 2023

Name of Co-Author	Gary S Taylor		
Contribution to the Paper	Provided project guidance and edited the manuscript		
Signature		Date	/'3.,.2..023

Name of Co-Author	Chris P Madden		
Contribution to the Paper	Provided project guidance and edited the manuscript		
Signature		Date	J 7- 7 -,2.0.23

Name of Co-Author	Philip Weinstein		
Contribution to the Paper	Provided project guidance and edited the manuscript		
Signature		Date	19/07/2023

TRANSACTIONS OF THE ROYAL SOCIETY OF SOUTH AUSTRALIA
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RESEARCH ARTICLE

A likely association of damselflies with the habitat heterogeneity provided by the freshwater swamp lily, *Ottelia ovalifolia*, in Eyre Peninsula granite rock-holes, with a review of potential threats to this ephemeral habitat

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ABSTRACT
The granite rock-holes (sometimes called gnammas) across northern Eyre Peninsula (EP), South Australia, are a unique but poorly studied ephemeral freshwater habitat containing a complex invertebrate community. Macroinvertebrate predator occurrence is often sporadic, both spatially and temporally. We aimed to determine if environmental conditions might predict predator occurrence in EP rock-holes. A total of 14 rock-holes were sampled across five granite outcrops along the Eyre Highway. Extensive dip-net sampling was undertaken and nymphs of three damselfly species were recorded from the rock-holes: *Austrolestes annulosus* (Lestidae), *Ischnura aurora*, and *Xanthagrion erythroneurum* (both Coenagrionidae), all in a single rock-hole at Pildappa Rock. This sole rock-hole contained a prominent floating-leaved, rooted aquatic macrophyte: the swamp lily, *Ottelia ovalifolia* (Hydrocharitaceae), which forms a complex, three-dimensional vegetative structure. Damselflies were hypothesised to be associated with the presence of *O. ovalifolia*, possibly as a result of the plant providing both suitable oviposition sites for the adults and habitat for nymphs throughout the water column, opportunities not afforded by the aquatic vegetation present in shallow rock-holes. Our findings contribute to the limited ecological information regarding EP rock-holes. We also briefly review potential threats to these ecosystems, an understanding of which will be critical to their management and conservation.

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KEYWORDS
Hydrocharitaceae; Zygoptera; Odonata; species interaction; ephemeral; rock-holes

Introduction

The northern Eyre Peninsula (EP) sits along the southern border of the South Australian arid climate zone (<250 mm average annual rainfall) and more Mediterranean lands to the south (~350 mm average annual rainfall) that are highly seasonal (Bureau of Meteorology, 2019; Twidale et al., 1985). Within this boundary zone, a series of granite inselbergs along the Eyre Highway (Figure 1a) provide locations for the temporary collection and storage of rainfall in rock-holes, referred to as "gnammas" by the

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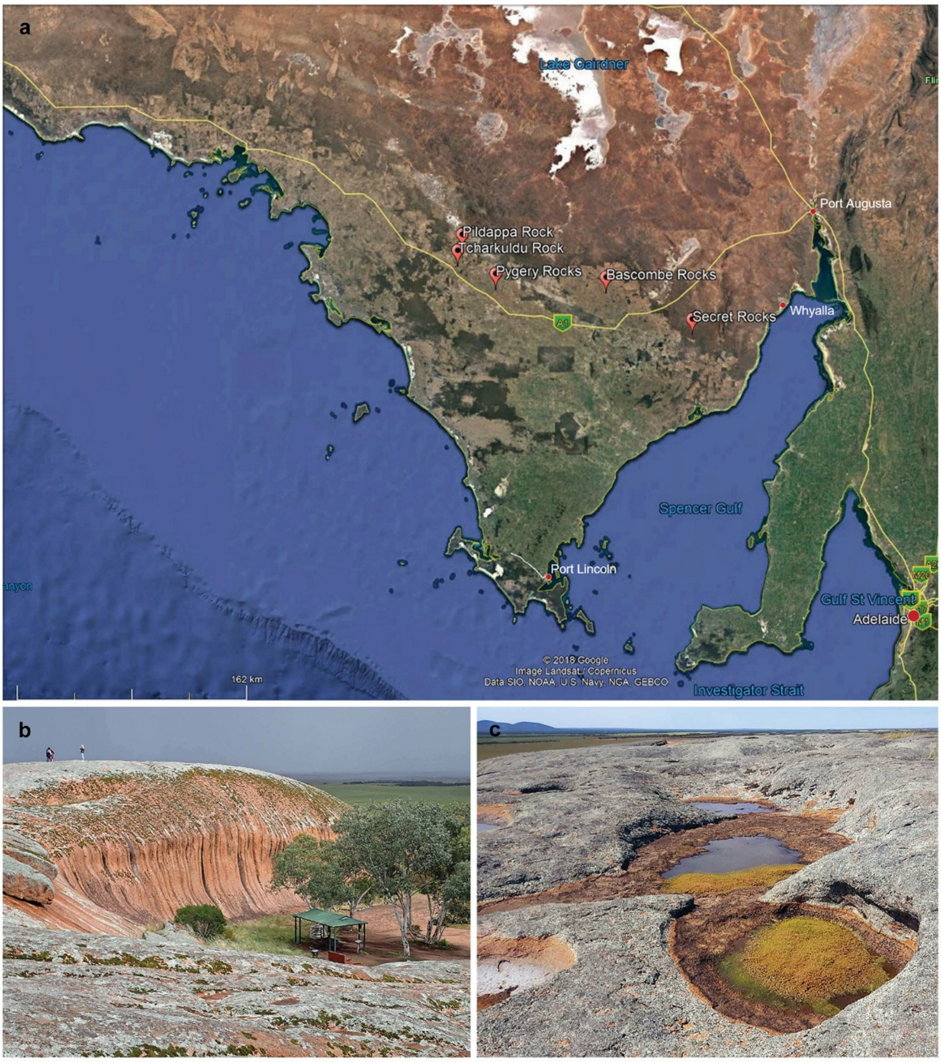

Figure 1. Locality details. a. Map of the Eyre Peninsula region showing the location of the granite inselbergs surveyed during the study; b. Pildappa Rock; c. Seasonally flooded gnamma holes on top of Pildappa Rock.

Noongar Aboriginal people of Western Australia (Bayly, 1997). These rock-holes fall into two broad categories: pits and pans (Timms, 2014). Pan rock-holes are typically shallow (≤20 cm deep) with gradually sloping walls, whereas pit rock-holes are typically deeper (>20 cm deep) with steeply sloping to vertical walls, sometimes with an overhang. These variably sized rock-holes are a reliable, albeit ephemeral, freshwater habitat in what is at times a completely dry landscape (Bureau of Meteorology, 2019), and are habitat for a complex community of native and invasive flora and fauna. These communities can be categorised as either residing permanently (present throughout both the dry and wet phases) or opportunistically (present only at inundation) (Bayly, 1997).

Despite having regular periods without standing water (Timms, 2014), EP rock-holes nevertheless contain several aquatic plants, which are present consistently during wet periods (usually during winter). In particular, there are two aquatic alismatid monocots: the swamp lily (or Gurragurra in the Yuwaalayaay language), *Ottelia ovalifolia* (R.Br.) Rich., Hydrocharitaceae, and a species of water mat: *Althenia* cf. *australis* (J. Drumm. ex Harv.) F. Muell., Potamogetonaceae, previously identified either as *Najas tenuifolia* R.Br., Zannichelliaceae, or *Lepilaena australis* Drumm. ex Harv., Potamogetonaceae (Timms, 2017), have been observed regularly over several seasons at the EP granite outcrop named Pildappa Rock (Figure 1b,c), although the latter taxon is also known from rock-holes on other nearby outcrops and pools in rocky ephemeral creeks in the Gawler Ranges National Park (J. G. Conran, unpubl. obs.).

The relatively recent self-establishment of *O. ovalifolia* in New Zealand (McCullough, 1997) suggests that the species can disperse long distances and is capable of colonising isolated freshwater bodies opportunistically. Furthermore, *O. ovalifolia* is cleistogamous in the deeper rock-holes and generally only produces open flowers when the water is shallow (usually at draw-down when the rock-holes are nearly dry; a feature seen in several members of the genus), with an estimated 90–95% of *O. ovalifolia* seeds the result of cleistogamous self-pollination (Cook et al., 1984; Ernst-Schwarzenbach, 1956). These flexible reproductive strategies are possibly an adaptation for the establishment and persistence of *O. ovalifolia* in ephemeral waterbodies from arid and semi-arid regions, where populations are small, widely separated, and where pollinators may be limiting.

Damselflies are invertebrate predators during both aquatic nymphal stages and their aerial adult phase. Although not commonly regarded to be of economic importance, damselflies are capable of regulating densities of both agricultural pests (May, 2019) and potential mosquito disease vectors (Weathered & Hammill, 2019), as well as preventing trophic cascades (De Omena et al., 2017). Predators such as damselflies are particularly important in isolated waterbodies such as rock-holes due to the lack of larger vertebrate predators such as fishes and in many cases amphibians (Brendonck et al., 2002). In the EP rock-holes, the most common predatory species are generally aquatic insects such as damselflies, dragonflies (Odonata) and diving beetles (Coleoptera), and tadpoles (Timms, 2014), and tadpoles. Small invertebrate predators often provide the only source of top-down ecosystem pressure and their presence and activity can be a major driver for the population dynamics of individual species and community structure in ecological systems lacking fish (May, 2019). It is important that unique ecosystems, such as rock-holes, be the focus of targeted conservation programs, particularly given their vulnerable ephemeral nature.

This study examines the apparently exclusive co-occurrence of damselfly nymphs with *O. ovalifolia* in the EP rock-holes, exploring the possible ecological drivers behind this observation. The specific aims were: (1) to determine the significance of the type of rock-hole and whether this can be used to predict the presence of complex macrophytes such as *O. ovalifolia*; (2) to investigate whether the presence of *O. ovalifolia* is a possible indicator for the presence of damselfly nymphs; and (3) to review the threats that may impact the biodiversity associated with the EP granite rock-holes.

Materials and methods

Study site

Five granite inselbergs were surveyed along the Eyre Highway in South Australia during October (2016) (Figure 1). The five outcrops were distributed across a longitudinal gradient, approximately 200 km in length. These inselbergs ranged from single structures protruding to >20 m above the surrounding area, to fragmented structures only exposed sporadically. The inselbergs were often surrounded by agricultural land and some have areas where low stone walls had been erected to retain rainwater. Vertebrate density was observed to be high, with many kangaroos being present on outcrops at dawn and dusk, and evidence (primarily scats) of other vertebrates such as emus, rabbits and various ungulates. In total, 14 rock-holes were surveyed across the five outcrops, but only rock-holes with standing freshwater present were surveyed.

Data collection

Physical characteristics were recorded for each rock-hole including, rock-hole type, length, width, depth, and sediment type. Repeated sweeps of the ponds using a fine-mesh dip net was undertaken across all depths to determine the presence of damselfly nymphs and any other taxa in the pools. Specimens were identified tentatively in the field and observed abundance of each taxon was recorded for each rock-hole. Specimens were then placed into 100% ethanol in 1.5 ml plastic tubes for further identification and imaging. The aquatic plant communities for each rock-hole were also identified and photographed in the field, with vouchers deposited in the State Herbarium of South Australia (AD). Odonata nymphs were identified using Hawking and Theischinger (1999) and Theischinger and Endersby (2009).

Results

The 14 rock-holes surveyed across the five inselbergs were variable in their length, width, depth, and type (Table 1). Pan rock-holes were generally circular, oval or irregularly-shaped. Pit rock-holes were generally circular or oval-shaped. Nymphs from three damselfly species were identified from the rock-hole survey: the common blue damselfly *Austrolestes annulosus* (De Sélys-Longchamps, 1862) (Lestidae), the golden dartlet, *Ischnura aurora* (Brauer, 1865) (Coenagrionidae) (Figure 3a), and the red and blue damsel, *Xanthagrion erythroneurum* De Sélys-Longchamps, 1876, (Coenagrionidae) (Figures 3b, c). These were all collected from a single pit rock-hole (Figure 2a, Table 2), but were present only as nymphs during the survey, with no exuviae or adults observed in the surrounding area. This rock-hole was also the only location at which the floating-leaved, rooted aquatic macrophyte *O. ovalifolia* was present (Figure 2), out of the 14 surveyed across the five sites.

Other predatory aquatic species were also recorded, including four diving beetles (Dytiscidae): *Allodessus bistrigatus* (Clark, 1862), *Eretes australis* (Erichson, 1842), *Rhantus suturalis* (Macleay, 1825), and *Sternopriscus multimaculatus* (Clark, 1862), with individuals of at least one species present in most rock-holes (Table 2). Tadpoles of varying size were recorded in eight of 14 rock-holes and at all outcrops except for the

Table 1. Summary of physical characteristics of the gnammas surveyed during the 2016 field survey. Latitude and longitude are presented in decimal degrees.

Location	Site	Date	Latitude	Longitude	Type	Substrate	Length (cm)	Width (cm)	Depth (cm)
Bascombe Rocks	1.1	29/10/2016	−33.0057	136.2694	pan	mud	300	220	4
Bascombe Rocks	1.2	29/10/2016	−33.0057	136.2693	pan	mud	620	310	4.5
Bascombe Rocks	1.3	29/10/2016	−33.0057	136.2692	pan	mud	550	450	7
Bascombe Rocks	1.4	29/10/2016	−33.0057	136.2689	pan	mud	380	340	8
Bascombe Rocks	1.5	29/10/2016	−33.0048	136.2689	pan	mud	820	780	4
Pygery Rocks	2.1	29/10/2016	−33.9861	135.4706	pit	algal detritus	198	337	60
Pildappa Rock	3.1	30/10/2016	−33.7515	135.2313	pan	mud	180	170	4
Pildappa Rock	3.2	30/10/2016	−33.7513	135.2308	pit	gravel	460	250	55
Pildappa Rock	3.3	30/10/2016	−33.7513	135.2300	pit	gravel	280	240	70
Pildappa Rock	3.4	30/10/2016	−33.7509	135.2292	pan	gravel + mud	310	85	6
Tcharkuldu Rock	4.1	30/10/2016	−33.8464	135.1977	pit	mud	90	120	30
Secret Rocks	5.1	30/10/2016	−33.1874	136.8413	pit	mud	96	64	52
Secret Rocks	5.2e	30/10/2016	−33.1872	136.8412	pit	mud	340	110	38
Secret Rocks	5.2w	30/10/2016	−33.1872	136.8412	pit	mud	630	90	44
Secret Rocks	5.3	30/10/2016	−33.1870	136.8412	pan	mud	480	120	17

single rock-hole at Pygery Rocks (Table 2). These were not identified to species but were most likely to be the spotted grass frog, *Limnodynastes tasmaniensis* Günther (S.C. Donnellan, pers. comm.).

The *O. ovalifolia* at Pildappa Rock was the only plant present in the EP rock-holes that provided a complex, three-dimensional vegetative structure, both floating and submerged, across the whole water column of a deep, long-lived pit rock-hole (Figure 2a,b).

Other common aquatic macrophytes at the site included the relatively short, permanently submerged *Althenia* cf. *australis*, mostly in deeper rock-holes, as well as the locally very common, shallow-growing, and mat-forming emergent herb: swamp stonecrop, *Crassula helmsii* (Kirk) Cockayne (Crassulaceae; Figure 1c). Other, less common aquatic macrophytes, such as *Callitriche stagnalis* Scop. (Brassicaceae), *Elatine gratioloides* A. Cunn. (Elatinaceae), *Limosella australis* R.Br. (Scrophulariaceae), and species of *Glossostigma* Wight & Arn. (Phrymaceae) were only seen in shallow ponds.

Figure 2. a. *Ottelia ovalifolia* showing floating-leaved habit at Pildappa Rock; b. Same, detail of floating leaves with long, submerged petioles; c. Emergent flower produced at draw-down (cultivated plant); d. Submerged cleistogamous flower produced in deep water (cultivated plant).

Discussion

Three species of damselfly (Odonata: Zygoptera) from two families (Coenagrionidae and Lestidae) were recorded from the EP rock-holes. Globally and in eastern Australia, adult narrow-winged damselflies (Coenagrionidae) are usually associated with still or slow-flowing freshwater bodies (Watson & O'Farrell, 1991). Two widespread Australian species of similar body size, *Ischnura aurora* and *X. erythroneurum*, have overlapping distributions. *Ischnura aurora* and *X. erythroneurum* have been recorded consistently as adults near granite outcrops in areas that lack other permanent sources of surface freshwater.

Figure 3. Damselfly nymphs associated with *Ottelia ovalifolia* at Pildappa Rock. a. *Ischnura aurora*; b, c. *Xanthagrion erythroneurum.*

Ischnura aurora is often windborne and known to be highly dispersive (Watson & O'Farrell, 1991) and associated with temporary pools (Rowe, 1978). As a result, it has been recorded from widely separated and isolated locations, including small oceanic islands, granite rock-holes and other ephemeral desert water bodies (Watson & O'Farrell, 1991). *Xanthagrion erythroneurum* is the only member of its genus and known to form large, non-territorial colonies (Watson & O'Farrell, 1991).

Similar to coenagrionid damselflies, adult spread-winged damselflies (Lestidae), are also associated primarily with still freshwater bodies (Watson & O'Farrell, 1991). Generally larger than coenagrionids, their greater wing size might suggest a greater dispersal capacity (Swaegers et al., 2014), although Bacca et al. (2021) defined both lestid and coenagrionid damselflies as weak fliers. *Austrolestes annulosus* has been reported previously from granite outcrops, but current records indicate that it is largely restricted to southern Australia and has a more limited range than either *I. aurora* or *X. erythroneurum*.

Table 2. Presence of submerged and emergent macrophytes (indicated by "x"), observed abundance for the three damselfly species, and potential predators (Dytiscidae), and presence of tadpoles in gnammas on five Eyre Peninsula granite outcrops during the 2016 field survey. * denotes rock-holes with a depth of greater than 50 cm.

	Outcrop and site															
	Bascombe Rocks					Pygery Rocks	Pildappa Rock				Tcharkuldu Rock	Secret Rocks			
Taxon	1.1	1.2	1.3	1.4	1.5	2.1*	3.1	3.2*	3.3*	3.4	4.1	5.1*	5.2e	5.2 w	5.3
Macrophytes															
Althenia sp.	0	0	0	0	0	0	0	0	X	0	0	0	0	0	0
Crassula helmsii	0	0	0	0	0	0	X	0	0	X	0	0	0	0	0
Ottelia ovalifolia	0	0	0	0	0	0	0	X	0	0	0	0	0	0	0
Damselfly species															
Ischnura aurora	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Xanthagrion erythroneurum	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0
Austrolestes annulosus	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Other potential predators															
Allodessus bistrigatus	1	4	0	0	3	0	2	4	0	1	2	0	0	0	1
Eretus australis	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Rhantus suturalis	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Sternopriscus multimaculatus	0	0	0	0	1	0	0	0	0	1	0	0	2	0	0
Tadpoles	0	0	X	0	0	0	X	0	X	0	X	X	X	X	X

Although our results are limited, they suggest that the presence of the macrophyte *O. ovalifolia* might be a potential predictor for damselfly occurrence in rock-holes on the EP. Abiotic physical characters, such as depth, have been shown previously to impact community composition within rock-holes in the area (Timms, 2014) and in regions of similar climate (Bayly et al., 2011; Timms, 2017). This is the first study that suggests that predator occurrence in EP rock-holes could also be influenced by biotic physical characters, such as macrophyte presence. Interestingly, this observation is consistent with a similar study undertaken on freshwater pond assemblages in France where invertebrate predators mostly occurred where macrophytes were present (Le Gall et al., 2018).

This apparent association of damselfly nymphs with emergent macrophytes may be related to three biological needs: feeding, oviposition, and respiration. Damselfly nymphs, like other aquatic predators, are "ambush predators" of other macroinvertebrates in the water column and sometimes small vertebrates, lying in wait behind vegetation before striking prey (Schultz & Kruschel, 2010; Watson et al., 1991). It is therefore possible that damselflies in rock-holes without complex three-dimensional plant structure are less efficient at catching prey (Klecka & Boukal, 2013). It is also possible that such plant structure is crucial in providing habitat for prey numbers to reach necessary thresholds for damselfly survival. Similarly, although many odonates oviposit directly into the water column (Rowe, 1988), some Australian species oviposit into vegetation above, or emergent from, temporary freshwater bodies (Watson et al., 1991). The floating leaves of *Ottelia* could thus provide a site for egg oviposition and/or a "landing pad" for egg-carrying adult females. In addition, the final aquatic nymphal instars of most damselflies are air-breathing prior to emergence (Watson et al., 1991). It is possible that the presence of emergent macrophytes allow late-stage damselfly nymphs to climb to the surface for air, rather than expending energy swimming through the water column (where they might also be exposed to predation), as well as providing aerial environments for the adults to emerge. *Ottelia* may also provide some protection to damselfly nymphs from predation by birds.

Due to the remoteness of the sample sites (>600 km from Adelaide) and the difficult terrain often present between granite outcrops, only 14 rock-holes, across five outcrops could be surveyed in this study, with the result that only a single rock-hole was observed containing either *O. ovalifolia* or damselfly nymphs. Nevertheless, distribution data for the plant suggest that it is uncommon in the region. The population at Pildappa rock is one of only three South Australian records for this species west of Port Augusta, all of which are from a few deep long lasting granite outcrop rock-holes (Bayly & Elliot, 2013; CHAH, 2020).

The rock-holes ranged from 4 cm deep in the shallowest (pan) rock-hole to the deepest (pit) rock-hole at 70 cm (where the *Ottelia* was growing). As such, the difference in hydroperiod between the rock-holes would be considerable, as it will obviously be affected by both water depth and volume (Jocque et al., 2010). Although 14 rock-holes represents only a small proportion of the total number present on the EP, we are nevertheless confident that those sampled are representative of their natural variability in size and shape.

Timms (2014) found that biogeography explained much of the variability in invertebrate species composition between rock-holes. However, the designation of pit versus pan rock-holes, seemed to impact community composition greater than this biogeographical signal, with distant pits observed to display greater similarity of community composition than to nearby pans (Timms, 2014). Also, as for the current study, the designation of “pan” rock-hole versus “pit” rock-hole discounts much of the geomorphological variability observed in Western Australian granite rock-holes, where 10 types of pit have been recognised (Timms, 2013b).

In previous studies, insects were found to be more species-rich in pit rock-holes than in pans, with 66% of taxa in pits and only 45% in pans (Pinder et al., 2000; Timms, 2013a). Habitat structure was correlated with the presence of certain invertebrates, although not odonates (Timms, 2012). Although odonate nymphs sometimes occur in pans, it was noted that they dried out before the nymphs could mature (Timms, 2012). Strong-flying insects (such as damselflies) have been observed to respond to climate and fine-scale spatial factors (Bacca et al., 2021). Rock-holes in both south-western Western Australia, and South Australia display seasonal variation amongst life stages of invertebrate taxa, and overall community composition (Timms, 2012, 2014), suggesting that regular sampling is required to better our understanding these systems. The flora observed in rock-holes at Terrick Terrick (Victoria) was similar to that at Pildappa rock, although no damselflies were reported (Timms, 2017) and it is possible that these rock-holes were eutrophic.

Both shallow pan rock-holes and deep pit rock-holes on the EP have diverse and abundant invertebrate communities (Hedges, 2018; Timms, 2014, 2017), so it is unlikely that prey abundance is a limiting factor for damselfly occurrence. The relatively low densities of large predators observed in the majority of the EP rock-holes sampled here also suggest that damselfly occurrence may not be impacted strongly by top-down affects. However, tadpoles were present in many rock-holes, and those at Secret Rocks (Table 1) had larger tadpoles, potentially increasing predation, given their observed, actively omnivorous behaviour (including cannibalism at some sites).

Our observations imply a potential relationship between the nature of the EP rock-hole habitats and the occurrence of the damselflies. The three species were the only damselflies recorded in this study, although previous rock-hole surveys in the region have also recorded the common bluetail, *Ischnura heterosticta* (Burmeister, 1839) (Coenagrionidae), as well as several dragonfly species (Timms, 2014), although previous surveys did not report if any plants were associated with these records. The fact that damselfly nymphs were only present in the single rock-hole with *O. ovalifolia*, even though there were other deep pit rock-holes, suggests that there may be factors other than just pool structure and sediment influencing the biota.

Several other aquatic plant species are known to occur, although none of these was emergent in the deeper EP rock-holes. The submergent *Althenia* cf. *australis* observed during this study, was found mainly in deep, longer-lived rock-holes, and its taxonomy is the subject of ongoing research (J.G. Conran, pers. comm.). Similarly, *Crassula helmsii* is not emergent in deep water. *Callitriche* sp., *Elatine* sp., *Limosella australis,* and *Glossostigma* sp. have also been proposed to occur in the rock-holes, although neither are likely to be emergent beyond 30 cm depth and were not observed during this study. Depth does not appear to be a predictor of damselfly presence (Tables 1 & 2), however, due to the absence of *O. ovalifolia* from other EP rock-holes at the time of our survey, it is not possible to verify this hypothesis statistically. It seems likely that *O. ovalifolia* will need deep rock pools with a hydroperiod of >2 months, to allow completion of its life cycle, but longer-term survey data are needed to confirm this. Supporting this current study, in the Northern Hemisphere odonata nymph presence is often dependent on vegetation structure and organic matter content (Le Gall et al., 2018; Remsburg & Turner, 2009) and coenagrionid damselflies require submerged plant stems for successful oviposition (Fincke, 1986). Plant structure can affect ecdysis (moulting) by providing cover when nymphs are more vulnerable to predation (Büsse et al., 2019).

Future examination of these questions using *in situ* mesocosm experiments could simulate characteristics of habitat structures, such as depth, the presence of landing platforms, a three-dimensional matrix, and the presence of *O. ovalifolia* or other submerged aquatic plants, thus allowing the nature of any damselfly interactions with these parameters to be assessed.

Threats to southern Australian ephemeral freshwater rock-holes

Like other small, disconnected ephemeral freshwater bodies, rock-holes are susceptible to a range of threats. Increased invasion rates of foreign taxa resulting from human activity have impacted similar systems globally, as well as in Victoria, where some rock-hole communities are dominated by the aquatic weed *Callitriche stagnalis* (see Timms, 2017). Invasion events can cause large changes in ephemeral freshwater system algal communities (Buchberger & Stockenreiter, 2018), macrophyte biomass (Carreira et al., 2014) and invertebrate populations (Devereaux & Mokany, 2006) and even single-species additions or subtractions can result in large changes to community composition (Jonsson, 2006). Large invasive mammals, such as feral camels and pigs can also impact ecosystem health and quality, when present near systems similar to the rock-holes, due to disturbance and nutrient input from faeces (Box et al., 2016; Doupé et al., 2010), as well as over-abundant native species, such as kangaroos.

Land use for agricultural purposes is correlated with disturbances and declines in native plant species richness generally and, more specifically, impacts species richness and diversity in ephemeral waterbodies (Bouahim et al., 2014; Hall et al., 2004). EP rock-hole communities are likely to be impacted by agricultural activities (Bruno et al., 2016; Dimitriou et al., 2006; Kerezsy et al., 2014) due to high input of windblown insecticides, fertilisers and which may result in detrimental effects on biodiversity or lead to potential trophic cascades (Boggs et al., 2007). Livestock, such as cattle may also impact water quality directly, increasing local nitrogen and turbidity levels (Canals et al., 2011).

In addition to the above mentioned threats, due to the occurrence of rain-fed ephemeral waterbodies being regulated by rainfall (Kneitel, 2016; Krieger, 2003), climate change poses a direct threat to their ecology. Climate change has already been identified as having induced shifts in distribution and phenology of species typically associated with similar ephemeral freshwater habitats (Ewald et al., 2013). Furthermore, changing climate regimes have been observed to impact associated plants, invertebrates (Frisch, 2001), and vertebrates (Chessman, 2011; Howard et al., 2016). Inter-species interactions have been recorded to change with increased water temperatures (Ewald et al., 2013), suggesting that the effects of climate change may result in shifts in community composition for the EP rock-holes, depending on the thermal tolerance of individual species.

Due to the predicted drying climate over the next 100 years for the EP (IPCC, 2013), the long-term viability and security of the EP rock-holes and their associated biota is uncertain. As all the species observed in this study are dependent on the regular wetting and drying regime for completion of their life cycles, the rock-hole ecosystem must be regarded as vulnerable. It is therefore important that the conservation of *O. ovalifolia* and other native rock-hole vegetation be considered as part of any efforts to conserve the invertebrate faunas associated with the EP rock-holes. The provision of a diversity of habitat types within the rock-holes can only be achieved by maintaining a healthy population of macrophytes. In addition to providing habitat, freshwater macrophyte species also play a role in regulating water quality and the density of phytoplankton populations (Barrow et al., 2019), as well as limiting the occurrence of algal blooms (Bakker et al., 2010).

Conclusions

Damselfly occurrence in EP rock-holes appears to be associated with the presence of the floating-leaved, rooted aquatic macrophyte, *Ottelia ovalifolia*. However, this plant is apparently rare in EP rock-holes, and it is therefore possible that damselfly presence and abundance in this system is impacted similarly. Unfortunately, the sparse occurrence of this plant means that the results of the present study were not amenable to statistical analysis. Nevertheless, given the potential stresses that the EP rock-holes may be experiencing currently and are projected to occur under ongoing climate change, these sites must be viewed to be "at-risk". It is therefore recommended that EP rock-holes be considered for listing as a threatened ecological community under the *Environment Protection and Biodiversity Conservation Act 1999* (Australian Government Department of the Environment, 1999), thereby facilitating the development of conservation management plans for the protection of these unique habitats. In the first instance, management should be directed to facilitate and conserve the small populations of

O. ovalifolia that remain, to promote diverse invertebrate communities. Such management options include alleviating current stresses such as the control of over-abundant grazers (primarily kangaroos, but also sheep and cattle on pastoral land), drift of agricultural chemicals, and loss of greater remnant vegetation and connectivity.

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Data availability statement

All data and material discussed in this paper are included in this paper.

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Appendix B. Supporting publication 2

Publication: Detection of vertebrates from natural and artificial inland water bodies in a semi-arid habitat using eDNA from filtered, swept, and sediment samples

Statement of Authorship

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Principal Author

Name of Principal Author	Rupert McDonald		
Contribution to the Paper	Led data curation, methodological design, data analysis and production of manuscript		
Overall percentage (%)	90%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a thi inclusion in this thesis. I am the primary author of this paper		
Signature		Date	19/7/23

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate in include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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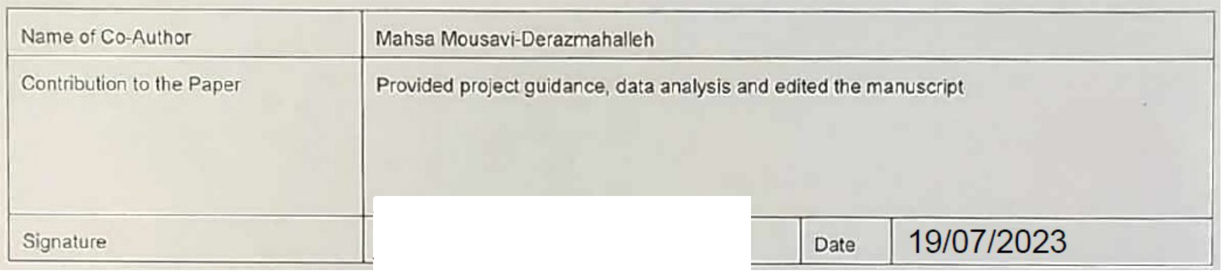

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RESEARCH ARTICLE

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Detection of vertebrates from natural and artificial inland water bodies in a semi-arid habitat using eDNA from filtered, swept, and sediment samples

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Abstract

Biomonitoring is vital for establishing baseline data that is needed to identify and quantify ecological change and to inform management and conservation activities. However, biomonitoring and biodiversity assessment in arid environments, which are predicted to cover 56% of the Earth's land surface by 2100, can be prohibitively time consuming, expensive, and logistically challenging due to their often remote and inhospitable nature. Sampling of environmental DNA (eDNA) coupled with high-throughput sequencing is an emerging biodiversity assessment method. Here we explore the application of eDNA metabarcoding and various sampling approaches to estimate vertebrate richness and assemblage at human-constructed and natural water sources in a semi-arid region of Western Australia. Three sampling methods: sediment samples, filtering through a membrane with a pump, and membrane sweeping in the water body, were compared using two eDNA metabarcoding assays, 12S-V5 and 16smam, for 120 eDNA samples collected from four gnammas (*gnamma*: Australian Indigenous Noongar language term–granite rock pools) and four cattle troughs in the Great Western Woodlands, Western Australia. We detected higher vertebrate richness in samples from cattle troughs and found differences between assemblages detected in gnammas (more birds and amphibians) and cattle troughs (more mammals, including feral taxa). Total vertebrate richness was not different between swept and filtered samples, but all sampling methods yielded different assemblages. Our findings indicate that eDNA surveys in arid lands will benefit from collecting multiple samples at multiple water sources to avoid underestimating vertebrate richness. The high concentration of eDNA in small, isolated water bodies permits the use of sweep sampling that simplifies sample collection, processing, and storage, particularly when assessing vertebrate biodiversity across large spatial scales.

KEYWORDS
arid lands, biodiversity, biomonitoring, environmental DNA, Fauna surveys, metabarcoding, vertebrates

TAXONOMY CLASSIFICATION
Applied ecology

1 | INTRODUCTION

Semi-arid and arid lands comprise more than one third of the Earth's land surface and include ecosystems such as forests, woodlands, savannas, shrublands, grasslands, and deserts (Lemons & Victor, 2003). Despite limited water availability due to a combination of low rainfall, high temperatures, and low humidity, these environments harbor a remarkably diverse biota, high levels of endemism and some of the world's most endangered species (Durant et al., 2012). Semi-arid and arid ecosystems have received less attention than other ecosystems, irrespective of their extent, ecological and social value, and the threats they face (Durant et al., 2012; Razgour et al., 2018). The extent of arid habitats is increasing globally due to climate change, and it is predicted they will cover 56% of the Earth's land surface by 2100 (Lemons & Victor, 2003; O'Farrell et al., 2010). Habitat degradation is a major issue for existing semi-arid and arid lands (Lemons & Victor, 2003; O'Farrell et al., 2010) and poses a significant threat to ecosystem function and services, upon which the livelihoods of more than 250 million people rely (James et al., 2013).

Biomonitoring is vital for establishing baseline data that is needed to identify and quantify ecological change and to inform management and conservation activities in semi-arid, arid, and other habitats (Campbell et al., 2002; Epanchin-Niell et al., 2012; Herrick et al., 2006). Conventional techniques such as observational surveys (direct observation, camera traps, audio recordings), recording field signs (hair/feathers, scats, tracks, feeding signs) and live trapping can be costly, labor intensive and may have ethical considerations (e.g., Cross et al., 2020; Furlan et al., 2020; Waudby et al., 2019). Some species may also have lower detectability than others, leading to biases in survey data (e.g., Einoder et al., 2018; Fernandes et al., 2018; Ji et al., 2013; Thompson & Newmaster, 2014). As a result, conventional monitoring techniques have some limitations, particularly detecting species of high priority for conservation or management, which are often rare, endangered, invasive, or elusive (Barnes & Turner, 2016; Harper, Handley, et al., 2019; Kelly et al., 2014; Rodgers & Mock, 2015). Therefore, there is an urgent need for biomonitoring practices that can generate detailed and current environmental information on arid ecosystems (Kelly et al., 2014).

Environmental DNA (eDNA) metabarcoding is a relatively new biomonitoring tool that has potential to overcome some of the disadvantages of more conventional approaches (Barnes & Turner, 2016). Metabarcoding involves sequencing small regions of DNA isolated from substrate samples and comparing the sequences against a library of taxonomically identified sequences to determine the species present in the sample (Taberlet et al., 2012). High-throughput sequencing has revolutionized the application of eDNA, enabling ecological communities to be characterized at a relatively low cost due to the simultaneous analysis of many samples (Furlan et al., 2020; Kelly et al., 2014). Metabarcoding of eDNA has proved highly successful in freshwater and marine systems (Egeter et al., 2018; Furlan et al., 2020; Harper, Handley, et al., 2019; Palacios et al., 2020; Thomsen et al., 2012; Ushio et al., 2017, Wang et al., 2021). However, despite rapid advancements in the technology, it is currently difficult to apply metabarcoding to terrestrial biomonitoring where eDNA may not be well preserved (van der Heyde et al., 2020), appropriate sampling substrates may be limited (Fahner et al., 2016; van der Heyde et al., 2020, 2021), consistent protocols to sample substrates are not well established (Harrison et al., 2019; van der Heyde et al., 2022), and reference databases for arid habitat taxa are incomplete (Bradford et al., 2010; Carrasco-Puga et al., 2021; Egeter et al., 2018; Palacios et al., 2020; van der Heyde et al., 2020). As a result, the application of eDNA for biomonitoring in arid lands is rare.

In water-limited environments, both natural and human-constructed (artificial) water bodies are critical water sources for animals (Davies, 1972; Hedges et al., 2021; James et al., 1999; Redfern et al., 2003). Consequently, these water bodies may be particularly rich sources of eDNA and important for biodiversity monitoring as they can provide a broad snapshot of biodiversity over a wide area given that animals may migrate over vast distances to water(. Studies have shown the applicability of eDNA metabarcoding in identifying fauna using water holes (Farrell et al., 2022; Furlan et al., 2020; Harper, Handley, et al., 2019; Mas-Carrió et al., 2021) but, to date, no studies have explored the use of different sampling methods (e.g., filtering vs sweeping vs sediment). Sample volume protocols designed for marine environments (i.e., 1–5 L) are typically applied to freshwater habitats (Egeter et al., 2018; Palacios et al., 2020; Takahashi et al., 2023) despite distinct differences in water characteristics. Water bodies in semi-arid and arid lands are often of a relatively small volume and a greater turbidity (due to high concentrations of algae, sediment, and organic debris) than marine or other freshwater environments (Harper, Buxton, et al., 2019). Attempts to apply sampling protocols for marine environments to studies of water bodies in semi-arid and arid habitats have been hindered by pump blockages during filtering of water for analysis, reducing the number of samples processed (Egeter et al., 2018; Klymus et al., 2017). Techniques to reduce sample turbidity (e.g., centrifugation, increased filter membrane pore size, pre-filtering) may reduce eDNA capture, especially when eDNA is bound to suspended particles (Turner et al., 2014), and increase costs and contamination risk (Klymus et al., 2017; Takahara et al., 2012). Turbidity issues can be avoided by collecting sediments rather than water, with the additional benefits of reduced eDNA decay in sediment compared to water (Buxton et al., 2018; Turner et al., 2015) and a high yield of eDNA due to binding of DNA to particulate matter (Turner et al., 2015). However, there is evidence that humic substances in sediment inhibit PCR amplification (e.g., Stoeckle et al., 2017) and that differences in eDNA viability between substrates can lead to a failure to detect all the components of the faunal community (Palacios et al., 2020).

Biomonitoring using eDNA metabarcoding has been used previously to detect terrestrial vertebrates in artificial water sources (Rodgers & Mock, 2015), but no studies to date have compared detection of eDNA from natural and artificial water sources in the same ecosystem. Here, we sampled water from both natural and artificial water bodies. While both water bodies are accessed by numerous fauna, there are likely to be differences in the community composition between them due to the location, structure, size, and immediate surroundings (e.g., Korine et al., 2016; Letnic et al., 2014; Schneider & Griesser, 2009). Here we tested the hypothesis that vertebrate eDNA can be detected in water and sediment samples collected from both gnammas (natural water sources) and cattle troughs (artificial water sources) in the semi-arid Great Western Woodlands, Western Australia. We investigated whether the vertebrate taxonomic richness and community assemblages detected with eDNA metabarcoding techniques differed for natural and artificial water sources and examined the effect of sampling methodology on these variables. We compared results from (1) sediment samples, (2) water samples filtered through membranes (filtered samples) and (3) unfiltered samples collected on membranes dipped in the water (swept samples; Bessey et al., 2021) and evaluated the effect of sample replication on detected taxonomic richness. Our overall aim was to expand the scope of eDNA metabarcoding to biomonitoring in environments previously considered suboptimal for the application of eDNA approaches, thereby enhancing the potential of eDNA techniques for conservation and management.

2 | MATERIALS AND METHODS

2.1 | Study sites

Fifteen samples were collected from each of four gnammas and four cattle troughs ($n = 120$; see below), located in a 96 km^2 area of the Fraser Range, 380 km NNE of Esperance, Western Australia. The study site has a semi-arid climate with 268 mm of annual rainfall occurring year-round. Vegetation communities are dominated by *Eucalyptus* woodlands and *Acacia* shrublands and herb lands. The Fraser Range is within the Great Western Woodlands (GWW), the world's largest semi-arid to arid woodland (Newbey et al., 1984), which covers 16 million hectares and supports an exceptional native flora (>3300 plant species) and fauna (49 native mammal, 11 feral mammal, 138 reptile, 14 frog, and 215 bird species; Department of Parks and Wildlife, 2013; Fox et al., 2016).

2.2 | Sample collection

Water samples, sediment samples, and samples from membranes swept through the water body were collected in May 2021 (late Autumn) from the four gnammas and four cattle troughs. Five replicate samples of each type were taken from each water source. Gnammas ranged from 30 cm to 300 cm in width, and 20 cm to 100 cm in depth. Cattle troughs were 130 cm wide and 60 cm deep. Five 50 mL water samples were collected into falcon tubes at five random locations in each water source, at a depth of 5–15 cm from the surface. Five Supor 47 mm 0.45 μm pore-size filter membranes (Pall Corporation) were submerged at a depth of 5–15 cm at five random locations from each water source, and “swished” around for 15 s before being placed immediately into individual zip-lock bags. Finally, five randomly chosen sediment samples were scooped from the bottom of each water source into a 50 mL falcon tube. Disposable gloves were worn during sampling and were changed between every sample at each location. Samples were placed on ice immediately after collection. Water samples (50 mL) were filtered within 24 h through a Supor 47 mm 0.45 μm pore size filter membrane (Pall Corporation) using a Sentino Microbiology Pump (Pall Corporation). Equipment was decontaminated between samples in a 10% bleach solution for 10 min and rinsed thoroughly before subsequent use. Two filtering control samples were taken by pumping 500 mL of local tap water through filter membranes. All samples were kept on ice from the time of collection or processing and were frozen at −20°C at Curtin University within 72 h of collection.

2.3 | Sample processing and DNA extraction

Prior to DNA extraction, samples were defrosted in a refrigerator (4°C) overnight. DNA was extracted from filter membranes using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). Filter membranes were cut up into 2 mm wide strips and incubated in 540 mL buffer ATL and 60 μL proteinase K at 56°C for 3 h. Sediment samples were homogenized using a TissueLyser (Qiagen) and DNA was extracted from 250 mg of sediment using the DNeasy PowerLyser Powersoil Kit (Qiagen), which contains steps to remove inhibitors from the extracts. Samples were then extracted using the QIAcube automated platform (Qiagen) and eluted to 100 μL using the manufacturer's protocol. DNA extracts were immediately frozen at −20°C. DNA extraction blanks (negative controls) were processed with each batch of 30 samples ($n = 4$) using the extraction reagents only.

2.4 | Assessment of DNA extracts

Due to the degraded nature of eDNA, metabarcoding primers typically target short barcode regions to improve amplification success (Yu et al., 2012). The primers used were the 12Sv5-F/R targeting the mitochondrial 12S gene (F: 5'-TAGAACAGGCTCCTCTAG-3'; R: 5'-TTAGATACCCCACTATGC-3', ~98 bp (Riaz et al., 2011) and the mammal specific primers 16Smam1/2 targeted the mitochondrial 16S ribosomal gene (F: 5'-CGGTTGGGGTGACCTCGGA-3'; R: 5'-GCTGT TATCCCTAGGGTAACT-3', ~135 bp (Taylor, 1996). Both assays target regions conserved across vertebrates (12S-V5) or mammals (16Smamm) and can be recovered from degraded DNA (Kitano et al., 2007; Sarri et al., 2014; Staats et al., 2016). Quantitative polymerase chain reaction (qPCR) was used to detect the quality and quantity of DNA in each extract, and extract and verify the optimum DNA input for metabarcoding (Murray et al., 2015). Here, qPCR assays were run on all samples using the 12S-V5 F/R primers on the neat extract, 1/5 and 1/10 dilution to screen for PCR inhibitors in the reaction. The extraction of sediment samples included inhibitor removal steps because those tend to be more abundant in sediments, but all sample types were screened. The majority of water/passive samples were found to have an optimum dilution in the realm of neat, 1/5 or 1/10, whereas the majority of sediment samples had to be diluted to 1/100. Using the optimum dilutions determined by qPCR with 12S-V5 primers, a supplementary qPCR assay was run on a subset of 20 samples to determine whether the 16smam forward/reverse primers were as effective at amplifying DNA at these dilutions. In some instances, primer bias can differentially amplify eDNA at sites with different community composition (Aird et al., 2011). To mitigate these effects the combination of a vertebrate primer and a specific mammal primer were used. In addition, 12S and 16S are broadly used for mammal detection in eDNA metabarcoding studies and there is a greater availability of reference sequences available online for taxonomic assignment (Deagle et al., 2014; Valentini et al., 2016).

The polymerase chain reaction (PCR) mix for quantification contained: 2.5 mM MgCl2 (Applied Biosystems), 10 × PCR Gold buffer (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific), 0.4 mg/mL bovine serum albumin (Fisher Biotec), 0.4 μmol/L forward and reverse primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.6 μL of a 1:10,000 solution of SYBR Green dye (Life Technologies). All PCR amplification was conducted on a StepOne Plus (Applied BioSystems) real-time qPCR instrument with the following conditions: 5 min at 95°C, 50 cycles of 95°C for 30 s, 30 s at the annealing temperature (58°C) and 45 s at 72°C, followed by 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C during the melt curve stage, ending with 10 min of elongation at 72°C. Contamination was minimized by preparing the PCR mixes in a dedicated clean laboratory, and then adding DNA extract in a separate laboratory, inside specialized ultraviolet hoods.

2.5 | DNA amplification and sequencing

Based on qPCR results, fusion tagging was performed on samples that contained adequate amplifiable DNA by assigning each sample a unique combination of fusion tag primers. Each fusion tag primer combination contained a unique multiplex identifier between 6 and 8 bp in length, Illumina's sequencing adaptors (i.e., P5 and P7) and the gene-specific primer (described above). A single-step fusion protocol was carried out with unique index combinations before qPCR was used to generate amplicons of each fusion-tagged sample using the same reagents and cycling conditions as described above. The fusion-tagged amplicons were generated in duplicates for each biological replicate to maximize amplicon numbers for sequencing and reduce the chances of non-detections and the effects of PCR stochasticity (Murray et al., 2015). PCR replicates were then pooled, amplicons cleaned using the QIAquick PCR Purification Kit (Qiagen) and then quantified using the QIAxcel Advanced System (Qiagen). Based on this quantification, the DNA library for sequencing was made from pools, combined in approximate equal concentrations. A Pippin Prep (Sage Science) was used to size-select the amplicons in this library, and the library was then cleaned using the QIAquick PCR Purification Kit (Qiagen). A Qubit fluorometer (Thermo Fisher Scientific) was used to quantify the final DNA library, before sequencing as per Illumina's sequencing protocols for single-end sequencing, using Illumina's single direction MiSeq 300 V2 Reagent Kit on the Illumina MiSeq platform (Illumina).

2.6 | Sequence analysis and taxonomic assignment

Using a high-performance computing cluster (Pawsey Supercomputing Centre), sequences were analyzed with the eDNAFlow automated pipeline (Mousavi-Derazmahalleh et al., 2021), which performed the following tasks: sequence quality was checked with FASTQC (Andrews, 2010) and filtered with AdapterRemoval v2 (Schubert et al., 2016) for Phred quality score lower than 20 and trimming sequences with Ns as enforced in eDNAFlow by—trimns and—trimqualities parameters. Remaining trimmed sequences were demultiplexed and sequences smaller than expected minimum amplicon length were trimmed (12S-V5 minimum length 50 bp; 16smam minimum length 25 bp) using OBITools' ngsfilter and obigrep tools, respectively (Boyer et al., 2016). Unique sequences, zero-radius operational taxonomic units (ZOTUs-denoised sequences) and an abundance table were generated using the USEARCH (Edgar, 2010) commands fastx-uniques, unoise3, (minsize 8) and otutab, respectively. The ZOTUs generated from both assays were queried against the nucleotide database Genbank (https://www.ncbi.nlm.nih.gov/genbank/) in October 2021 using the following parameters in Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990): perc_identity ≥90, evalue ≤1e−3, best_hit_score_edge 0.05, best_hit_overhang 0.25, qcov_hsp_perc 100, max_target_seqs = 10. Taxonomic identification was then assigned with more strict parameters using the eDNAFlow Lowest Common Ancestor (LCA) script (Mousavi-Derazmahalleh et al., 2021) with a minimum percentage identity (%identity) of ≥95, and if a ZOTU had multiple blast assignments where the difference between their %identity was equal to or smaller than 1, then that ZOTU was assigned to the nearest common taxonomic level otherwise a species level assignment was returned.

The results of the LCA script were compared against existing taxonomic data for the sampling area (Department of Parks and Wildlife, 2013), and if necessary, manually curated to ensure that the taxa assigned are known to occur in the sampling area. While the majority of ZOTUs were assigned to species level, the LCA script dropped a few ZOTUs to the nearest common taxonomic level (e.g., Meliphagidae *sp.*). Where ZOTUs were assigned to taxa that are not local to the sampling area, the ZOTUs were either reassigned to sister species, for example, black-flanked rock-wallaby (*Petrogale lateralis*) and Australian shelduck (*Tadorna tadornoides*) known to occur in the area, or dropped to the nearest common taxonomic level that currently exists in the sampling area, for example, vesper bats, (Vespertilionidae *sp.*), that is, ZOTUs were manually assigned to locally known species if all other species for that ZOTU were exotic to the sampling area: Australian magpie (*Gymnorhina tibicen*), yellow-throated miner (*Manorina flavigula*), Australian magpie-lark (*Grallina cyanoleuca*), euro (*Osphranter robustus*), and sheep (*Ovis aries*). In cases where ZOTUs were assigned to family level and multiple exotic species were attributed to a ZOTU with 100% identity, the ZOTUs were reassigned to generic rank of species known to be native to the sampling area (e.g., *Corvus* sp.). All ZOTUs assigned to "chordata environmental samples" were removed from the data set, and all ZOTUs assigned to exotic canid spp. (i.e., *Nyctereutes viverrinus*, *Canis lupus rufus*, *Canis lupus*) were reassigned to *Canis lupus familiaris*. ZOTUs assigned to exotic "Artiodactyla spp." (i.e., *Bison bonasus*, *Bos javanicus*, *Bos mutus*, *Cephalophus dorsalis*, *Muntiacus* sp., *Pudu puda*, *Syncerus caffer*, *Tragelaphus eurycerus*) were reassigned to domestic cow (*Bos taurus*).

2.7 | Statistical analysis

We generated a presence–absence matrix for the two assays (12S-V5 and 16smam) for both sources (gnammas and cattle troughs) and all sampling methods (swept, filtered, and sediments). This matrix was used to calculate taxon richness for each sample. The effect of source and sampling method was evaluated using a two-way analysis of variance (ANOVA) achieved using StatistiXL v 2.0 (www.statistiXL.com). The presence–absence matrix was also used to examine differences in biological assemblages identified from the various sources and sampling methods, using the PERMANOVA+ software add-on in PRIMER7 (Clarke & Gorley, 2015). To avoid unassigned resemblance values, two gnamma-sediment samples (GS1_3 and GS2_4) and one gnamma-filter sample (GW2_2) for which no taxa were detected were removed from the matrix, before a Jaccard distance matrix was generated between samples. Using these data, a PERMANOVA was run using Type III sums of squares, unrestricted permutation of raw data and significance determined by 9999 permutations of the pseudo-F statistic (Clarke & Gorley, 2015). PRIMER7 was used to visualize and estimate pair-wise tests to determine how the sources and sampling methods compared using non-metric multidimensional scaling (nMDS).

We used the "BiodiversityR" (Kindt & Coe, 2005) and "drc" packages (Knezevic et al., 2007) with R 3.5.1 (R Core Team, 2018) to generate accumulation curves for taxa at each site and with each sampling method. Asymptotic regression rarefaction curves were generated for each sampling method and models were visualized using the package "ggplot2" (Chiarucci et al., 2008; Wickham & Sievert, 2016). Curves represent the order-free accumulation of mean taxa detections calculated from random permutations of all possible orderings of taxa detections. We then used an EcoTest (Cayuela et al., 2015) to statistically compare rarefaction curves representing each sampling method for each source respectively, testing the null hypothesis that three samples were drawn from a single assemblage and any differences in their rarefaction curves reflect only sampling effects. Using the package "rareNMtests" (Cayuela et al., 2015) the abundances of all taxa detections were summed to generate a pooled composite curve. The test statistic (Z) was calculated from the cumulative summed areas of difference between the three individual curves representing each sampling method and the composite curve. The observed value of this Z-statistic was compared to a null model distribution constructed from rarefaction curves generated from 200 random permutations of all possible orderings of taxa detections in each set of replicates across the three sampling methods and significance assessed at $\alpha = 0.05$. A "leave one out" analysis was used to subsequently determine how z-values and significance changed when sampling methods were individually omitted from the analysis, after Cayuela et al. (2015).

3 | RESULTS

There was successful eDNA amplification from 110 and 111 of 120 samples using the 12S-V5 and 16smam assays, respectively. Sequencing yielded 9,740,203 (mean per sample = 74,924 ± 4395 standard error; SE) sequences for the 12S-V5 assay and 12,530,593 (mean per sample = 96,389 ± 5762 SE) for the 16smam assay sequences in total. A small proportion of sequence reads were present in the field and extraction controls, highest in the 16smam assay (3.09% of total reads) and lowest in the 12S-V5 (2% of total reads). A proportion of sequence reads were assigned to humans, which were highest in the 16smam assay (27.05%) and lowest in the 12S-V5 assay (13.84%); ZOTUs representing humans were not included in the statistical analysis. One ZOTU assigned to *Gallus* amplified with the 12S-V5 assay accounted for 0.7% of total reads for that assay and was removed as a likely contaminant as *G. gallus* DNA is used in the laboratory as a positive control.

3.1 | Vertebrate taxon richness detected across sources and sampling methods

12S-V5 and 16smam assays produced a total of 9190 and 3203 ZOTUs respectively, from which 6584 and 1144 were assigned. A total of 26 unique taxa were detected from both assays combined, representing 12 orders and 21 families including 11 mammal, 15 bird and 1 amphibian taxa (Table 1). The 12S-V5 assay detected 25 unique taxa and the 16smam assay nine unique taxa. Eight common taxa were detected by both assays (Table 1). There were significant differences for total vertebrate richness between gnammas (3.55 ± 0.23 taxa per sample, 20 taxa total) and cattle troughs (4.65 ± 0.34 taxa per sample, 22 taxa total; $F_{5,\ 114} = 7.9$, $p = .006$) and between sampling methods ($F_{5,\ 114} = 7.4$, $p = .001$; Figure 1). The highest total richness was detected in swept (4.3 ± 0.36 taxa per

TABLE 1 Vertebrate taxa detected from five samples each from four gnamma holes (granite rock pools) and four cattle troughs in the Great Western Woodlands, Western Australia, indicating sampling method (sediment, sweeping, and filtering) and assay (12S-V5 and 16smam). Site indicates which of the gammas (G1–G4) and troughs (T1–T4) the taxa were detected in.

		Gnamma			Trough			Site		
		Sediment	**Swept**	**Filtered**	**Sediment**	**Swept**	**Filtered**	**Sediment**	**Swept**	**Filtered**
Amphibia	*Neobatrachus pelobatoides*	16S	16S	16S				G1/G3	G3	G1/G2/G3
Mammalia	*Macropus fuliginosus*					12S			T4	
	Petrogale lateralis				12S	12S	12S	T1	T1/T4	T1/T4
	Osphranter robustus	12S				12/16S		G2/G4		
									T1/T4	T1/T4
	Oryctolagus cuniculus				16S		16S	T4		T4
	Vespertilionidae sp.	12S	12S		12S	12S	12S	G3/G4	G2/G3	
								T1	T1/T4	T3
	Felis catus					12S			T1/T4	
	Canis lupus familiaris	12/16S	12/16S	12/16S	12/16S	12/16S	12/16S	G1/G2/G3/G4	G1/G2/G4	G1/G3/G4
								T1/T2/T3	T1/T2/T3/T4	T1/T2/T3/T4
	Sus scrofa	16S	12/16S	16S	12/16S	12S		G1	G1/G2/G3	G1/G2/G3/G4
								T1/T3/T4	T1	
	Camelus dromedarius	12/16S	16S	12/16S	16S		16S	G1/G2/G4	G1/G4	G1/G2/G3/G4
								T1/T2		T1/T4
	Bos taurus	12/16S	12/16S	12/16S	12/16S	12/16S	12/16S	G1/G2/G3/G4	G1/G2/G3/G4	G1/G2/G3/G4
								T1/T2/T3/T4	T1/T2/T3/T4	T1/T2/T3/T4
	Ovis aries					12S	12/16S		T4	T4
Aves	*Dromaius novaehollandiae*		12S	12S		12S	12S		G2/G4	G4
									T4	T2/T4
	Falco peregrinus		12S	12S					G2	G2
	Tadorna tadornoides			12S						G1
	Eolophus roseicapilla	12S		12S	12S	12S	12S	G1/G3/G4	G3/G4	
								T1/T2/T4	T1/T2/T3/T4	T1/T2/T3/T4
	Northiella haematogaster			12S		12S	12S			G1
									T1/T2/T4	T1/T2
	Ocyphaps lophotes	12S	12S	12S	12S	12S	12S	G1	G1	G1/G2/G4
								T1/T2/T3	T1/T2	T1/T2/T4
	Phaps chalcoptera	12S	12S	12S	12S	12S	12S	G1	G1/G2/G4	G1/G2/G3
								T1/T2/T3	T1/T2/T3/T4	T1/T2/T3/T4

TABLE 1 (Continued)

	Gnamma			Trough			Site		
	Sediment	Swept	Filtered	Sediment	Swept	Filtered	Sediment	Swept	Filtered
Gymnorhina tibicen		12S	12S		12S			G4	G4
								T2	
Manorina flavigula			12S		12S	12S			G2/G3
								T2	T2
Pardalotus striatus			12S					G2	
Meliphagidae sp.		12S	12S		12S	12S		G3/G4	G1/G2/G3/G4
									T1/T2/T3
Corvus sp.		12/16S	16S		12S	12S		G1/G3	G4
								T2	T2/T4
Grallina cyanoleuca					12S	12S		T2	T2
Cinclosoma clarum	12S					12S	G4		
									T3

sample; 23 taxa total) and filtered samples (4.9 ± 0.39 taxa per sample; 22 taxa total) while significantly fewer taxa were detected from sediment samples (3.1 ± 0.29 taxa per sample; 13 taxa total; $p \leq .016$; Figure 1 and Figure 2). No unique taxa were detected in sediment samples compared to water samples (both swept and filtered), while 13 taxa were only detected in water samples. Thirteen bird taxa were detected overall and nine were detected only in water samples and not in sediment. Similarly, 11 mammal taxa were detected and only three (*Ovis aries*, *Macropus fuliginosus* and *Osphranter robustus*) were detected in water and not in sediment (Figure 2).

3.2 | Source and sampling effects on vertebrate taxon assemblages

There was a significant effect of source ($pseudo\text{-}F = 9.44$; $p < .001$) and sampling method ($pseudo\text{-}F = 3.86$; $p < .001$) on the vertebrate assemblages detected and a significant interaction between these factors ($pseudo\text{-}F = 2.32$; $p = .002$;). All sampling techniques resulted in identification of different species assemblages for gnammas ($p \leq .013$), but for cattle troughs the assemblages detected by swept samples did not differ from those detected by the filtered and sediment samples ($p \geq .050$).

Differences between water sources appear to be driven by higher variation in amphibian and bird assemblages in gnamma samples and higher variation in mammal assemblages in cattle troughs (Table 1, Figure 2). For both sources there was a higher variation in the bird assemblage in both swept and filtered samples compared to sediment samples while the higher variation in the mammal assemblage in swept samples compared to filtered samples appeared to be driving the unique groupings observed in these sampling methods (Figure 2).

For gnammas, where the various treatments yielded statistically different species assemblages, a number of native birds were detected (i.e., *Manorina flavigula*, *Northiella haematogaster*, and *Tadorna tadornoides*) in filtered samples compared to swept samples where *Eolophus roseicapilla* (Australian galah) and a bat (Vespertilionidae sp.) were detected (Table 1). In contrast, *Osphranter robustus* and a few bird species (*Corvus* sp., *Cinclosoma clarum*, *Dromaius novaehollandiae*, *Falco peregrinus*, *Gymnorhina tibicen* and Meliphagidae sp.) were detected from sediment samples (Table 1). Meanwhile, eDNA metabarcoding of cattle trough filtered samples detected a few unique birds (*Corvus* sp., *D. novaehollandiae*, *Grallina cyanoleuca*, *Gymnorhina tibicen*, *Manorina flavigula*, *Northiella haematogaster* and Meliphagidae sp.) and mammals (*Felis catus*, *Ovis aries*, and *Osphranter robustus*) in contrast to sediment samples which yielded other mammals also detected in water samples (*Oryctolagus cuniculus* and *Camelus dromedarius*; Table 1).

3.3 | Vertebrate taxa richness accumulation with replication effort

Rarefaction EcoTests indicate that accumulation curves differed between sampling methods for both gnammas ($z = 20.18$; $p = .040$)

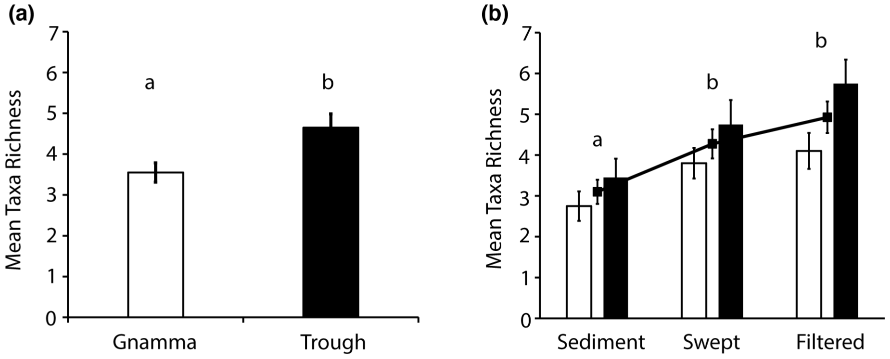

FIGURE 1 Vertebrate taxon richness detected in (a) gnammas (rock holes) and cattle troughs, and (b) using sediment, sweeping, and filtering sampling methods from the two sources in the Great Western Woodland, Western Australia. Letters indicate significant differences ($\alpha = 0.05$) between factors. Values are mean ± SE for $n = 5$ from each of four gnammas and four troughs.

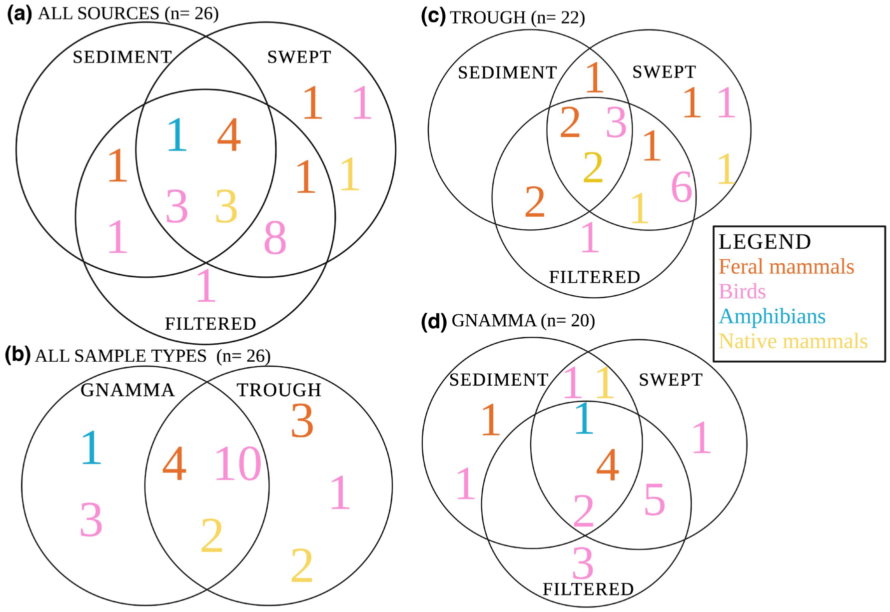

FIGURE 2 Tallies of feral mammals, birds, native mammals, and amphibians detected from sediments, swept water samples, and filtered water samples collected from gnamma holes (granite rock pools) and cattle troughs. Venn diagrams display the relationship of these tallies in (a) filtered ($n = 22$), swept ($n = 23$), and sediment ($n = 13$) samples from gnamma holes and cattle troughs, (b) gnamma holes ($n = 20$) and cattle troughs ($n = 22$) across all sampling methods (c) filtered ($n = 18$), swept ($n = 19$), and sediment ($n = 10$) samples collected from cattle troughs, (d) filtered ($n = 15$), swept ($n = 15$), and sediment samples ($n = 11$) collected from gnamma holes. Four gnammas and four troughs were sampled.

and cattle troughs ($z = 30.2$; $p = .045$; Figure 3). However, when sediment was omitted from the analysis there was no difference between curves representing filter and sweeping sampling methods for either source ($z = 2.82$; $p = .800$). For both gnammas and cattle troughs, mean vertebrate taxon richness per sample was greatest for filtered samples, followed by swept and sediment samples. None of the curves approached an asymptote by the fifth replicate but the gradients in curves from cattle trough samples were lower at the fifth replicate than were those from gnamma samples (Figure 3).

4 | DISCUSSION

We detected a variety of mammals, birds, and amphibians from both artificial and natural semi-permanent freshwater sources in the semi-arid Great Western Woodlands, Western Australia. Our results indicate that sediment samples, filtered water samples and membranes swept through the water are all appropriate sampling approaches for detecting vertebrates in waterbodies in a semi-arid environment using eDNA metabarcoding. However, the overall community structure detected varied both with the type of water source and the sampling technique. We observed that five replicates per water source was insufficient to detect all species interacting with these water sources. These findings are consistent with previous studies that have assessed the impact of sample collection on eDNA-based terrestrial biodiversity assessment (e.g., Newton et al., 2022; van der Heyde et al., 2020) and demonstrate that this approach is most effective when replicate samples are collected from multiple water sources using a variety of sampling methods and substrates.

We detected only a relatively small proportion (10.2% of mammals, 6.5% of birds, and 7.15% of amphibians) of the vertebrate diversity recorded from the Great Western Woodland region

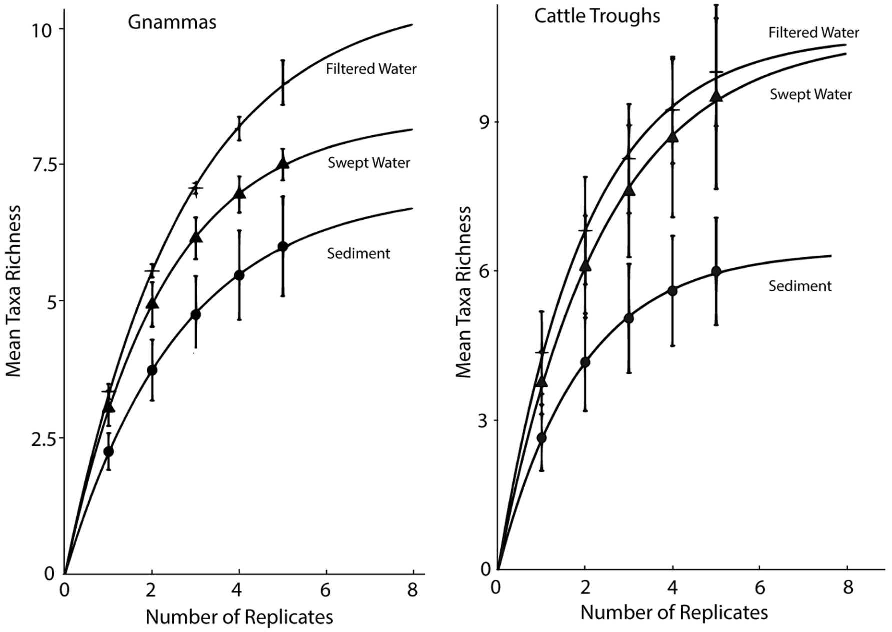

FIGURE 3 Rarefaction curves of vertebrate taxa detected with environmental DNA as a function of sampling effort in four gnammas and four cattle troughs in the Great Western Woodlands, Western Australia from samples collected from sediments, swept water samples, and filtered water samples.

(Department of Parks and Wildlife, 2013) but we did detect numerous threatened and elusive taxa (e.g., *Cinclosoma clarum*, *Petrogale lateralis*, *Northiella haematogaster*), as well as feral species (36.4% of total feral diversity in GWW) that threaten native biota. These results indicate that eDNA metabarcoding from water sources can contribute to biomonitoring in semi-arid lands, particularly if sample collection involves numerous replicate samples, multiple sampling sources and techniques, and occurs over large spatial scales.

Overall, we detected more vertebrate taxa in samples from artificial water bodies than from natural water bodies (Figure 1 and Figure 2), despite them being found in similar habitats. Differences in accessibility of gnammas and cattle troughs for fauna may account for some of the variation in vertebrate richness and assemblage or that animals may preferentially use certain water bodies. For example, gnammas may present an injury risk with an uneven, slippery surface around the perimeter (Bayly, 1999) and the relative lack of plant cover on outcrops may result in greater vulnerability to predation while drinking for some species (Crosmary et al., 2012; Votto et al., 2022). Terrestrial mammals may therefore prefer to drink from above-ground cattle troughs where the water surface is higher, reducing the risk of falling, and facilitating vigilance. This may be reflected by the greater diversity of mammals we detected from troughs. In contrast, we detected a greater diversity of birds from gnammas than from troughs (Figure 2). It is possible that diurnal birds may avoid cattle troughs due to the prevalence of large mammals, including predators (Fontaine et al., 2006) and humans (Meager et al., 2012) at these artificial water sources, especially since access is less of an issue for flighted birds.

Differences in ambient conditions in artificial and natural water sources related to shape and volume (e.g., diameter, depth, surface area, and edge complexity) may also influence persistence of vertebrate eDNA. For instance, ultraviolet exposure can increase eDNA degradation (Green et al., 2011 but see Bae & Wuertz, 2009) and high temperature denatures DNA molecules both directly and by enhancing microbial activity and enzyme kinetics (Fu et al., 2012; Okabe & Shimazu, 2007). Ledges, overhangs, and a smaller surface to volume ratio may provide protection from the impacts of temperature and ultraviolet for eDNA in gnammas compared to troughs, improving detection of small species which may deposit less eDNA, for example, birds compared to mammals. Our observations are consistent with previous studies that compare detection of terrestrial vertebrate taxa between multiple eDNA sources (Newton et al., 2022; van der Heyde et al., 2020) and support the conclusion that, when aiming to characterize arid or semi-arid vertebrate biodiversity using eDNA metabarcoding, samples should be collected from both natural and artificial water sources to maximize the taxa detected. It is worth noting also that while eDNA is often proposed as a replacement of conventional methods, concomitant use of technology such as camera traps or conventional survey methods will value-add to eDNA data (Newton et al., 2023; Ryan et al., 2022), particularly with regard to behavior of taxa near water holes, for example, just drinking or also bathing in the source (Harper, Handley, et al., 2019; Mas-Carrió et al., 2021).

Sampling protocol can have a major impact on the biological community detected with eDNA metabarcoding (e.g., Stoeckle et al., 2017). Our results for vertebrate assemblages in a semi-arid habitat support this finding. Sediment samples yielded significantly lower vertebrate taxonomic richness (50% of total richness) than did swept (88% of total richness) and filtered (84% of total richness) water samples (Figure 1); no unique taxa were detected in sediment samples while 13 taxa were only detected in water (swept and filtered) samples (Table 1 and Figure 2). Our findings are consistent with a previous study comparing eDNA detection in water and sediments for a single species, the great crested newt (*Triturus cristatus*; Buxton et al., 2018), but contradict some previous studies comparing eDNA detection in water and sediments (including those from arid lands; Palacios et al., 2020), which found that sampling sediments yielded higher biodiversity than water samples (Palacios et al., 2020; Turner et al., 2015). Environmental DNA can degrade rapidly in water, and the role of sediments in slowing eDNA degradation is well documented (Barnes et al., 2014; Hou et al., 2014; Romanowski et al., 1991). However, a variety of abiotic and biotic factors determine the rate of eDNA degradation and eDNA can be detectable in water for periods of only days to as long as weeks (Barnes et al., 2014) depending on environmental conditions. Our data suggest that either the rates of eDNA degradation in the water at our two sites were low, or, that the species we detected visited the water frequently enough to contribute detectable levels of eDNA still suspended in the water when sampling occurred.

To our knowledge, this is the first successful trial of swept eDNA collection from terrestrial water sources. Filtration through a membrane using a pump is the most common aquatic eDNA sampling method, typically processing large volumes of water rapidly. This approach is considered essential for accurately detecting the breadth of taxa present (Shu et al., 2020) but can be time consuming if the water contains particulates and/or organics. Our findings indicate that comparable taxonomic richness can be detected from filtered samples and swept samples from small semi-arid habitat water sources (Figure 2). Similar findings were reported for fish detection in marine systems, where sample membranes were submerged for 4–24 h (Bessey et al., 2021). Interestingly, we only submerged the membranes for 15 s but still detected considerable faunal diversity. High concentrations of eDNA in isolated water bodies (Buxton et al., 2017) could account for the high vertebrate richness detected by eDNA metabarcoding of water samples collected by sweeping. To better understand the efficacy of sweeping we recommend future studies compare sweeping to filter volumes of 50 mL, 500 mL, and 1000 mL to address whether our results demonstrate effective sampling of different volumes with filtration and sweeping. We recognize that the 50 mL of filtered water we sampled here is considerably less than for most sampling protocols (Hunter et al., 2019 but see Day et al., 2019), but collecting small sample volumes was necessary due to the small volumes present at many of our study sites, and our results confirm that is enough for high eDNA concentration water bodies like the ones in this study. Additionally, the target substrate should also be considered since DNA in open water is more readily recovered using the current methods compared to DNA in sediment bound to clay particles, which requires a targeted approach (Sakata et al., 2020).

Our finding of comparable taxonomic richness between swept and filtered sampling approaches has profound implications for sampling turbid water, sampling in remote regions and for the logistics of collecting and transporting large volumes of samples. Optimal water filtering procedures are among the most important considerations when collecting and filtering turbid water samples. Particulates block water pumps, increasing the time it takes to filter samples and reducing the total number of samples that can be processed (Egeter et al., 2018; Hunter et al., 2019; Klymus et al., 2017; Turner et al., 2014). This issue is eliminated with sweeping. Membranes swept in water can also facilitate effective sample collection in remote regions where transporting pumps and/or large volumes of water samples and sourcing power to run pumps or freeze large volumes of water can be logistically challenging.

The application of eDNA metabarcoding as a biomonitoring tool to assess biological richness in terrestrial environments is becoming more frequent in the literature (e.g., van der Heyde et al., 2022) and commercially (e.g., Gold et al., 2021). However, the level of replication required to ensure that richness is not underestimated remains largely unexplored. Other studies assessing biological richness from arid zone water sources have used 1–12 biological replicates per environmental sample (e.g., Egeter et al., 2018; Furlan et al., 2020; Palacios et al., 2020) with optimal replication varying with species and context (Ficetola et al., 2015). Here we have demonstrated that five biological replicates collected from isolated water sources are insufficient to detect the true vertebrate richness at our study site (Figure 3). Even for the best-performing water samples, rarefaction curves representing the accumulation of vertebrate richness with increasing replication effort continued to increase beyond the fifth replicate, particularly for samples collected from gnammas (Figure 3). Increasing replication reduces false-negative results but may increase the probability of false positives stemming from contamination and inflates workload and analysis costs (Ficetola et al., 2015; Furlan et al., 2020). A need for high sample numbers for eDNA metabarcoding reduces the relative benefits of this approach as a rapid, cost-effective biomonitoring tool compared to more conventional approaches (Ficetola et al., 2015; Furlan et al., 2020).

5 | CONCLUSIONS

Our findings indicate that eDNA-based monitoring of vertebrates in a semi-arid habitat was most effective when samples were collected from both natural and artificial water sources using multiple sampling methods. We demonstrated that the water source significantly influenced determination of taxonomic richness and assemblage and that sediment samples had significantly less utility for determining vertebrate richness and were not associated with the identification of unique taxa compared to water samples either filtered or swept. We recommend that future studies should explore the use of sample storage buffers and eDNA extraction protocols that are tailored to reduce inhibition, especially in sediment samples. In contrast, we detected no difference in vertebrate richness between sweeping and filtering approaches for water samples. This finding has profound implications for the application of eDNA methods to inland regions and water sources, especially in remote areas, as sweep sampling dramatically reduces the time and logistics required to process samples. Finally, our observations demonstrated that increasing replication increased the number of vertebrate taxa detected, but >5 replicate samples are required to accurately sample small inland water bodies. In conclusion, eDNA metabarcoding for biomonitoring holds promise for applications in semi-arid and arid lands but the design and execution of these studies must be carefully considered to overcome the unique limitations of this method in these environments.

AUTHOR CONTRIBUTIONS

Rupert Mcdonald: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Bill Bateman:** Conceptualization (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Christine Cooper:** Conceptualization (equal); formal analysis (equal); investigation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Mieke Van Der Heyde:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Mousavi-Derazmahalleh Mahsa:** Data curation (supporting); formal analysis (supporting). **Brock Hedges:** Investigation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Michelle Guzik:** Investigation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Paul Nevill:** Conceptualization (equal); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (lead); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

Sequencing data and dada2 script are available at the Dryad Digital https://doi.org/10.5061/dryad.wdbrv15rb.

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Appendix C. Supporting publication 3

Publication: Time capsules of biodiversity: Future research directions for groundwater-dependent ecosystems of the Great Artesian Basin.

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Principal Author

Name of Principal Author	Perry G Beasley-Hall
Contribution to the Paper	Led data curatlon, methodological design, data analysis and production of manuscript
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a [redacted] in its inclusion in this thesis. I am the primary author of this paper.
Signature	[redacted] Date n 11 J2.3

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By signing the Statement of Authors[redacted]

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contfibution.

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Contribution to the Paper	Provided project guidance and edited the manuscript

Name of Co-Author	Rachael A King		
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Signature		Date	12/or /2023

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Time capsules of biodiversity: Future research directions for groundwater-dependent ecosystems of the Great Artesian Basin

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The Great Artesian Basin of Australia represents one of the largest and deepest basins of freshwater on Earth. Thousands of springs fed by the Basin are scattered across Australia's arid zone, often representing the sole sources of freshwater for thousands of kilometers. As "islands" in the desert, the springs support endemic fauna and flora that have undergone millions of years of evolution in almost total isolation. Here, we review the current body of knowledge surrounding Great Artesian Basin springs and their significance from ecological, evolutionary, and cultural perspectives using South Australian spring wetlands as a case study. We begin by identifying the status of these springs as critical sources of groundwater, the unique biodiversity they support, and their cultural significance to the Arabana people as Traditional Custodians of the land. We then summarize known threats to the springs and their biota, both exogenous and endogenous, and the potential impacts of such processes. Finally, considering the status of these at-risk habitats as time capsules of biodiversity, we discuss lessons that can be learnt from current conservation and management practices in South Australia. We propose key recommendations for improved biodiversity assessment and monitoring of Great Artesian Basin springs nationwide, including 1) enhanced legal protections for spring biota; 2) increased taxonomic funding and capacity; 3) improved biodiversity monitoring methods, and 4) opportunities for reciprocal knowledge-sharing with Aboriginal peoples when conducting biodiversity research.

KEYWORDS

Great Artesian Basin, groundwater-dependent ecosystems, springs, biodiversity, taxonomy, aquifers, climate change, eDNA

1 Introduction

The Great Artesian Basin (GAB) is an expansive aquifer spanning an area of 1.7 million km^2 in arid and semi-arid regions of Australia. In addition to representing one of the world's largest sources of groundwater the GAB feeds around 6,300 individual springs, approximately 80% of which occur in South Australia (SA) (National Parks South Australia, 2017) with the remainder in Queensland and New South Wales. GAB springs, also called mound springs in certain states (Fensham et al., 2010), are often the only permanent bodies of freshwater for thousands of kilometers in the arid interior of the country. Springs have persisted for upwards of 1 million years at certain sites (Love et al., 1993) and represent immeasurable value from both biodiversity and cultural perspectives (e.g. the *Environment Protection and Biodiversity Conservation Act 1999,* hereafter EPBC Act; *Dodd v State of South Australia* [2012] FCA 519; *Malone v State of Queensland* [2019] FCA 2115), supporting extraordinary numbers of endemic species not found elsewhere on Earth (Murphy et al., 2015a; CAPAD, 2016; Rossini et al., 2018) and serving as the foci of ancient songlines vital to Aboriginal cultural identity (Nursey-Bray et al., 2020).

The GAB springs have been managed using traditional practices by Aboriginal peoples for at least 5,000 years (Florek, 1993). However, substantial modifications have been made to these water sources since European colonization, particularly *via* the sinking of bores, which have lowered aquifer pressure and reduced emergent water from springs. Contemporary threats to the springs include extraction of water for industrial and pastoral practices, grazing and trampling of wetlands by livestock, the presence of invasive (or overabundant native) fauna and flora, disruption of habitat by tourists, and climate change (Brake, 2020). All of these processes have the potential to impact spring environments in the long term. Indeed, changes have already been observed at certain sites: these include declines and extinctions of fauna, decreased water flow rate, and disturbance of spring sediment (Mudd, 2000; Noack, 2003; Guzik et al., 2012). Unfortunately, the exceptionally diverse GAB spring fauna and flora are not adequately protected in the face of these threats under existing federal Australian environmental legislation (Rossini, 2020), and many springs of high biodiversity and cultural value sit outside of protected areas such as conservation parks (Rossini et al., 2018; Habermehl, 2020a). The cultural heritage of GAB springs have also not been appropriately respected or recognised, either by government legislation or industrial stakeholders (Parliament of Australia, 2021).

The GAB springs clearly require our renewed attention and protection. However, the majority of existing conservation efforts to ameliorate threats to GAB springs have primarily focused on improving artesian pressure—and therefore the flow of emergent water—and the fencing of wetlands, to varying degrees of success (Fatchen and Fatchen 1993; Fensham et al., 2019; GABCC 2019). At the heart of these efforts is a national strategic management plan that has facilitated the rehabilitation of over 700 bores through a GAB-wide capping program (Great Artesian Basin Coordinating Committee, 2019). The rehabilitation of bores has led to increased water flow rates at certain springs (Fensham et al., 2019), associated with healthier wetlands (Fensham and Laffineur, 2022). However, approximately a third of bores still flow uncontrollably and other remaining threats, such as surface disturbances caused by livestock, have received comparatively little attention to date (Brake, 2020). As a result, an adaptive management plan was recently proposed to ensure the future survival of GAB springs (Brake et al., 2020). This integrated approach advocates for a range of measures, including a stronger evidence base related to spring environmental characteristics, the ongoing monitoring of spring condition, and improved biodiversity assessments. Nonetheless, major knowledge gaps mean that our capacity to improve and implement biodiversity assessment and monitoring is limited due to poor understandings of GAB spring biodiversity in the first place. A comprehensive biodiversity assessment of GAB springs is critical because species reliant on such vulnerable habitats are at risk of decline themselves. This absence of taxonomic reference information has led to a paucity of biodiversity knowledge for the GAB springs more broadly and is a well-recognized "taxonomic impediment" to robust understandings of similarly underexplored environments (Ficetola et al., 2019). If the biotic inventory of a habitat is poorly known, such an impediment can render any spring bioassessment or monitoring program lacking in ecological sensitivity. Extreme vulnerabilities therefore exist for GAB spring endemic fauna in the face of the above-mentioned habitat degradation, as the vast majority cannot and have not been effectively assessed regarding their extinction risk (Rossini et al., 2018; Rossini, 2020). Further, without formal names and/or identification methods, these fauna also cannot be effectively protected by environmental legislation such as the EPBC Act, exacerbating their vulnerability. Ultimately, one of the most fundamental issues facing scientists, managers, and legislators is that a unified mechanism by which GAB spring biodiversity value can be assessed and monitored does not currently exist.

Several robust reviews on GAB springs as "oases of life" have been conducted from diverse angles in response to concerns surrounding their survival. These have included retrospectives, papers on legal mechanisms of protection, the cultural significance of springs, spring hydrogeology, and the biogeography and biodiversity of ecological communities dependent on springs (Nursey-Bray and Arabana Aboriginal Corporation, 2015a; Nursey-Bray and Arabana Aboriginal Corporation, 2015b; Rossini et al., 2018; Habermehl, 2020a; 2020b; Harris, 2020; Kerezsy, 2020; Moggridge, 2020; Nursey-Bray et al., 2020; Pointon and Rossini, 2020; Rossini, 2020). Clear issues and gaps in knowledge have been identified, primarily in relation to management directives, awareness, and conservation of the system as a whole or endemic species therein. However, most reviews on spring biodiversity have primarily concentrated on Queensland wetlands, in stark contrast to the fact that approximately 80% of GAB springs are found in South Australia (Rossini et al., 2017, 2018, 2020; Pointon and Rossini, 2020). The ecological communities reliant on GAB springs in South Australia are some of the best-characterized in the system from a taxonomic and genetic standpoint. However, even in these springs true measures of diversity of fauna and flora are no doubt vastly underestimated due to the widespread presence of undescribed species that cannot be distinguished from one another morphologically (i.e., cryptic species) (Murphy et al., 2009; 2015b; Guzik et al., 2012; Guzik and Murphy, 2013). Advancements towards better understandings of these biota offer a roadmap ahead for characterizing and conserving the biodiversity of springs in other states. In South Australia, much of the GAB spring fauna consists of invertebrates that are ultra-short-range endemics (i.e., with distributions <100 km^2) (Guzik et al., 2019) and at risk of decline, though the majority remain undescribed and lack formal names. Springs in Queensland are similarly ecologically diverse, but almost no genetic or taxonomic research has been conducted on that fauna to examine the presence of cryptic or undescribed species (Rossini 2020). To our knowledge no such work has been conducted on the New South Wales springs.

In addition to being well-characterized from a biodiversity standpoint, a substantial body of knowledge exists regarding the Aboriginal significance of South Australian GAB springs. The involvement of Aboriginal peoples in natural resource management, including the GAB springs, has been intentionally limited on a systematic level in Australia (Parliament of Australia, 2021; Samuel, 2021). However, this status quo is slowly changing. In the case of the GAB springs, Aboriginal names and stories associated with springs have been collated on a fine geographic scale (Hercus and Sutton, 1985)—of note as many Australian Aboriginal languages are threatened with extinction (McConvell and Thieberger, 2001)—and the potential and realized cultural impacts of spring loss have been documented in collaboration with Traditional Custodians (Nursey-Bray et al., 2020). Frameworks have also been proposed for the co-management of GAB springs that utilize Aboriginal knowledge alongside Western scientific understandings (Nursey-Bray and Arabana Aboriginal Corporation, 2015a, 2015b). As such, South Australian GAB springs provide an opportunity to amplify Aboriginal voices and develop understandings of spring biodiversity in partnership with Aboriginal knowledge.

FIGURE 1
A Great Artesian Basin-fed spring vent (foreground) in the Elizabeth North spring group (Kati Thanda–Lake Eyre supergroup), South Australia with a distinctive distal “tail” (background) formed by the overflow of groundwater. Tails support wetlands that house endemic fauna and flora and act as refuges for migratory species such as waterbirds. Photo credit Rachael King.

Compared to other springs fed by the GAB nationwide, those found in South Australia are some of the best-studied from biodiversity and taxonomic standpoints. This is particularly the case for cryptic species, which to our knowledge have not been characterized in other states and hint at a much more diverse biota than is currently known. With this in mind, we believe South Australian GAB springs represent an underutilized resource for the development of a roadmap ahead to conserve and characterize groundwater-dependent ecosystems GAB-wide. In the current review, we use South Australian GAB (hereafter SA GAB) springs as a case study to propose future directions for GAB spring biota within the state and beyond. We begin by providing an overview of the biodiversity of the SA GAB springs and their cultural significance. We then discuss current threats to SA springs, the potential impacts of such threats, and lessons that can be applied to the GAB as a broader system, particularly with respect to biodiversity and taxonomic research. In doing so, we provide recommendations to facilitate future research on groundwater-dependent ecosystems supported by the GAB by proposing: 1) enhanced legal protections of GAB spring taxa under federal environmental legislation; 2) increased taxonomic funding and capacity to ensure members of these communities are named; 3) mechanisms for effective monitoring of such taxa using novel, less invasive technologies compared to traditional surveys; and finally 4) opportunities for collaboration with Aboriginal peoples to inform spring management and biodiversity research.

FIGURE 2
View of vegetation in the Jersey spring group (Kati Thanda–Lake Eyre supergroup) dominated by bore-drain sedge (*Cyperus laevigatus*). Photo credit Rachael King.

2 SA GAB spring terminology

Great Artesian Basin-fed springs can either be generally categorized as recharge springs, which form due to overspilling of groundwater at recharge points, or discharge springs, which form at geological faults (Fensham and Fairfax, 2003). Springs fed by the GAB in South Australia are predominantly the latter and, being at the extreme westerly margin of the GAB, are reliant on water which has traveled thousands of kilometers through aquifers from the western slopes of the Great Dividing Range in eastern Australia. South Australian GAB springs are generally grouped together using a hierarchical framework established in the 1980s (Ponder, 1986) and standardized relatively recently by the Allocating Water and Maintaining Springs in the Great Artesian Basin (AWMSGAB) project (Lewis et al., 2013). Discrete discharge points of water from the GAB, also called vents, are the smallest unit used when referring to springs from a policy standpoint and are joined together by permanent wetland vegetation to form springs. In some cases, springs are represented by a single vent. Tails are the distal ends of wetlands that flow away from vents (see Figure 1). Springs are clustered together to form spring groups, which themselves form spring complexes. Supergroups represent the broadest geographic classification of GAB springs. Figure 2.

The above hierarchical classification also considers hydrogeological factors: spring groups and complexes cluster spring units together that broadly share water chemistry, geomorphology, or source water from the same fault or structure (Fatchen and Fatchen, 1993). For consistency we here employ the spring hierarchy and naming conventions of the AWMSGAB project. However, other classification systems or clustering definitions have been used historically (Habermehl, 1982; Zeidler and Ponder, 1989; Kinhill Engineers, 1997; Fensham and Fairfax, 2003), and may also differ when referring to discharge points of the GAB in other Australian states (Fensham and Price, 2004; Rossini et al., 2018). Three spring supergroups exist in South Australia: Dalhousie, Lake Eyre, and Lake Frome (Figure 3). We note that since 2012, the salt lake basin Lake Eyre has been officially known by the dual name Kati Thanda–Lake Eyre in acknowledgement of the traditional lands of the Arabana people (detailed below). We will therefore refer to that supergroup as Kati Thanda–Lake Eyre. Per the naming system of Lewis et al. (2013), the three South Australian supergroups contain 22 spring complexes, which themselves encompass 169 individual spring groups. Prior to the above-mentioned standardization, other complexes or groups were infrequently recognized in the literature but have since been subsumed within other sites: for example, the Mount Hopeless complex in the Lake Frome supergroup is now split into the Petermorra, Reedy, and Lake Blanche complexes (Social and Ecological Assessment Pty Ltd, 1986).

3 Biodiversity

The SA GAB springs are a critical source of freshwater in an otherwise arid landscape for a diverse biota including invertebrates, birds, mammals, fishes and a wide range of unique wetland vegetation. Springs also support a large number of species representing short-range endemics, with some restricted to single spring groups (Murphy et al., 2015a; Guzik et al., 2019). A preliminary checklist of species endemic to the GAB springs Australia-wide has shown a large number of putatively undescribed species exist in springs (Rossini et al., 2018), but the South Australian fauna and flora (both endemic and non-endemic) remain to be systematically collated and

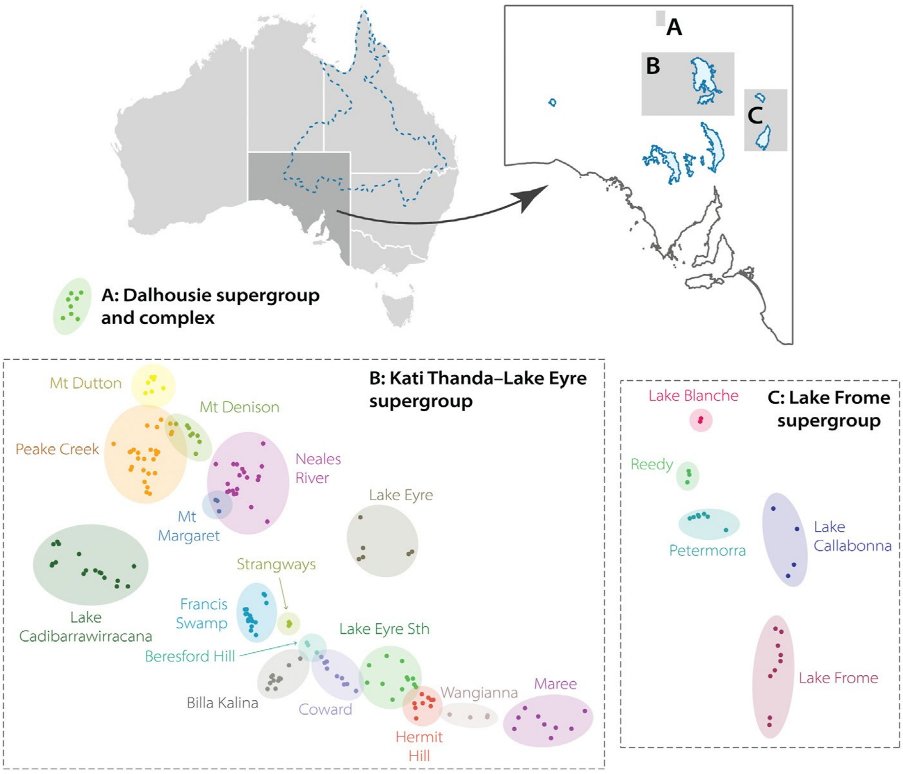

FIGURE 3
Location and naming of the three Great Artesian Basin (GAB) spring supergroups in South Australia following Lewis et al. (2013): (A) Dalhousie, (B) Kati Thanda–Lake Eyre, (C) Lake Frome. The approximate extent of the GAB is indicated top-left. Spring complexes (coloured circles) contain spring groups (points). The Maree complex is also referred to as *Marree* under certain classifications.

standardized. Here, we outline the current body of knowledge regarding SA GAB taxa (summarized in Figure 4) including their conservation and taxonomic status. A more detailed description of taxonomic groups mentioned here are provided in the Supplementary Material.

South Australian GAB-fed springs can be viewed as “museums of biodiversity, preserving lineages that would have otherwise gone extinct” (Murphy et al., 2015a). Research over the last 15 years has demonstrated that fauna endemic to the springs have undergone millions of years of evolution in almost complete isolation, reflected in an extraordinary genetic diversity and degree of local endemism, adaptation, and mutational change (Murphy et al., 2009, 2012, 2013, 2015a; Guzik et al., 2012, 2019; DeBoo et al., 2019). Due to the extent of this endemism, it is also likely that in the event of local extinction, particular sites might represent complete species extinctions in certain cases. There are currently nine described endemic animal taxa of formal conservation concern in the SA GAB. Three fish, the Dalhousie goby (*Chlamydogobius gloveri*), hardyhead (*Craterocephalus dalhousiensis*), and gudgeon (*Mogurnda thermophila*) are critically endangered and restricted to the Dalhousie spring supergroup/complex (Hammer et al., 2019; Unmack et al., 2019; Whiterod et al., 2019). Six snail taxa in the genera *Trochidrobia* and *Fonscochlea*, which may represent many additional species based on molecular data (Ponder et al., 1995; Rossini et al., 2018), are either endangered or vulnerable (Ponder, 1996a, 1996b, 1996c; Mollusc Specialist Group, 1996; Ponder et al., 1996; Clark, 2011). However, no insect or crustacean in the SA GAB, the system’s two most speciose groups containing endemics, has been evaluated on a per-taxon basis to determine if it is at risk of decline, either at an Australian federal legislative or global level (e.g. the EPBC Act or The International Union for Conservation of Nature’s Red List of Threatened Species). It is likely that many other species associated with GAB springs are also at risk of decline in SA but have not been formally investigated in this context.

In addition to the described species presently recognised as endemic to the SA GAB springs, a significant number of undescribed species have been identified from molecular studies (Murphy et al., 2009; 2015b; Guzik et al., 2012; Guzik and Murphy, 2013) However, three major limiting factors impede our ability to quickly and effectively describe species from GAB springs. Firstly, the rigors of formal taxonomic work and a global lack of taxonomic expertise mean the description of

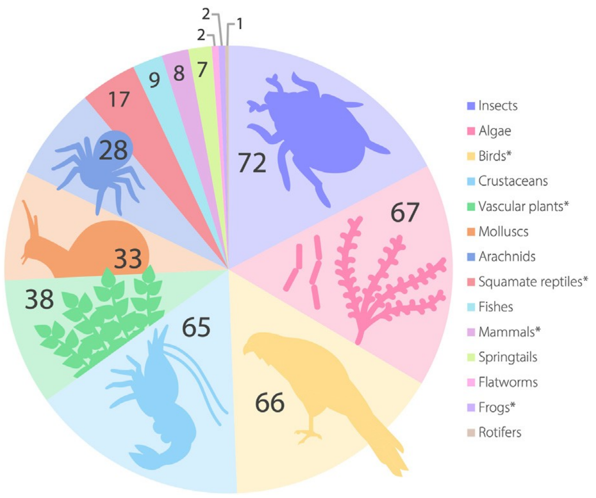

FIGURE 4
Broad taxonomic groups known to occur in the SA GAB springs. Taxon numbers are listed for each group; divergent genetic lineages or morphological forms explicitly stated as putatively undescribed species in the literature are included as separate taxa following Rossini et al. (2018). A full summary of the taxa detailed here is supplied in the electronic supplementary material. Here, algae refer to a paraphyletic grouping including both micro-algae and filamentous algae (see text). *Group is not known to contain representatives endemic to SA GAB springs.

species often occurs decades after their discovery. For example, 32 putative species from SA GAB springs need formal taxonomic descriptions at the time of writing; almost all are divergent lineages of isopods and molluscs discovered using molecular data (Rossini et al., 2018) though this list also includes beetles (DeBoo et al., 2019), sedges (Department of Climate Change, Energy, the Environment and Water, 2022a), and microalgae (Ling et al., 1989). All but two of these taxa are not known to occur outside of SA GAB springs. Secondly, a large number of putatively undescribed endemics are morphologically cryptic and can only be discriminated using robust DNA sequence data rather than morphological characters (Fišer et al., 2018), as demonstrated by the *Wangiannachiltonia guzikae* King, 2009 cryptic species complex (Murphy et al., 2015b). Finally, microbial biodiversity of springs is likely to be high but remains poorly characterized for the SA GAB (Love et al., 1993; Andrews and Patel, 1996; Byers et al., 1998; Kimura et al., 2005; Ogg and Patel, 2009).

Understandings of determinants of GAB spring species distributions and their biogeography are limited. A publicly available database cataloging environmental attributes of GAB springs only exists for such habitats in Queensland (http://data.qld.gov.au/dataset/springs). The lack of a system-wide dataset currently represents a major impediment to our knowledge of these ecosystems. Broad environmental factors (e.g., spring vent morphology, mean annual rainfall) have been explored as determinants of species distributions of GAB springs in the past (Rossini et al., 2018), but no strong relationship was found when examining supergroups across the GAB as a whole. At the smaller scale of spring complexes, distributions of endemic snails are correlated with sections of wetlands they occupy (Rossini et al., 2017), and wetland size is associated with invertebrate taxonomic richness (Negus and Blessing, 2022).

4 Cultural significance

There are two groups of peoples indigenous to Australia: Torres Strait Islanders from the Torres Strait Islands north of Cape York in Queensland and Aboriginal peoples from all other parts of Australia. Collectively, these groups may be referred to as Aboriginal and Torres Strait Islander peoples. The alternate term Indigenous peoples is sometimes discouraged in an Australian context as it is seen as generalizing the two groups (Australian Institute of Aboriginal and Torres Strait Islander Studies, 2020), though it may be used as a broader term to refer to First Nations peoples worldwide (e.g., Bennett, 2022). Traditional Custodians refer to descendants of the group of Aboriginal and Torres Strait Islander peoples that occupied a region prior to European colonization. Country is a mass noun used by Aboriginal and Torres Strait Islander peoples to describe their traditional land and seas and their relationship with them. Dreaming(s) refer to unique stories and beliefs, such as world-creation narratives or personal totemic symbols, held by different Aboriginal and Torres Strait Islander groups. All of the above terms are capitalized by convention. Here, we will use Aboriginal and Torres Strait Islander peoples when referring to First Nations peoples of all of Australia, Aboriginal peoples when referring to First Nations peoples to the exclusion of the Torres Strait, and Indigenous when referring to First Nations peoples in a global context. Where possible, in this review we will use Aboriginal names of springs, as contributed by Arabana, Kuyani, Southern Aranda, Wangkangurru, and Yarluyandi people throughout South Australia (Hercus and Sutton, 1985), alongside English names.

The Arabana people are the Traditional Custodians of much of the area containing the SA GAB springs and, alongside the South Australian Government Department for Environment and Water, co-manage almost 69,000 km^2 of land spanning from Maree in the southeast, Oodnadatta in the west, Kati Thanda–Lake Eyre (including the Wabma Kadarbu Mound Springs Conservation Park) and the boundary of the Macumba River in the north (Nursey-Bray and Arabana Aboriginal Corporation, 2015b). A Native Title Determination Application was filed by the Arabana people in 1998 and they were officially recognised as Traditional Custodians of their Country at Finniss Springs in 2012. Here, we will focus on the Arabana people as the Traditional Custodians of the bulk of the SA GAB springs but acknowledge the Dieri people are native title holders of Country encompassing a portion of the Maree spring complex (National Native Title Tribunal, 2022) and that the

SA GAB springs are also an important water resource to the Antakarinja, Dhirari, Karangura, Kokatha, Kuyani, Lower Southern Arrernte, Ngamini, Pirlatapa, Southern Aranda, Thirrari, Wangkangurru, and Yandruwandha peoples (Hercus and Sutton, 1985; Brake et al., 2020). Certain Aboriginal groups not holding native title should not be interpreted as that group lacking strong cultural connections to GAB springs, nor the Country encompassing them.

The Arabana people have deep and ongoing cultural connections to the SA GAB springs. Springs are the only permanent source of freshwater for thousands of kilometers in the arid South Australian interior and the Arabana have relied on them for water, food, and other resources, such as reeds to weave baskets and nets, for generations (National Parks South Australia, 2017). The springs, which Arabana people describe as "like a living pulse that provides life for everything", are also a central theme in Dreaming stories that have been exchanged for thousands of years to facilitate trade and extend cultural ties (National Parks South Australia, 2017). Individual spring groups usually have their own names and unique Dreaming stories (Hercus and Sutton, 1985; Nursey-Bray and Arabana Aboriginal Corporation, 2015b). Springs are visited and used for cultural activities such as knowledge sharing, which includes the expression of collective memory and the transmission of culturally sensitive or privileged knowledge (Nursey-Bray and Arabana Aboriginal Corporation, 2015b). Western archaeological evidence suggests widespread use of the SA GAB springs as places central to social and ancestral identity by Arabana people began upwards of 5,000 years before present. Prior to European colonization, the Arabana people managed threats to the SA GAB springs with burning and excavation practices to ensure access to water (Davies, 2005; Lewis and Packer, 2020). This management continued until the mid-19th century, when many springs were appropriated by pastoralists and for the laying of railway lines (Hughes and Lampert, 1985; Harris, 1992; Florek, 1993). Today, this degradation has continued through the removal of water from aquifers for mining and pastoral purposes by the sinking of bores, decreasing the pressure of emergent water from vents and therefore the volume of water in springs (known as drawdown). Drawdown is a key concern for the Arabana with respect to the conservation of SA GAB springs (Nursey-Bray et al., 2020) and one of the major threats to the system we identify in this review.

5 Threats to SA GAB springs

5.1 Water use by industry

Contemporary use of SA GAB springs is not as intensive as in the past with respect to the volume of extracted water, but it is still a major threat to springs due to pastoral, agricultural, and mining practices. Intensive extraction of water *via* bores by pastoralists for livestock and irrigation represents a significant source of drawdown for the SA GAB springs. At present, the volume of stock-accessible water across the GAB is not formally metered and generally estimated based on regional characteristics such as stocking rates. In South Australia, approximately 10 megaliters (ML) of water per annum is allocated to stock access (Frontier Economics, 2016), but due to a lack of regulation the real volume may be much higher than estimated and/or reported. Notably, a significant proportion of water extracted for pastoralism has historically been wasted: in the mid-1990s, up to 80% or more was left to evaporate, in part due to the uncontrolled flow of high-yield bores (Hillier, 1996); many on pastoral land have now been capped, substantially curtailing water wastage. Water is also extracted from the GAB for agriculture and irrigation. In South Australia, approximately 115 ML of water is thought to be extracted from the GAB for irrigation, which far exceeds the license volumes of up to ~76 ML/year in other states (Frontier Economics, 2016).

The largest known user of water extracted from the GAB in South Australia is the mining industry, representing approximately 24,200 ML a year (Frontier Economics, 2016). Compared to pastoral impacts, mining-associated water extraction in the southern GAB, whilst substantial, is currently strictly licensed with allocations to individual mining companies. However, these allocations do not necessarily account for possible future expansions of mines and still have the potential to substantially reduce spring flow and pressure in local aquifers, especially around extraction wellfields. For example, the Olympic Dam mine managed by BHP is the largest mine in the state and, accordingly, the largest single user of SA GAB water (Frontier Economics, 2016). In springs outside of the company's two primary borefields, BHP predicted spring flow declines of up to 20% due to drawdown and observed actual declines of up to 10% between 2016 and 2018 (BHP, 2018). While increases in flow rate in those same springs have occurred since (BHP, 2021), it is also important to note that pastoral bores tend to be spread diffusely across the landscape and are of low water yield.

A relatively new industry to South Australia is the extraction of coal seam gas (CSG) (Cosens, 2015). CSG is currently much more prevalent in other Australian states and territories and uses large amounts of groundwater for the life of such projects; as of 2016 in Queensland, for instance, annual estimated GAB water use for CSG was almost double that of irrigation and agriculture (de Rijke et al., 2016; Frontier Economics, 2016). Use of GAB water in South Australia by such operations may increase in coming years to facilitate the extraction of natural gas from the underlying geological Cooper Basin (Menpes and Hill, 2012). Whilst there is an extraction component to CSG processes which may impact flow rates of SA GAB springs, an additional issue is the reinjection of water into GAB aquifers *via* surface bores

(Moran and Vink, 2010). Re-injection aims to replace water extracted for CSG, thereby mitigating aquifer drawdown and avoiding decreases in artesian pressure. However, it is unknown whether re-injected water would reach discharge points at spring vents and if so, how this might impact GAB spring faunal communities. More robust field data related to the hydrological processes of GAB springs, as well as long-term monitoring programs of fauna and flora within these habitats, is needed to better understand potential cumulative impacts on groundwater resources by CSG developments (Williams et al., 2012). The extraction of CSG has been identified as an "emerging challenge" for GAB preservation nationwide (Great Artesian Basin Coordinating Committee, 2019).

5.2 Invasive or overabundant species

Overabundant native and introduced species form a significant threat to the SA GAB springs. This is chiefly due to the fact that most springs fall outside of areas that are protected from stock and pest animals, e.g., national/conservation parks (in which grazing is prevented), fenced areas on pastoral lease land, or areas that have been de-stocked (Rossini et al., 2018; Harris, 2020; Lewis and Harris, 2020). Stock and large-bodied feral animals including cattle, donkeys, goats, pigs, and camels can graze on or trample tail vegetation and disturb spring substrate, causing clogging of spring vents and impacting natural spring flows. Large vertebrates are also known to impact water quality in arid zone freshwater bodies more directly through the input of dung, which can lead to water fouling and negatively impact macroinvertebrate communities (Brim-Box et al., 2010; McBurnie et al., 2015). The above impacts may have already led to local extinctions of spring fauna and flora such as fish (Kodric-Brown et al., 2007), spiders (Kovac and Mackay, 2009), and salt pipewort (Fatchen and Fatchen, 1993; Davies, 2005) at a number of sites. To reduce the negative effects of trampling on wetland communities, it is possible to fence springs such that stock and other herbivores are excluded (Dobkin et al., 1998; Yates et al., 2000). Depending on the method of fencing used, species diversity in GAB spring wetlands has substantial capacity for recovery following the removal of disturbance pressures from invasives (Lewis, 2001; Gotch et al., 2016; Peck, 2020).

In addition to terrestrial vertebrates, the invasive mosquitofish *Gambusia holbrooki* is also likely to pose a significant risk to the fauna of the SA GAB springs. The impact of mosquitofish incursion has not been robustly assessed in South Australia, though it is currently known in four spring complexes within the Kati Thanda–Lake Eyre supergroup (Gotch et al., 2016; Rossini et al., 2018). Mosquitofish has also been implicated in the demise of Australia's most endangered freshwater fish, the red-finned blue-eye, from Queensland GAB springs (Kerezsy, 2015). In that state, the red-finned blue-eye has been translocated to springs without mosquitofish to avoid further extirpations and fencing has been employed to prevent subsequent overland dispersals of mosquitofish after rain (Kerezsy, 2015). Mosquitofish are known to prey on several of the invertebrate groups represented as endemics in the SA GAB springs—for example, hydrobiid snails and crustaceans—and their aggressive behavior towards other fish may negatively impact endemics like the desert goby (*Chlamydogobius eremius*) in much the same way Queensland species have been affected (Gotch et al., 2016).

Although fencing protects springs from damage caused by stock and other invasives, it is not without its drawbacks. For example, fences can cause more damage to sites than if a wetland was not fenced at all if invasives are unable to escape those areas and become trapped (Negus et al., 2019). The long-term effects of stock removal on wetland vegetation communities within arid regions *via* fencing are also poorly understood (Lewis and Packer, 2020) and a reduction in grazing pressure can lead to undesirable expansion of plant species such as the native *Phragmites* reed and the invasive date palm (Fatchen, 2001). These species can cause significant change to spring habitats by outcompeting other flora and limiting available habitat for aquatic species. In extreme cases, *Phragmites* can effectively suck springs dry by greatly increasing evapotranspiration and/or forming mats of rotting stems that soak up water and risk blocking vents (Fatchen, 2000; Harris and Lewis, 2006; Munro et al., 2009; Witjira National Park Co-management Board et al., 2018). *Phragmites* overgrowth can also push certain GAB spring fauna to the margin of wetlands, exposing them to increased grazing pressures (Gotch et al., 2016). Whether species such as *Phragmites* have always played such a large role in SA GAB spring ecosystems is unclear. However, oral histories and archaeological evidence suggest Aboriginal peoples controlled *Phragmites* growth with traditional burning regimes (Hercus and Sutton, 1985; Boyd, 1990; Boyd, 1994; Davies, 2005). Following European colonization this shift, concurrent with the introduction of invasive grazers and a reduction in water flow due to drawdown, may have exacerbated the impacts of overabundant plant species (Lewis and Packer, 2020). Local managers consider a mixed management scheme of temporary stock grazing valuable in maintaining spring flow and reducing *Phragmites* populations (Lewis and Packer, 2020).

5.3 Climate change

Under all likely emissions scenarios, the Earth is expected to experience a 3°C increase in mean global surface temperatures by 2,100 compared to pre-industrial levels (Allan et al., 2021; Australian Academy of Science, 2021). Arid Australia is likely to experience more frequent heatwaves, often of longer duration compared to historical trends (Australian Academy of Science, 2021). Annual rainfall will decline while rare, major (1-in-20-year) rainfall events will become more intense, potentially leading to flooding (Australian Academy of Science, 2021). Southern arid zone rainfall patterns are also likely to shift from being predominantly dominated by low-intensity winter events, to higher-intensity events during the summer (Alexander and Arblaster, 2009; Malerba et al., 2022). These changes have high potential to negatively impact the biota of the SA GAB springs. Indeed, some of the most widespread extinctions of local plant and animal populations have been observed in freshwater habitats as a result of extreme climate events (Pörtner et al., 2022).

The climate of arid Australia is generally characterized by long periods of dry conditions lasting months or years, interspersed by unpredictable rainfall events caused by tropical monsoon systems from the north and north-west of the continent (Morton, 2022). In the SA arid zone, rainfall varies greatly and ranges from 125 to 250 mm a year (Ponder, 1995). Unfortunately, research on the impacts of climate change on the Australian arid zone and its freshwater systems is lacking. Based on early predictions, the southern portion of the GAB may experience substantial drying while the northern basin will be subject to higher intensity rainfall and recharge (Cosens, 2015). As the GAB is recharged by rainfall from a wetter climatic zone in the east of the continent, the volume and pressure of emergent water in the SA springs is therefore not necessarily dependent on local rainfall at short timescales (Davis et al., 2013). However, on a long enough time scale decreasing annual rainfall in recharge areas of the GAB could potentially lead to groundwater extraction levels exceeding replenishment, lowering groundwater levels and leading to a reduction in flow rate of SA GAB springs (Welsh et al., 2012). The level of emergent groundwater being decoupled from local rainfall in the short term also does not mean that SA GAB spring biota will be protected from other impacts of climate change, such as flooding or rising surface temperatures.

The response of GAB spring endemic fauna to flooding is complex. Historically, major flooding events of SA GAB springs have occurred approximately once per decade (Ponder, 1986) and resulted in greater temporary connectivity between springs. However, these events can also lead to the extinction of populations (particularly invertebrates) due to springs essentially being flushed clean. Floods have been proposed as a mechanism that encourages gene flow between otherwise isolated GAB spring populations, but evidence for this is mixed (Worthington Wilmer and Wilcox, 2007; Wilmer et al., 2011). Dramatic population crashes have been observed in SA GAB invertebrates post-flood, with populations requiring years to recover (Wilmer et al., 2011). Even so, genetic data from spring invertebrates indicates a resilience to disturbance given the extent of old and new genetic diversity in major phylogeographic groups (Guzik et al., 2012; Murphy et al., 2015a), suggesting a climatic buffering of species over evolutionary time. Fishes fare better in such precarious habitats during flooding events compared to smaller animals like invertebrates (Larson, 1995) and it has been proposed they move between GAB springs during these periods (Kodric-Brown and Brown, 1993) albeit with subsequent population declines (Fairfax et al., 2007). Flooding is responsible for well-documented "boom and bust" cycles for vertebrates in Australia's arid zone (Kingsford et al., 1999; Roshier et al., 2002), and as such is likely to also impact species with strongholds outside of these regions that use SA GAB wetlands to breed, such as waterbirds (Badman, 1985).

Higher local temperatures in Australia's arid lands have the potential to increase spring evaporative rates and water temperatures to the detriment of spring-dependent ecosystems. Increases in evapotranspiration stemming from higher ground temperatures, for instance, may negatively impact springs (particularly those with low flows) and the taxa they support by increasing salinity or drying wetlands entirely (Ponder, 1986; Gotch et al., 2016). The impact of changing temperatures on spring endemics and their life cycles is poorly known and will depend on baseline temperatures in specific spring groups. For most GAB springs, water temperatures are generally stable close to spring vents (Ponder, 1986). Away from the vent, spring water temperatures approach ambient temperatures and can vary within a 24-h period and across seasons and geographic locations (Smith, 1989; Keppel et al., 2011, 2012; Rossini et al., 2017). In South Australia's warmest springs, at the Dalhousie supergroup/complex, temperatures can exceed 40°C due to warmer groundwater from the Pedirka Basin partially contributing to spring flows (Fensham et al., 2010; Wolaver et al., 2013; Gotch et al., 2016). Hence, a more immediate threat to the high temperature-dependent fauna at Dalhousie is in fact a *reduction* in water temperature posed by drawdown from the Pedirka Basin through coal or mining developments (Gotch et al., 2016). Taxa from the comparatively milder Lake Eyre and Lake Frome supergroups (≤30°C) may be more negatively affected by the warming of spring water *via* increased ambient temperatures (Ponder, 1986; Keppel et al., 2012).

5.4 Tourist and visitor activities

Inappropriate visitor activity has the potential to negatively impact the GAB springs, especially at sites endorsed as tourist destinations. Three key protected areas overlap with springs in South Australia: Witjira National Park (the entire Dalhousie supergroup/complex), Wabma Kadarbu Mound Springs Conservation Park (a subset of the Coward complex, Kati Thanda–Lake Eyre supergroup), and Kati Thanda–Lake Eyre National Park (a subset of the Lake Eyre and Lake Eyre South complexes, Kati Thanda–Lake Eyre supergroup). Of these three groups, only springs in the Kati Thanda–Lake Eyre National Park are not tourist destinations.

Swimming is permitted at the Main Pool spring of Dalhousie in the Witjira National Park, which has led to the erosion of substrate at spring banks (Noack, 2003) and caused concern for Aboriginal peoples and conservationists (Ah Chee, 2002; Harris, 2020). Long-term impacts of these practices on GAB spring chemistry or the disturbance of spring sediment remains unknown. Tourist activities are supported by a campground at Dalhousie, which is thought to have released potentially contaminated runoff into the springs and impacted resident populations of spike-rush plants (Noack, 2003). The National Park's management plan does not acknowledge these threats beyond noting that "swimming is [generally] not compatible with maintaining healthy mound springs" and cites the Main Pool as an exception due to its size and flow (Witjira National Park Co-management Board, 2022).

Further south in the Wabma Kadarbu Mound Springs Conservation Park, the above activities are forbidden, and the Park is restricted to day-visitors. Nonetheless, springs such as Pirdali-nha (The Bubbler) and Thirrka/Thirka (Blanche Cup) have boardwalks installed to enable tourists to view active springs at a distance. Visitors have the capacity to come into contact with spring water, compact soil adjacent to springs as a result of foot traffic and degrade ecologically significant wetland tails. These risks are explicitly listed as a threat to the Conservation Park's protection in its management plan (National Parks South Australia, 2017) and World Heritage assessment (Morton et al., 1995). Overall, such behavior from visitors—either deliberate or accidental—has the potential not only to degrade springs from an ecological standpoint, but compromise sites of immense cultural significance.

6 Discussion

Here, we have used the South Australian GAB springs as a case study to illustrate the immense biodiversity and cultural value of artesian springs in arid and semi-arid Australia. The threatening processes detailed here pose similar risks to the remainder of GAB springs across Queensland and New South Wales. The well-studied nature of the South Australian GAB wetlands provides valuable lessons applicable to GAB springs nationwide and may inform their future study and custodianship. What might become of these habitats if the above threats eventuate? In light of the current state of SA GAB spring management, below we discuss potential, and in some cases already realized, impacts of these threatening processes and propose recommendations to aid the ongoing monitoring and conservation of ecological communities within GAB springs.

6.1 Impact of threats on GAB springs

6.1.1 Risk of extinction of endemic taxa

Arguably the most pressing environmental and conservation impact of the threats above is the risk of extinction of GAB spring endemic species. Most at risk are the endemic invertebrates, which have some of the smallest distributions of GAB spring taxa yet are not well-monitored or managed, and for which a systematic data deficiency exists (Rossini, 2020). This deficiency is of particular concern due to the slow pace of invertebrate species discovery, the overlooking of invertebrates in conservation legislation, and the fact that preliminary data suggest invertebrates are proxies for GAB spring health (Lydeard et al., 2004; Cardoso et al., 2011; Eisenhauer et al., 2019). All of these factors have the strong potential to lead to invertebrate species going extinct before they can be described and adequately conserved. Indeed, in the SA GAB springs a localized extinction, if not a probable species extinction, has already occurred concerning the isopod *Phreatomerus latipes* from the Hergott spring bore in Maree (Figure 5). Recent collections by the authors failed to find *P. latipes* at its type locality and revealed the original bore it was described from had been capped. A subsequent molecular analysis of almost 500 specimens from additional locations indicated the existence of up to 10 evolutionarily distinct lineages representing putative species, all isolated within separate spring groups (Guzik et al., 2012). Thus, the population from which type specimens of this species were collected may represent a separate species that has since become extinct. Given the geographic isolation and exceptional levels of phylogeographic structure observed in *P. latipes* (Guzik et al., 2012), it is unlikely that the "original" species exists elsewhere and has little chance of recolonisation in its type locality, irrespective of reinvigorated water flow.

FIGURE 5
The isopod *Phreatomerus latipes*, a species endemic to the SA GAB springs that might represent a localized, if not species-wide, extinction as a result of the degradation of springs *via* threats detailed in this review. Photo credit Rachael King.

Although invertebrates are perhaps at the highest risk of extinction in GAB wetlands, processes which threaten these ecosystems do not occur in a vacuum and act at the whole-community level. Some degree of loss of local populations might be recoverable over time *via* migration and re-colonisation (Fairfax et al., 2007; Kodric-Brown et al., 2007), but new populations of colonizers, such as fish (Fatchen and Fatchen, 1993), have not been observed to survive long-term and instead losses are likely to represent permanent extinctions of evolutionarily distinct species with little to no chance of recovery to their original state. The probability of local extinctions is inversely proportional to spring size, and can also be driven by major changes in habitat (e.g., large increases in the abundance or area of *Phragmites*), decreases in the area and volume of open water, and increases in evaporation, all factors influenced by the threats we have discussed above (Kodric-Brown and Brown, 1993; Kodric-Brown et al., 2007).

6.1.2 Cultural ramifications of spring water loss

The possibility of spring extinction or loss has the potential to have deeply negative impacts on Traditional Custodians. For the Arabana people in South Australia, water is embedded in identity. Water is not distinguished as belonging to different ecosystems, per Western scientific classifications, but instead understood by the Arabana as a single, unified resource (Nursey-Bray and Arabana Aboriginal Corporation, 2015a); any loss of GAB water may therefore be culturally catastrophic. The Arabana have expressed a great loss in response to spring flow rate decreasing in SA GAB springs, with particular concern about current mining practices. A respondent from Maree described this decrease as “terrible, really sad to go out there now in terms of water. It is not only The Bubbler [...] but [the] other one at Finniss there - it is very dry but used to run.” (Nursey-Bray et al., 2020). Further loss of SA GAB springs would lead to an erosion of cultural identity for the Arabana, the destruction of key foci of dreaming stories, and ultimately a potentially permanent disconnection from traditional lands: “People can not live in or go back to *dead* Country.” (Nursey-Bray and Arabana Aboriginal Corporation, 2015b). The negative impacts of this loss will not be restricted to native title holders such as the Arabana and Dieri, but affect all Traditional Custodians with connections to Country encompassing GAB springs. While less published information is available regarding the Aboriginal cultural significance of GAB springs in Queensland and New South Wales, Western archaeological evidence suggests Aboriginal peoples in those states have, like the Arabana, relied upon these water sources for thousands of years (Robins, 1995; Noble et al., 1998; Powell and Powell, 2012; Powell et al., 2015).

6.2 A roadmap ahead for GAB spring-dependent ecosystems

6.2.1 Enhanced legal protections for GAB spring biota

Since 2001, the biological community of native species dependent on GAB springs has been recognised as endangered under the EPBC Act. While we note the Act does not concern all GAB springs Australia-wide (Habermehl, 2020a), a corresponding recovery plan has been published with the aim of enhancing groundwater supplies to spring wetlands, maintaining or increasing habitat area and health, and increasing populations of endemic organisms (Fensham et al., 2010). Nonetheless, the listing of these communities as a whole (as opposed to on a per-species basis) has been criticized as offering insufficient protection of endemic species, particularly the invertebrates (Rossini, 2020). Listing of species enables a concentrated direction of conservation and policy efforts, without which they may go extinct (Stuart et al., 2010). We agree with the assessment of Rossini (2020) that these species deserve individual EPBC assessments, and argue this issue is symptomatic of a broader trend that sees invertebrates overlooked in conservation legislation and threatened species lists.

Species formally considered at risk of extinction, such as *via* the List of Threatened Fauna in Australia’s EPBC Act and the IUCN Red List, are overwhelmingly vertebrates (Eisenhauer et al., 2019; Department of Climate Change, Energy, the Environment and Water, 2022a). In contrast, many invertebrate groups contain a large amount of undescribed genetic and taxonomic diversity but are not often listed as species of conservation concern. In part, this is likely due to a general lack of data, funding, knowledge, and support for experienced invertebrate taxonomists capable of intergenerational knowledge transfer (Cardoso et al., 2011). In Australia, legal structural issues have reinforced this bias. The EPBC Act replicates the IUCN’s criteria to assess species as being at risk with two exceptions: 1) the Act does not allow species to be listed as Data Deficient, ignoring that for invertebrates this tends to be the norm, and 2) species cannot be listed as vulnerable on the basis of short range endemism alone, despite the fact that short range endemics are more likely than not to be invertebrates (Harvey, 2002; Braby, 2018; Taylor et al., 2018). Progress towards equitable listings of GAB spring invertebrate species should begin with the assessment of taxa on a case-by-case basis where possible, but will also require a review of the way Australian conservation legislation is currently designed (Pointon and Rossini, 2020). A better understanding of species diversity and distributions will also significantly contribute to this undertaking, detailed below.

6.2.2 Taxonomic funding and capacity

Around 70% of Australian and New Zealand species are thought to yet be discovered, formally described, or documented (Taxonomy Decadal Plan Working Group, 2018). Without formal acknowledgement and description of species *via* this taxonomic process, the undescribed majority is essentially invisible—i.e., not included or acknowledged by stakeholders outside the research sphere, especially under existing legislation—and this has serious implications for their conservation. Tackling such an impediment is imperative if we are to gain a greater understanding of current environmental crises and apply sound scientific evidence to industry, conservation, and management practices moving forward. For every dollar invested, a thirty-five-fold return has been estimated if all remaining Australian species were discovered, potentially representing tens of billions of dollars in economic benefits (Deloitte Access Economics, 2020). Such an undertaking, as proposed by the Taxonomy Australia initiative (taxonomyaustralia.org.au), could bring value to sectors including biosecurity, drug development, industry, and society more broadly by stimulating connections to nature (Taxonomy Decadal Plan Working Group, 2018). Even outside the conservation sphere, there is a clear incentive to invigorate taxonomic research in Australia and this would hugely benefit the protection and management of GAB springs nationwide. A comprehensive understanding of the biodiversity and ecology of the GAB springs will not be possible without rigorous and rapid taxonomic research underpinned by strong species delimitation methods. To assist in breaching this gap, here we make recommendations for the use of genetic data in distinguishing cryptic species and the translation of those data into taxonomic descriptions.

Virtually all ecological monitoring studies require species-level identifications. However, these are almost impossible for some spring taxa due to the taxonomic impediment mentioned previously earlier. This barrier has the potential to impact environmental impact assessment, monitoring, and legislative protections at all levels due to 1) a paucity of formal taxonomic descriptions for most faunal groups; 2) morphological homogeneity amongst cryptic species; 3) low quality, or absent, identification keys and 4) few accurate databases of occurrence records. Genetic data can resolve these roadblocks and in doing so, illuminate population genetic structure, distinct phylogeographic lineages, putative cryptic species, and as-yet unknown biodiversity within springs. Much foundational genetic data already exists for GAB-dependent ecosystems (e.g., Murphy et al., 2009; Murphy et al., 2012, 2013, 2015a; Guzik et al., 2012, 2019; DeBoo et al., 2019) but is yet to be implemented at the level of species descriptions or bioassessment protocols (Brake et al., 2019).

Existing genetic surveys of endemic macro-invertebrate spring fauna have demonstrated evidence of at least 42 evolutionarily significant units (ESUs) in SA GAB springs (Murphy et al., 2009; 2015b; Guzik et al., 2012; Guzik and Murphy, 2013). ESUs represent genetic diversity equivalent to species for conservation purposes, and in many cases, they are readily distinguishable from one another using morphological characters. In order to translate these groupings into taxonomic descriptions, adoption of an abbreviated or faster taxonomy is required (Riedel et al., 2013). In recent studies (Murphy et al., 2015b; King et al., 2022), robust examples of a total evidence approach to species description from genetic lineages, especially in situations of cryptic species complexes, have been provided. Recommendations in such studies have included: standard generic descriptions to establish a strong taxonomic framework; use of integrative approaches (combining DNA, morphological characters, and locality data) to describe species; and Open Access publication practices for efficient dissemination. Macro-invertebrate taxa that require taxonomic attention based on current evidence include, but are certainly not limited to, chiltoniid amphipods, *Phreatomerus* isopods, *Trochidrobia* snails, *Ngarawa* ostracods, *Fonscochlea* snails, and hydraenid beetles.

For cryptic species that are not morphologically distinguishable from others, a multi-tiered approach of genetic data can be implemented for taxonomic diagnosis. This may include the use of genomic data for species-level delineation (e.g., whole mitochondrial genomes, genome skimming approaches), and population genetic and phylogeographic analyses to differentiate fauna from different geographic locations (e.g., the use of single nucleotide polymorphisms). Other approaches—such as metabarcoding, which we detail in Section 6.2.3—may be required to characterize GAB spring fauna more holistically, e.g., in the case of the minimally-known meio- and micro-faunal communities (Love et al., 1993; Andrews and Patel, 1996; Byers et al., 1998; Kimura et al., 2005; Ogg and Patel, 2009). In addition to an increased use of genetic data for species discovery, we also recommend that biodiversity assessment becomes a proactive, government-funded priority at the state and federal level in Australia. A comprehensive biodiversity collecting framework would permit planned collections, curation of specimens in state institutions, and a long-term biobank record of endemic fauna from a range of broad geographic areas. Current practices couple biodiversity assessment with land-use applications and their environmental assessments. This approach unfortunately often leads to discrete and limited sampling as well as specimens and data that can take years to be appropriately housed within museums and similar institutions, potentially limiting their value for future molecular research. Biodiversity assessment and monitoring practices can also be assisted by next-generation DNA sequencing methods, which can feed back into the species discovery process.

6.2.3 Improving biodiversity monitoring methods

The emergence of next-generation DNA sequencing (hereafter NGS) platforms over the last decade has resulted in the capacity to produce large amounts of DNA sequence data in rapid timeframes. Metabarcoding is one method reliant on NGS that can be applied to ecological questions of organismal detection and involves the simultaneous identification of multiple species from a single complex sample, whether the sample contains entire organisms or fragments of DNA (Taberlet et al., 2012). Metabarcoding can be applied at a relatively low cost in a wide range of contexts, ranging from the detection of microbiota in gut samples to that of higher organisms which have “shed” DNA (also known as environmental DNA, or eDNA) into their environment, e.g., in water, leaf litter, and soil samples. eDNA metabarcoding methods have clear applications for biomonitoring, in which the resulting DNA sequence data can be translated into ecological inventories of known and putative species (Brandt et al., 2021; Mousavi-Derazmahalleh et al., 2021). These approaches also represent a major advancement in the speed and accuracy of the identification and characterisation of ecological communities in diverse and difficult-to-sample environments (Zepeda Mendoza et al., 2015).

Instances in which eDNA metabarcoding has been successfully used in Australian groundwater-dependent ecosystems such as the GAB springs include the calcrete aquifers of Western Australia (Saccò et al., 2020), alluvial aquifers in eastern Australia (Korbel et al., 2017), karst systems on Christmas Island (West et al., 2020), and Beetaloo sub-basin environments in the Northern Territory (Oberprieler et al., 2021). In these habitats, rare and elusive species and their distributions have been examined in a way that has previously been impossible, such as the detection of the rare Pilbara blind cave eel (White et al., 2020) and the olm in Croatia (Vörös, 2017) in deep groundwater. eDNA metabarcoding can also offer invaluable insights into groundwater microbial communities (Smith et al., 2015; Smith et al., 2018) and their potential associations with macro-fauna (Smith et al., 2016). This rapid and cost-effective methodology has the potential to advance the monitoring of GAB spring dependent fauna by providing a complimentary, standardized, and affordable biomonitoring tool in addition to traditional survey-based approaches. eDNA is increasingly recognized as a powerful, but underutilized, tool for biomonitoring of delicate ecosystems (Saccò et al., 2022). We propose that eDNA metabarcoding has the potential to revolutionize the characterisation and monitoring of taxa present in the SA GAB springs by enabling the detection of at-risk species, overcoming several impediments to the taxonomic process and accelerating species discovery efforts in the process.

Accurate detection of species from eDNA amplicon data relies on matching millions of anonymous reads (fragments of sequenced DNA) to a reference collection of verified sequences with known taxonomic identities. For eDNA metabarcoding to be an effective tool for biomonitoring, an accurate barcode reference library is required: a database containing DNA sequences annotated with taxonomic information such that new sequences may be referenced against it to determine the identity of the organism they originated from. For GAB spring taxa, much of existing publically available DNA sequence data corresponds to the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) mitochondrial gene, limiting the ease of taxonomic identification of eDNA data represented by other genes or loci (i.e., nuclear or other mitochondrial genes, commonly 12S, 16S, or 18S rRNA) or for organisms lacking a mitochondrion such as bacteria (Page et al., 2007; King, 2009; Murphy et al., 2009; Guzik et al., 2012; Murphy et al., 2013; King et al., 2014; Murphy et al., 2015a; Guzik et al., 2019; Stringer et al., 2019). If eDNA metabarcoding were to be implemented as a biomonitoring tool in GAB springs, DNA sequencing for other barcoding genes would be required to establish robust and taxonomically accurate barcode reference libraries (Saccò et al., 2022).

6.2.4 Partnership with Traditional Custodians

In this section we defer to published Indigenous expertise to summarize how Traditional Custodians are currently recognized in GAB spring management practices, frameworks co-developed with Aboriginal peoples to improve this status quo, and ways in which reciprocal information-sharing can be facilitated by the incorporation of traditional knowledge into biodiversity and taxonomic research.

Aboriginal and Torres Strait Islander peoples have been systematically excluded from yielding power in natural resource management decisions in Australia. A key example is the EPBC Act, a core piece of federal environmental legislation containing a framework for the protection of biodiversity and locations of natural and cultural significance nationwide. In a recent independent review, the Act was criticized for consistently devaluing Aboriginal and Torres Strait Islander knowledge and values in its decision-making processes, failing Aboriginal and Torres Strait Islander peoples with respect to protection of their cultural heritage, and creating unbalanced power relations with leaseholders of protected areas (Samuel, 2021). Unfortunately, frameworks designed to conserve the GAB springs have been similarly lacking. In South Australia, for instance, authorisation of water use from the GAB is at ministerial discretion (*Aboriginal Heritage Act 1988* (SA) s14 (2)) provided consultation is conducted with the Arabana people, even if the Arabana do not permit such a decision. Any subsequent damage to springs only incurs a maximum penalty of $50,000 AUD, at odds with the cultural and environmental significance of these ecosystems (Parliament of Australia, 2021). The chair of the Arabana Aboriginal Corporation has stated that a discussion has been initiated on a national level regarding how the protection of SA GAB springs from ecological, cultural, and spiritual standpoints might be implemented (Parliament of Australia, 2021).

The Arabana people have developed a proposal in collaboration with non-Aboriginal researchers for the co-development of cultural indicators for water sites in the Kati Thanda–Lake Eyre region, including the SA GAB springs (Nursey-Bray and Arabana Aboriginal Corporation, 2015a). As stressed by the Arabana people and their co-authors, Aboriginal and Torres Strait Islander traditional ecological and cultural knowledge should be recognized as a separate body that is equally powerful, testable, and able to guide conservation practices *in concert* with existing Eurocentric scientific systems, "link[ing] the two systems like the sleepers on the railway lines that snake across their country" (Nursey-Bray and Arabana Aboriginal Corporation, 2015a; Nursey-Bray et al., 2020). Collaboration with Traditional Custodians should not be limited to the management of the GAB springs. For example, one way in which Traditional Custodians may be acknowledged is by the incorporation of Aboriginal names or words in species epithets by taxonomists. The use of Indigenous names can act as conduits of traditional knowledge, encourage a sense of ownership over the species and epithet itself, and affirm the expertise of Indigenous peoples, a much-needed contrast to the overwhelmingly Eurocentric history of biodiversity and taxonomy research (Veale et al., 2019; Gillman and Wright, 2020; Knapp et al., 2020; Galbreath, 2021; Sterner and Elliott, 2022).

Best practices do not currently exist regarding how taxonomic research may be conducted in partnership with Aboriginal peoples in Australia. However, recommendations have been published regarding the involvement of Indigenous Māori peoples in taxonomic research in *Aotearoa*/New Zealand (Veale et al., 2019). A key recommendation therein was that Indigenous names should not only be considered in the taxonomic process, but that active discussions with Indigenous peoples should take place to facilitate the selection of species names and reciprocal learning. In the absence of such discussions, epithets may prove insulting to Indigenous peoples if they are poorly researched or constructed (Veale et al., 2019). These recommendations provide a robust starting point for a similar framework in Australia. In addition to the co-development of cultural indicators of health for the GAB springs, the co-designing of species epithets with Traditional Custodians is one way in which Aboriginal and Western bodies of knowledge can exist together and ensure groundwater-dependent communities are adequately cared for into the future.

7 Concluding remarks

Here, we have reviewed current understandings of the significance of South Australian Great Artesian Basin-fed springs, remaining knowledge gaps, and a way forward regarding biodiversity research on the groundwater-dependent ecosystems they support. We have also outlined the cultural significance of these environments to Traditional Custodians of the land, namely the Arabana people, and discussed ways in which Aboriginal knowledge can be affirmed and acknowledged in future studies of these ecosystems. There are clear threatening processes that pose impending–and in some cases, already realized–risks to these habitats. Not only is a holistic characterization of the fauna and flora that rely on GAB springs necessary, but so too are additional protective mechanisms to buffer springs against such processes. Here we have largely concentrated on the former, as GAB spring ecosystems cannot be adequately protected if we do not have a detailed understanding of their biota in the first place.

In our view, key barriers exist to a robust understanding of the ecological communities reliant on GAB-fed springs: 1) spring fauna are not adequately protected under Australian federal environmental legislation and 2) a substantial taxonomic impediment exists to robustly naming and characterizing GAB springs fauna, particularly short-range endemic species. The way in which Australian fauna and flora are listed under legislation needs to be revisited such that short range endemics and data deficient species, the norm for many GAB spring-reliant taxa, are considered. An increase in taxonomic funding and capacity, in line with nationwide programs such as Taxonomy Australia, would enable new species to be described well before extinctions occur. Taxonomic and biodiversity research also represents an opportunity for reciprocal knowledge-sharing with Traditional Custodians.

The GAB springs are hotspots of biodiversity supporting unique ecological communities that have evolved in isolation for millions of years. In many cases, species housed in these vulnerable habitats are not found elsewhere on Earth. If we are to effectively conserve these plants and animals they must first be characterized, and in many cases their existence cannot be confirmed without the use of emerging genetic techniques as detailed here. A robust understanding of the species supported by these "oases of life" will ensure their corresponding ecological communities can be effectively managed and monitored into the future. The GAB springs hold universal biodiversity and cultural value, and the conservation management and characterization of the ecosystems they support should ultimately be a social responsibility shared by all stakeholders.

Author contributions

PBH and MG conceived the study. PBH provided expertise on spring endemic flora and fauna, the cultural significance of springs, and tourism impacts. MG provided expertise on short-range endemic spring taxa more broadly. NM provided expertise regarding industrial water use from the Great Artesian Basin. RK provided expertise regarding spring endemics, with specific focus on the crustaceans, and the cultural significance of springs. BH, SC, and AA provided expertise regarding the impact of climate change on freshwater bodies. All authors edited the manuscript and approved the final draft. The authors declare that they have no competing interests.

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Conflict of interest

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

Publisher's note

Supplementary Material

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Appendix D: Supplementary Materials for Chapter 2

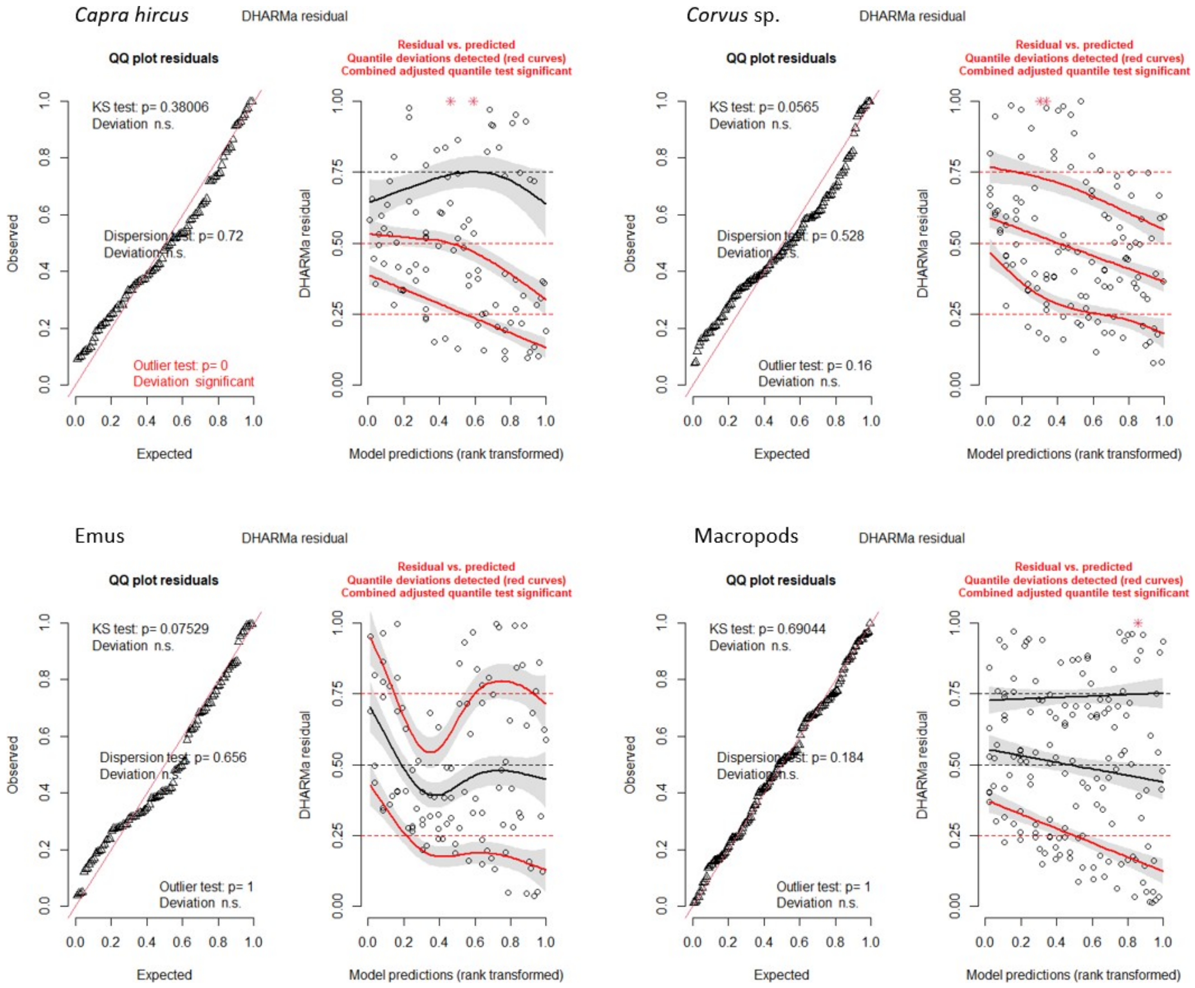

Figure D1. Residual diagnostics for generalised additive models Estimated response in visitation (number of captures) by goats (*C. hircus*); crows and ravens (*Corvus* spp.); emus (*D. novaehollandiae*); and kangaroos and wallaroos (macropods) to the number of days since a significant rainfall event (0.9 > mm) modelled with generalised additive models.

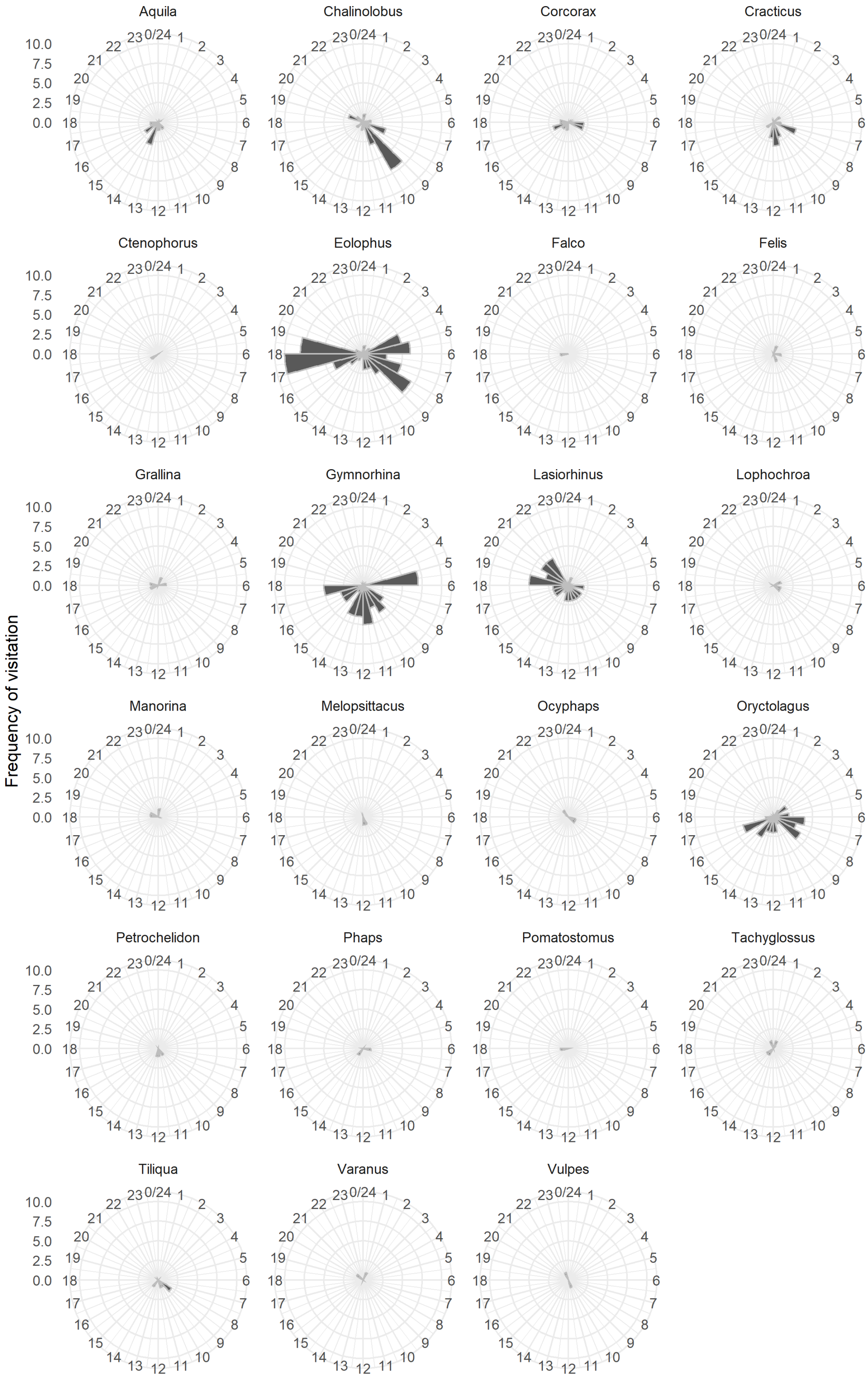

Figure D2. Circular distribution of vertebrate visitation patterns for 23 animal groups attending rock-holes at Hiltaba Nature Reserve.

Table D1. Summary statistics for generalised additive models. Effective degrees of freedom = edf, reference degrees of freedom = Ref.df, Chi-square value = Chi.sq, R-squared value = R-sq, Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

	Model summary				Approximate significance of smooth terms				Model Fit and Information		
Taxon	Estimate	Standard error	z value	p-value	edf	Ref.df	Chi.sq	p-value	R-sq	Deviance	n
Capra hircus	-0.6565	0.0753	-8.719	<2e-16 ***	1	1	0.169	0.681	0/0543	0.236%	94
Corvus sp.	-0.37304	0.07503	-4.972	6.64e-07 ***	1	1	20.67	6.33e-06 ***	-0.0109	15.9%	122
Dromaius novaehollandae	-0.26325	0.08481	-3.104	0.00191 **	1.502	1.849	44.58	<2e-16 ***	0.362	34.7%	103
Macropods	0.03535	0.06525	0.542	0.588	1.945	2.435	55.13	<2e-16 ***	0.346	33.6%	153

Table D2. Comparison between survey methods for vertebrate detection visiting rock-holes, with all known vertebrate species records for HNR. The column Bush Blitz reports species recorded during or prior to the bush blitz survey summarised in Commonwealth (2015a), WCT refers to species recorded by my wildlife camera traps in Chapter 2, and eDNA refers to species detected using the 16S vertebrate eDNA metabarcoding in Chapter 3. Species marked with * are invasive, exotic or introduced livestock species in Australia. Species marked with ^ are either EPBC Act listed, or NPW Act listed.

Family	Species name	Common name	Bush blitz	WCT	eDNA
Mammals					
Bovidae	*Capra hircus* *	Goat	✓	✓	✓
	Ovis aries *	Sheep	✓	✗	✓
Burramyidae	*Cercartetus concinnus*	Western Pygmy-possum	✓	✗	✗
Canidae	*Canis lupus* *	Dog or dingo	✗	✗	✓
	Vulpes vulpes *	Red Fox	✓	✓	✓
Dasyuridae	*Ningaui yvonneae*	Southern Ningaui	✓	✗	✗
	Sminthopsis crassicaudata	Fat-tailed Dunnart	✓	✗	✗
	Sminthopsis dolichura	Little Long-tailed Dunnart	✓	✗	✗
	Sminthopsis macroura	Stripe-faced Dunnart	✓	✗	✗
	Sminthopsis murina	Common Dunnart	✓	✗	✗
	Sminthopsis ooldea	Ooldea Dunnart	✓	✗	✗
Felidae	*Felis catus* *	Cat	✓	✓	✓
Leporidae	*Oryctolagus cuniculus* *	European Rabbit	✗	✓	✓
Macropodidae	*Macropus fuliginosus*	Western Grey Kangaroo	✓	✓	✓
	Macropus robustus	Common Wallaroo	✓	✓	✓
	Macropus rufus	Red Kangaroo	✓	✓	✓
	Petrogale xanthopus ^	Yellow-footed Rock-wallaby	✓	✗	✗
Muridae	*Mus musculus**	House Mouse	✓	✗	✗
	Pseudomys bolami	Bolam's Mouse	✓	✗	✗
	Pseudomys hermannsburgensis	Sandy Inland Mouse	✓	✗	✗
Tachyglossidae	*Tachyglossus aculeatus*	Short-beaked Echidna	✓	✓	✗
Vespertilionidae	*Chalinolobus gouldii*	Gould's Wattled Bat	✓	✗	✗
	Chalinolobus morio	Chocolate Wattled Bat	✓	✓	✗
	Nyctophilus geoffroyi	Lesser Long-eared Bat	✓	✗	✗
	Nyctophilus major	Central Long-eared Bat	✓	✗	✗
	Vespadelus baverstocki	Inland Forest Bat	✓	✗	✗
	Vespadelus regulus	Southern Forest Bat	✓	✗	✗
Vombatidae	*Lasiorhinus latifrons*	Southern Hairy-nosed Wombat	✓	✓	✓
Birds					
Acanthizidae	*Acanthiza apicalis*	Inland Thornbill	✓	✗	✗
	Acanthiza chrysorrhoa	Yellow-rumped Thornbill	✓	✗	✗
	Acanthiza iredalei iredalei ^	Slender-billed Thornbill	✓	✗	✗
	Acanthiza uropygialis	Chestnut-rumped Thornbill	✓	✗	✗
	Aphelocephala leucopsis	Southern Whiteface	✓	✗	✗
	Smicrornis brevirostris	Weebill	✓	✗	✗
Accipitridae	*Accipiter fasciatus*	Brown Goshawk	✓	✗	✗
	Accipter virgatus	Besra	✗	✗	✓
	Aquila audax	Wedge-tailed Eagle	✓	✓	✗
Aegothelidae	*Aegotheles cristatus*	Australian Owlet-nightjar	✓	✗	✗
Anatidae	*Anas gracilis*	Grey Teal	✓	✗	✗
	Chenonetta jubata	Australian Wood Duck	✓	✗	✗
Artamidae	*Artamus cinereus*	Black-faced Woodswallow	✓	✗	✗
	Artamus cyanopterus	Dusky Woodswallow	✓	✗	✗
	Artamus minor	Little Woodswallow	✓	✗	✗
	Gymnorhina tibicen	Australian Magpie	✓	✓	✗

	Cracticus torquatus	Grey Butcherbird	✓	✓	✗
	Strepera versicolor	Grey Currawong	✓	✗	✗
Cacatuidae	*Eolophus roseicapillus*	Galah	✓	✓	✗
	Lophochroa leadbeateri ^	Pink Cockatoo	✓	✓	✗
Campephagidae	*Coracina maxima*	Ground Cuckoo-shrike	✓	✗	✗
	Coracina novaehollandiae	Black-faced Cuckoo-shrike	✓	✗	✗
	Lalage sueurii	White-winged Triller	✓	✗	✗
Caprimulgidae	*Eurostopodus argus*	Spotted Nightjar	✓	✗	✗
Casuariidae	*Dromaius novaehollandiae*	Emu	✓	✓	✓
Charadriidae	*Vanellus tricolor*	Banded Lapwing	✓	✗	✗
Climacteridae	*Climacteris affinis* ^	White-browed Treecreeper	✓	✗	✗
	Climacteris rufa	Rufous Treecreeper	✓	✗	✗
Columbidae	*Ducula melanochroa*	Black Imperial Pigeon	✗	✗	✓
	Ocyphaps lophotes	Crested Pigeon	✓	✓	✗
	Phaps chalcoptera	Common Bronzewing	✓	✓	✗
Corcoracidae	*Corcorax melanorhamphos*	White-winged Chough	✓	✓	✗
Corvidae	*Corvus bennetti*	Little Crow	✓	✗	✗
	Corvus coronoides	Australian Raven	✓	✓	✓
	Corvus mellori	Little Raven	✓	✓	✗
Cuculidae	*Cacomantis flabelliformis*	Fan-tailed Cuckoo	✓	✗	✗
	Cacomantis pallidus	Pallid Cuckoo	✓	✗	✗
	Chrysococcyx basalis	Horsfield's Bronze-cuckoo	✓	✗	✗
Falconidae	*Falco berigora*	Brown Falcon	✓	✓	✗
	Falco cenchroides	Nankeen Kestrel	✓	✗	✗
	Falco longipennis	Australian Hobby	✓	✗	✗
	Falco peregrinus ^	Peregrine Falcon	✓	✗	✗
Hirundinidae	*Cheramoeca leucosterna*	White-backed Swallow	✓	✗	✗
	Hirundo neoxena	Welcome Swallow	✓	✗	✗
	Petrochelidon ariel	Fairy Martin	✓	✓	✗
	Petrochelidon nigricans	Tree Martin	✓	✓	✗
Maluridae	*Amytornis merrotsyi*	Short-tailed Grasswren	✓	✗	✗
	Malurus lamberti	Variegated Fairy-wren	✓	✗	✗
	Malurus leucopterus	White-winged Fairy-wren	✓	✗	✗
	Malurus splendens	Splendid Fairy-wren	✓	✗	✗
Megaluridae	*Cincloramphus cruralis*	Brown Songlark	✓	✗	✗
Megapodiidae	*Leipoa ocellata* ^	Malleefowl	✓	✗	✗
Meliphagidae	*Acanthagenys rufogularis*	Spiny-cheeked Honeyeater	✓	✗	✗
	Anthochaera carunculata	Red Wattlebird	✓	✗	✗
	Epthianura albifrons	White-fronted Chat	✓	✗	✗
	Epthianura tricolor	Crimson Chat	✓	✗	✗
	Gavicalis virescens	Singing Honeyeater	✓	✗	✗
	Gliciphila melanops	Tawny-crowned Honeyeater	✓	✗	✗
	Manorina flavigula	Yellow-throated Miner	✓	✓	✗
	Melithreptus brevirostris	Brown-headed Honeyeater	✓	✗	✗
	Nesoptilotis leucotis	White-eared Honeyeater	✓	✗	✗
	Ptilotula ornatus	Yellow-plumed Honeyeater	✓	✗	✗
	Ptilotula plumula	Grey-fronted Honeyeater	✓	✗	✗
	Purnella albifrons	White-fronted Honeyeater	✓	✗	✗
Meropidae	*Merops ornatus*	Rainbow Bee-eater	✓	✗	✗
Monarchidae	*Grallina cyanoleuca* ^	Magpie-lark	✓	✓	✗
	Myiagra inquieta ^	Restless Flycatcher	✓	✗	✗
Motacillidae	*Anthus novaeseelandiae*	Australasian Pipit	✓	✗	✗
Nectariniidae	*Dicaeum hirundinaceum*	Mistletoebird	✓	✗	✗
Neosittidae	*Daphoenositta chrysoptera*	Varied Sittella	✓	✗	✗
	Colluricincla harmonica	Grey Shrike-thrush	✓	✗	✗

Pachycephalidae	*Oreoica gutturalis*	Crested Bellbird	✓	✗	✗
	Pachycephala inornata ^	Gilbert's Whistler	✓	✗	✗
	Pachycephala rufiventris	Rufous Whistler	✓	✗	✗
Pardalotidae	*Pardalotus striatus*	Striated Pardalote	✓	✗	✗
Petroicidae	*Eopsaltria griseogularis*	Western Yellow Robin	✓	✗	✗
	Melanodryas cucullata	Hooded Robin	✓	✗	✗
	Microeca fascinans	Jacky Winter	✓	✗	✗
	Petroica goodenovii	Red-capped Robin	✓	✗	✗
Phalacrocoracidae	*Microcarbo melanoleucos*	Little Pied Cormorant	✓	✗	✗
Phasianidae	*Coturnix pectoralis*	Stubble Quail	✓	✗	✗
	Gallus gallus domesticus	Chicken	✗	✗	✓
Podargidae	*Podargus strigoides*	Tawny Frogmouth	✓	✗	✗
Podicipedidae	*Poliocephalus poliocephalus*	Hoary-headed Grebe	✓	✗	✗
	Tachybaptus novaehollandiae	Australasian Grebe	✓	✗	✗
Pomatostomidae	*Pomatostomus superciliosus*	White-browed Babbler	✓	✓	✗
Psittacidae	*Barnardius zonarius*	Australian Ringneck	✓	✗	✗
	Glossopsitta porphyrocephala	Purple-crowned Lorikeet	✓	✗	✗
	Melopsittacus undulatus	Budgerigar	✓	✓	✗
	Neophema splendida	Scarlet-chested Parrot	✓	✗	✗
	Neopsephotus bourkii	Bourke's Parrot	✓	✗	✗
	Northiella haematogaster	Blue Bonnet	✓	✗	✗
	Platycercus eximius	Eastern Rosella	✗	✗	✓
	Psephotus varius	Mulga Parrot	✓	✗	✗
Rallidae	*Tribonyx ventralis*	Black-tailed Native-hen	✓	✗	✗
Rhipiduridae	*Rhipidura albiscapa*	Grey Fantail	✓	✗	✗
	Rhipidura leucophrys	Willie Wagtail	✓	✗	✗
Strigidae	*Ninox novaeseelandiae*	Southern Boobook	✓	✗	✗
Timaliidae	*Zosterops lateralis*	Silvereye	✓	✗	✗
		Reptiles			
Agamidae	*Ctenophorus cristatus*	Bicycle Lizard	✓	✗	✗
	Ctenophorus fionni	Peninsula Dragon	✓	✓	✗
	Ctenophorus pictus	Painted Dragon	✓	✗	✗
	Moloch horridus	Thorny Devil	✓	✗	✗
	Pogona minor	Dwarf Bearded Dragon	✓	✗	✗
	Tympanocryptis lineata	Lined Earless Dragon	✓	✗	✗
Boidae	*Morelia spilota* ^	Carpet Python	✓	✗	✗
Carphodactylidae	*Underwoodisaurus milii*	Barking Gecko	✓	✗	✗
Diplodactylidae	*Diplodactylus furcosus*	Ranges Stone Gecko	✓	✗	✗
	Diplodactylus vittatus	Eastern Stone Gecko	✓	✗	✗
	Diplodactylus wiru	Desert Wood Gecko	✓	✗	✗
	Lucasium damaeum	Beaded Gecko	✓	✗	✗
	Strophurus elderi	Jewelled Gecko	✓	✗	✗
	Strophurus intermedius	Eastern Spiny-tailed Gecko	✓	✗	✗
Elapidae	*Brachyurophis semifasciatus*	Southern Shovel-nosed Snake	✓	✗	✗
	Demansia reticulata	Desert Whipsnake	✓	✗	✗
	Parasuta nigriceps	Mitchell's Short-tailed Snake	✓	✗	✗
	Parasuta spectabilis	Mallee Black-headed Snake	✓	✗	✗
	Pseudechis australis	Mulga Snake	✓	✗	✗
	Pseudonaja mengdeni	Western Brown Snake	✓	✗	✗
	Pseudonaja modesta	Ringed Brown Snake	✓	✗	✗
	Simoselaps bertholdi	Jan's Banded Snake	✓	✗	✗
Gekkonidae	*Cnemaspis nilagrica*	Nilgiri Day Gecko	✗	✗	✓

	Gehyra lazelli	Southern Rock Dtella	✓	✗	✗
	Gehyra purpurascens	Purplish Dtella	✓	✗	✗
	Gehyra variegata	Tree Dtella	✓	✗	✗
	Heteronotia binoei	Bynoe's Gecko	✓	✗	✗
Pygopodidae	*Delma australis*	Marble-faced Delma	✓	✗	✗
	Delma butleri	Unbanded Delma	✓	✗	✗
	Lialis burtonis	Burton's Snake-lizard	✓	✗	✗
	Pygopus lepidopodus	Common Scaly-foot	✓	✗	✗
Scincidae	*Cryptoblepharus australis*	Inland Snake-eyed Skink	✓	✗	✗
	Cryptoblepharus plagiocephalus	Péron's Snake-eyed Skink	✓	✗	✗
	Ctenotus atlas	Southern Mallee Ctenotus	✓	✗	✗
	Ctenotus orientalis	Eastern Ctenotus	✓	✗	✗
	Ctenotus pantherinus	Leopard Ctenotus	✓	✗	✗
	Ctenotus robustus	Robust Ctenotus	✓	✗	✗
	Ctenotus schomburgkii	Barred Wedgesnout Ctenotus	✓	✗	✗
	Ctenotus uber	Spotted Ctenotus	✓	✗	✗
	Cyclodomorphus branchialis	Common Slender Blue-tongue	✗	✗	✓
	Cyclodomorphus melanops	Spinifex Slender Blue-tongue	✓	✗	✗
	Egernia stokesii	Gidgee Skink, Stokes' Skink	✓	✗	✗
	Egernia striolata	Tree Skink	✓	✗	✗
	Eremiascincus richardsonii	Broad-banded Sand-swimmer	✓	✗	✗
	Hemiergis millewae	Triodia Earless Skink	✓	✗	✗
	Lerista edwardsae	Edwards' Slider	✓	✗	✗
	Lerista labialis	Southern Sandslider	✓	✗	✗
	Lerista muelleri	Wood Mulch-slider	✓	✗	✗
	Lerista terdigitata	Robust Mulch Slider	✓	✗	✗
	Lerista timida	Timid Slider	✓	✗	✗
	Menetia greyii	Common Dwarf Skink	✓	✗	✗
	Morethia boulengeri	South-eastern Morethia Skink	✓	✗	✗
	Morethia butleri	Woodland Morethia Skink	✓	✗	✗
	Tiliqua rugosa	Shingle-back Lizard	✓	✓	✓
Varanidae	*Varanus gouldii*	Gould's Goanna	✓	✓	✗
	Varanus tristis	Black-headed Monitor	✓	✗	✗
		Amphibians			
Myobatrachidae	*Neobatrachus pictus*	Painted Frog	✓	✗	✓
	Neobatrachus sudellae	Sudell's Frog	✓	✗	✓

Appendix E: Supplementary Materials for Chapter 3

Table E1. A presence absence matrix indicating which rock-holes each 100% OTU was detected within. Species marked with * are considered false positives due to contamination or primer bias.

	Man01	Man02	Tad01	Mid01	Roun01	PP01	PP02
Accipiter virgatus	✗	✗	✗	✓	✗	✗	✗
*Bos indicus**	✗	✗	✗	✗	✗	✓	✗
Canis lupus	✗	✓	✓	✗	✗	✗	✗
Capra hircus	✓	✓	✓	✓	✗	✗	✗
Cnemaspis nilagirica	✓	✓	✓	✓	✓	✓	✓
Corvus coronoides	✓	✗	✗	✓	✗	✗	✗
Cyclodomorphus branchialis	✓	✗	✗	✗	✗	✗	✗
Dromaius novaehollandiae	✓	✓	✓	✗	✓	✓	✗
Ducula melanochroa	✗	✓	✗	✗	✗	✗	✗
Felis catus	✗	✗	✓	✗	✗	✗	✗
Gallus gallus	✗	✗	✗	✓	✗	✗	✗
Lasiorhinus krefftii	✗	✓	✗	✗	✗	✗	✗
Macropus fuliginosus	✓	✓	✓	✓	✓	✗	✗
Macropus giganteus	✓	✓	✗	✗	✓	✗	✗
Macropus robustus	✓	✓	✓	✓	✓	✗	✗
Macropus rufus	✓	✓	✓	✓	✗	✓	✗
*Natator depressa**	✗	✗	✗	✗	✓	✗	✗
Neobatrachus pictus	✓	✗	✓	✗	✗	✓	✗
Neobatrachus sudelli	✓	✗	✗	✗	✗	✗	✗
Oryctolagus cuniculus	✓	✗	✗	✗	✗	✗	✗
Osphranter rufus	✗	✗	✗	✗	✓	✗	✗
Ovis aries	✗	✗	✗	✗	✓	✗	✗
Platycercus eximius	✗	✓	✓	✓	✗	✗	✗
Rupicapra pyrenaica *	✓	✗	✗	✗	✗	✗	✗
Saguinus niger *	✗	✗	✗	✗	✗	✗	✓
Tamiops maritimus *	✓	✗	✓	✗	✗	✗	✓
Tiliqua rugosa	✓	✗	✗	✗	✗	✗	✗
Vulpes vulpes	✗	✗	✗	✗	✓	✗	✗

Appendix F: Supplementary Materials for Chapter 4

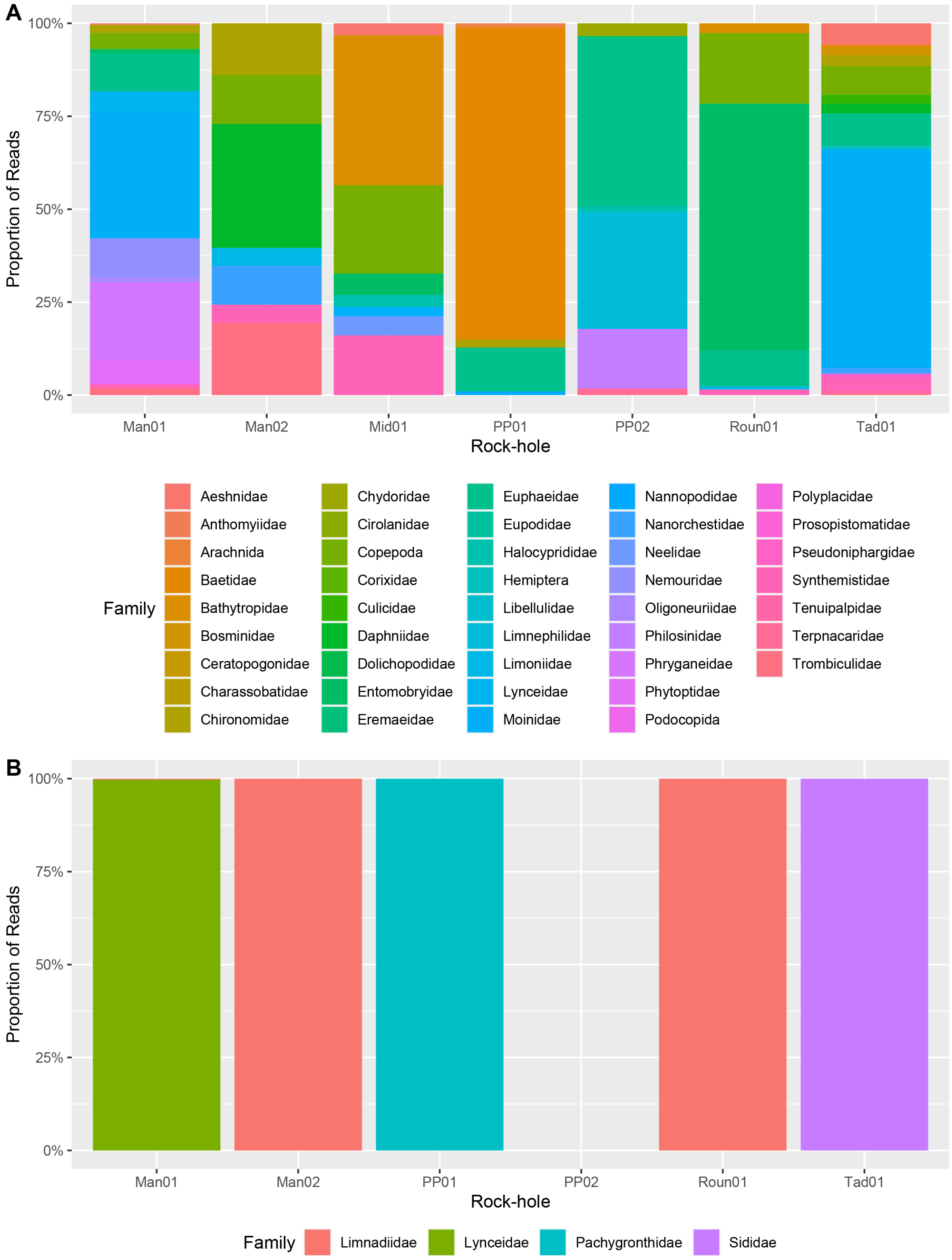

Figure F1. Stacked bar plots depicting the proportion of sequences assigned to family for each rock-hole for A) COI and B) 16S rRNA. The full dataset is presented in Table F1, and stacked bar charts for the distribution of reads by order can be seen in Figure 4.2.

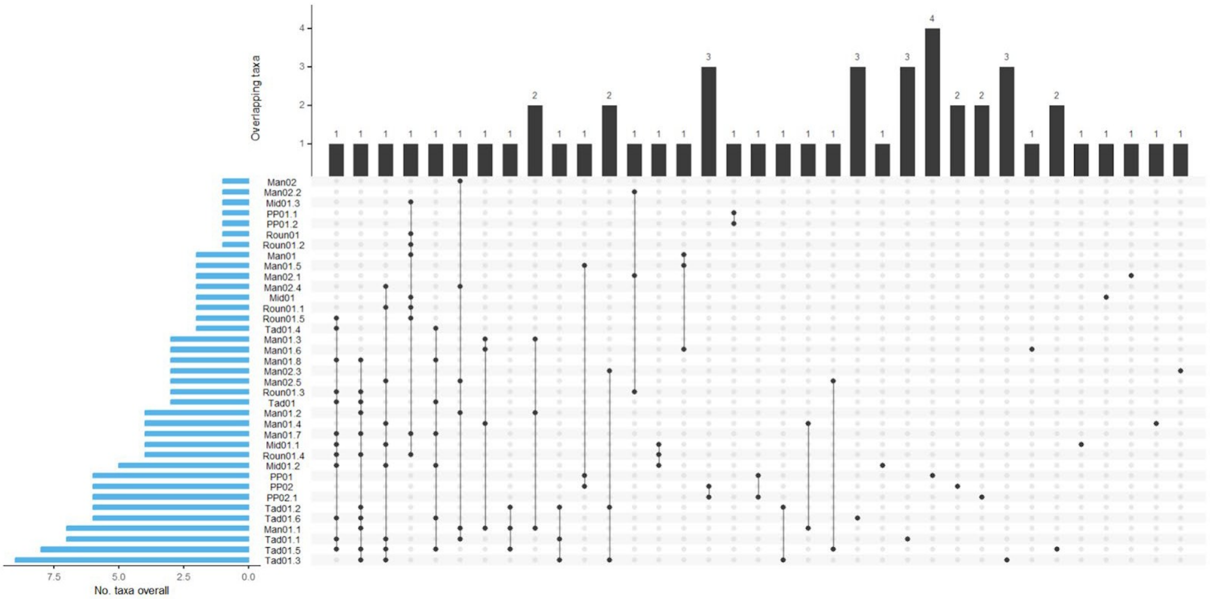

Figure F2. UpSet plots showing the number of ZOTUs shared between rock-holes for the uncollapsed CO1 eDNA metabarcoding data. Blue bars represent the total number of taxa within a single site. Black bars represent taxa that are shared between each site with black circles indicating which sites these taxa are shared with.

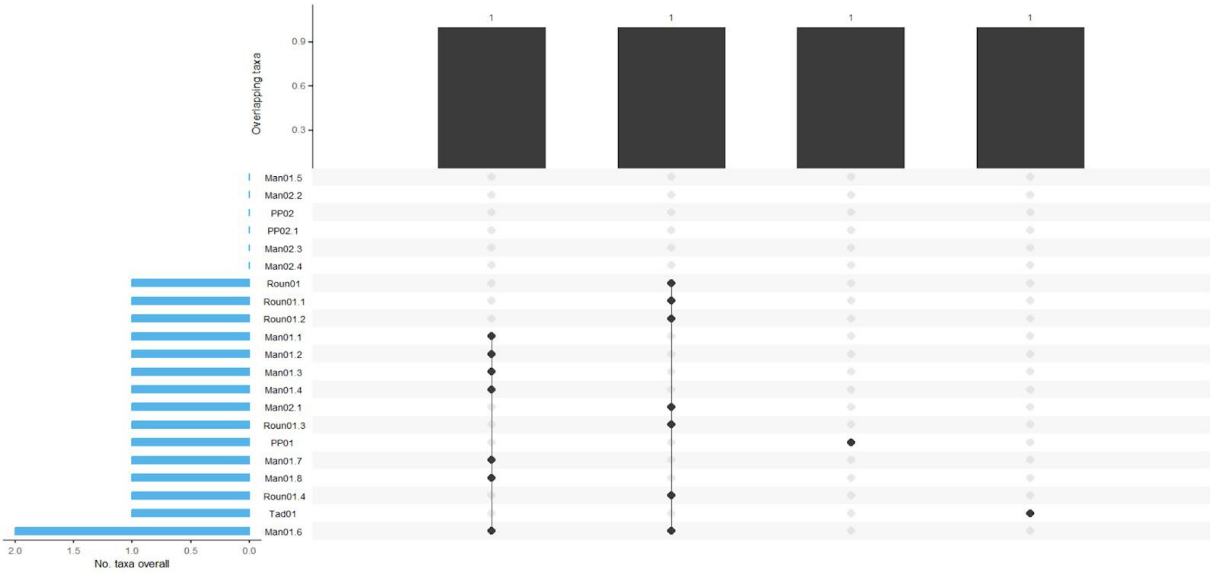

Figure F3. UpSet plots showing the number of ZOTUs shared between rock-holes for the uncollapsed 16S eDNA metabarcoding data. Blue bars represent the total number of taxa within a single site. Black bars represent taxa that are shared between each site with black circles indicating which sites these taxa are shared with.

Table F1. BLASTN results for showing assignment of ZOTUs for the COI dataset. Species marked in green are considered likely to be freshwater taxa and were included in downstream analysis, species marked in red are considered unlikely to be freshwater taxa and were excluded from downstream analysis. EC1 refers to experimental control, 'P1' and 'P2' refer to positive controls 1 and 2 respectively and 'Blank' refers to field blanks.

BLAST species name	**Man01**	**Man02**	**Mid01**	**PP01**	**PP02**	**Roun01**	**Tad01**	**EC1**	**P1**	**P2**	**Blank**
Acompus rufipes	0	0	0	0	0	2	0	0	0	0	0
Acontia aprica	0	0	0	0	0	0	6	0	0	0	0
Aedes alboannulatus	0	0	0	0	0	0	33	0	0	0	0
Aeshna juncea	2	0	0	0	0	0	82	0	0	0	0
Agathis sp.	0	0	0	0	0	0	3	0	0	0	0
Aleyrodidae sp.	0	0	236	0	0	43	0	0	0	0	0
Alona sp.	0	0	0	0	18	0	0	0	0	0	0
Altiverruca sp.	0	0	16	0	0	33	0	0	0	0	0
Ambohima ranohira	0	0	0	0	0	0	6	0	0	0	0
Anisostephus betulinus	26	0	0	0	0	0	0	0	0	0	0
Anoplodactylus stictus	0	0	0	0	0	0	8	0	0	0	0
Anzygina sp.	0	0	0	20	0	0	0	0	0	0	0
Aphididae sp.	0	0	0	0	7	0	0	0	0	0	0
Apoidea sp.	0	13	0	0	0	0	0	0	0	0	0
Arachnida sp.	2	0	0	0	0	0	0	0	0	0	0
Austrogynacantha heterogena	0	0	5	0	0	0	0	0	0	0	0
Baetidae sp.	0	0	0	766	0	0	0	0	0	0	0
Baetis rhodani	0	0	0	0	0	0	8	0	0	0	0
Brachypterus glaber	5	0	0	0	0	0	0	0	0	0	0
Brommella punctosparsa	2	0	3	25	0	0	0	0	0	0	3
Callilepis nocturna	0	0	0	4	0	0	0	0	0	0	0
Caloptilia cuculipennella	0	0	0	0	0	0	25	0	0	0	0
Caloptilia sp.	0	0	0	54	0	0	0	0	0	0	119
Campylaspis sulcata	0	0	0	6	0	0	0	0	0	0	0
Carapoia sp.	0	0	0	0	6	0	0	0	0	0	0
Cecidomyiidae sp.	0	23	0	0	0	0	0	0	0	0	30
Ceratina apacheorum	3	0	0	0	0	0	0	0	0	0	0
Ceratopogonidae sp.	0	0	0	0	0	0	4	0	0	0	0
Chihuahuanus crassimanus	0	0	0	0	3	0	0	0	0	0	0
Chironomidae sp.	10	0	0	0	0	0	0	0	0	0	0
Chironomus bernensis	0	0	0	18	0	0	0	0	0	0	3
Chironomus cloacalis	0	0	0	0	0	0	4	0	0	0	0

Chironomus tepperi	0	0	0	0	0	0	39	0	0	0	0
Chrysomelidae sp.	0	0	0	0	0	0	30	0	0	0	0
Chthamalus moro	207	0	0	0	0	0	0	0	0	0	0
Cicada barbara	0	0	0	0	4	0	0	0	0	0	0
Cicadellidae sp.	0	0	0	0	19	0	0	0	0	0	0
Cicadidae gen.	0	0	0	3	0	0	0	0	0	0	0
Cleptoria bipraesidens	0	0	0	0	10	0	0	0	0	0	0
Coenobita cavipes	0	0	0	0	20	0	0	0	0	0	0
Coleoptera sp.	2	0	0	7	12	0	0	0	0	0	0
Colopha sp.	0	119	0	0	0	0	0	0	0	0	0
Copepoda environmental	20	0	37	0	0	65	100	0	0	0	0
Coptotettix longtanensis	25	0	0	0	0	0	0	0	0	0	0
Crustacea environmental	0	19	0	0	0	0	8	0	0	0	0
Cryptinae sp.	0	0	0	0	3	0	0	0	0	0	0
Daphnia angulata	0	48	0	0	0	0	37	0	0	0	0
Delia tenuiventris	0	0	0	0	8	0	0	0	0	0	0
Dicellarius bimaculatus	0	0	12	0	0	0	110	0	0	0	0
Dichotomius geminatus	16	8	0	0	0	16	4	0	0	0	0
Diptera sp.	0	0	0	0	2	0	0	0	0	0	0
Draconarius himalayaensis	6	0	0	0	0	0	0	0	0	0	0
Drassodes pubescens	0	0	0	33	11	0	0	0	0	0	0
Dysphaea dimidiata	0	0	0	99	237	0	0	0	0	0	0
Ecphanthacris mirabilis	0	0	0	0	0	59	0	0	0	0	0
Ectopsocus sp.	0	0	0	0	0	15	4	0	0	0	0
Eidmannella pallida	0	0	0	4	0	0	0	0	0	0	0
Elachista sp.	0	0	0	0	3	0	0	0	0	0	0
Empoasca sp.	0	0	0	0	0	0	3	0	0	0	0
Endoclita minanus	1211	0	0	0	0	0	158	0	0	0	0
Enoplops sibiricus	0	0	9	0	0	0	0	0	0	0	0
Epeolus carolinus	0	0	11	0	0	0	0	0	0	0	0
Erdelyia saucra	0	0	0	0	0	7	0	0	0	0	0
Eremaeidae sp.	0	0	0	0	3	0	0	0	0	0	0
Eremocosta spinipalpis	3	0	0	0	0	2	0	0	0	0	0
Eubosmina huaronensis	0	0	0	0	0	0	28	0	0	0	0
Eucelatoria charapensis	0	0	386	0	0	6	0	0	0	0	0
Eulaira obscura	0	0	0	0	9	0	0	0	0	0	0
Euphaea formosa	50	0	0	0	0	33	123	0	0	0	0
Eupodidae sp.	0	0	0	0	0	0	2	0	0	0	0

Eutrichota tunicata	0	0	0	3	0	0	0	0	0	0	0
Excirolana mayana	2	0	0	0	0	0	0	0	0	0	0
Eysarcoris aeneus	0	0	5	0	0	0	19	0	0	0	0
Fahrenholzia ehrlichi	0	0	0	0	0	0	4	0	0	0	0
Heliophanus termitophagus	7	0	0	0	0	0	108	0	0	0	0
Hemimysis margalefi	0	0	0	0	0	0	12	0	0	0	0
Hemiptera sp.	0	0	0	0	0	0	3	0	0	0	0
Hilaphura varipes	0	0	0	0	0	0	142	0	0	0	0
Hymenoptera sp.	2	0	3	0	0	11	0	0	0	0	0
Ichneumonidae sp.	0	0	0	5	0	0	0	0	0	0	13
Lachlania sp.	6	0	0	0	0	0	0	0	0	0	0
Lacinipolia olivacea	0	7	0	0	0	0	0	0	0	0	0
Lamprochernes nodosus	0	0	0	0	0	0	2	0	0	0	0
Larentiinae sp.	0	0	0	0	0	0	9	0	0	0	0
Leptogenys sp.	0	0	0	51	0	0	0	0	0	0	13
Leptonetela lineata	8	0	0	0	0	0	0	0	0	0	0
Leptotrombidium deliense	9	28	0	0	0	0	6	0	0	0	0
Libellulidae sp.	0	0	0	0	0	0	9	0	0	0	0
Limnephilus ignavus	0	0	0	0	165	0	0	0	0	0	0
Litoscalpellum discoveryi	0	0	301	0	0	0	0	0	0	0	0
Lophoturus molloyensis	0	0	12	0	0	0	0	0	0	0	0
Loxosceles sp.	0	0	0	0	0	0	9	0	0	0	0
Lynceus macleyanus	25	0	0	0	0	0	0	0	0	0	0
Macrophya bifasciata	2	2	0	22	17	5	0	0	0	0	0
Mamilloecia mamillata	0	0	5	0	0	0	0	0	0	0	0
Mangatangi sp.	9	3	0	0	0	0	21	0	0	0	0
Megachile (Pseudocentron)	1031	6	0	0	0	0	2	0	0	0	0
Megachile velutina	8	0	0	0	0	0	0	0	0	0	0
Megalothorax sp.	0	0	8	0	0	0	0	0	0	0	0
Meimuna kuroiwae	0	0	0	6	0	0	0	0	0	0	0
Melanotus senilis	71	2	0	0	0	5	0	0	0	0	0
Melitta tricincta	0	0	0	108	0	0	0	0	0	0	0
Merodon pumilus	33	93	0	0	0	0	39	0	0	0	0
Mesocallis pteleae	17	0	0	0	0	0	0	0	0	0	0
Mesochorinae sp.	0	0	0	0	2	0	0	0	0	0	0
Mesochorus nuncupator	15	539	0	0	0	0	0	0	0	0	0
Mesochorus sp.	0	0	0	0	3	0	0	0	0	0	0

Metidiocerus elegans	0	0	2	0	0	0	0	0	0	0	0
Mikroconchoecia sp.	0	0	0	0	7	0	0	0	0	0	0
Moina sp.	169	0	4	0	0	0	828	0	0	0	0
Molophilus pullus	0	7	0	0	0	3	0	0	0	0	0
Mycetophilidae sp.	0	0	0	0	0	0	3	0	0	0	0
Nannopus dimorphicus	0	0	0	8	0	0	0	0	0	0	0
Nanorchestes sp.	0	11	0	0	0	0	0	0	0	0	0
Nanorchestidae sp.	0	4	0	0	0	0	22	0	0	0	0
Nemoura stratum	52	0	0	0	0	0	0	0	0	0	0
Neotroponiscus littoralis	0	0	63	0	0	9	0	0	0	0	0
Nephilengys sp.	0	41	0	0	0	0	0	0	0	0	0
Nesophrosyne sp.	0	0	0	0	5	0	0	0	0	0	0
Nitiditermes sp.	0	0	0	0	6	0	0	0	0	0	0
Nysius sp.	0	0	0	4	0	0	0	0	0	0	0
Odontotermes mathuri	0	0	234	0	0	3	0	0	0	0	0
Opilo whitei	0	0	0	0	0	0	22	0	0	0	0
Orosius orientalis	0	0	0	0	0	0	5	0	0	0	0
Orosius sp.	7	0	0	0	0	0	0	0	0	0	0
Pagurus arcuatus	0	0	0	0	79	0	0	0	0	0	0
Parameioneta tricolorata	0	0	0	6	2	2	0	0	0	0	0
Paramenexenus laetus	0	10	0	0	0	0	0	0	0	0	0
Paramyia nigritarsi	7	5	2	0	0	0	7	0	0	0	0
Paraphaenocladius sp.	0	20	0	0	0	0	0	0	0	0	0
Philosina buchi	0	0	0	0	84	0	0	0	0	0	0
Phryganea japonica	103	0	0	0	0	0	0	0	0	0	0
Phytoptus monthalensis	32	0	0	0	0	0	0	0	0	0	0
Platygastridae sp.	0	0	0	6	0	0	0	0	0	0	3
Poeta denotalis	0	0	34	0	0	0	0	0	0	0	0
Psechrus clavis	0	0	0	0	9	0	0	0	0	0	0
Pseudoniphargus cupicola	0	0	0	0	0	0	9	0	0	0	0
Pteromalinae sp.	51	0	6	0	0	0	0	0	0	0	0
Raoiella sp.	0	0	0	0	0	0	12	0	0	0	0
Rhysodesmus zapotecus	6	0	0	0	0	0	0	0	0	0	0
Robertus borealis	0	0	0	0	0	0	28	0	0	0	0
Rudiloria kleinpeteri	0	16	0	0	0	6	137	0	0	0	0
Rudiloria trimaculata	0	0	0	0	8	0	0	0	0	0	0
Sciomyzidae sp.	114	187	0	0	0	0	271	0	0	0	3
Scolopendra subspinipes	0	0	55	0	0	0	59	0	0	0	0

Sigara lateralis	0	0	0	0	0	0	2	0	0	0	0
Sigmella ectobioides	0	0	0	0	3	0	0	0	0	0	0
Sphaerobelum sp.	0	68	152	0	0	2	198	0	0	0	0
Sphingonotus pamiricus	0	0	25	0	0	180	0	0	0	0	0
Synthemis eustalacta	5	7	25	0	0	5	50	0	0	0	0
Teleogryllus emma	35	0	0	13	71	0	268	0	0	0	13
Terpnacaridae sp.	0	0	0	0	9	0	0	0	0	0	0
Tetraclita singaporensis	0	0	0	0	4	0	0	0	0	0	0
Tetragonisca angustula	422	0	0	0	0	26	303	0	0	0	0
Tettigettalna afroamissa	0	0	16	0	0	12	34	0	0	0	0
Tettigidea lateralis	0	0	0	0	0	111	0	0	0	0	0
Thrips sp.	0	0	0	7	0	0	0	0	0	0	4
Thymopides grobovi	0	0	8	0	0	87	0	0	0	0	0
Tineidae sp.	0	0	6	0	0	4	0	0	0	0	0
Trichothurgus aterrimus	0	0	7	0	0	0	0	0	0	0	0
Trigonopterus subglabratus	16	0	0	0	0	0	0	0	0	0	0
Trioza erytreae	0	0	0	0	14	0	0	0	0	0	0
Tumulitermes recalvus	0	2	0	0	0	0	0	0	0	0	0
Tympanistalna gastrica	9	0	5	0	0	63	4	0	0	0	0
Ulolonche sp.	10	0	0	0	0	0	0	0	0	0	0
UNVERIFIED: Arachnida	0	0	0	7	0	0	0	0	0	0	0
UNVERIFIED: Liposcelis	36	0	0	0	0	0	0	0	0	0	0
UNVERIFIED: Molytinae	0	0	0	0	0	0	3	0	0	0	0
Willowsia nigromaculata	6	0	9	0	0	227	0	0	0	0	0
Wiseana cervinata	0	0	0	0	0	4	0	0	0	0	0
Zelotes longipes	0	0	0	0	3	0	0	0	0	0	0
Zodarion sp.	0	0	0	0	3	0	0	0	0	0	0
Only present in blanks and positive controls											
Charassobates aff.	0	0	0	0	0	0	0	0	0	0	2
Coptotermes frenchi	0	0	0	0	0	0	0	0	0	0	7
UNVERIFIED: *Menippe*	0	0	0	0	0	0	0	0	702	2117	0
UNVERIFIED: *Euproctis*	0	0	0	0	0	0	0	0	0	0	7
UNVERIFIED: Hemiptera	0	0	0	0	0	0	0	0	0	0	3
Tonsilla sp.	0	0	0	0	0	0	0	0	0	0	4
Tetraclita ehsani	0	0	0	0	0	0	0	0	0	0	124

Sympetrum semicinctum	0	0	0	0	0	0	0	0	0	0	3
Salticidae sp.	0	0	0	0	0	0	0	0	0	0	3
Prosopistoma oronti	0	0	0	0	0	0	0	1	0	0	0
Podocopida sp.	0	0	0	0	0	0	0	0	0	0	3
Leiophron sp.	0	0	0	0	0	0	0	0	0	0	7
Oligostomis pardalis	0	0	0	0	0	0	0	0	0	0	4
Hydrophorus alboflorens	0	0	0	0	0	0	0	0	0	0	3
Impatiophila trifurcatosternata	0	0	0	0	0	0	0	0	0	0	115
Fannia ciliatissima	0	0	0	0	0	0	0	0	0	0	7
Galathea leporis	0	0	0	0	0	0	0	0	0	0	2
Geocoris pallidipennis	0	0	0	0	0	0	0	0	0	0	4
Epeolus basili	0	0	0	0	0	0	0	0	0	0	7

Table F2. Blast results for showing assignment of ZOTUs for the 16S rRNA dataset. Species marked in green are considered likely to be freshwater taxa and were included in downstream analysis, species marked in red are considered unlikely to be freshwater taxa and were excluded from downstream analysis. EC1 refers to experimental control, 'P2' and 'P3' refer to positive controls 2 and 3 respectively and 'Blank' refers to field blanks.

BLAST species name	**Man01**	**Man02**	**Roun01**	**PP02**	**PP01**	**Tad01**	**Blank**	**P2**	**P3**
Acizzia uncatoides	0	0	0	0	100519	0	0	0	0
Amitermes sp.	3	0	0	0	0	0	0	0	0
Apis mellifera	0	27635	28	0	0	0	0	0	0
Drepanotermes sp.	0	6264	0	0	0	0	0	0	0
Eulimnadia braueriana	101	2	216151	0	0	0	0	0	0
Lynceus macleyanus	36702	0	0	0	0	0	0	0	0
Orosius orientalis	14946	0	8334	0	0	0	0	0	0
Orosius sp.	1572	0	3431	1673	0	0	10238	0	0
Penilia avirostris	0	0	0	0	0	2	0	24836	26838
Stenophyella sp.	0	0	0	0	1263	0	0	0	0
Tanystigma sp.	0	0	0	50	0	0	0	0	0
Only present in blanks and positive controls									
Melicertus plebejus	0	0	0	0	0	0	0	5062	0
Monomia argentata	0	0	0	0	0	0	3	0	0
Lithadia brasiliensis	0	0	0	0	0	0	0	0	839
Pinnotheres excussus	0	0	0	0	0	0	0	3257	0
Processa japonica	0	0	0	0	0	0	0	623	0
Sympagurus pictus	0	0	0	0	0	0	0	356	0

Table F3. Comparison of orders and families between those recovered from Eyre Peninsula granite rock-holes by Timms (2014) and by our eDNA metabarcoding.

	Detected by Timms (2014)	Detected by our eDNA metabarcoding
Order		
Amphipoda	✗	✓
Anomopoda	✓	✓
Arachnida	✗	✓
Calanoida	✓	✗
Coleoptera	✓	✓
Copepoda	✗	✓
Cyclopoida	✓	✗
Ctenopoda	✗	✓
Diptera	✓	✓
Endeostigmata	✗	✓
Entomobryomorpha	✓	✓
Ephemeroptera	✓	✓
Halocyprida	✗	✓
Harpacticoida	✗	✓
Hemiptera	✓	✓
Hydrocarina	✓	✗
Isopoda	✗	✓
Laevicaudata	✓	✓
Neelipleona	✗	✓
nematoda	✓	✗
Odonata	✓	✓
Planarian (dark)	✓	✗
Planarian (green)	✓	✗
Plecoptera	✗	✓
Podocopida	✓	✗
Prostigmata	✗	✓
Sarcoptiformes	✗	✓
Spinicaudata	✓	✓
Trichoptera	✓	✗
Trombidiformes	✗	✓
Family		
Aeshnidae	✓	✓
Anthomyiidae	✗	✓
Arachnida	✗	✓
Baetidae	✓	✓
Bathytropidae	✗	✓
Bosminidae	✗	✓
Centropagidae	✓	✗
Ceratopogonidae	✓	✓
Chironomidae	✓	✓
Chydoridae	✓	✓

Cirolanidae	✗	✓
Coenagrionidae	✓	✗
Corduliidae	✓	✗
Corixidae	✓	✓
Culicidae	✓	✓
Cyclopidae	✓	✗
Cyprididae	✓	✗
Daphniidae	✓	✓
Dytiscidae	✓	✗
Entomobryidae	✗	✓
Eremaeidae	✗	✓
Euphaeidae	✗	✓
Eupodidae	✗	✓
Halocyprididae	✗	✓
Hydropilidae	✓	✗
Leptoceridae	✓	✗
Lestidae	✓	✗
Libellulidae	✓	✓
Limnadiidae	✓	✓
Limnephilidae	✗	✓
Limoniidae	✗	✓
Lynceidae	✓	✗
Macrothricidae	✓	✗
Micronectidae	✓	✗
Moinidae	✗	✓
Nannopodidae	✗	✓
Nanorchestidae	✗	✓
Neelidae	✗	✓
Nemouridae	✗	✓
Notonectidae	✓	✗
Oligoneuriidae	✗	✓
Pachygronthidae	✗	✓
Philosinidae	✗	✓
Phryganeidae	✗	✓
Phytoptidae	✗	✓
Polyplacidae	✗	✓
Pseudoniphargidae	✗	✓
Sididae	✗	✓
Synthemistidae	✗	✓
Tabanidae	✓	✗
Tenuipalpidae	✗	✓
Terpnacaridae	✗	✓
Tipulidae	✓	✗
Trombiculidae	✗	✓

Appendix G: Supplementary Materials for Chapter 5

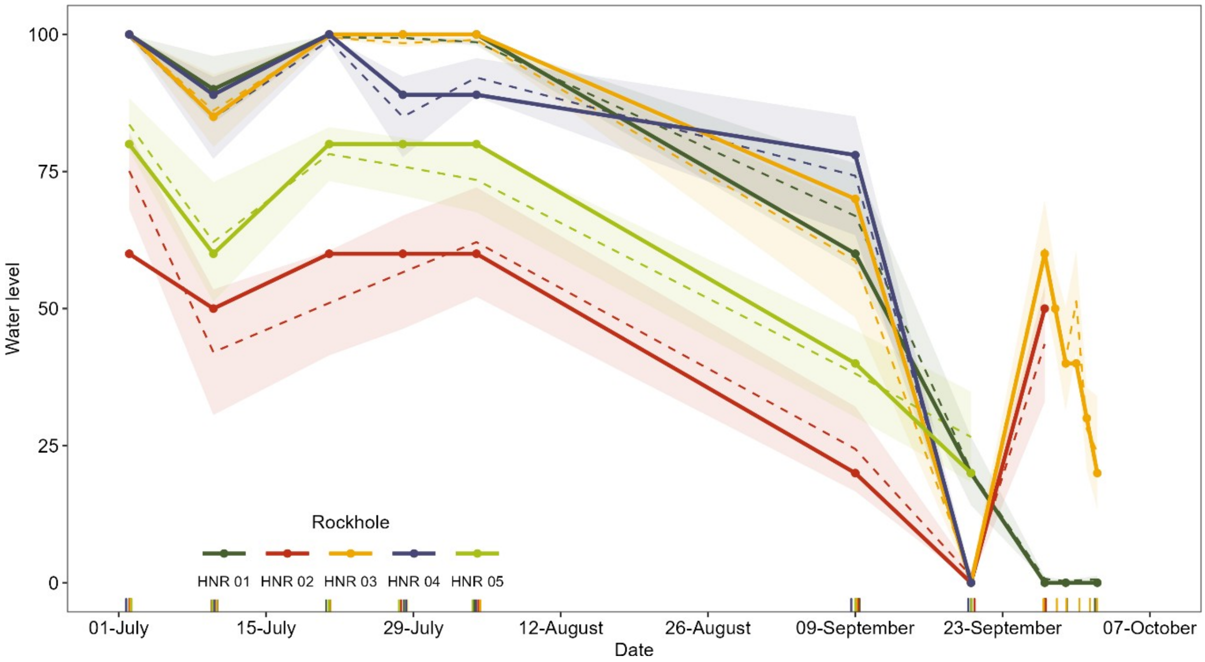

Figure G1. Hydrological models of rock-hole drying for five HNR rock-holes. Coloured points indicate observed water levels within rock-holes and solid bars indicate observed drying rate. Dashed lines indicate model prediction for water level, with coloured bands indicating 95% confidence intervals.

Table G1. Summary of GLMs testing the effect of scenario on the percentage of tanks with hatching success for *D. clarinata* and *Sarscypridopsis* sp.

Scenario	Species	Z-value	*p*
1	*Daphnia clarinata*	2.62 (79)	> 0.05
	Sarscypridopsis sp.	0 (79)	> 0.05
2	*Daphnia clarinata*	-2.36 (79)	0.018
	Sarscypridopsis sp.	0 (79)	> 0.05
3	*Daphnia clarinata*	-2.36 (79)	0.018
	Sarscypridopsis sp.	0 (79)	> 0.05
4	*Daphnia clarinata*	0.01 (79)	> 0.05
	Sarscypridopsis sp.	0.01 (79)	> 0.05
5	*Daphnia clarinata*	-1.36 (79)	> 0.05
	Sarscypridopsis sp.	0 (79)	> 0.05

Table G2. Pair-wise test results of the univariate PERMANOVA analysis for *D. clarinata* and *Sarscypridopsis* sp.

Test	**Days to hatching**		**Days to max. abundance**		**Max. population growth rate**		**Max. relative abundance**	
Test	*t*	*p* (perm)	*t*	*p* (perm)	*t*	*p* (perm)	*t*	*p* (perm)
				Daphnia clarinata				
Climate Scenario								
Current day, RCP 4.5	7.45 (34)	0.001	2.26 (34)	0.024	-	-	-	-
Current day, RCP 8.5	9.22 (31)	0.001	2.78 (31)	0.01	-	-	-	-
RCP 4.5, RCP 8.5	1.48 (37)	0.16	<0.0001 (37)	0.977	-	-	-	-
Year								
2050, 2090 (RCP 4.5)	1.88 (20)	0.101	1.37 (20)	0.206	-	-	-	-
2050, 2090 (RCP 8.5)	3.90 (17)	0.001	2.96 (17)	0.017	-	-	-	-
				Sarscypridopsis sp.				
Climate Scenario								
Current day, RCP 4.5	13.22 (42)	0.001	n/a	n/a	3.66 (42)	0.001	-	-
Current day, RCP 8.5	11.38 (43)	0.001	n/a	n/a	4.68 (43)	0.001	-	-
RCP 4.5, RCP 8.5	1.24 (57)	0.197	n/a	n/a	0.53 (57)	0.788	-	-
Year								
2050, 2090 (RCP4.5)	6.84 (28)	0.001	n/a	n/a	2.73 (28)	0.004	1.90 (28)	0.069
2050, 2090 (RCP 8.5)	4.93 (29)	0.001	n/a	n/a	3.02 (29)	0.001	2.03 (29)	0.007

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